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Antoine Couto

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LABORATOIRE EVOLUTION GENOMES COMPORTEMENT ECOLOGIE

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Structure et dynamique des systèmes vivants

Spécialité de doctorat : Sciences de la Vie et de la Santé

Par

Antoine Couto

Etude neurophysiologique et comportementale de la perception olfactive chez une espèce invasive, le frelon *Vespa velutina*

Thèse présentée et soutenue à Gif-sur-Yvette, le 13 juin 2016 :

Composition du Jury :

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Contexte de la thèse

Les événements majeurs survenus sur terre comme les changements de relief, les variations chimiques de l'atmosphère ou les extinctions massives d'espèces sont inscrits dans les strates sédimentaires qui ont permis de définir l'échelle des temps géologiques. La composition physico-chimique des roches et les fossiles qu'elles renferment renseignent alors sur le paysage et la biodiversité à une époque donnée. En 2002, Paul Crutzen, prix Nobel de chimie (1995), proposa l'ajout d'une nouvelle époque géologique marquée par les conséquences de l'activité humaine, l'Anthropocène (Crutzen, 2002 ; Zalasiewicz *et al*., 2008). Ce terme suggère que l'Homme et ses activités seraient devenus la plus grande contrainte géophysique et évolutive agissant sur la planète. L'accroissement de l'érosion des sols, la découverte de plastiglomérats, la perturbation du cycle du carbone et les bouleversements biotiques sont autant d'empreintes anthropiques dès à présent observables (Vitousek *et al*., 1997a ; Corcoran *et al*., 2014).

Bien que l'interaction de l'Homme avec son environnement soit complexe, les activités humaines semblent bien engendrer depuis quelques années une perte massive de la biodiversité, identifiable comme la sixième grande crise biologique (Barnosky *et al*., 2011). L'anthropisation des paysages urbains et agricoles fragmente l'habitat des espèces sauvages et agit conjointement avec la domestication par l'Homme pour appauvrir et homogénéiser la biodiversité globale (McKinney & Lockwood, 1999 ; Diamond, 2002). Un rapport publié en 2014 par le Fond Mondial pour la Nature (World Wildlife Fund), en association avec la Société Zoologique de Londres, estime qu'entre 1970 et 2010 les effectifs des espèces sauvages auraient décliné d'environ 52% (McRae *et al*., 2014). Des phénomènes récents causés par la massification des échanges internationaux qui propagent les pathogènes et les espèces non-natives à travers le monde, amplifient cette perte d'endémisme (Olden *et al*., 2004 ; Olden, 2006). L'urgence de la mise en place de mesures de protection et de conservation de la biodiversité est aujourd'hui très largement admise et leurs coûts financiers pourraient s'avérer extrêmement importants (McCarthy *et al*., 2012).

Cette thèse financée par la Région Ile-de-France dans le cadre d'un domaine d'intérêt majeur (DIM) du réseau de recherche sur le développement soutenable (R2DS), vise à étudier la biologie d'une espèce invasive en France, le frelon *Vespa velutina*. Dans une approche fondamentale, nous avons cherché à acquérir une meilleure connaissance du système nerveux et du comportement de cette espèce, afin de trouver des solutions permettant de lutter durablement contre cet insecte qui pourrait avoir un impact très négatif sur l'apiculture et nos écosystèmes.

INTRODUCTION

I. Les invasions biologiques

1) Définition

Sous l'effet d'une mondialisation croissante et du développement des flux internationaux de personnes et de marchandises, le phénomène d'invasion biologique est en constante augmentation depuis le début du XXe siècle (Elton, 1958 ; Vitousek *et al*., 1996 ; Vitousek *et al*., 1997b ; van der Velde *et al*., 2006). Une **invasion biologique** survient lorsqu'une espèce exotique est implantée dans une zone géographique située en dehors de son aire de répartition naturelle. L'introduction peut se faire de manière accidentelle ou volontaire, mais elle est toujours dépendante d'une intervention humaine et peut potentiellement engendrer des perturbations environnementales, économiques, et/ou de santé humaine. Ainsi le phénomène d'invasion biologique se distingue de l'évolution d'une aire de répartition, car cette dernière s'effectue par l'intermédiaire d'un vecteur naturel de dispersion, au cours d'une période de temps plus longue.

Au sens strict, une espèce est considérée comme invasive lorsque l'introduction se traduit par une explosion démographique de cette espèce dans son nouvel écosystème et par une tendance à se disperser à partir de ce point d'introduction (Allendorf & Lundquist, 2003). Il est fréquemment observé que ces espèces engendrent une perturbation de la biodiversité en entrant en compétition avec les espèces autochtones pour l'habitat et/ou les ressources alimentaires ou en exerçant une prédation de ces espèces locales (van der Velde *et al*., 2006). Les invasions biologiques sont ainsi considérées par l'Union Internationale pour la Conservation de la Nature (UICN), comme la deuxième cause de perte de biodiversité dans le monde, après la dégradation des milieux (pollution, fragmentation des habitats, etc.) (Walker & Steffen, 1997 ; Mooney & Hobbs, 2000 ; Park 2004 ; Clavero & Garcia-Berthou, 2005). Les problèmes écologiques et économiques qui y sont liés font de ces invasions une préoccupation majeure dans le monde entier (Park 2004 ; Pimentel *et al*., 2005). Par conséquent, des méthodes de lutte visant à contrôler et à gérer les populations invasives sont activement recherchées.

2) Processus

Le processus d'invasion biologique est habituellement décrit en trois étapes (Kolar & Lodge, 2001 ; Allendorf & Lundquist, 2003 ; Colautti & MacIsaac, 2004): **l'introduction**, **l'établissement** et la **propagation** (**Fig. 1**). A chaque étape, les caractéristiques de l'écosystème d'accueil et de l'organisme invasif définissent le succès de ce dernier et son passage à l'étape suivante (Williamson & Fitter, 1996a). Les espèces introduites ne deviennent donc pas toutes envahissantes. Seulement une espèce introduite sur dix parvient à s'établir dans son nouvel habitat, et seulement une espèce établie sur dix devient invasive (Williamson & Fitter, 1996b). Lors de ce processus, l'écosystème est perturbé par

l'invasion de la nouvelle espèce et l'éventuelle disparition d'espèces autochtones. Une action de contrôle visant à empêcher l'invasion ou à limiter son impact est envisageable lors de chacune des trois étapes du processus.

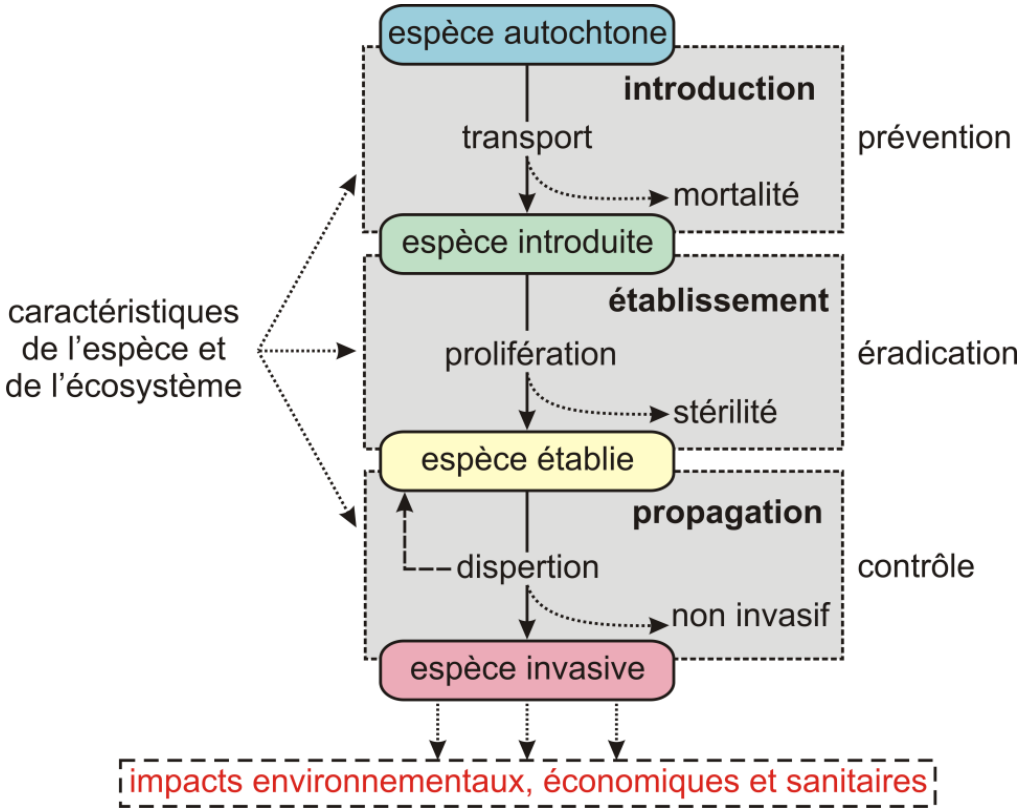


Figure 1 : Etapes du processus d'invasion biologique
A chaque étape du processus, les caractéristiques de l'écosystème et de l'organisme introduit peuvent favoriser ou entraver la réussite de l'invasion. Différentes actions de lutte (prévention, éradication, contrôle) peuvent être envisagées pour endiguer l'invasion. (Adapté d'Allendorf & Lundquist, 2003 et de Kolar & Lodge, 2001).

- **L'introduction** correspond à un apport d'individus, dans un nouveau milieu situé hors de son aire d'origine. C'est lors de cette étape que l'intervention de l'homme, volontaire ou accidentelle, est fondamentale, puisqu'elle permet à l'espèce de franchir les barrières géographiques. Un des moyens de lutte possible contre la multiplication des invasions biologiques serait d'anticiper toute future introduction grâce à des programmes de prévention visant par exemple à informer les populations sur les risques d'introduction et à intensifier les contrôles aux frontières (IUCN 2000 ; Genovesi, 2005).
- **L'établissement** nécessite que les individus introduits atteignent le stade de population viable sans apport de nouveaux individus. Pour cela, l'espèce introduite doit être capable, au sein de son nouvel environnement, de se reproduire et de produire des descendants sur le long terme. Les espèces généralistes et à fécondité élevée, ou présentant des soins parentaux, semblent s'établir plus facilement (Cassey *et al.*, 2004). A ce stade, il est possible de stopper l'invasion en

éradiquant l'espèce ou en interférant avec les individus reproducteurs (Courchamp *et al.*, 2003 ; Myers *et al.*, 2000).

- **La propagation** est le dernier stade de l'invasion biologique, au cours duquel les nouvelles populations viables se dispersent et colonisent de nouveaux territoires. Ainsi l'aire géographique envahie s'étend jusqu'à ce qu'elles rencontrent de nouveaux obstacles environnementaux (biotiques ou abiotiques). A ce stade, l'éradication de l'espèce s'avère plus compliquée à mettre en œuvre mais des moyens de lutte visant à limiter sa démographie peuvent être appliqués.

Ainsi, pour qu'une espèce introduite devienne invasive, celle-ci doit passer sans encombre par toutes les étapes du processus. La probabilité de succès à chaque étape dépend principalement des interactions de l'espèce introduite avec son nouvel environnement. La similarité des climats entre les aires d'origine et d'introduction, en adéquation avec l'adaptabilité de l'espèce, peut favoriser sa survie. La disponibilité d'une niche écologique au sein du nouvel écosystème et une position élevée de l'espèce dans la chaîne trophique (absence de prédateur), favorisent sa prolifération, alors que les facteurs abiotiques et la capacité de prolifération de l'espèce vont favoriser sa dispersion. Les espèces robustes, généralistes et à forte prolifération font donc d'excellents candidats pour la réussite d'une invasion. La **pression de propagule,** qui prend en compte à la fois le nombre d'événements d'introduction et les effectifs introduits, peut également influencer le succès invasif (Williamson, 2006). Ces facteurs agissent notamment sur la diversité génétique des populations invasives. En effet, les individus introduits portent seulement une petite portion de la diversité génétique des populations d'origine (Lockwood *et al.*, 2005). Il résulte de ce goulot d'étranglement génétique que les populations invasives ont souvent un fort taux de consanguinité, pouvant diminuer leurs capacités d'adaptation à leur nouvel environnement.

3) Impacts

L'impact direct des invasions biologiques sur l'environnement varie fortement en fonction des espèces et des biotopes concernés. En théorie, certaines espèces peuvent occuper des niches écologiques vacantes et ainsi avoir un impact relativement faible sur les écosystèmes envahis. A contrario, les espèces invasives peuvent aussi provoquer des perturbations à des échelles très variées, allant du génome à l'écosystème (Strayer *et al.*, 2006). Par conséquent, l'environnement et le comportement des espèces natives sont susceptibles d'être modifiés jusqu'à la réorganisation complète des réseaux trophiques (Mooney & Cleland, 2001). Cependant, les interactions interspécifiques au sein des écosystèmes sont souvent difficiles à établir car elles passent simultanément par plusieurs processus tels que la **prédation**, la **compétition**, **l'hybridation** et le **parasitisme**.

- L'introduction d'un nouveau **prédateur** dans un écosystème peut engendrer un déplacement et une réduction importante des communautés subissant la pression de prédation. Par exemple, l'introduction de la truite *Salmo trutta* en Nouvelle-Zélande a directement perturbé les populations endémiques de petits poissons d'eau douce, tel que les Galaxiidae (Townsend & Crowl, 1991). Cette prédation à eu également des conséquences indirectes en provoquant l'accroissement des populations d'algues dont les Galaxiidés se nourrissent (Townsend, 1996). Lorsque la pression de prédation est très importante, elle entraîne parfois l'extinction des espèces natives. Ainsi la perche du Nil, *Lates niloticus,* introduite dans le lac Victoria à la fin des années 1950 pour la pêche sportive, a provoqué l'extinction de plus de 50% des 600 espèces endémiques (Kaufman, 1992 ; Schofield & Chapman, 1999).
- L'introduction d'un **compétiteur** peut avoir un impact similaire sur la biodiversité en monopolisant les ressources de l'écosystème envahi. A titre d'exemple, le tapis dense de la Jacinthe d'eau *Eichhornia crassipes* maintes fois introduite comme plante ornementale, empêche la pénétration de la lumière et diminue la photosynthèse des autres espèces végétales aquatiques, comme le phytoplancton qui est à la base des chaînes alimentaires. De plus la décomposition des feuilles mortes de cette plante rend le milieu anoxique, privant les autres espèces d'oxygène et entraînant leur raréfaction dans les territoires envahis (Villamagna & Murphy, 2010).
- Les invasions biologiques peuvent aussi être à l'origine d'**hybridations** entre espèces natives et introduites. Dans certains cas des hybrides fertiles présentant une forte prolifération et une bonne tolérance aux conditions locales apparaissent. Ils peuvent alors entrer en compétition avec les espèces natives, et finir par les remplacer. L'hybridation peut aussi engendrer une pollution génétique qui diminue l'adaptabilité ou la valeur sélective (fitness) de l'espèce indigène et peut menacer son intégrité (introgression) (Rhymer & Simberloff, 1996).
- Enfin, les populations introduites peuvent être **vecteurs de maladies et de parasites,** jusque là absents du milieu, et qui vont infecter les populations locales. Ainsi, l'acarien *Varroa destructor,* parasite de l'abeille *Apis cerana*, était initialement confiné en Asie. A la suite d'un changement d'hôte vers l'abeille européenne *Apis mellifera* et d'exportations de colonies infectées, ce parasite s'est dispersé à travers le monde. Il est actuellement présent sur la quasi-totalité de l'aire de distribution d'*Apis mellifera* et est considéré comme la principale menace pour l'apiculture (Rosenkranz *et al*., 2010 ; Nazzi & Le Conte, 2016).

L'ensemble de ces processus participe à une modification profonde et souvent irréversible de l'environnement et du fonctionnement des écosystèmes dont les activités humaines dépendent. De cette façon, les invasions biologiques perturbent l'agriculture, la pêche, le transport et le tourisme, ce qui se répercute finalement sur l'économie. Le coût des pertes économiques engendrées par les

espèces invasives a été estimé à environ 120 milliards de dollars par an aux Etats-Unis (Pimentel *et al.*, 2005). D'autre part, l'introduction d'espèces peut avoir des conséquences sanitaires importantes. Au Moyen-âge, l'introduction du rat noir, vecteur de parasites, fut responsable de la diffusion du basile de la peste parmi les populations européennes (pandémie de peste noire du XIV siècle). Plus récemment, l'arrivée du moustique tigre *Stegomyia albopicta* en Europe inquiète les autorités sanitaires en raison des maladies qu'il est susceptible de propager comme le chikungunya ou la dengue (Enserink, 2007).

Cependant, les introductions d'espèces n'aboutissent pas obligatoirement à des nuisances. La réintroduction du loup gris dans le parc national de Yellowstone (USA) a induit, grâce à la prédation qu'il exerce sur les autres espèces, une cascade trophique bénéfique à la biodiversité (Ripple & Beschta, 2012). Les cervidés subissant la prédation ont évité les gorges et les vallées où ils pouvaient facilement être piégés (Mao *et al.*, 2005). La végétation de ces lieux s'est alors régénérée permettant l'installation d'oiseaux et de petits mammifères (Ripple & Beschta, 2003). Parmi ces mammifères, les castors ont proliféré et créé des barrages qui ont fourni un habitat adapté à d'autres espèces aquatiques (Marshall *et al.*, 2013). Par ailleurs, de nombreuses espèces invasives sont quotidiennement exploitées dans l'agriculture, par exemple comme auxiliaires des cultures, ou dans la pêche. La Perche du Nil introduite dans le lac Victoria est aujourd'hui intensivement exploitée comme ressource alimentaire exportée vers l'Europe. Les notions de nuisance et de bénéfice sont donc subjectives et les considérations écologiques et économiques peuvent entrer en conflit.

4) Méthodes de lutte

Pour limiter l'impact des espèces invasives, plusieurs stratégies sont envisageables et leurs applications nécessitent de prendre en compte les caractéristiques biologiques et écologiques des espèces concernées. Lorsqu'une espèce invasive est introduite, sa détection précoce et une intervention rapide augmentent les chances de succès des mesures de contrôle mises en place (Myers *et al.*, 2000 ; Byers *et al.*, 2002). La régulation des effectifs d'une espèce invasive jusqu'à son extermination peut se faire suivant différentes approches : **mécanique**, **chimique** ou **biologique**.

- Le contrôle **mécanique** d'une espèce végétale (arrachage) ou animale (piégeage, chasse) est une solution coûteuse car elle nécessite une main d'œuvre importante. Cette méthode est efficace pour éradiquer l'espèce lorsque celle-ci est encore au stade de l'établissement et que l'écosystème est peu envahi. A partir de la phase de propagation, cette méthode s'avère moins efficace, bien qu'elle puisse tout de même représenter une solution pour contrôler localement l'effectif des populations invasives et limiter leur impact.
- La lutte **chimique** comme l'utilisation d'herbicides et de pesticides, représente une solution plus globale grâce à la possibilité d'effectuer des épandages sur de grandes surfaces. Cependant, ces

traitements manquent généralement de spécificité et leur importante toxicité peut aussi détériorer l'environnement, et notamment la qualité de l'eau. Par exemple, les insecticides utilisés pour lutter contre les moustiques affectent aussi les oiseaux, les abeilles et les invertébrés aquatiques (Rose, 2001). De plus, cette solution ne donne parfois que des résultats limités et/ou temporaires du fait de l'apparition de résistances chez l'espèce ciblée (Hemingway & Ranson, 2000 ; Rose, 2001).

- La lutte **biologique** consiste à introduire un pathogène ou un prédateur afin de réguler l'abondance des populations invasives. Cette méthode permet de retrouver un équilibre des réseaux trophiques mais nécessite des précautions sur le choix de l'agent de contrôle. Un agent de contrôle peu spécifique ou à forte capacité de prolifération peut perturber la diversité locale et devenir invasif à son tour (Howarth, 1991). Ainsi, le gastéropode carnivore *Euglandina roseai,* introduit dans les îles du Pacifique pour lutter contre l'escargot africain *Achatina fulcia,* est responsable de l'éradication d'un grand nombre de mollusques endémiques à ces îles (Simberloff & Stiling, 1996 ; Henneman & Memmott, 2001).

Toutes ces méthodes peuvent être utilisées dans une approche intégrative visant à exploiter leur complémentarité et à optimiser leur usage en fonction du contexte. Cependant, leur application nécessite une bonne connaissance de l'espèce ciblée afin d'en optimiser l'efficacité et la spécificité. Puisque les espèces envahissantes ne sont généralement pas à l'origine de perturbations dans leur milieu d'origine, il est rare que celles-ci aient fait l'objet d'investigations approfondies. Il est donc parfois préférable de retarder la mise en place des mesures de lutte afin de pouvoir élaborer une stratégie efficace. Il est également important de prendre en compte les facteurs biotiques et abiotiques des écosystèmes envahis pour limiter les altérations de l'environnement que peuvent engendrer ces différentes mesures de lutte (Buckley, 2008). Dans cette thèse nous avons donc cherché à acquérir des connaissances sur la biologie d'une nouvelle espèce invasive en Europe, le frelon à pattes jaunes *Vespa velutina,* afin de pouvoir proposer des solutions de lutte qui ne perturberaient pas les écosystèmes locaux.

II. L'invasion de *Vespa velutina*

1) Les insectes sociaux invasifs

De par leur petite taille, les insectes peuvent aisément être transportés d'un point à un autre par inadvertance. Par exemple, aux Etats-Unis, un grand nombre d'insectes exogènes, principalement dissimulés dans des fruits, ont été interceptés dans les bagages de passagers des lignes aériennes (Liebhold *et al*., 2006 ; Beggs *et al*., 2011). De plus, du fait de leur rôle central dans les réseaux trophiques, certains insectes ont été volontairement introduits comme agents de contrôle biologique. Par exemple, les parasitoïdes peuvent être utilisés pour lutter contre les ravageurs de cultures (Howarth, 1991). Ainsi, le programme DAISIE (Delivering Alien Invasive Species Inventory for Europe) référençait en 2009 plus de 1300 espèces d'insectes invasifs en Europe (Roques *et al*., 2009).

Des aspects intrinsèques au comportement des insectes peuvent également faciliter l'invasion et accentuer son impact, notamment les différentes formes de coopération intraspécifique qui rendent l'espèce plus compétitive (Holway & Suarez, 1999). Une haute organisation sociale représente donc un avantage pour la dominance écologique (Wilson, 1990 ; Chapman & Bourke, 2001). L'**eusocialité** est le niveau le plus élevé d'organisation sociale, essentiellement présente dans deux ordres d'insectes, les Hyménoptères (abeilles, fourmis et guêpes) et les Isoptères (termites), et qui est définie par 3 critères : une coopération des soins aux jeunes, un chevauchement des générations et une division du travail reproducteur. Les insectes sociaux vivent en colonies populeuses, organisées suivant un système élaboré d'attribution des tâches, ce qui leur confère la capacité d'exploiter efficacement les ressources de l'environnement (Wilson & Hölldobler, 2005). Ils bénéficient en outre de réponses de groupe offrant une défense efficace contre les prédateurs et rendant la perte de quelques individus peu néfaste pour la colonie. Enfin, chaque colonie est susceptible de produire de nombreux individus reproducteurs favorisant la dispersion de l'espèce (Moller, 1996 ; Chapman & Bourke, 2001). Les insectes sociaux figurent ainsi parmi les animaux les plus invasifs, causant souvent d'importantes perturbations dans les écosystèmes envahis. Les fourmis illustrent bien ce propos puisque 140 espèces ont été identifiées à travers le monde comme étant en dehors de leur habitat d'origine (McGlynn, 1999). La fourmi d'Argentine, *Linepithema humile,* aujourd'hui présente sur les 6 continents, possède un comportement social contribuant à un potentiel dispersif extrêmement important (Suarez *et al*., 2001 ; Holway & Case, 2000). Le goulot d'étranglement génétique inhérent aux invasions biologiques, à cause du faible nombre d'individus introduits, semble avoir favorisé l'instauration d'une organisation unicoloniale dans les zones envahies (Suarez *et al*., 1999 ; Tsutsui *et al*., 2001 ; Pedersen *et al*., 2006). Cette organisation unicoloniale, définie comme l'absence d'agressivité intraspécifique et de frontière coloniale, favorise

l'exploitation efficace des ressources et la prolifération de l'espèce grâce aux échanges d'ouvrières et de reines fertiles entre nids. En Europe, la fourmi d'Argentine forme ainsi deux supercolonies polygynes qui s'étendent de l'Espagne à l'Italie, en passant par la France, et qui causent d'importants dégâts environnementaux (Giraud *et al.*, 2002).

Ainsi, un niveau élevé d'organisation sociale confère des avantages pour la conquête de nouvelles niches écologiques puisqu'il apporte une flexibilité propre au mode de reproduction et permet d'être compétitif quant à l'exploitation des ressources alimentaires (Chapman & Bourke, 2001). Parmi les insectes sociaux, les Vespidés possèdent un fort potentiel de nuisance lorsqu'ils sont introduits dans un nouvel environnement. En effet, le plus souvent ce sont des prédateurs généralistes pouvant fortement perturber l'entomofaune des territoires envahis (Snyder & Evans, 2006). Nous allons donc nous intéresser plus spécifiquement à cette famille d'insecte qui, dans le contexte d'une invasion biologique, peut déstabiliser les écosystèmes.

2) Les Vespidae

a. Taxonomie

La famille des Vespidae regroupe plus de 5000 espèces de guêpes (ordre : Hyménoptères), possédant un aiguillon terminal (infra-ordre : Aculéates). Celles-ci sont distribuées dans 6 sous-familles affichant un large éventail de comportements sociaux, de solitaire à eusocial (**Fig. 2**), ce qui en fait un groupe de choix pour l'étude de l'évolution de la socialité (Jeanne, 1980 ; Hunt, 2007 ; Pickett & Carpenter, 2010).

- Les **Euparagiinae** sont des guêpes solitaires qui ne représentent actuellement que 10 espèces connues. Elles sont présentes uniquement dans des régions arides d'Amérique centrale et du Nord.
- Les **Masarinae** regroupent 344 espèces de guêpes solitaires qui nourrissent leurs larves de pollen et de nectar. Cette alimentation les distingue des autres Vespidae qui sont généralement carnivores.
- Les **Eumeninae** désignent 3579 espèces de guêpes au comportement solitaire et présocial. Le plus souvent, elles construisent des nids de terre ou de matière végétale mâchée.
- Les **Stenogastrinae** rassemblent 58 espèces de guêpes tropicales montrant différents degrés de socialité (eusociaux facultatifs). Elles nichent dans des nids en papier mâché qu'elles construisent à des emplacements abrités.
- Les **Polistinae** présentent 958 espèces de guêpes eusociales qui bâtissent des nids en papier mâché sans enveloppe externe.
- Enfin, les **Vespinae** regroupent 69 espèces de guêpes eusociales possédant de vastes aires de répartition. On compte parmi celles-ci, les guêpes au sens strict, présentes en zone tempérée de

l'Hémisphère Nord (les genres *Dolichovespula* et *Vespula*), 3 espèces nocturnes tropicales (*Provespa*) endémiques du Sud-Est asiatique et 22 espèces de frelons du genre *Vespa*.

Le genre ***Vespa*** (frelons) comprend des espèces de grande taille qui affichent une large diversité de colorations. Le Sud-Est asiatique semble être leur centre de diversification car 19 des 22 espèces recensées y sont présentes et s'étendent des zones tempérées aux zones tropicales (Carpenter & Kojima, 1997). Seules deux espèces se sont naturellement dispersées en dehors de l'Asie : *Vespa crabro* en Europe, et *Vespa orientalis* au Moyen-Orient et autour de la Méditerranée (Afrique et Europe) (Matsuura & Yamane, 1990 ; Carpenter & Kojima, 1997).

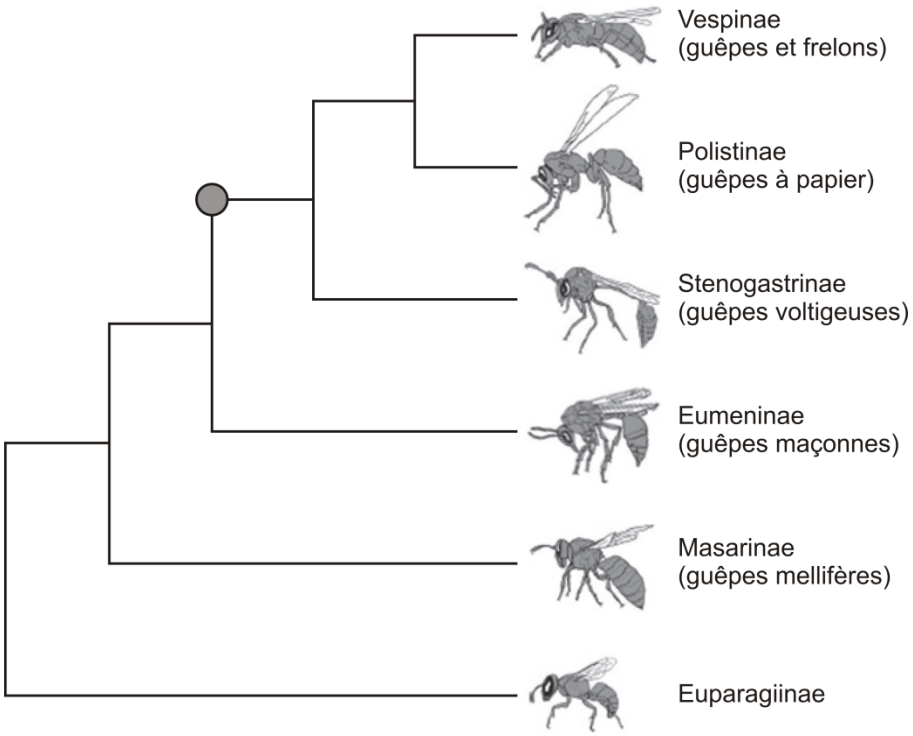


Figure 2 : Phylogénie des Vespidae
Arbre phylogénétique basé sur des caractères morphologiques et comportementaux des 6 sous-familles de Vespidae. Le rond gris indique une possible apparition de la socialité chez un ancêtre commun aux Vespinae, Polistinae et Stenogastrinae. (D'après Pickett & Carpenter, 2010 et Bruschini *et al*., 2010).

b. Les invasions biologiques

A travers le monde, 34 espèces de Vespidés ont été recensées comme étant volontairement ou accidentellement introduites. Parmi celles-ci, 23 (soit 68%) sont eusociales alors que les guêpes eusociales ne représentent que 20% des Vespidés. De plus, tous les Vespidés considérés comme véritablement invasifs, c'est-à-dire ayant franchis les étapes de l'introduction et de l'établissement, sont eusociaux (Beggs *et al*., 2011). Bien que l'eusocialité semble favoriser le potentiel invasif de ces espèces, une majorité d'entre elles bénéficie également d'un comportement de prédation opportuniste et généraliste qui facilite leur intégration dans les réseaux trophiques (Snyder & Evans, 2006). L'introduction des Vespidés peut donc affecter de nombreuses communautés d'insecte, tels

que les phytophages, les carnivores, les parasitoïdes et les pollinisateurs puisque ces prédateurs généralistes ont de multiples connections au sein de la chaîne alimentaire. Par exemple *Vespula vulgaris* et son espèce sœur, *Vespula germanica* (**Fig. 3, A** et **B**), sont toutes deux invasives en Nouvelle-Zélande et causent d'importants dégâts à l'entomofaune locale (Clapperton *et al.*, 1989). Au même titre que la fourmi d'Argentine, *Linepithema humile,* le succès invasif de ces guêpes pourrait être dû à un changement de structure sociale entre l'aire d'origine, où les colonies sont monogynes et annuelles, et l'aire d'introduction où des colonies polygynes et pérennes ont été observées (Plunkett *et al.*, 1989 ; Donovan *et al.*, 1992).

La guêpe *V. germanica* est invasive dans de nombreux territoires (Nouvelle-Zélande, Australie, Tasmanie, Afrique du Sud, île de l'Ascension, Etats-Unis, Canada, Chili et Argentine) et a donné lieu à de nombreuses études. En Nouvelle-Zélande, *V. germanica* a connu une expansion très rapide, et semble parfaitement adaptée aux écosystèmes locaux. En revanche, les populations invasives de Patagonie semblent souffrir d'une compétition beaucoup plus rude avec les espèces indigènes, dont les espèces locales de fourmis généralistes (Masciocchi *et al.*, 2010). Ainsi, en dépit d'un régime alimentaire globalement similaire dans les deux aires envahies, les populations invasives d'Amérique du Sud sont maintenues à de faibles densités et ne représentent pas une menace majeure pour les espèces locales (Sackmann *et al.*, 2000 ; Sackmann *et al.*, 2008).

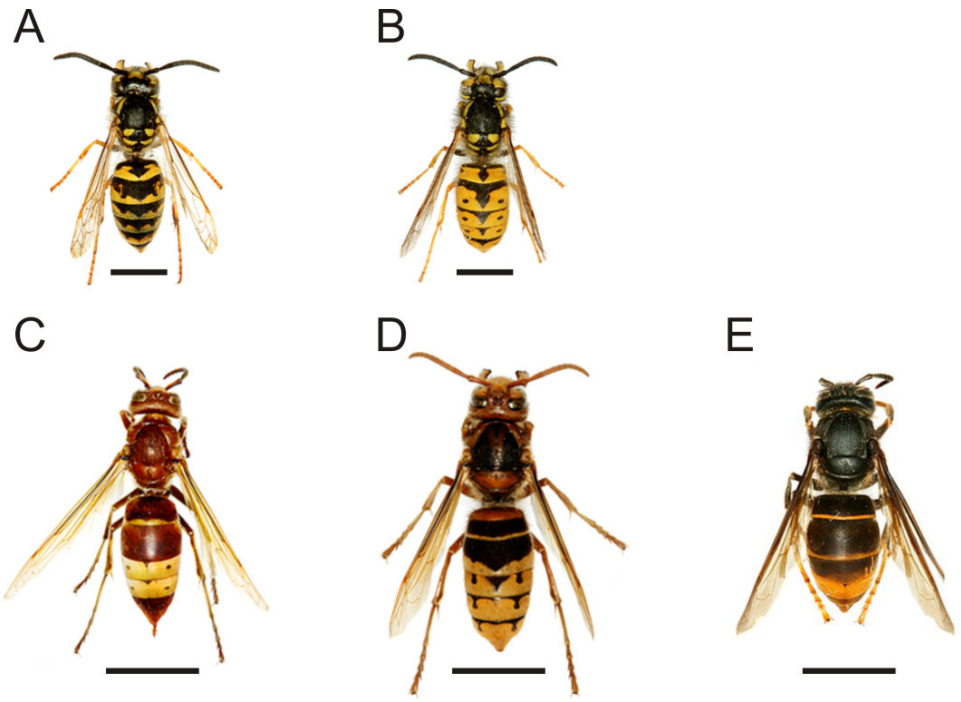


Figure 3 : Ouvrières de différentes espèces de la sous-famille des Vespinae
A) *Vespula vulgaris*. **B**) *Vespula germanica*. **C**) *Vespa orientalis*. **D**) *Vespa crabro*. **E**) *Vespa velutina*. Les Vespinae européens à coloration majoritairement jaune-orangée (A, B, C et D) se distinguent facilement du frelon *V. velutina* à coloration majoritairement noire. Les barres d'échelle en A et B représentent environ 0.5 cm, alors que celles en C, D et E représentent environ 1 cm (Photographies de Josef Dvořák : http://www.biolib.cz).

Parmi les différentes espèces de frelons introduites en dehors de leur aire de distribution naturelle, peu d'entre elles ont réussi à s'établir (**Fig. 4**). Le frelon oriental, *Vespa orientalis* (**Fig. 3, C**), a été accidentellement introduit en Belgique et au Royaume-Uni dans des caisses de fruits en provenance de Chypre, sans pour autant devenir invasif (Edwards, 1982). Il a aussi été observé au Mexique, à Madagascar et en Chine sans que l'acclimatation de l'espèce ne soit confirmée (Dvořák, 2006). *Vespa simillima*, espèce originaire d'Asie orientale, a été identifiée au Canada mais ne s'y est jamais établie (Carpenter & Kojima, 1997). Toutefois, Le frelon européen *V. crabro* (**Fig. 3, D**), introduit aux Etats-Unis (**Fig. 4**) dans le cadre d'une lutte biologique contre des chenilles défoliatrices, y est maintenant bien implanté (Shaw & Weidhaas, 1956). A ce jour, la seule espèce de frelon à s'être implantée avec succès dans plusieurs zones géographiques différentes est le frelon, *Vespa velutina* (**Fig. 3, E**), invasif en Europe et en Corée (**Fig. 4**). Dans cette thèse, nous allons nous concentrer sur l'étude de la biologie de ce frelon qui semble avoir un fort caractère invasif et dont l'introduction peut engendrer énormément de nuisances.

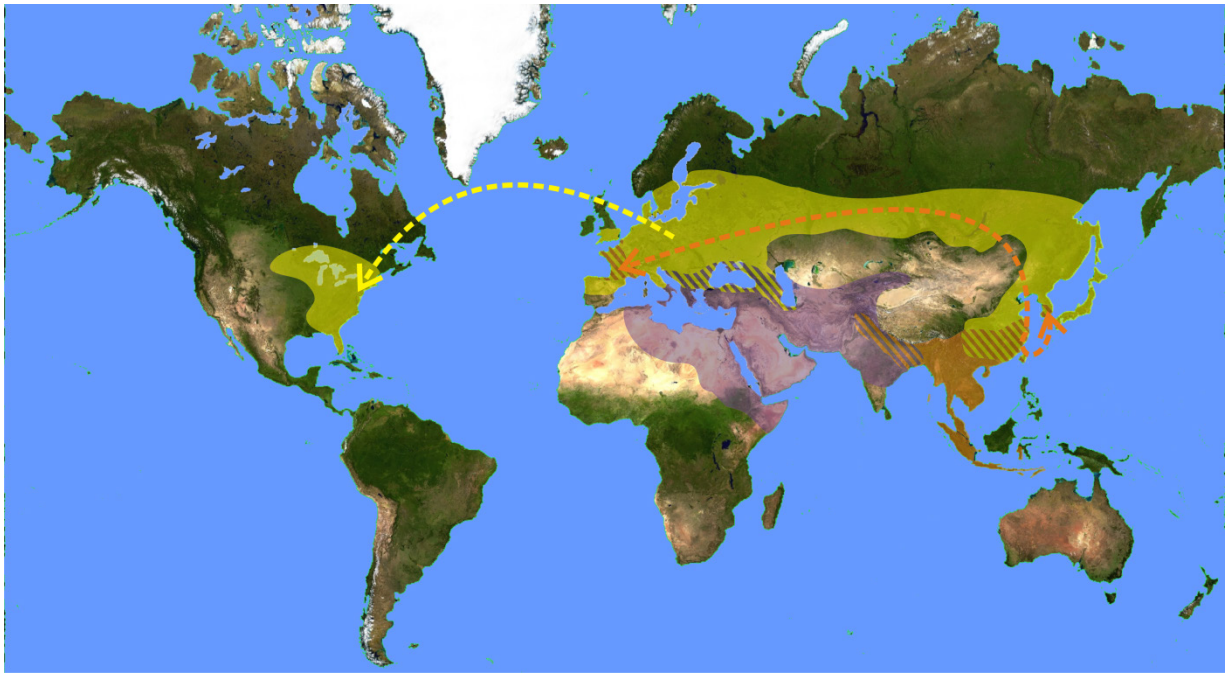


Figure 4 : Répartition mondiale des 3 espèces de frelon présentes en Europe
Les frelons *V. crabro* (jaune) et *V. orientalis* (mauve) sont naturellement présents en Europe. Le frelon asiatique *V. velutina* (orange), originaire du Sud-Est asiatique à été introduit dans plusieurs zones géographiques. Les parties hachurées correspondent à un chevauchement des aires de répartition de ces espèces. Les flèches montrent les introductions ayant abouti à des invasions biologiques.

3) Le frelon à pattes jaunes, *Vespa velutina*

a. Biologie du frelon

Vespa velutina est un frelon dont l'aire de répartition naturelle se situe dans les régions tropicales et subtropicales d'Asie du Sud-Est. Il s'étend du Népal et du Nord de l'Inde jusqu'à l'Est de la Chine, la péninsule Indochinoise et l'archipel Indonésien (**Fig. 4**). Les individus introduits en Europe

semblent appartenir à une sous-population (lignée), appelée *Vespa velutina nigrithorax* provenant de l'Est de la Chine (Arca *et al.*, 2015). La lignée nigrithorax a effectivement une coloration majoritairement noire en face dorsale, mais elle est aussi connue sous le nom de « frelon à pattes jaunes » car le quatrième segment de l'abdomen et les extrémités des pattes sont entièrement jaunes (Perrard *et al.*, 2014). De par sa livrée majoritairement foncée, ce frelon se distingue facilement des Vespinae Européens dont la coloration est majoritairement jaune (**Fig. 3, E**).

Avant son introduction, très peu d'études se sont intéressées à la biologie et à l'écologie de ce frelon. Néanmoins, un certain nombre de connaissances ont été acquises sur d'autres espèces des genres *Vespa* et *Vespula* et semblent pouvoir être transposées à *V. velutina*. Charles Janet, inventeur du nid artificiel de fourmis (présenté à l'Exposition Universelle de Paris en 1900), et dont les travaux de chimie et d'histologie ont été redécouverts au début du XXIe siècle, fut le premier à s'intéresser au comportement du frelon (Stewart, 2010 ; Billen & Wilson, 2008). En 1895, il décrivait pour la première fois l'activité d'un nid de *V. crabro* depuis son origine jusqu'à son terme (**Fig. 5**) (Janet, 1895).

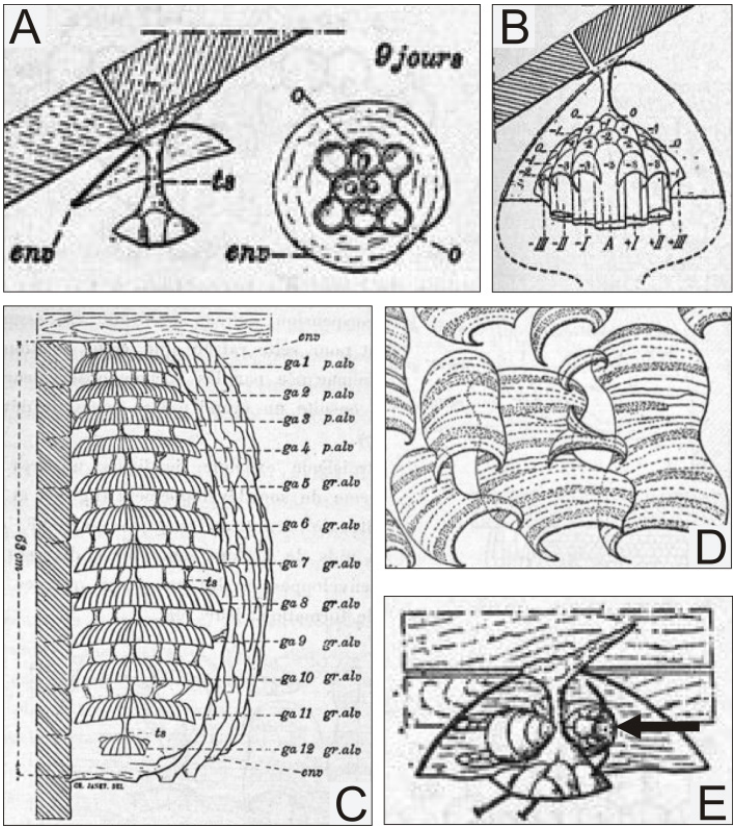


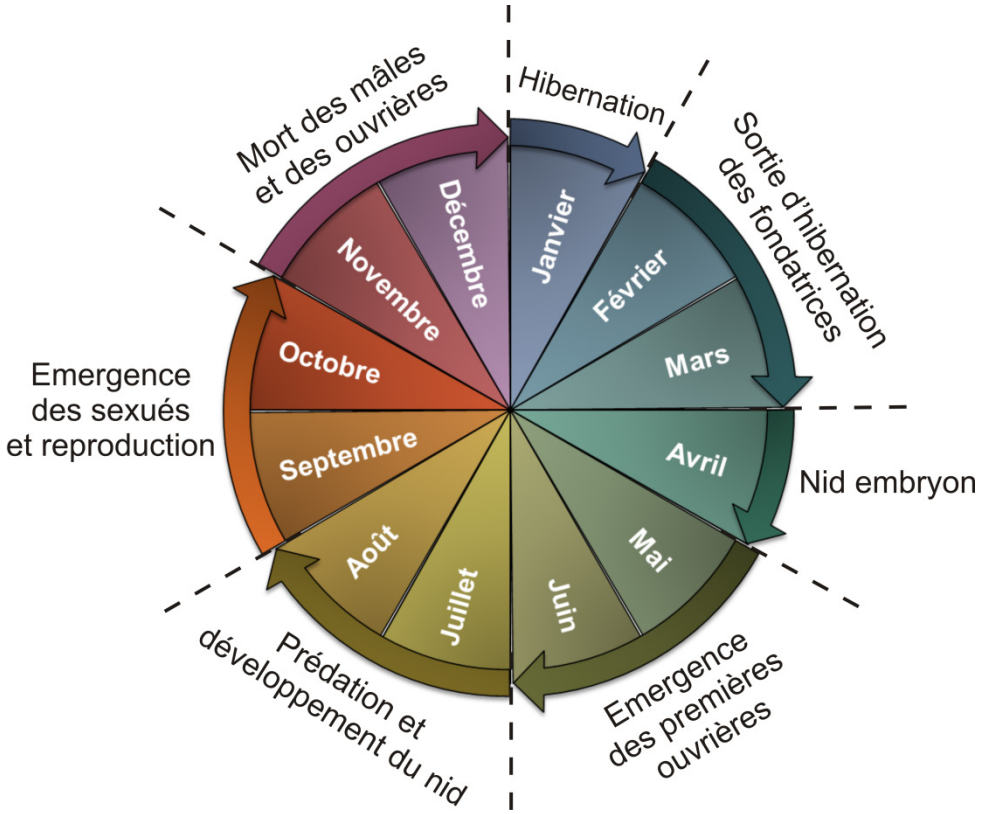
Figure 5 : Illustration de Charles Janet (1895) sur l'architecture d'un nid de frelon *V. crabro*
A) Nid embryon (9 jours) dans lequel la fondatrice pond ses premiers œufs (ts : tige de suspension ; env : enveloppe ; o : œuf). **B**) Etat du nid (55 jours) à la veille de l'éclosion de la première ouvrière. **C**) Etat du nid en fin de saison (ga : galette ; p.alv : petite alvéoles ; gr.alv : grandes alvéoles). **D**) Enchevêtrement de lames formant l'enveloppe externe du nid. **E**) Position de la fondatrice (flèche noire) régulant la température du couvain avant l'émergence des premières ouvrières.

Les nids sont bâtis à l'aide de matières ligneuses que les frelons récoltent et mâchent pour les transformer en pâte. Ils étalent alors cette pâte en couches successives de façon à former des galettes de cellules hexagonales orientées vers le bas, au sein desquelles se développent les larves. Ces galettes sont entourées d'un enchevêtrement de lames formant des sortes de boursouflures qui

s'accumulent les unes sur les autres et constituent une enveloppe protectrice favorable à la conservation de la chaleur (**Fig. 5, D**). Alors que certaines espèces de frelon bâtissent leur nid dans des cavités, il a été observé que *V. velutina* initie préférentiellement ses nids en haut des arbres (jusqu'à 20 m de hauteur) (Nakamura & Sonthichai, 2004), ce qui rend leur détection précoce extrêmement difficile à cause du feuillage présent à cette époque de l'année.

Chez les frelons, les femelles sont divisées en deux castes plus ou moins distinctes morphologiquement selon les espèces (Perrard *et al.*, 2012) : les femelles reproductrices (reines ou fondatrices) qui fondent les colonies et pondent les œufs, et les femelles stériles (ouvrières) qui participent à la construction du nid, à l'approvisionnement et à la défense de la colonie. Contrairement à la majorité des espèces de fourmis champignonnistes, aucun polyéthisme de caste, c'est-à-dire une division du travail basée sur des individus morphologiquement différenciés, n'a été observée au sein des colonies de Vespa sp. Cependant, les tâches pourraient être réparties entre les ouvrières selon un polyéthisme d'âge (Monceau *et al.*, 2013a) comme chez les abeilles du genre *Apis*. Les mâles, quant à eux, sont produits par la reine ou des ouvrières non-fécondées et ne participent pas au développement de la colonie mais attendent l'émergence des femelles reproductrices en vue de l'accouplement (Janet, 1895 ; Matsuura & Yamane, 1990 ; Monceau *et al.*, 2014).

Les colonies sont fondées et périclitent suivant un cycle annuel dont la durée varie en fonction du milieu (Edwards, 1980 ; Matsuura & Yamane, 1990). En milieu tropical, les colonies se développent tout au long de l'année de manière asynchrone d'une espèce à l'autre. En milieu tempéré, les colonies se développent selon un rythme saisonnier à la fin duquel seules les fondatrices survivent à l'hiver en hibernation (**Fig. 6**). Au printemps (entre Février et fin Mars), les fondatrices fécondées sortent d'hibernation. Chacune initie (jusqu'en Avril) un nid embryon de quelques alvéoles dans lesquelles elles pondent leurs premiers œufs (**Fig. 5, A**). Elles vont ensuite s'occuper du couvain en l'alimentant en proies collectées à l'extérieur et en régulant la température au sein du nid (**Fig. 5, E**). Lorsque les premières ouvrières émergent (entre Mai et Juin), la reine diminue progressivement ses activités hors du nid pour se consacrer exclusivement à la ponte. Les ouvrières se chargent alors d'alimenter le couvain, d'agrandir la structure du nid et de défendre la colonie. Pendant l'été (entre Juillet et Août), la taille du nid et le nombre d'ouvrières augmentent jusqu'à atteindre leur maximum en fin de saison (automne). C'est à ce moment (entre Septembre et Octobre) que la reine commence à pondre des œufs destinés à devenir des individus sexués (femelles fertiles et mâles). Toutefois, il a aussi été observé, en Europe, que des males diploïdes peuvent être produit avant l'automne (Darrouzet *et al.*, 2015). Chez *V. velutina,* chaque nid est susceptible de produire environ 300 futures fondatrices et 900 mâles (Rome *et al.*, 2015). Finalement, les nouvelles fondatrices vont s'accoupler et rejoindre un abri pour passer l'hiver avant de commencer un nouveau cycle au printemps suivant (**Fig. 6**). Avec le froid des périodes hivernales, l'ancienne colonie décline rapidement jusqu'à la mort de tous les individus.



<u>Figure 6</u> : Cycle de vie de *V. velutina* en milieu tempéré
Les mois sont donnés à titre indicatif et les différentes étapes peuvent être décalées d'une année à l'autre en fonction des températures. (D'après Monceau *et al.*, 2014).

b. Régime alimentaire et comportement de prédation

Pour survivre, les frelons ont besoin de s'alimenter en glucides et en protéines. Les ouvrières collectent les glucides de manière opportuniste aussi bien sur des ressources naturelles que sur des aliments de nature anthropogéniques (Spradbery, 1973). Ces glucides sont le plus souvent directement consommés par les ouvrières mais peuvent aussi être apportés au nid pour être distribués par trophallaxie aux autres adultes et au couvain de la colonie. Les Vespinae semblent pouvoir consommer toutes les sources de sucres disponibles dans l'environnement comme du nectar de fleur, de la sève, du miellat ou de la pulpe de fruit (Raveret Richter, 2000). Il a même été observé en Nouvelle-Zélande que la guêpe *Vespula vulgaris* est capable de s'alimenter en consommant l'élaïosome des graines (riche en lipides et protéines) de plantes myrmécophiles, participant de la sorte à leur dissémination (Jules, 1996). Les guêpes et les frelons peuvent aussi être attirés par les glaces et les boissons sucrées consommées par l'Homme, ce qui leur a valu une réputation d'insectes nuisibles (Spradbery, 1973).

Les protéines consommées par les frelons sont, quant à elles, majoritairement d'origine animale. Les chairs animales collectées sont transformées en boulettes alimentaires et rapportées au nid afin de nourrir les larves. Lorsqu'une ouvrière se présente avec une boulette alimentaire, la larve rejette la tête en arrière et dégorge un liquide riche en acides aminés dont l'ouvrière se nourrit

(Janet, 1895 ; Matsuura & Yamane, 1990). En vertu du fait que les frelons ne peuvent pas stocker de nourriture dans les alvéoles en papier mâché, ce liquide nutritif riche en acides aminés pourrait être indispensable à la survie des membres adultes de la colonie (Abe *et al*., 1991). Quelques observations laissent supposer que les Vespidae dont *Vespa velutina* sont également susceptibles de collecter des protéines d'origine végétale, comme du pollen (Hunt *et al*., 1991 ; Ueno, 2015). Dans la plupart des cas, les frelons sont des prédateurs et des charognards généralistes. Ils prélèvent dans l'environnement une large diversité d'insectes, jouant ainsi un rôle important dans la régulation des populations nuisibles pour l'agriculture et les forêts (Edwards 1980 ; Matsuura & Yamane 1990). Cependant, quelques espèces semblent être spécialisées dans la chasse d'un type de proie en particulier. Par exemple, *Vespa tropica* chasse presque exclusivement des guêpes polistes, en effectuant des raids sur leur nids (Matsuura, 1984).

Les ressources alimentaires disponibles, et la compétition existant pour les obtenir, changent en fonction du biotope et exercent une forte pression de sélection sur le comportement de prédation des frelons. Ainsi le frelon géant, *Vespa mandarinia,* qui cohabite souvent avec plus de 5 autres espèces de frelons, est capable d'adopter une stratégie de prédation très efficace dans laquelle il recrute ses congénères pour opérer à des attaques « *en masse* » sur les essaims d'autres Hyménoptères sociaux (Matsuura, 1984 ; Ono *et al*., 1995). Ce comportement n'a pour l'instant pas été observé chez d'autres espèces de frelons, dont certaines, comme le frelon européen *V. crabro,* semblent chasser de manière plus solitaire (Baracchi *et al*., 2010). En revanche, les ouvrières de la guêpe *Vespula germanica* font preuve d'un niveau de coopération intermédiaire, car celles-ci sont attirées par leurs conspécifiques sur les sources de nourritures, grâce à un processus de recrutement appelé accentuation locale, ou « *local enhancement* » en anglais (Overmyer & Jeanne, 1998 ; D'Adamo *et al*., 2000 ; D'Adamo *et al*., 2003 ; D'Adamo & Lozada, 2005). Les Vespinae affichent donc une diversité de comportements de prédation, des plus solitaires aux plus coopératifs.

Dans le cas de *V. velutina*, des enregistrements vidéos ont permis de constater que, au plus fort de la saison, jusqu'à 20 ouvrières peuvent chasser simultanément à l'entrée d'une même ruche d'abeilles (Monceau *et al*., 2013b). Elles attendent, en vol stationnaire, l'opportunité d'attraper une abeille dont elles vont découper les membres pour ne porter à la colonie que la partie la plus riche en protéines, le thorax (contenant les muscles alaires). Bien qu'il ne soit pas clairement établi que les ouvrières *V. velutina* coopèrent dans leur activité de prédation, des expériences de piégeage semblent mettre en évidence une attraction conspécifique comparable à ce qui a été observé chez *Vespula germanica* (O. Bonnard, communication personnelle). Ces comportements coopératifs de prédation constituent un avantage notable pour l'exploitation des ressources de l'environnement, et, dans le contexte d'une invasion biologique, amplifient la vulnérabilité des proies au sein des écosystèmes envahis (D'Adamo *et al*., 2004).

c. Historique de l'invasion

Le frelon asiatique *V. velutina* a été introduit et s'est établi avec succès en Corée (en 2003), au Japon (en 2012) et en Europe (Choi *et al*., 2012 ; Ueno, 2014 ; Arca *et al*., 2015). La première colonie européenne a été recensée en 2004 par un producteur de bonzaïs dans le Sud-Ouest de la France (Agen). L'analyse des séquences d'ADN microsatellites des populations invasives et des populations naturelles d'Asie a révélé qu'une seule reine fécondée, originaire de l'Est de la Chine, aurait été introduite en France (Arca *et al*., 2015). L'hypothèse la plus vraisemblable pour expliquer son arrivée impliquerait donc l'importation de poteries en provenance de Yixing, ville de la province de Jiangsu (Chine) où le producteur de bonzaïs s'approvisionnait. Une fondatrice aurait été accidentellement introduite dans les cartons de poteries et aurait été transportée en bateau pendant la période d'hivernation (Arca *et al*., 2011 ; Arca, 2012 ; Arca *et al*., 2015). Depuis son arrivée, le frelon asiatique s'est largement répandu et serait maintenant (début 2016) présent dans plus de 66 départements de France métropolitaine (http://inpn.mnhn.fr/) (**Fig. 7**).

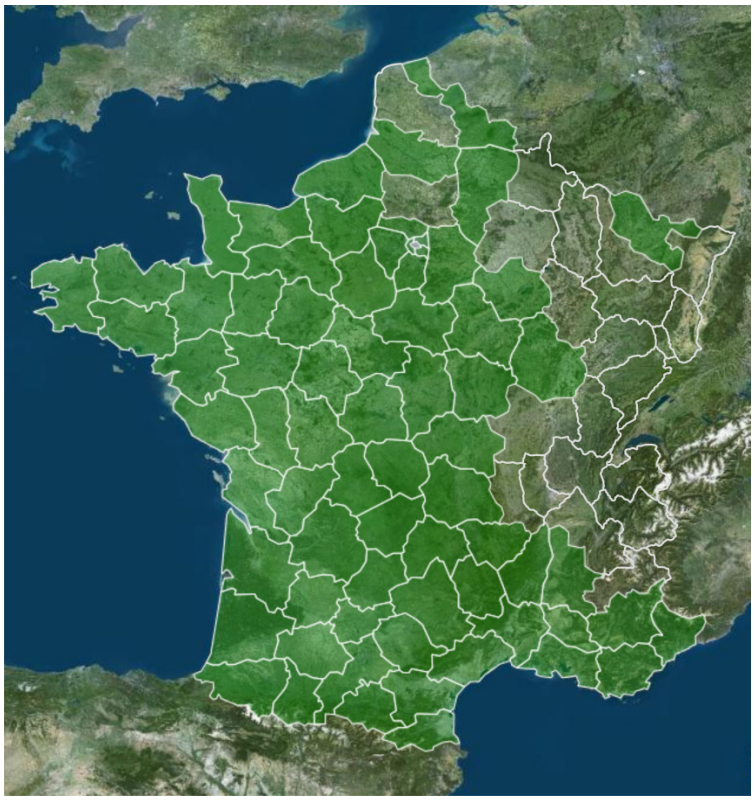


Figure 7 : Distribution de *V. velutina* en France métropolitaine (10/09/2015)
Cette carte représente en vert les départements dans lesquels au moins un individu *V. velutina* a été identifié. De 2004 à 2015 plus de la moitié du territoire français a été envahie. (Source : http://inpn.mnhn.fr/)

V. velutina a aussi été observé dans d'autres pays Ouest-Européens comme l'Espagne en 2010, le Portugal en 2011, la Belgique en 2011 et l'Italie en 2014 (López *et al*., 2011 ; Grosso-Silva & Maia, 2012 ; Bruneau, 2011 ; Demichelis *et al*., 2014). Des simulations basées sur des données bioclimatiques des aires d'origine et d'introduction prédisent que la propagation de *V. velutina* pourrait s'étendre vers l'Est, jusqu'en Grèce et en Roumanie et au Nord jusqu'aux pays baltes (Villemant *et al*., 2011 ; Barbet-Massin *et al*., 2013).

A l'heure actuelle, on ne connaît en France aucun antagoniste biologique susceptible de ralentir la progression du front d'invasion. Seul un cas de prédation du nid de *V. velutina* par un oiseau insectivore, la bondrée apivore *Pernis apivorus*, a été rapporté (Monceau *et al*., 2014). De même, seulement 2 nids dans lesquels les fondatrices étaient mortes à la suite de l'infestation par un parasitoïde Européen (*Conops vesicularis*), ont été enregistrés dans la région de Tours (Darrouzet *et al*., 2014). L'Europe représente donc un territoire propice à l'invasion du frelon asiatique car celui-ci semble bien adapté aux conditions climatiques et ne souffre pas d'antagonismes délétères à sa propagation.

d. Impact de l'invasion biologique

A la suite de son introduction en France, le frelon asiatique a rapidement défrayé la chronique du fait des multiples interractions qu'il peut avoir avec les activités humaines. En effet, *V. velutina* est capable d'initier des nids aussi bien en zone agricole qu'en zone urbaine, souvent à proximité des habitations où il peut représenter un risque pour l'homme. Bien que son venin ne soit pas plus toxique que celui d'autres Hyménoptères, plusieurs décès survenus en France à la suite de piqûres ont participé à l'instauration d'un climat de psychose. En réalité, il s'agit de personnes allergiques ayant fait une importante réaction anaphylactique mais le nombre de piqûres d'hymenoptères ne semble pas avoir augmenté significativement dans les départements envahis (de Haro *et al*., 2010). Ce prédateur et charognard s'est également fait remarquer dans les marchés de plein air, où il vient couper des morceaux de chair sur les étals des bouchers et des poissonniers.

Les colonies de *V. velutina* sont généralement très populeuses, au moins trois fois plus que celles du frelon Européen *V. crabro*, ce qui implique qu'un grand nombre d'individus sont chargés d'approvisionner le nid en nourriture (Rome *et al*., 2015). Pour cela, les frelons chassent les insectes disponibles en grande quantité pendant toute la saison. L'analyse des boulettes alimentaires collectées en France à l'entrée de plusieurs nids montre que le spectre des proies capturées par les ouvrières de *V. velutina* est assez diversifié (Rome *et al*., 2011). Il comprend notamment des espèces d'abeilles et de Diptères qui jouent un rôle central dans la pollinisation. La dégradation de ce service écologique vital à l'agriculture et aux plantes sauvages, pourrait avoir un impact très néfaste sur la biodiversité locale (Potts *et al*., 2010). Les proies récoltées sont différentes en fonction du milieu où se situe le nid (urbain, agricole ou forestier) mais en France, les Apidés constituent souvent la majorité des captures. En effet, dans son aire d'origine comme en zone envahie, *V. velutina* est un prédateur d'abeilles notoire (Li *et al*., 2008).

Contrairement à l'abeille asiatique *A. cerana* qui a coévolué en Asie avec un grand nombre d'espèces de frelons et qui possède des comportements de défense efficaces, l'abeille Européenne *A. mellifera* semble être beaucoup plus vulnérable à leurs attaques (Tan *et al*., 2007, 2012, 2013 ; Arca *et al*., 2014). Les abeilles *A. cerana* se défendent en s'agglutinant sur les frelons qui attaquent leurs

colonies. Elles forment ainsi une balle contenant des centaines d'abeilles, de sorte que la température augmente fortement en son centre, jusqu'à devenir létale pour l'assaillant. Cette stratégie de défense appelé « heat-balling » (boule thermique) semble moins bien organisée chez l'abeille Européenne *A. mellifera*, ce qui rend leur comportement de défense inefficace. Cependant, cette technique peut être utilisée par les abeilles chypriote*, Apis mellifera cypria*, pour asphyxier leur prédateur, *Vespa orientalis* (Papachristoforou *et al.*, 2007).

Les ruchers français sont ainsi victimes de cette prédation car ils concentrent une grande quantité de proies particulièrement vulnérables et disponibles pendant toute la saison. De plus, la pression de prédation qu'exerce *V. velutina* atteint son maximum annuel à la fin de l'été lorsque la population des colonies d'abeilles décroît afin de se préparer à l'hivernage. Lorsque les colonies d'abeilles sont particulièrement affaiblies à cause de la pression de prédation, les frelons parviennent à entrer dans la ruche et se livrent alors à un pillage du couvain et des provisions de miel. En dépit d'un manque d'études de l'impact réel du frelon sur l'activité apicole, il apparait que cette prédation diminue fortement l'activité de butinage des ouvrières et donc la production de miel (Monceau *et al.*, 2013b). Le frelon asiatique remplit donc tous les critères qui définissent une espèce invasive puisqu'il prolifère en France, déséquilibre les écosystèmes par son impact sur l'entomofaune, bouleverse les activités économiques apicoles et provoque des perturbations sanitaires et sociales.

e. Méthodes de lutte

Face à cette menace, plusieurs stratégies de lutte reposant principalement sur des initiatives individuelles ont été envisagées et employés au niveau local (Monceau *et al.*, 2014). Mais ce n'est qu'en 2012, soit 8 ans après la première identification de *V. velutina* sur le territoire français, que le gouvernement le classa dans la liste des espèces nuisibles de deuxième catégorie, c'est-à-dire présentant des dangers sanitaires pour lesquels il peut être nécessaire, pour l'intérêt collectif, de mettre en œuvre des mesures de lutte et/ou de prévention (Journal officiel de la République Française, 2012). Il est toutefois important de noter que, dans le monde, la majorité des tentatives d'éradication des Vespidés invasifs ont échoué (Beggs *et al.*, 2011). En effet, le haut potentiel dispersif de ces espèces, lié aux nombreux individus reproducteurs produits en fin de saison, suggère qu'une éradication complète de *V. velutina* est probablement impossible, bien que des mesures de contrôle visant à diminuer la densité de population et la pression de prédation sur les colonies d'abeilles puissent tout de même être considérées. Ainsi, pour lutter efficacement contre l'invasion de *Vespa velutina* il convient de prendre en compte les particularités de son cycle de vie afin d'élaborer des stratégies liées au développement des colonies. Trois grandes phases, au cours desquelles différentes castes sont présentes au sein de la colonie, peuvent être distinguer (**fig. 8**).

Au printemps ou à l'initiation du nid (phase 1), seule la fondatrice est présente (zone bleue, **Fig. 8**). Ceci rend les colonies extrêmement vulnérables, car la mort de ces seuls adultes conduirait à

une rapide agonie du couvain (Spradbery, 1973). En France, les associations apicoles préconisent notament d'articuler les méthodes de lutte autour de l'élimination des fondatrices au cours de cette phase, pour réduire le nombre de colonies initiées (Blot, 2009). Cependant l'efficacité de cette stratégie est controversée car de nombreuses fondatrices sont produites dans chaque nid, et une seule reine est suffisante pour créer une nouvelle colonie. En effet, des études sur le taux de survie des guêpes *Vespula vulgaris* et *Vespula germanica* au cours de l'hiver ont montré que seuls 0.01% des reines produitent survivent jusqu'au printemps, ce qui n'empêche pas ces espèces d'être relativement abondantes (Edwards, 1980 ; Thomas, 1960). L'élimination d'une partie des fondatrices ne suffirait donc pas, pour diminuer radicalement l'effectif des populations invasives.

A la phase suivante du cycle de vie de *V. velutina* (zone verte, **Fig. 8**), de nombreuses femelles stériles (ouvrières) ont émergé et recherchent activement des ressources alimentaires pour approvisionner la colonie. Lors de cette phase, la destruction des ouvrières vise principalement à réduire la pression de prédation qu'elles exercent sur l'entomofaune car il est alors plus difficile d'atteindre la reine, qui reste cloîtrée dans le nid et pond continuellement de nouveaux œufs. Cette solution ne permettant d'éliminer qu'une partie des individus n'est efficace que localement et ne devrait donc perturber significativement ni la dispersion ni l'abondance de l'espèce (Monceau *et al.*, 2014).

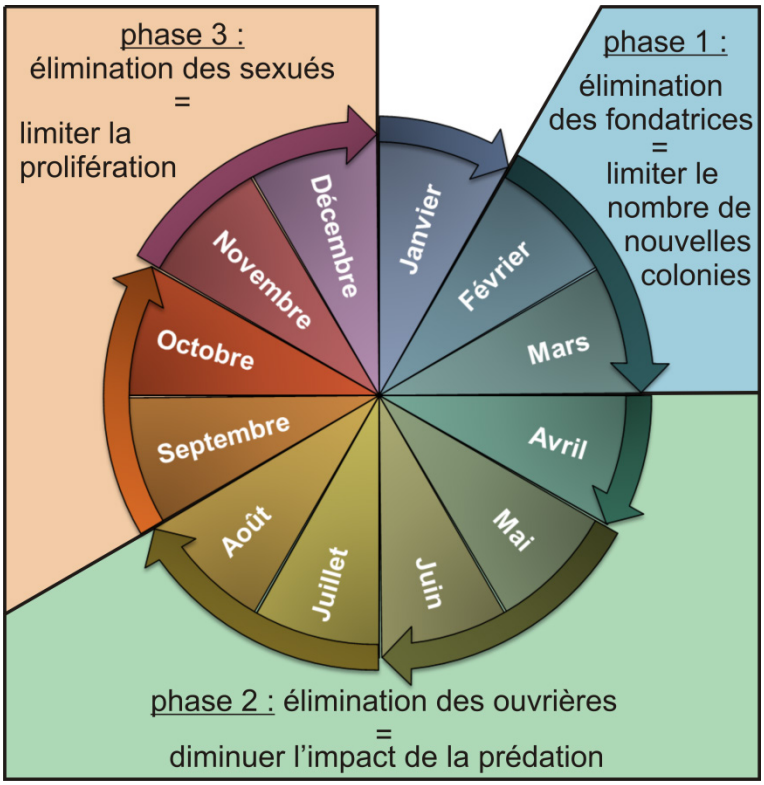


Figure 8 : Phases de lutte contre *V. velutina* en fonction de son cycle de vie
Lors de la phase d'initiation des nids (phase 1), seules les fondatrices sont présentes. Le piégeage de celles-ci peut éventuellement limiter le nombre de nouvelles colonies créées. Lors de la phase 2, les ouvrières sont particulièrement actives et chassent des insectes pour nourrir leur colonie. Leur piégeage pendant cette période vise à réduire la pression de prédation qu'elles exercent. Lors de la phase 3, les individus sexués cherchent à se reproduire. Employer une approche de confusion sexuelle pourrait limiter le nombre de reines fécondées au printemps suivant.

Lors de la troisième phase du cycle (zone orange, **Fig. 8**) qui débute en automne, les individus sexués (mâles et fondatrices) émergent. On retrouve alors dans la nature aussi bien des ouvrières en

prédation que des fondatrices et des mâles cherchant à se reproduire. Le contrôle de *V. velutina* au cours de cette phase vise donc principalement à éliminer les mâles avant qu'ils n'aient fécondé les futures fondatrices, ou à éliminer ces fondatrices avant qu'elles n'entrent en hibernation. Au même titre que l'élimination des fondatrices au printemps (phase 1), l'efficacité de cette stratégie est controversée car chaque nid produit de nombreux indivisdus sexués et la survie de quelques uns suffi pour maintenir les effectifs des populations invasives.

La destruction des nids, assimilable à un contrôle mécanique, peut être appropriée pour diminuer localement l'abondance de l'espèce. A cet effet, en 2013, l'Agence Nationale de Sécurité Sanitaire (l'Anses) autorisa provisoirement l'utilisation du dioxide de soufre (SO_2). Injecté sous pression directement dans un nid, il permet d'éliminer les individus qui y sont présents (Anses, 2013). Néanmoins cette technique ne s'avère réellement efficace que si toutes les castes, y compris la reine, sont détruits. Dans le cas contraire, le nid peut simplement être relocalisé. Il est aussi préférable de détruire les nids précocément, avant l'émergence et le départ des castes sexuées pour les raisons explicités ci-dessus. Cette stratégie se confronte donc au problème de la localisation précoce des colonies car, comme mentionné précédemment, celles-ci sont fréquemment localisées à la cîme des arbres où le feuillage peut empêcher leur détection avant l'automne.

Une autre stratégie de lutte relativement répandue contre les insectes nuisibles consiste à interférer avec leurs comportements olfactifs grâce à des appâts odorants (Foster *et al.*, 1997 ; Cook *et al.*, 2007). En effet, pour lutter contre les ravageurs des cultures, l'utilisation de pièges attractifs à base de phéromones s'est largement développée à partir des année 90 comme alternative à l'épandage de pesticides (Howse *et al.*, 1998). Par exemple, la stratégie de « confusion sexuelle » utilise les phéromones sexuelles d'une espèce pour attirer les mâles pendant la période de reproduction et ainsi diminuer l'effectif des populations de ravageurs (Witzgall *et al.*, 2010). Lorsque l'écologie chimique d'une espèce est particulièrement bien connue, il est possible d'utiliser une stratégie intégrée visant à attirer les individus quels que soient leur sexe et leur caste. C'est ainsi qu'en utilisant à la fois des phéromones d'aggrégation, attractives pour les femelles, et des phéromones sexuelles, attractives pour les males, les populations de scolytinae (coléoptères ravageurs pour la sylviculture) ont pu être considérablement réduites lors de campagnes d'éradication menées en Scandinavie et en Amérique du Nord (Howse *et al.*, 1998).

A l'heure actuelle, les appâts utilisés pour lutter contre les guêpes sociales sont principalement élaborés à base d'aliments riches en glucides ou en protéines, et qui sont susceptibles d'émettre des odeurs attractives pour ces insectes (Dvořák & Landolt 2006 ; Dvořák, 2007). Cette méthode a notamment été utilisée pour étudier la dynamique de prédation et d'apparition des castes au cours du cycle de vie de *V. velutina* (Monceau *et al.*, 2012 , 2013c). Il peut être envisagé de mélanger les appâts à des insecticides qui empoisonneront la colonie lorsque que les ouvrières les ramènent au nid (Spradbery, 1973 ; Edwards, 1980). Cette technique intensivement appliquée en Argentine et en

Nouvelle-Zélande a permis de considérablement réduire les populations invasives de *Vespula germanica* (Sackmann *et al.*, 2001 ; Spurr, 1991). Par contre, si cette méthode n'est pas appliquée régulièrement, les populations peuvent retrouver en quelques saisons leur densité initiale. De plus, ces attractifs sont très peu spécifiques et peuvent fortement perturber l'entomofaune locale (Rome *et al.*, 2011). La solution la plus efficace pour lutter spécifiquement contre *V. velutina* sans altérer pour autant la biodiversité locale serait donc de disposer d'appats très attractifs et spécifiques de l'espèce comme le peuvent être les phéromones. En raison des particularités du cycle de vie de *V. velutina,* différentes phéromones devraient alors être utilisées en fonction de la période de l'année.

Ainsi toutes les stratégies de lutte envisagées pour lutter contre l'invasion de *V. velutina* semblent avoir des limites. Cependant, une stratégie intégrée reposant sur des appâts attractifs et spécifiques, utilisés judicieusement en fonction des périodes de l'année, pourrait permettre de réduire significativement la densité des populations invasives, ainsi que leur impact sur l'apiculture et l'entomofaune (Monceau *et al.*, 2014). La stratégie intégrée consisterait donc à capturer les fondatrices au printemps pour limiter la création de nouvelles colonies, protéger les ruches de la prédation des ouvrières en été et limiter la reproduction des sexués en pratiquant de la confusion sexuelle en automne. Toutes ces stratégies reposent sur l'utilisation de composés odorants attractifs et spécifiques du frelon. Pour les mettre en oeuvre, une meilleur connaissance du système olfactif de cet insecte, ainsi que des composés odorants impliqués dans son comportement, semble indispensable. Ceci a constitué le sujet principal des recherches de cette thèse.

III. L'olfaction

1) Le sens olfactif

La perception sensorielle est une faculté biophysique vitale qui fait office d'interface entre un organisme et son environnement. Elle permet à l'organisme de détecter les signaux internes et externes afin d'adapter son comportement face à une situation donnée. Il existe différents types de signaux sensoriels tels que les signaux mécaniques, électromagnétiques ou chimiques dont la prévalence varie selon l'espèce, le milieu de vie et le contexte. Ces signaux sont détectés par les organismes grâce à des cellules sensitives capables de capter et traduire la qualité et l'intensité des stimuli en influx nerveux.

Le sens olfactif permet la détection de substances chimiques volatiles présentes dans l'air pour les organismes terrestres, ou solubles dans l'eau pour les organismes aquatiques. Son importance fondamentale fut soulignée en 2004 par l'attribution du prix Nobel de Physiologie et Médecine à Linda Buck et Richard Axel pour leurs travaux sur la caractérisation des récepteurs olfactifs. Ces chercheurs mirent en évidence qu'une famille de très nombreux gènes est impliquée dans l'expression des fonctions olfactives alors que les autres modalités sensorielles fonctionnent avec un nombre de récepteurs beaucoup plus restreint (Buck & Axel, 1991). La nature de la tâche olfactive, à savoir la détection et la discrimination d'un grand nombre de molécules, sollicite sans doute ce choix coûteux génétiquement. En effet, l'olfaction est considérée comme une fonction indispensable à la survie de nombreuses espèces car elle fournit des informations essentielles à l'exécution des tâches les plus fondamentales comme la nutrition ou la reproduction. Des modifications au sein de ce système sensoriel peuvent donc, dans certains cas, agir de manière conséquente sur la spéciation des espèces. Par exemple, les mouches de l'espèce *Rhagoletis pomonella* utilisaient initialement l'odeur des cenelles (fruit de l'aubépine) pour trouver leur site de ponte. Un changement de préférence olfactive, apparu il y a moins de 150 ans, pour l'odeur des pommes serait à l'origine de la spéciation sympatrique de *Rhagoletis pomonella* (Linn *et al*., 2003). De manière similaire, des changements de préférences olfactives concernant les phéromones sexuelles pourraient participer à l'isolement reproducteur de certaines espèces (Bacquet *et al.*, 2015).

Le système olfactif confère la possibilité de percevoir un nombre très important de nuances au sein de notre environnement chimique. Tout d'abord, une odeur est définie par sa qualité perceptuelle. C'est ce qui lui donne son identité et permet de la distinguer des autres. Une étude récente prédit que l'homme serait capable de discriminer jusqu'à plus de 10^{12} stimuli olfactifs différents (Bushdid *et al*., 2014). Mais les odeurs naturelles sont le plus souvent des mélanges de molécules volatiles dont il est difficile pour l'organisme d'identifier les composants élémentaires. De ce fait, ces mélanges peuvent parfois revêtir une qualité perceptuelle propre, indépendante de celles

de ses composés élémentaires (Coureaud *et al.*, 2008, 2009 ; Barkat *et al.*, 2012). Ainsi, l'odeur caractéristique des roses est le fruit d'un mélange de plus de 30 molécules qui peuvent varier selon les espèces (Kim *et al.*, 2000). Ensuite, une odeur est perçue avec plus ou moins d'intensité en fonction de la concentration des composés volatiles. Mais la qualité et l'intensité ne sont pas des notions indépendantes car elles interfèrent en permanence. En effet, la qualité perçue d'un stimulus olfactif est susceptible de changer selon son intensité (Gross-Isseroff & Lancet, 1988). Enfin, en condition naturelle les odeurs se diffusent dans l'air suivant une mécanique complexe que les animaux peuvent mettre à profit en adoptant des comportements de recherche particulier afin d'en localiser la source (Farkas & Shorey, 1972 ; Murlis *et al.*, 1992 ; Vickers, 2000).

Pour une espèce animale donnée, on distingue communément plusieurs types d'odeurs. Les odeurs dites **générales** ne sont pas activement libérées dans l'air mais fournissent des informations essentielles sur l'environnement proche ou sur la localisation et la qualité d'une source de nourriture. Les substances **sémiochimiques** sont, quant à elles, activement produites par un organisme et ont valeur de signal (communication) pour un autre organisme (**Fig. 9**). Les **phéromones** sont des composés chimiques émis par un individu et qui induisent un comportement spécifique (phéromones incitatrices) ou un changement physiologique (phéromones modificatrices) chez un individu de la même espèce (Wilson, 1963). Chez de nombreux animaux, ces signaux sont notamment utilisés dans le recrutement de congénères ou l'attraction sexuelle. Ils sont particulièrement importants chez les insectes sociaux qui nécessitent des systèmes de communication efficaces pour maintenir une cohésion sociale (Vander Meer, 1998). Lorsque la substance émise agit sur une autre espèce au bénéfice de l'émetteur, on parle d'**allomone** alors que si elle bénéficie à l'espèce réceptrice, on parle de **kairomone**. Les composés odorants jouent donc un rôle crucial dans les interactions intra- et interspécifiques. L'illustration la plus parlante est la fonction centrale des odeurs florales dans l'interaction mutualiste qui relie les angiospermes à leurs pollinisateurs et joue donc à la fois le rôle d'allomone et de kairomone (Raguso, 2008). Les signaux odorants peuvent aussi bénéficier à certaines espèces pour détecter la présence d'un prédateur (kairomone). La souris possède par exemple une aversion innée pour les odeurs présentes dans l'urine de chat (Ferrero *et al.*, 2011). Il a même été observé que le comportement olfactif peut être exploité dans des relations de parasitisme. Le protozoaire agent de la toxoplasmose, *Toxoplasma gondii*, tire avantage des capacités de détection des souris, en changeant leur aversion pour l'odeur d'urine de chat en attraction, afin de faciliter son passage d'un hôte à l'autre (Vyas *et al.*, 2007). Ainsi, les signaux chimiques ont souvent un rôle prépondérant dans les interactions entre les êtres vivants. Une bonne connaissance des fonctions olfactives peut donc permettre de mieux appréhender l'écologie d'une espèce donnée, et dans le contexte d'une invasion biologique, de pouvoir interférer avec son comportement.

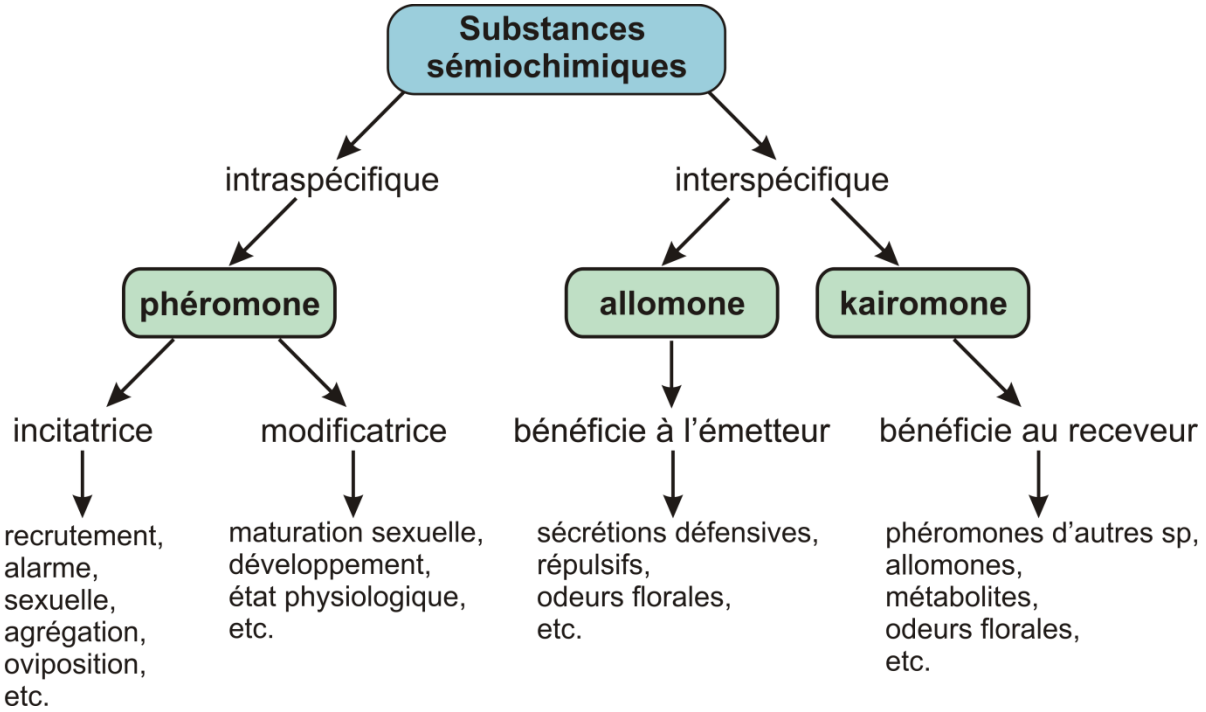


Figure 9 : Les différentes catégories de substances sémiochimiques
Les substances sémiochiques à effet intraspécifique sont appelées phéromones (incitatrice ou modificatrice). Lorsque l'effet est interspécifique au bénéfice de l'espèce émettrice, on parle d'allomone alors qu'un effet au bénéfice du receveur correspond à une kairomone. Les odeurs florales sont utilisées comme exemple à la fois pour les kairomones et les allomones car elles sont impliquées dans une interaction mutualiste plante-pollinisateur. (D'après Howse *et al.*, 1998).

2) Structure générale du système olfactif

Il existe des similarités frappantes dans l'organisation du système olfactif entre espèces divergentes. En effet, depuis la détection périphérique des odeurs jusqu'au système nerveux central, les insectes et les Vertébrés présentent des structures et des étapes de traitement neuronal analogues (Ache & Young, 2005 ; Kay & Stopfer, 2006). A ce jour, on ne sait pas encore si ce système est apparu relativement tôt dans l'évolution et a été conservé, ou si les similarités observées sont issues de processus de convergence évolutive (Eisthen, 2002). Quoi qu'il en soit, ces analogies semblent indiquer qu'il existe une solution efficace pour résoudre les problèmes liés à la détection et à la discrimination d'un grand nombre de molécules odorantes.

Les animaux détectent les odeurs qu'ils rencontrent à l'aide de récepteurs olfactifs (RO), présents sur la membrane des neurones sensoriels olfactifs (NSO), eux-mêmes situés au niveau de l'épithélium olfactif des Vertébrés ou des sensilles antennaires des insectes. Dans la majorité des cas, chaque neurone sensoriel olfactif exprime un seul type de récepteur olfactif (Mombaerts *et al.*, 1996 ; Vosshall *et al.*, 2000), qui possède une affinité particulière pour une gamme de molécules odorantes (Duchamp-Viret *et al.*, 1999 ; Hallem & Carlson, 2006). Les molécules odorantes sont différentiables sur la base d'une combinaison de nombreuses caractéristiques telles que le (ou les) groupe(s) fonctionnel(s), la longueur ou le niveau de saturation de la chaîne carbonée, etc. (Haddad

et al., 2008). Ainsi, plusieurs molécules peuvent activer le même RO sensible à l'une de leurs caractéristiques communes, et réciproquement une molécule peut activer différents RO, sensibles aux différentes caractéristiques de cette molécule (**Fig. 10**). Par conséquent, chaque odorant active une combinaison spécifique de récepteurs, façonnant ainsi l'information olfactive dès la périphérie du système nerveux (Malnic *et al.*, 1999 ; Schmuker *et al.*, 2007). Le nombre de combinaisons possibles de RO activés est extrêmement important, ce qui permet au système olfactif de distinguer au moins autant de composés odorants différents.

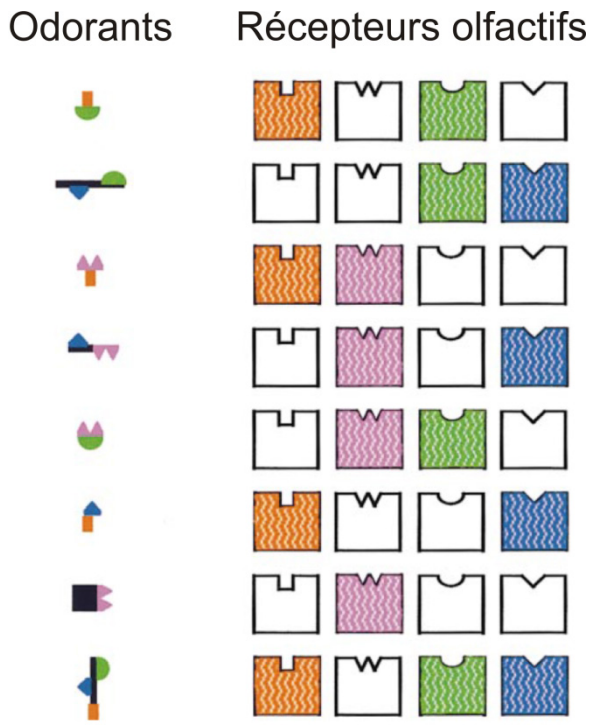


Figure 10 : Codage combinatoire au niveau des récepteurs olfactifs.
Chaque couleur représente une caractéristique chimique d'une molécule odorante (colonne de gauche) reconnu par un type de récepteur (colonne de droite). L'identité d'une molécule est encodée par l'activation d'une combinaison de récepteurs olfactifs. Chaque RO peut participer au codage combinatoire de plusieurs odeurs. Compte tenu des nombreuses combinaisons possibles, ce modèle permet de discriminer un nombre très important d'odorants différents. (D'après Malnic *et al.*, 1999).

Les axones des NSO se projettent dans le premier centre de traitement de l'information olfactive, le **bulbe olfactif** des Vertébrés (BO) ou le **lobe antennaire** (LA) des insectes, où ils forment des connections synaptiques avec des neurones de second ordre. Au sein du LA ou du BO, les projections des NSO exprimant le même RO convergent dans une structure sphérique dense en contacts synaptiques, appelée **glomérule** (Mombaerts *et al.*, 1996 ; Vosshall *et al.*, 2000). Le nombre, la position et la forme des glomérules est généralement invariable entre individus de la même espèce (Anton & Homberg, 1999 ; Hansson & Anton, 2000). Le premier centre de l'information olfactive est donc spatialement compartimenté en unités morphologiques et fonctionnelles (les glomérules), présents au nombre de 1800 chez la souris (Mori *et al.*, 2006), 160 chez l'abeille (Galizia *et al.*, 1999a), et de 200 jusqu'à plus de 600 chez certaines espèces de fourmis (Kelber *et al.*, 2009). Les glomérules sont interconnectés entre eux par des populations de neurones confinés au LA ou au BO, appelés cellules périglomérulaires et granulaires chez les Vertébrés ou interneurones locaux chez les insectes (Kay & Stopfer, 2006). Ils sont de morphologie, connectivité et pharmacologie variables, et ont pour fonction d'affiner le codage des odeurs par des effets d'activation et/ou d'inhibition.

Après cette première étape de traitement, l'information olfactive est convoyée vers les aires de traitement supérieures par les neurones de second ordre, les neurones de projection des insectes ou les cellules mitrales et les cellules à panache des Vertébrés. Chez les insectes, les neurones de projection aboutissent principalement dans deux structures, les corps pédonculés et/ou la corne latérale (Galizia & Rössler, 2010). Chez les Vertébrés, ces neurones acheminent l'information vers l'amygdale corticale, le cortex entorhinal, le striatum ventral et le cortex olfactif (Wilson & Mainen, 2006). Ces structures sont considérées comme des centres supérieurs d'intégration de l'information olfactive, impliquées dans les émotions et la mémorisation.

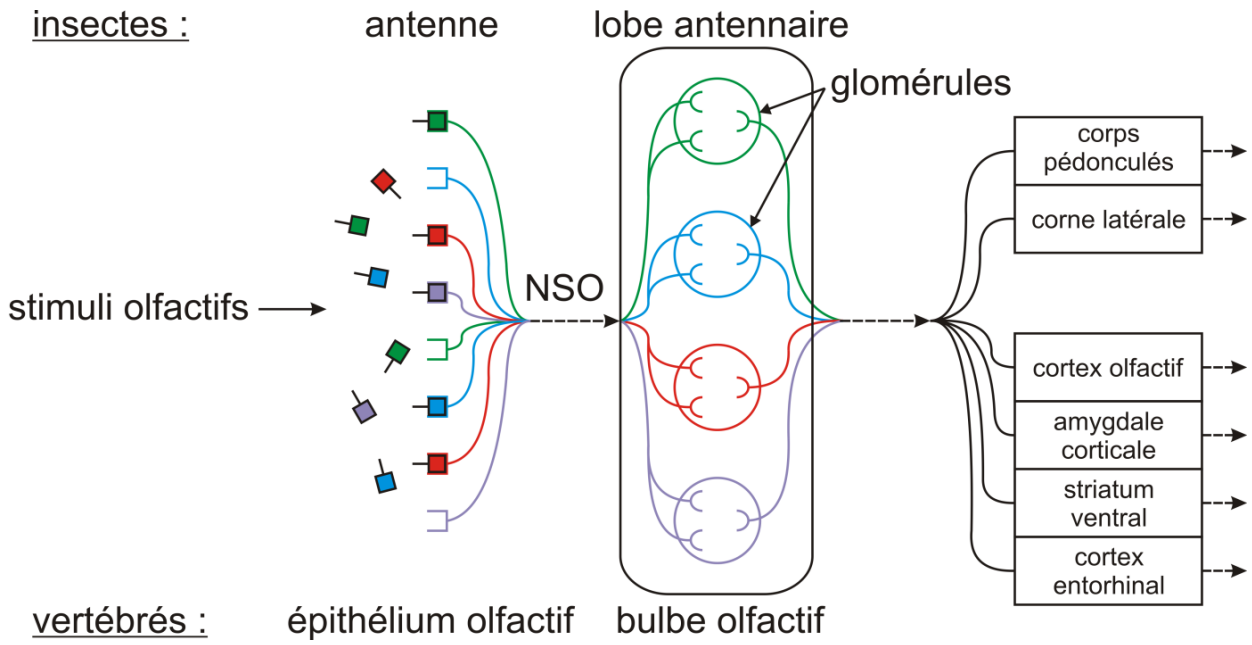


Figure 11 : Anatomie générale du système olfactif des insectes et des Vertébrés
Les stimuli olfactifs sont détectés par les neurones sensoriels olfactifs (NSO) au niveau de l'antenne des insectes et de l'épithélium olfactif des vertébrés. Les NSO acheminent ensuite l'information vers le lobe antennaire des insectes ou le bulbe olfactif des vertébrés, tous deux composés de glomérules. Puis l'information olfactive est envoyée vers les aires supérieures, au nombre de deux chez les insectes et plus nombreuses chez les vertébrés (D'après Tanaka *et al.*, 2004).

3) Le système olfactif des insectes

a. Détection périphérique des odeurs

Chez les insectes, l'antenne est un organe sensoriel primordial capable de détecter les signaux olfactifs mais également les signaux gustatif, thermo, hygro- et mechanosensoriels (Schneider, 1964). Les antennes peuvent avoir une morphologie extrêmement variable en fonction de l'espèce et de son écologie (**Fig. 12, A**), allant d'une forme simple et filiforme (ex : cafard) à une forme complexe et plumeuse (ex : moustique). Cependant, les antennes présentent généralement toutes 3 articles, le scape, le pédicelle et le flagelle (Schneider, 1964). Le scape est une structure mobile qui relie la tête de l'insecte au reste de l'antenne. Le second segment, le pédicelle, est un article mobile qui contient

des scolopidies (mécanorécepteurs) formant l'organe de Johnston, impliqué dans la détection des vibrations et des mouvements de l'antenne (Schneider, 1964). Enfin, le flagelle est la partie sensorielle principale de l'antenne, subdivisée en plusieurs segments, ou flagellomères. Il n'est que passivement mobile et suit les déplacements du pédicelle. Les flagellomères sont recouverts de soies, appelés sensilles, abritant des neurones sensoriels.

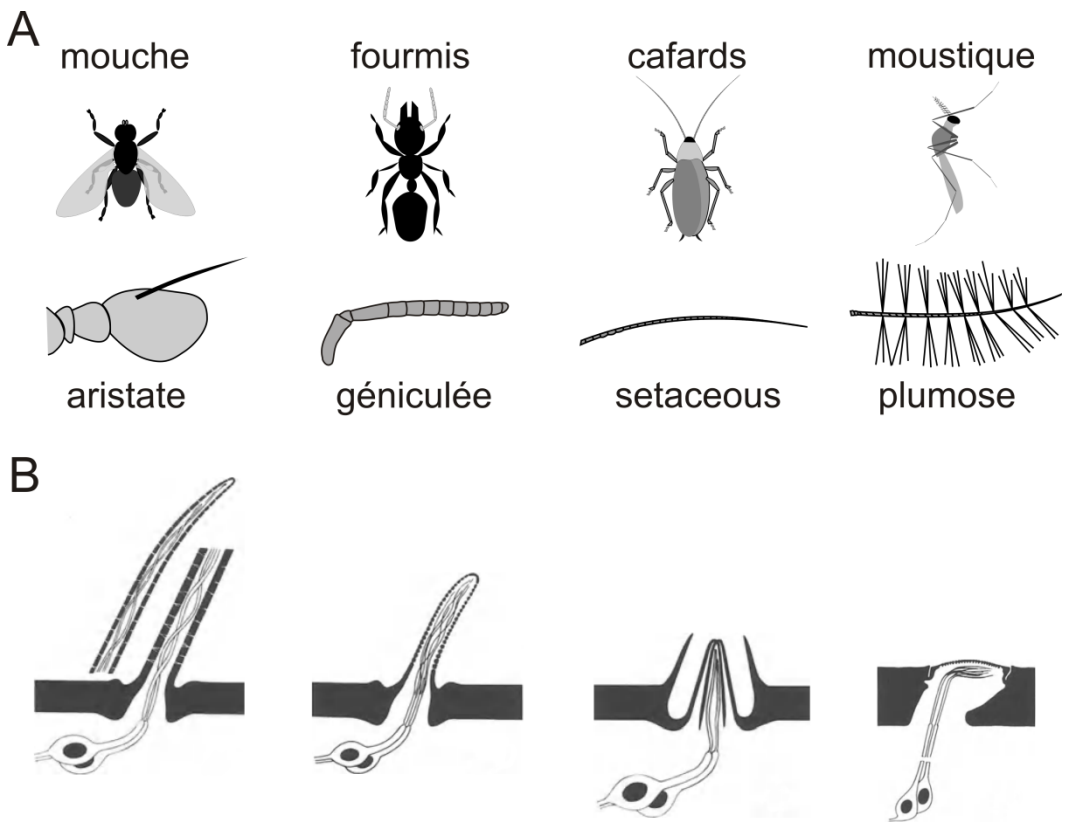


Figure 12 : Variabilité morphologique de l'antenne et des sensilles olfactives chez les insectes.
A) L'antenne des insectes peut avoir une morphologie très variable en fonction de l'espèce. Par exemple, les mouches ont des antennes de type aristate alors que les moustiques qui appartiennent au même ordre (Diptère) ont des antennes plumeuses. **B**) Schémas représentant différents types sensillaires. De gauche à droite : sensille trochoïde, sensille basiconique, sensille coeloconique et sensille placodée. Malgré des formes différentes, elles comportent toutes des neurones sensoriels à leur base (D'après Keil, 1999).

Chez un même individu, plusieurs types sensillaires (**Fig. 12, B**) peuvent être identifiés sur la base de différences morphologiques et structurales (Callahan, 1975 ; Zacharuk, 1980 ; Altner & Loftus, 1985). Les sensilles chaétiques ont une enveloppe cuticulaire droite et rigide ; celles présentant un pore terminal sont considérées comme sensilles gustatives alors que des fonctions méchanosensorielles ont été attribuées à celles ne possédant pas de pores (Keil, 1999). Les sensilles ampullacées forment un creux à la surface de l'antenne, et des enregistrements électrophysiologiques indiquent qu'elles seraient sensibles aux variations de concentration de CO_2 (Kleineidam *et al.*, 2000). Les sensilles coeloconiques ont une double paroi cuticulaire se dressant au centre d'une cavité et ont une fonction olfactive ou hygro- et thermosensoriel (Altner *et al.*, 1977 ; Pophof, 1997 ; Yao *et al.*, 2005 ; Ruchty *et al.*, 2009). Les sensilles olfactives trichoïdes, basiconiques

et placodées sont différentiées par leur forme allongée, en cheville, ou aplatie **(fig 12, B)** mais possèdent toutes de nombreux pores de diamètre nanométrique qui permettent le passage des molécules volatiles vers l'intérieur de la sensille (Steinbrecht, 1997 ; Hallberg & Hansson, 1999).

A l'intérieur des sensilles olfactives, les dendrites d'un à plusieurs dizaines de NSO baignent dans une lymphe aqueuse sécrétée par des cellules accessoires (Zacharuk, 1980). Les molécules odorantes sont alors solubilisées dans cette lymphe ou prises en charge par divers complexes protéiques comme les protéines de transport appelées OBP (odorant binding protein), les protéines chimiosensorielles (CSP) ou des enzymes de dégradations (Zhou, 2010 ; Fan *et al.*, 2011 ; Pelosi *et al.*, 2014 ; Yi *et al.*, 2014 ; Younus *et al.*, 2014). Les OBP sont des molécules hydrophiles qui faciliteraient le transport des molécules hydrophobes vers les dendrites des NSO où sont exprimés les RO, alors que les enzymes de dégradation permettent d'éviter la saturation des RO (Leal, 2013). Ainsi le système olfactif périphérique des insectes est compartimenté en sensilles au sein desquelles des mécanismes moléculaires, mentionnés ci-dessus, augmentent et régulent la sensibilité de détection des odorants.

Les récepteurs olfactifs des insectes sont principalement des hétérodimères composés d'un récepteur (OR) à 7 domaines transmembranaires couplé à une protéine G, et d'un corécepteur ubiquitaire aux NSO, appelé Orco (Neuhaus *et al.*, 2005 ; Nakagawa *et al.*, 2005 ; Vosshall & Hansson, 2011 ; German *et al.*, 2013). L'OR est la partie sensible à la fixation des odorants et qui détermine le spectre de réponse du NSO dans lequel il est exprimé (Dobritsa *et al.*, 2003 ; Hallem & Carlson, 2006), alors que Orco formerait un canal cationique non sélectif et permettrait l'adressage du complexe à la membrane dendritique (Larsson *et al.*, 2004 ; Jones *et al.*, 2011 ; Nichols *et al.*, 2011 ; Stengl & Funk, 2013). Le mode de signalisation de ces hétérodimères reste sujet à controverse puisqu'aussi bien les propriétés ionotropiques (d'Orco) et métabotropiques (de l'OR) semblent être impliquées dans la transduction des signaux olfactifs (Sato *et al.*, 2008 ; Nakagawa & Vosshall, 2009). Dans un schéma général consensuel (**Fig. 13**), la fixation d'un odorant sur l'OR permettrait l'ouverture du canal cationique formé par Orco pour une détection rapide de l'odeur, alors que la signalisation métabotropique de l'OR permettrait une détection plus sensible et prolongée du signal olfactif grâce à l'amplification du signal par l'intermédiaire des protéines G (Wicher *et al.*, 2008).

Une seconde famille de récepteurs impliqués dans la détection des signaux olfactifs a également été identifiée chez plusieurs insectes (Olivier *et al.*, 2011 ; Rytz *et al.*, 2013). Les récepteurs ionotropiques (RI) sont des récepteurs olfactifs possédant un site de fixation du ligand, 3 domaines transmembranaires et un canal ionique (Benton *et al.*, 2009). Ces récepteurs sont considérés comme ancestraux par rapport aux OR puisqu'ils seraient apparus chez l'ancêtre commun aux Protostomiens et auraient dérivé des récepteurs ionotropiques au glutamate (Benton *et al.*, 2009 ; Croset *et al.*, 2010). Les études fonctionnelles menées sur les RI indiquent qu'ils seraient

impliqués dans la détection des composés hydrophiles comme les acides aminés (Ai *et al.*, 2010 ; Ai *et al.*, 2013 ; Min *et al.*, 2013).

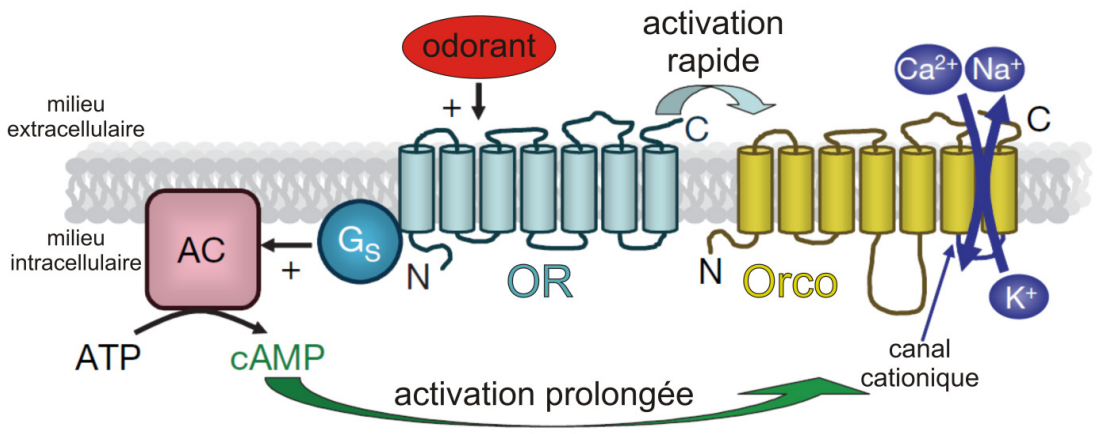


Figure 13 : Signalisation des hétérodimères OR-Orco
Lorsqu'une molécule odorante se fixe sur un récepteur olfactif (OR), elle induit l'ouverture du canal cationique formé par le co-récepteur (Orco) grâce à une voie de signalisation ionotropique et métabotropique. L'activation directe d'Orco par l'OR (flèche bleue) conduit à une ouverture rapide et transitoire du canal cationique. En parallèle la fixation de l'odorant sur l'OR induit l'activation de protéines Gs, qui vont activer à leur tour l'adénylate cyclase (AC), augmentant ainsi la production d'AMP cyclique (cAMP). L'AMP cyclique permet une activation lente et prolongée (flèche verte) du canal cationique d'Orco et aboutit à une dépolarisation de la membrane par échanges de cations entre les milieux extra- et intracellulaire (D'après Wicher *et al.*, 2008).

Ainsi, La liaison d'un odorant sur un récepteur olfactif provoque une variation du potentiel transmembranaire au niveau des dendrites du NSO, suivi de l'ouverture de canaux voltage-dépendants impliqués dans la génération des potentiels d'action. La fréquence de génération de ces potentiels d'action est à l'origine du codage des signaux olfactifs (de Bruyne & Baker, 2008).

La distribution des types fonctionnels de NSO dans les différents types sensillaires ne semble pas être aléatoire. En effet, il a été montré chez la mouche (*Drosophila melanogaster*) et le criquet pèlerin (*Schistocerca gregaria*) que les NSO exprimant les RI sont exclusivement présents dans les sensilles coeloconiques (Benton *et al.*, 2009 ; Guo *et al.*, 2014). De plus, les NSO des sensilles basiconiques chez les Diptères, les Lépidoptères et les Coléoptères semblent détecter préférentiellement les composés allélochimiques (de Bruyne *et al.*, 2001 ; Andersson *et al.*, 2009) alors que les NSO des sensilles trichoïdes sont plus souvent impliqués dans la détection des composés hydrophobes à chaîne longue, comme de nombreuses phéromones (Kurtovic *et al.*, 2007 ; Andersson *et al.*, 2009). Cependant la ségrégation des types fonctionnels de NSO sur la base de réponses à différents types d'odorants n'est pas drastique. Cette compartimentation pourrait également jouer un rôle dans la sensibilité de détection et le codage de l'information olfactive car il a été montré que des inhibitions latérales non-synaptiques (ephaptique) peuvent se produire entre NSO logés au sein d'une même sensille (Su *et al.*, 2012).

b. Rôle des NSO dans le traitement de l'information olfactive

Les axones des NSO se regroupent le long de l'antenne et se projettent dans le premier centre de traitement de l'information olfactive, le lobe antennaire. Les NSO exprimant un même RO convergent dans un même glomérule où ils forment des synapses avec des interneurones locaux et des neurones de projection (Gao *et al.*, 2000 ; Vosshall *et al.*, 2000 ; Couto *et al.*, 2005 ; Fishilevich & Vosshall, 2005 ; Rybak *et al.*, 2016). Ainsi, pour une espèce donnée, il y a généralement une forte correspondance entre le nombre d'OR et de glomérule (Vosshall *et al.*, 2000 ; Robertson & Wanner, 2006). Chez les insectes le nombre de NSO et de glomérules varie fortement entre espèces et parfois entre sexes au sein de la même espèce. Par exemple chez la Drosophiles le nombre de NSO et de glomérules est identique entre mâles et femelles avec environ 1200 NSO se projetant dans un peu moins de 50 glomérules (Stocker *et al.*, 1990). Par contre, l'abeille *Apis mellifera* présente un dimorphisme sexuel puisque les mâles possèdent environ 300 000 NSO pour environ 120 glomérules contre 65 000 NSO pour 160 glomérules chez les ouvrières (Esslen & Kaissling, 1976). Enfin, chez certaines espèces, le nombre de NSO est identique entre mâles et femelles mais ceux-ci diffèrent par leur spectre de réponse aux odorants, comme chez *Manduca sexta* où les deux sexes possèdent environ 300 000 OSNs pour 60 glomérules (Sanes & Hildebrand, 1976 ; Oland & Tolbert, 1988).

Dans la nature, les odorants se diffusent suivant une mécanique complexe en raison des turbulences de l'air (Murlis *et al.*, 1992). Par conséquent la réponse temporelle des NSO reflète principalement les variations des stimuli (Vickers *et al.*, 2001). En effet, il a été montré que les NSO peuvent présenter une résolution temporelle très élevée, pouvant suivre des fluctuations de stimulation à une fréquence excédant 100 Hz (Szyszka *et al.*, 2014). Cependant les NSO ont des propriétés intrinsèques variables en termes de réponse temporelle. Chez un même individu, des populations de NSO différentes peuvent avoir des réponses phasiques ou bien toniques. Ces réponses peuvent également être excitatrices ou inhibitrice et se prolonger après la stimulation ou bien s'arrêter au cours de la stimulation (Heinbockel & Kaissling, 1996 ; de Bruyne *et al.*, 2001 ; Getahun *et al.*, 2012 ; Cao *et al.*, 2016). Ainsi les glomérules reçoivent des informations complexes, structurées temporellement, ce qui participe aussi au codage de l'information olfactive (Laurent & Davidowitz, 1994 ; Wehr & Laurent, 1996 ; Laurent *et al.*, 1996 ; Brown *et al.*, 2005).

Le nombre limité de glomérules chez les insectes et leurs positions relativement constantes d'un individu à l'autre ont permis l'élaboration d'atlas glomérulaires chez de nombreuses espèces (Rospars, 1988), comme la drosophile (Stocker *et al.*, 1990 ; Laissue *et al.*, 1999), des moustiques (Ignell *et al.*, 2005), plusieurs Lépidoptères (Rospars & Hildebrand, 1992 ; Berg *et al.*, 2002 ; Greiner *et al.*, 2004 ; Huetteroth & Schachtner, 2005 ; Masante-Roca *et al.*, 2005 ; Skiri *et al.*, 2005 ; Couton *et al.*, 2009 ; Varela *et al.*, 2009), la blatte (Watanabe *et al.*, 2010) ou l'abeille domestique (Flanagan & Mercer, 1989 ; Galizia *et al.*, 1999a). Initialement ces descriptions morphologiques étaient réalisées sur la base de séries de coupes histologiques, mais les progrès en matière d'imagerie

confocale ainsi qu'en traitement et en stockage numérique des données informatiques ont permis la création d'atlas digitaux en 3 dimensions du lobe antennaire, et du cerveau de manière plus générale (Toga et Thompson, 2001 ; Rein *et al.*, 2002 ; Brandt *et al.*, 2005 ; Rybak *et al.*, 2010 ; Menzel, 2012). Chaque glomérule ou population neuronale peut ainsi être identifié ce qui améliore fortement la comparabilité des résultats entre groupes de recherche. Les données neuroanatomiques ont alors pu être intégrées aux réponses fonctionnelles enregistrées afin de décrypter le codage olfactif au sein du LA.

Au niveau périphérique (sensilles de l'antenne), le codage olfactif est typiquement étudié grâce à des enregistrements électrophysiologiques des réponses des NSO, ce qui permet de définir leurs spectres de réponse individuels (de Bruyne *et al.*, 2001 ; Hallem *et al.*, 2004). Dans le lobe antennaire, les techniques d'imagerie fonctionnelle permettent quant à elles de mesurer simultanément les réponses de nombreux glomérules. La technique la plus utilisée actuellement est l'imagerie calcique *in vivo* qui permet de mesurer les variations de concentration de calcium intracellulaire se produisant lorsqu'un neurone s'active (entrée de Ca^{2+} extracellulaire et sortie de Ca^{2+} du réticulum endoplasmique) (Joerges *et al.*, 1997 ; Berridge, 1998).

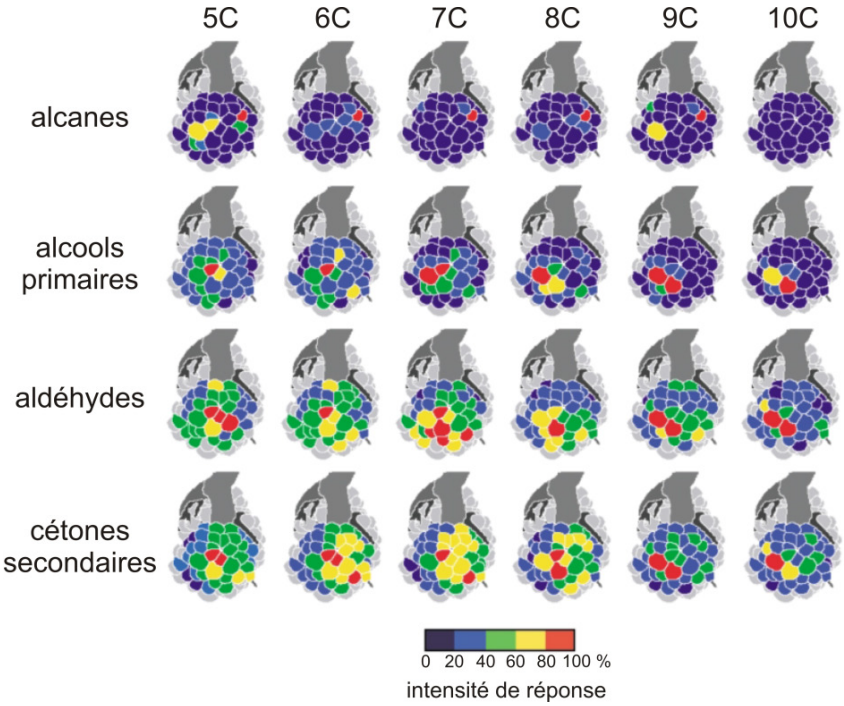


Figure 14 : Cartes d'activité glomérulaire dans le LA de l'abeille, *Apis mellifera*
La présentation d'odeurs possédant différents groupes fonctionnels (alcanes, alcools primaires, cétones secondaires, aldéhydes) et différentes longueurs de chaîne carbonée (de 5 à 10 carbones) induit des cartes d'activité différentes dans le LA de l'abeille (D'après Sachse *et al.*, 1999).

Il a pu être observé que de manière générale, chaque glomérule possède un spectre de réponse aux odorants qui lui est propre, ce qui confirme que les NSO exprimant le même RO convergent dans un unique glomérule (Sachse *et al.*, 1999 ; Carlsson *et al.*, 2002 ; Ng *et al.*, 2002 ; Fiala *et al.*, 2002 ; Wang *et al.*, 2003 ; Skiri *et al.*, 2004). D'autre part, la présentation d'une molécule odorante induit généralement l'activation de plusieurs de glomérules (**Fig. 14**) en raison du chevauchement des spectres de réponse des RO (Hallem & Carlson, 2006). Enfin, cette carte

d'activité spatiale est généralement conservée entre individus de la même espèce du fait de l'organisation stéréotypée des projections des NSO (Joerges *et al.*, 1997 ; Galizia *et al.*, 1999b ; Wang *et al.*, 2003). Ainsi la plupart des stimuli olfactifs déclenchent l'activité de plusieurs glomérules, résultant en un codage combinatoire des odeurs dans le lobe antennaire (**Fig. 14**).

Les NSO (comme leurs glomérules associés) diffèrent par leur spectre de réponse qui peut être plus ou moins large. Les premiers enregistrements électrophysiologiques des NSO du ver à soie, *Bombyx mori,* ont mis en évidence que certains RO sont particulièrement sensibles et spécifiques d'un composé chimique particulier, le Bombykol (Boeckh *et al.*, 1965). Cette détection spécifique conduit logiquement à l'activation exclusive d'un seul glomérule du LA, ce qui pourrait sembler aller à l'encontre du modèle combinatoire de l'information olfactive présenté plus haut. En fait, deux principes de codage possédant chacun leurs avantages et leurs inconvénients pourraient expliquer la représentation des odeurs au niveau central (Hansson, 2002 ; Sandoz *et al.*, 2007).

- Dans le modèle théorique d'une **ligne dédiée** ou « labeled-line », chaque récepteur est spécifique d'un stimulus et envoie l'information au système nerveux central par une voie directe (**Fig. 15, A**) (Binder *et al.*, 2009). Ce traitement fournit des informations précises mais sur un nombre très limité de stimuli car chaque glomérule est dédié à un odorant. Le codage en ligne dédiée est donc judicieux pour permettre à l'animal de reconnaître des stimuli d'importance cruciale pour sa biologie, comme les phéromones.
- Le modèle théorique du **codage combinatoire** ou « across-fiber pattern » propose que chaque récepteur (et donc chaque glomérule) réponde à une large gamme de molécules, comme expliqué précédemment, et que l'ensemble des réponses du LA forment le message olfactif (**Fig. 15, B**). Cette stratégie permet d'encoder un grand nombre de stimuli olfactifs mais peut s'avérer moins efficace en termes de contraste pour discriminer des composés chimiquement proches notamment à faible concentration.

Il a été démontré qu'en réalité les deux modèles coexistent et permettent de traiter efficacement différents types de stimuli (Hildebrand, 1996 ; Galizia & Menzel, 2000 ; Galizia *et al.*, 2000 ; Sandoz, 2006 ; Deisig *et al.*, 2012). Les odeurs dites générales, qui sont extrêmement nombreuses dans l'environnement, sont majoritairement traitées selon une stratégie combinatoire dans des glomérules de taille standard. Le codage en ligne dédiée a, quant à lui, été observé pour le traitement de composés particuliers comme le CO_2, les phéromones ou la geosmin (composé microbien) chez les Drosophiles (Hansson *et al.*, 1991 ; Agarwal & Isacoff, 2011 ; Grosjean *et al.*, 2011 ; Stensmyr *et al.*, 2012). Ainsi l'activité de certains glomérules hautement spécialisés dans le traitement de stimuli à forte pertinence écologique (CO2, phéromones) semblent avoir une relation étroite avec la valence de l'odeur et les comportements d'attraction ou de répulsion associés (Semmelhack & Wang, 2009).

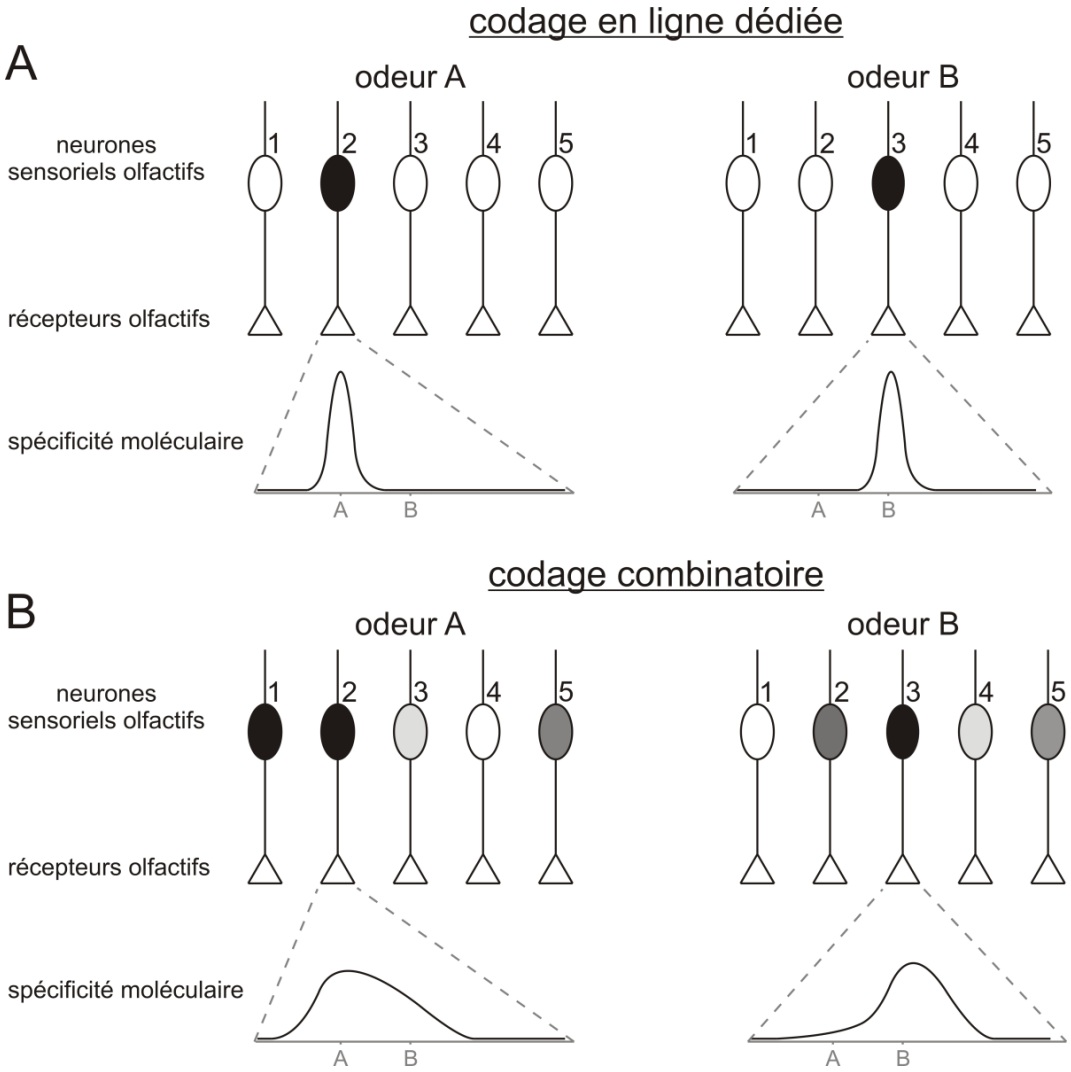


Figure 15 : Représentation schématique des deux principes de codage de l'information olfactive
Dans un modèle théorique de codage en ligne dédiée (**A**) chaque neurone sensoriel répond spécifiquement à un odorant (le NSO 2 est spécifique de l'odorant A, et le NSO 3 est spécifique de l'odorant B) et active une seule unité fonctionnelle. Les odorants A et B sont alors parfaitement discriminées mais ce système théorique ne peut différentier que 5 odorants différents. Dans le modèle théorique du codage combinatoire (**B**) les récepteurs peuvent être activés par plusieurs odorants. Les 5 récepteurs répondent alors à différentes gammes d'odorants avec différentes intensités. Dans cet exemple le récepteur 2 est fortement activé par l'odorant A et un peu moins par l'odorant B, alors que le récepteur 3 est fortement activé par l'odorant B mais très faiblement par l'odorant A. Les autres récepteurs répondent indépendamment en fonction de leurs affinités propres pour l'un ou l'autre des odorants. Ce système permet d'encoder un nombre important d'odorants différents mais leur discrimination fine nécessite d'autre étapes de traitement (D'après Sandoz *et al.*, 2007).

Le traitement en ligne dédié est particulièrement bien documenté en ce qui concerne les phéromones sexuelles des papillons de nuit (les hétérocères) (Hansson & Anton, 2000). Les NSO sensibles aux composés de la phéromone sexuelle sont localisés, au niveau de l'antenne, dans un type de sensilles morphologiquement distinct (les longues sensilles trichoïdes) qui n'est présent que chez les mâles alors que ceux répondant aux odeurs générales, se trouvent dans d'autres types sensillaires retrouvés aussi bien chez les mâles que chez les femelles (autre sensilles trichoïdes, basiconiques, coeloconiques) (Shields & Hildebrand, 2001). Les NSO phéromonaux se projettent sélectivement dans un groupe de glomérules très volumineux, appartenant au complexe

macroglomérulaire (CMG) et n'existant aussi que chez les mâles (**Fig. 16**) (Koontz & Schneider, 1987 ; Hansson *et al.*, 1992). Le CMG est donc composé de plusieurs glomérules hypertrophiés dont le nombre et la taille varient en fonction des espèces (Anton & Homberg, 1999). Chaque composé de la phéromone sexuelle est alors détecté par un seul type de RO et active spécifiquement un macroglomérule du CMG (Hansson *et al.*, 1991 ; Galizia *et al.*, 2000 ; Carlson *et al.*, 2002 ; Deisig *et al.*, 2012).

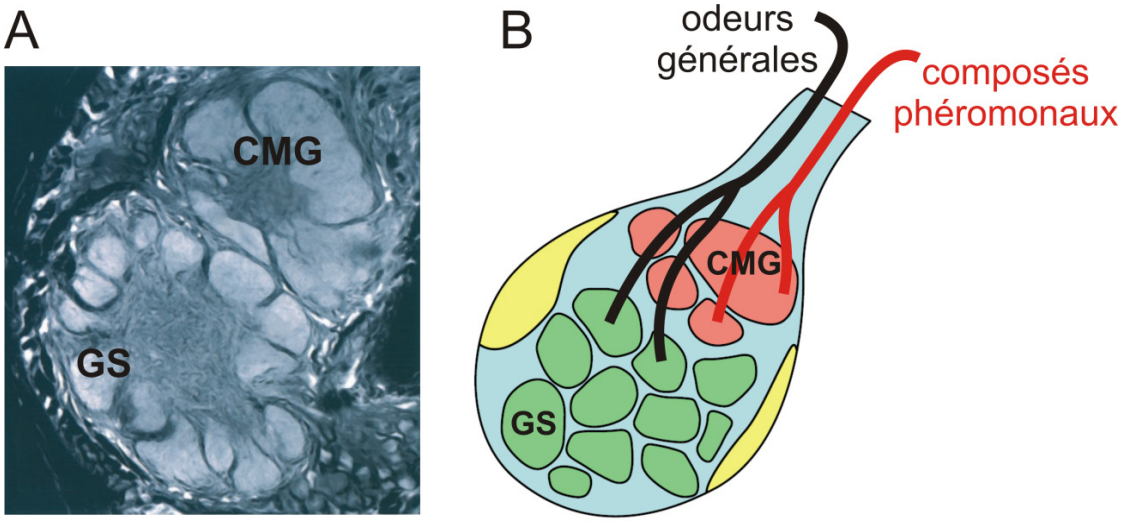


Figure 16 : Le lobe antennaire des hétérocères (papillons de nuit)
A) Section optique d'un lobe antennaire de *Bombyx mori* (Kanzaki *et al.*, 2003). **B**) Schéma d'un LA d'hétérocère (Hansson, 2002). Les NSO sensibles aux composés phéromonaux se projettent dans le complexe macroglomérulaire (CMG) alors que ceux répondants aux odeurs générales se projettent dans des glomérules standards (GS).

Alors que la majorité des glomérules du LA reçoivent les afférences des NSO dans leur cortex (périphérie), les macroglomérules (MG) sont souvent innervés de manière dense, aussi bien en leur périphérie qu'en leur cœur. Ces structures hypertrophiées ont été identifiées chez les mâles de plusieurs ordres d'insectes, tels que les Diptères, les Lépidoptères (papillons de nuit), les Coléoptères, les Blattoptères (blattes) et les Hyménoptères (abeilles, fourmis) (Burrows *et al.*, 1982 ; Arnold *et al.*, 1985 ; Hanson *et al.*, 1992 ; Kondoh *et al.*, 2003 ; Nishikawa *et al.*, 2008 ; Hu *et al.*, 2011). Ce dimorphisme sexuel indique que le système olfactif des insectes peut présenter des adaptations pour la détection et le traitement efficace de composés pertinents pour la survie de l'espèce.

c. Traitement de l'information olfactive au sein du LA

Le lobe antennaire ne représente pas uniquement une zone de relais de l'information olfactive entre les NSO et les neurones de projection (NP). En effet, les réponses obtenues en mesurant l'activité des NSO ne sont pas identiques à celles obtenues par mesure de l'activité des NP (Wilson *et al.*, 2004 ; Bhandawat *et al.*, 2007 ; Deisig *et al.*, 2010). Le signal est donc en partie transformé grâce

à l'intervention d'**interneurones locaux** qui sont définis comme formant des connections entre glomérules, restreintes au lobe antennaire. Le nombre de ces interneurones locaux est très variable d'une espèce à l'autre, et a été estimé à environ 200 chez la Drosophile (Seki *et al.*, 2010), 300 chez le cafard *Periplaneta americana* (Boeckh et al., 1984), 360 chez le papillon de nuit *Manduca sexta* (Homberg, 1988) et jusqu'à 4000 chez l'abeille *Apis mellifera* (Witthöft, 1967). Deux grandes classes d'interneurones locaux ont été identifiées grâce à leurs morphologies distinctes (Fonta *et al.*, 1993 ; Christensen *et al.*, 1993 ; Silbering & Galizia, 2007). Le premier type, le plus répandu chez les insectes, correspond aux **interneurones homogènes** qui innervent tous les glomérules du lobe antennaire de manière uniforme (**Fig. 17, A**). Ce sont des interneurones inhibiteurs le plus souvent GABAergiques, qui exerceraient un contrôle de gain, réduisant ainsi le bruit de fond provoqué par les odeurs ambiantes (Olsen & Wilson, 2008). De plus leur action inhibitrice globale pourrait éviter la saturation du système olfactif en cas de stimulation olfactive intense.

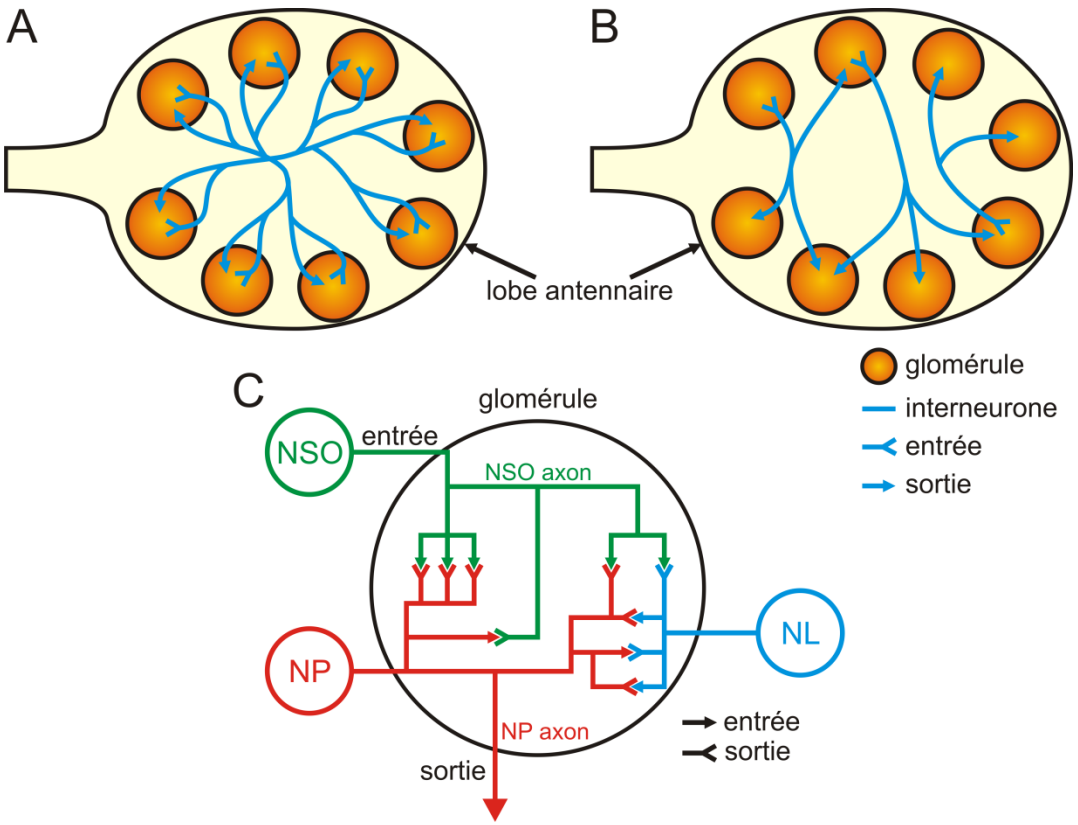


Figure 17 : réseaux neuronaux au sein du lobe antennaire
Deux grandes classes d'interneurones locaux ont été identifiées dans le lobe antennaire des insectes. Les interneurones homogènes (**A**) innervent uniformément les glomérules du lobe antennaire. Les interneurones hétérogènes (**B**) interconnectent les glomérules de façon asymétrique. Au sein d'un glomérule (**C**) les neurones sensoriels olfactifs (NSO), les neurones de projection (NP) et les interneurones locaux (NL) forment un réseau complexe. Les axones des NSO ont de multiples contacts synaptiques avec les NP et les NL. Les NP forment une boucle d'entrée/sortie avec les NSO et les NL (D'après Rybak *et al.*, 2016).

La seconde classe d'interneurones est qualifiée d'**interneurones hétérogènes** car ceux-ci interconnectent une combinaison de glomérules de façon asymétrique (**Fig. 17, B**). Ces neurones

innervent densément un glomérule dans lequel ils prendraient leur information et se projettent dans quelques glomérules de manière éparse (Fonta *et al.*, 1993 ; Galizia & Kimmerle, 2004). Les interneurones hétérogènes permettent l'inhibition latérale entre glomérules, augmentant ainsi le contraste des motifs d'activité glomérulaire induits par différentes odeurs (Sachse & Galizia, 2002 ; Wilson & Laurent, 2005 ; Girardin *et al.*, 2013). Ces deux classes d'interneurones ont donc été définies sur la base de leur motif d'innervation entre glomérules, mais des études ont montré que d'autres sous-populations peuvent être distinguées sur la bases de leurs propriétés électrophysiologiques et pharmacologiques (Seki *et al.*, 2010 ; Chou *et al.*, 2010 ; Tabuchi *et al.*, 2015). Par exemple, des interneurones glutamatergiques inhibiteurs et des interneurones cholinergiques excitateurs ont été observés dans le LA des drosophiles (Shang *et al.*, 2007 ; Liu & Wilson, 2013) alors que des interneurones inhibiteurs histaminergiques sont présents chez les Orthoptères, Hyménoptères et Blattoptères (Loesel & Homberg, 1999 ; Gebhardt & Homberg, 2004 ; Dacks *et al.*, 2010). Au sein des glomérules, les connections entre NSO, interneurones locaux et neurones de projection forment un réseau complexe (Rybak *et al.*, 2016). L'ensemble du réseau (**Fig. 17, C**) participe au traitement de l'information provenant des NSO, ce qui permet d'optimiser la discrimination des odeurs. Par exemple, les motifs d'activité glomérulaire induits par la présentation d'un mélange d'odorants n'est généralement pas égale à la somme des motifs induits par la présentation de ses composants élémentaires. Cette stratégie de codage permet une représentation unique des mixtures d'odorants sans saturer les informations afférentes aux neurones de second ordre, les neurones de projection.

d. Les tractus de neurones de projection

L'information olfactive provenant du LA est relayée aux centres nerveux supérieurs par les neurones de projection (NP), dont la morphologie a subi des transformations importantes au cours de l'évolution (Galizia & Rössler, 2010). Le nombre de neurones de projection est de l'ordre de 200 chez la drosophile (contre 1200 NSO), 860 chez *Manduca sexta* (contre 300 000 NSO) et environ 800 chez *Apis mellifera* (contre 65 000 NSO) ce qui réduit considérablement le nombre de neurones entre l'entrée (NSO) et la sortie (NP) du lobe antennaire. Les aptérygotes (ex : lépisme ou poisson d'argent) et les polynéoptères (ex : criquet) ne possèdent qu'un seul tractus principal de NP en position médiane (**Fig. 18**), appelé **m-ALT** (**medial Antennal Lobe Tract**) (Ignell *et al.*, 2001 ; Anton *et al.*, 2002 ; Strausfeld, 2009). Ce tractus est composé de NP multiglomérulaires, c'est-à-dire prenant l'information de plusieurs glomérules simultanément, qui acheminent l'information vers les corps pédonculés, puis vers la corne latérale. Parmi les polynéoptères, les orthoptères (ex : les criquets) pourraient posséder également un **tractus médio-latéral** (**ml-ALT**), composé de NP également multiglomérulaires, dont les projections dans les aires supérieures restent à déterminer.

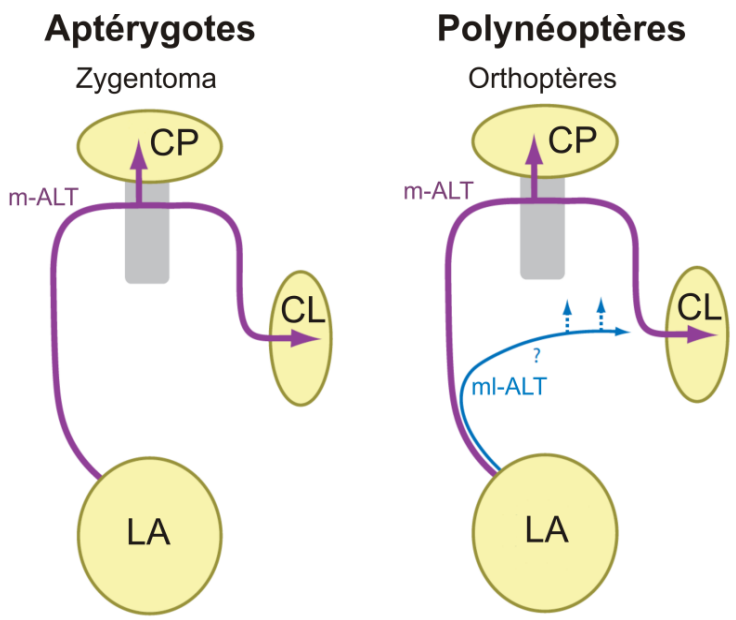


Figure 18 : Les tractus de neurones de projection des Aptérygotes et des Polynéoptères
Les aptérygotes et les polynéoptères possèdent un tractus de neurones de projection multiglomérulaires qui achemine l'information olfactive du lobe antennaire (LA) vers les corps pédonculés (CP) puis vers la corne latérale (CL). Il traverse la partie médiane du protocérébron (m-ALT). Un second tractus multiglomérulaire traversant le protocébron de sa partie médiane vers sa partie latérale (ml-ALT) pourrait exister chez les orthoptères (d'après Galizia & Rössler, 2010).

Les Holométaboles montrent une complexité plus importante des tractus de NP, révélant une nette évolution de leur morphologie. Les Lépidoptères (Homberg *et al.*, 1988 ; Rø *et al.*, 2007) et les diptères (Stocker *et al.*, 1990 ; Tanaka *et al.*, 2004) possèdent un tractus médian (m-ALT) de NP uniglomérulaires, c'est-à-dire arborisant chacun un seul glomérule, et un tractus médio-latéral se projetant soit uniquement vers la corne latérale (Lépidoptères) soit vers les deux aires supérieures (Diptères) (**Fig. 19**). De plus, on observe l'apparition d'un tractus secondaire situé en position latérale, (**lateral Antennal Lobe Tract, l-ALT**). Ce tractus de NP multiglomérulaires (et uniglomérulaires chez les Diptères) se projette principalement dans la corne latérale, avec une connexion secondaire dans les corps pédonculés chez les Lépidoptères.

Chez les Hyménoptères, les voies de projection de l'information olfactive montrent une organisation remarquablement plus complexe par rapport à celle des autres ordres d'insectes (Mobbs, 1982 ; Abel *et al.*, 2001 ; Zube *et al.*, 2008 ; Rössler & Zube, 2011). Ces insectes possèdent un tractus médian (m-ALT) de NP uniglomérulaires, similaire à celui des autres Holométaboles, acheminant l'information vers les corps pédonculés puis vers la corne latérale. Ils possèdent ensuite 3 tractus médio-latéraux de NP multiglomérulaires contre un unique tractus chez les autres Holométaboles. Enfin, ils possèdent un tractus latéral (l-ALT) de NP uniglomérulaires qui acheminent l'information dans le sens inverse du tractus médian, c'est-à-dire d'abord vers la corne latérale puis vers les corps pédonculés. Le tractus médian arborise uniquement les glomérules de la face dorsale du lobe antennaire alors que le tractus latéral arborise uniquement les glomérules de la face ventrale (Kirschner *et al.*, 2006 ; Zube *et al.*, 2008). Les deux voies se projettent de plus dans des zones partiellement ségrégées des aires supérieures, formant ainsi deux sous-systèmes dédiés au traitement de l'information olfactive.

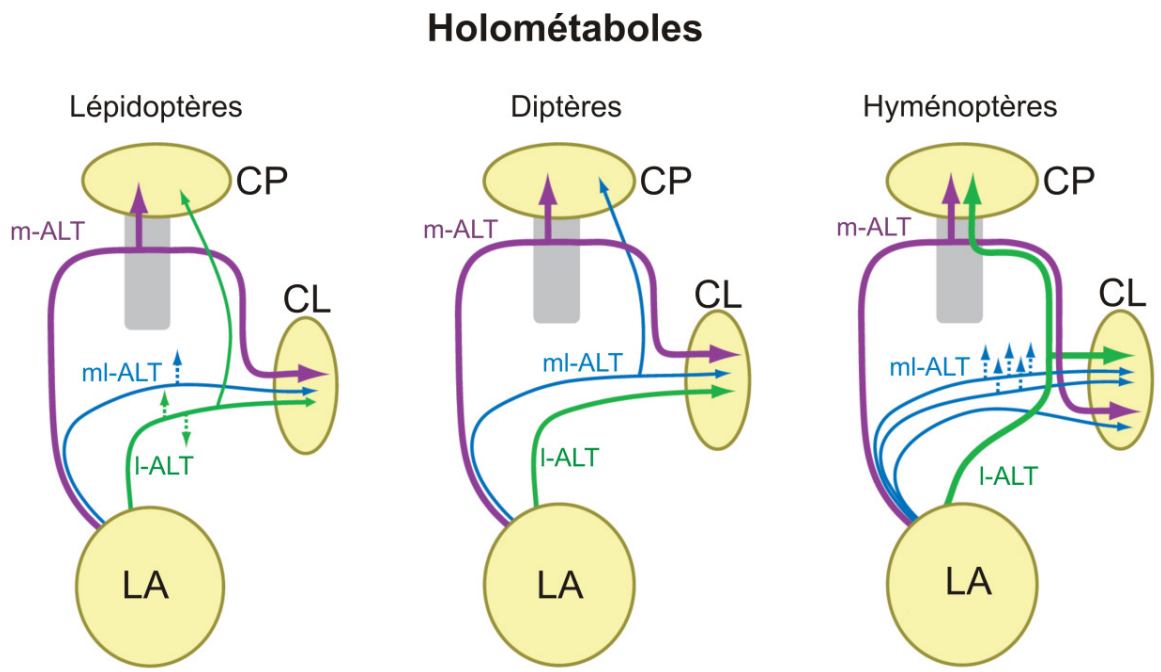


Figure 19 : Les tractus de neurones de projection des Holométaboles
Les Lépidoptères, les Diptères et les Hyménoptères possèdent un tractus de neurones de projection uniglomérulaires traversant la partie médiane du protocérébron (m-ALT) et se projetant dans les corps pédonculés (CP) puis dans la corne latérale (CL). Ils possèdent également un (ou 3 chez les Hyménoptères) tractus médio-latéral (ml-ALT) acheminant l'information uniquement dans la corne latérale (et dans les corps pédonculés chez les Diptères). Les Hyménoptères sont le seul ordre possédant un tractus latéral (l-ALT) d'égale importance par rapport au m-ALT, acheminant l'information vers la CL puis vers les CP (D'après Galizia & Rössler, 2010).

e. Les aires supérieures de traitement de l'information olfactive

L'information olfactive acheminée par les neurones de projection doit ensuite être intégrée aux autres modalités sensorielles et/ou mémorisée pour que l'animal puisse produire un comportement en adéquation avec son contexte interne (physiologie) et les informations externes (environnement). Chez les insectes, deux structures sont communément associées à ces fonctions, les corps pédonculés et la corne latérale (Tanaka *et al.*, 2004). Les **corps pédonculés** sont des structures symétriques situées dans la partie rostrale du cerveau de l'insecte, décrites pour la première fois en 1850 par Félix Dujardin. Dans une étude comparative, Il observa que les dimensions de ces structures sont bien plus importantes chez les insectes sociaux que chez les insectes solitaires (Dujardin, 1850). En considérant que « l'intelligence » d'un animal est liée à son niveau d'organisation sociale, il associa les corps pédonculés à cet attribut. La structure interne des corps pédonculés fut décrite plus tard, en 1896 par Frederick Kenyon qui donna son nom aux neurones qui les composent (Kenyon, 1896a, b). Les **cellules de Kenyon** sont donc des neurones intrinsèques aux corps pédonculés qui forment une ou deux coupes, appelées calices, dans chaque hémisphère cérébral. Chez la plupart des espèces, les calices reçoivent des afférences olfactives, provenant directement du lobe antennaire, et des afférences visuelles, souvent de manière indirecte (Strausfeld, 1998 ; Farris, 2005). Les corps cellulaires des cellules de Kenyon sont situés près des

calices, où les dendrites prennent leur information. Les axones de ces neurones se projettent en faisceaux parallèles dans le protocérébron, formant ainsi le pédoncule, puis se ramifient en général en 2 branches qui forment le lobe vertical (α) et le lobe horizontal (β). Chez différents insectes plusieurs classes de cellule de Kenyon (CK I, II et III) ont été identifiées grâce à leur innervation dans distincte régions des calices, et leur projection dans différents lobes ou sous-division des lobes (Strausfeld, 2002 ; Strausfeld *et al.*, 2003 ; Tanaka *et al.*, 2004 ; Farris, 2005 ; Fahrbach, 2006). Au niveau des lobes, les cellules de Kenyon contactent des neurones extrinsèques qui se projettent dans différentes régions du cerveau, telles que le protocérébron, le corps central ou la corne latérale (Rybak & Menzel, 1993 ; Ito *et al.*, 1998).

Au sein des calices, chaque NP olfactif forme des contacts synaptiques avec de nombreuses cellules de Kenyon, et chaque cellule de Kenyon reçoit les projections de nombreux NP (**Fig. 20, A**) (Heisenberg, 2003). Par exemple, il a été estimé chez le criquet *Schistocerca americana* qu'en moyenne chaque cellule de Kenyon est en contacte avec 50% des NP (Jortner *et al.*, 2007).

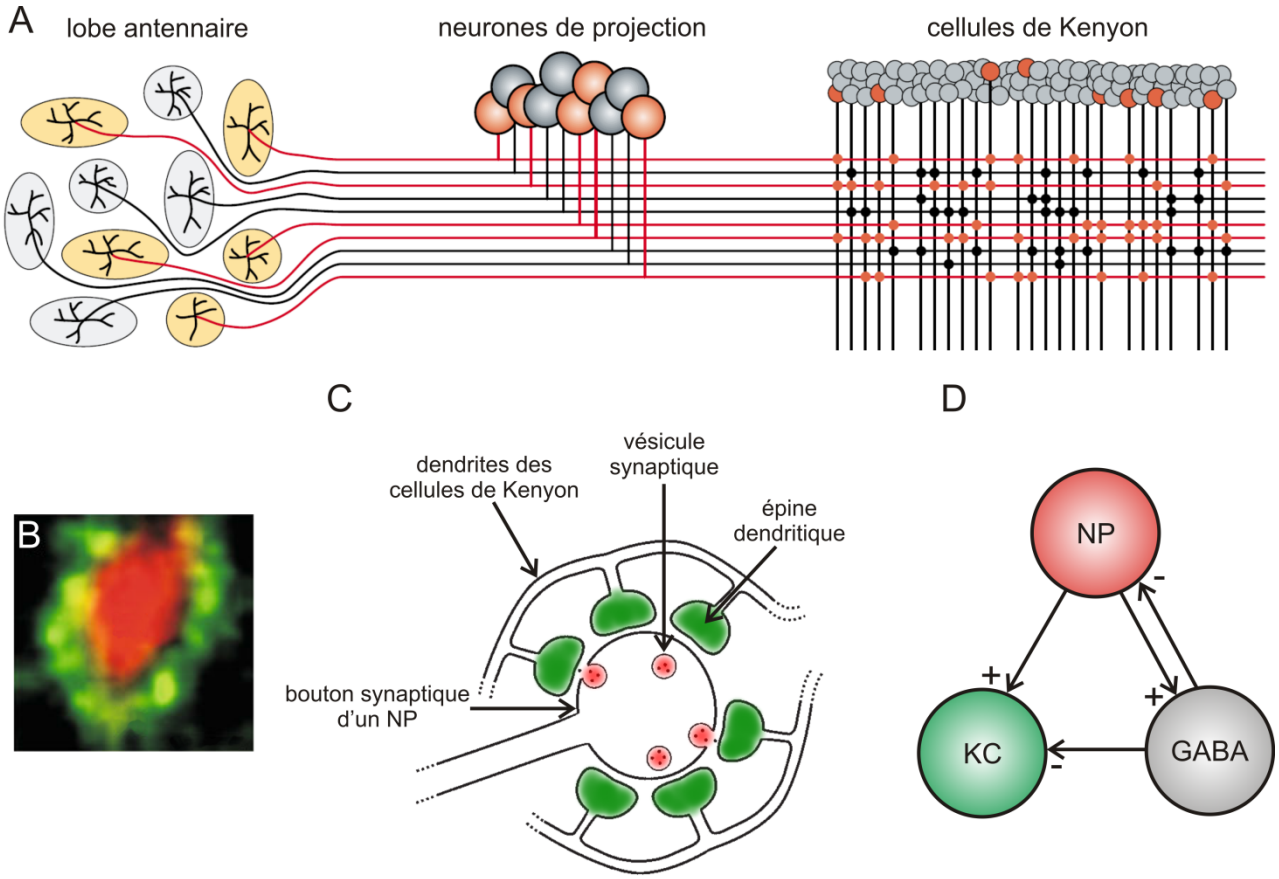


Figure 20 : Réseaux neuronaux au sein des corps pédonculés
A) Chaque neurone de projection entre en contact avec plusieurs cellules de Kenyon, et chaque cellule de Kenyon reçoit les projections de plusieurs NPs au sein des calices des corps pédonculés (D'après Heisenberg, 2003). Les cellules de Kenyon opèrent donc une lecture combinatoire des réponses des neurones de projection. **B**) Les terminaisons synaptiques des neurones de projection (NP) (rouge) entre en contact avec plusieurs cellules de Kenyon (vert) dans une structure appelée microglomérule (**C**). **D**) Ils y forment un réseau synaptique complexe avec des neurones rétrogrades GABAergic inhibiteurs (GABA) (D'après Ganeshina & Menzel, 2001).

L'observation des terminaisons synaptiques des NP en microscopie électronique révéla qu'ils y recrutent également des neurones extrinsèques GABAergiques (inhibiteurs) formant des boucles de retour qui constituent des réseaux synaptiques locaux appelés microglomérules (**Fig. 20, B, C**) (Ganeshina & Menzel, 2001 ; Yasuyama *et al.*, 2002). Les cellules de Kenyon sont peu actives spontanément, et ne peuvent être excitées que par l'activation concomitante de plusieurs NP (Turner *et al.*, 2008). Elles détectent ainsi une coïncidence d'activité entre plusieurs NP dans un réseau excitateur et inhibiteur d'une grande complexité (**Fig. 20, A, D**).

Cette configuration suggère que les microglomérules opèrent une lecture combinatoire des réponses des NP. Du fait du grand nombre de cellules de Kenyon contenues dans les corps pédonculés, cette opération rend la représentation olfactive dans les calices plus clairsemée (Laurent, 2002 ; Wang *et al.*, 2004 ; Szyszka *et al.*, 2005). Ainsi, alors qu'environ 60 % des neurones de projection répondent à une seule odeur, environ 92 % des cellules de Kenyon sont spécifiques d'une odeur (Szyska *et al.*, 2005). En clairsemant la représentation olfactive, le nombre important de cellules de Kenyon augmente le nombre de combinaisons envisageables et devrait permettre un codage spécifique de mélanges complexes (floraux, p. ex) ou de la concentration des odeurs. En plus de leur rôle important dans le codage de l'information olfactive, les corps pédonculés sont impliqués dans l'apprentissage et le stockage de la mémoire olfactive (Strausfeld, 1998 ; Pascual & Préat, 2001 ; Hourcade *et al.*, 2010 ; Heisenberg, 2003) ainsi que dans la résolution de tâches cognitives complexes (Devaud *et al.*, 2015).

La **corne latérale**, quant à elle est considérée comme un centre pré-moteur important pour l'exécution de comportements innés stéréotypés (Hammer, 1997 ; Jefferis *et al.*, 2007 ; Yamagata *et al.*, 2009 ; Li & Liberles, 2015). Cette structure reçoit les afférences des neurones de projection selon une organisation spécifique qui reflète la valence innée de l'odeur qu'ils codent (Seki *et al.*, 2005 ; Fisek & Wilson, 2014 ; Knaden & Hansson, 2014). Par exemple, chez la drosophile comme chez *Apis mellifera* ou *Bombyx mori* des enregistrements fonctionnels semblent indiquer que la représentation olfactive dans la corne latérale serait dépendante de la signification biologique des odeurs plutôt que de leur structure chimique (Kanzaki *et al.*, 2003 ; Roussel *et al.*, 2014). Notamment, les odeurs attractives (à valence positive) et les odeurs répulsives (à valence négative) induisent une activité dans des régions partiellement ségrégées de la corne latérale (Min *et al.*, 2013 ; Strutz *et al.*, 2014). Puisque cette structure reçoit généralement les afférences de NP multiglomérulaires, elle pourrait également être impliquée dans le codage précis de l'intensité des stimuli (Gupta & Stopfer, 2012). Les voies neuronales quittant la corne latérales sont, à l'heure actuelle, très peu connues, mais on pense que de manière indirecte elles entre en contact avec des neurones descendants pré-moteurs (Okada *et al.*, 2003).

4) Spécificité du système olfactif des Hyménoptères

L'ordre des Hyménoptères comprend de très nombreuses espèces (environ 230 000 décrites), aux modes de vie très variés comprenant aussi bien des espèces solitaires phytophages, des parasitoïdes (majoritairement entomophages) ou des espèces sociales vivant en colonie. Les pressions de sélection agissant sur les différentes caractéristiques du système olfactif varient en fonction du sexe et du mode de vie, ce qui engendre une grande diversité dans l'organisation et la complexité des voies olfactives des Hyménoptères (Strausfeld & Reisenman, 2009 ; Kleineidam & Rössler, 2009 ; Hansson & Stensmyr, 2011 ; Streinzer *et al.*, 2013). Environ 100 millions d'années d'évolution séparent les premiers Hyménoptères, dont l'apparition est estimé au Trias, et les Hyménoptères sociaux apparus au Crétacé (Grimaldi & Engel, 2005). Par conséquent, de nombreuses adaptations neuronales uniques parmi les insectes ont pu être observées chez les espèces de cet ordre.

a. Périphérie du système olfactif

A la périphérie du système olfactif, la forme, le nombre, la densité, et la distribution des différents types sensillaires sont extrêmement variables chez les Hyménoptères (Walther, 1983). Par exemple, chez *Syntexis libocedrii* (Symphyta ou mouche à scie) les sensilles sont distribuées sur l'antenne de façon très clairsemée alors qu'elles sont très densément distribuées sur l'antenne des Vespoidea (ex : fourmis, frelons) (Walther, 1983). Du point de vue de la structure, la morphologie des sensilles placodées présentes chez les abeilles et les guêpes aurait progressivement divergé en se redressant chez les fourmis pour donner des sensilles trichoïdes courbées, qui seraient donc homologues aux placodes (Basibuyuk & Quicke, 1999). De plus, alors que chez de nombreux insectes les sensilles olfactives contiennent un nombre limité d'OSN (de 1 à 10), les Hyménoptères présentent plusieurs types sensillaires abritant de très nombreux OSN. Ainsi, les sensilles placodées peuvent présenter plusieurs dizaines d'OSN (abeilles, Esslen & Kaissling, 1976) et certaines sensilles basiconiques contiennent plus d'une centaine d'OSN (fourmis, Nakanishi *et al.*, 2009).

En ce qui concerne les aspects moléculaires de la détection des odeurs, des expansions importantes du répertoire des récepteurs olfactifs semblent avoir eu lieu chez les Hyménoptères (Engsontia *et al.*, 2015 ; Zhou *et al.*, 2015). Cette observation découle aussi bien des études génomiques ou transcriptomiques proposant des listes d'OR (Robertson & Wanner, 2006 ; Smith *et al.*, 2011a, b ; Brand et *al.*, 2015 ; Zhou *et al.*, 2015), que des études de neuroanatomie comptant le nombre de glomérules présents chez différentes espèces, du fait de la relation 1 récepteur olfactif / 1 glomérule. A ce jour, le plus petit nombre de glomérules chez un Hyménoptère à été observé chez les *Neodiprion* (Symphyta) qui sont considérés comme représentatifs des Hyménoptères basaux, avec environ 44 glomérules (Dacks & Nighorn, 2011). Le nombre de glomérules, et donc de récepteurs olfactifs, semble ensuite avoir grandement augmenté car de nombreux Hyménoptères possèdent

plus de 100 glomérules, jusqu'à atteindre un maximum de 630 chez la fourmis *Apterostigma cf. mayri* (Flanagan & Mercer, 1989 ; Smid *et al.*, 2003 ; Kelber *et al.*, 2009 ; Roselino *et al.*, 2015 ; van der Woude & Smid 2015).

En théorie, une multiplication des récepteurs olfactifs peut être associée à une augmentation des capacités de discrimination olfactive de l'espèce (Kaupp, 2010). Puisque les insectes sociaux doivent être capables de discriminer un grand nombre de composés phéromonaux servant à la communication intraspécifique, l'augmentation des capacités de discrimination chez les Hyménoptères pourrait avoir un lien étroit avec les multiples apparitions de l'eusocialité au sein de cet ordre (Zhou *et al.*, 2015). Ainsi la structure et le fonctionnement du circuit olfactif ont fait l'objet de nombreuses études, principalement chez des Hyménoptères sociaux, comme les abeilles et différentes espèces de fourmis.

b. Le lobe antennaire

Similairement aux insectes de différents ordres, le lobe antennaire des Hyménoptères peut présenter un dimorphisme sexuel correspondant à des adaptations en lien avec à des différences comportementales entre les mâles et les femelles (Nishikawa *et al.*, 2008 ; Nishikawa *et al.*, 2012 ; Streinzer *et al.*, 2013). Par exemple, un macroglomérule observé chez les faux bourdons (mâles d'abeille *A. mellifera)* est spécifiquement activé par la présentation du 9-ODA (9-Oxodec-2-enoic acid), composé majoritaire de la phéromone sexuelle de reine, qui induit une attraction des mâles (**Fig. 21**) (Gary, 1962 ; Sandoz, 2006). L'hypertrophie spécifique de ce glomérule chez les mâles semble être lié à une multiplication des OSN (Esslen & Kaissling, 1976 ; Arnold *et al.*, 1985) ainsi qu'à une surexpression du récepteur olfactif sensible au 9-ODA (Wanner *et al.*, 2007). Cette adaptation du lobe antennaire répond sans doute à la nécessité pour les mâles de repérer efficacement les reines fertiles lors de leur vol nuptial.

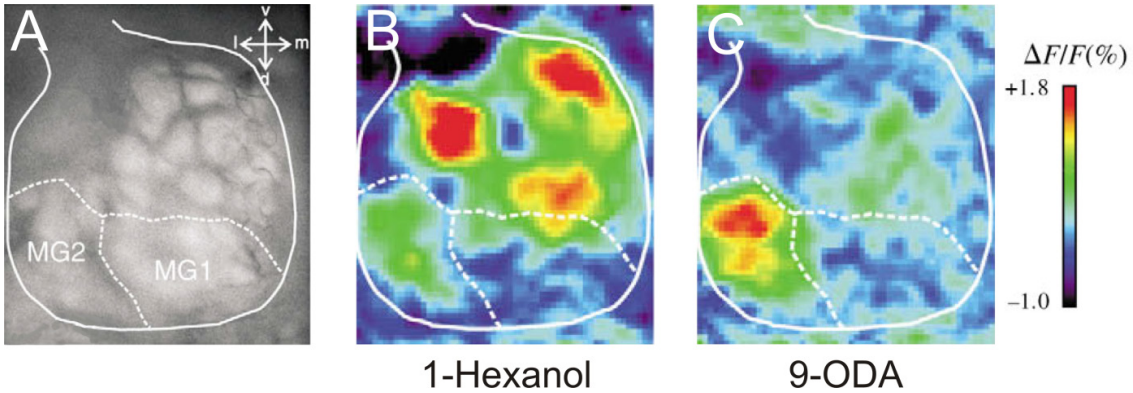


Figure 21 : Activation spécifique du macroglomérule des mâles d'abeille *A. mellifera*
Le lobe antennaire des mâles d'abeille (**A**) contient plusieurs macroglomérules. Ceux-ci ne sont pas activés par la présentation d'odeurs générales comme le 1-hexanol (**B**). En revanche, le composé majoritaire de la phéromone de reine, le 9-ODA, active spécifiquement le macroglomérule 2 (**C**). (D'après Sandoz, 2006).

Une particularité importante des espèces sociales est l'existence d'un polyéthisme correspondant à des rôles et à des comportements distincts entre les membres de la colonie. Chez les Hyménoptères, ces différences comportementales peuvent être associées à un polymorphisme du lobe antennaire. En effet, le dimorphisme entre individus du même sexe n'a, pour l'instant, été observé que chez des espèces sociales. Ainsi, chez les fourmis champignonnistes (*Atta sp.*), qui présentent des castes morphologiquement différenciées, la phéromone de piste est perçue différemment par les grandes et les petites ouvrières (Kleineidam *et al*., 2007). Il a été observé que seules les grandes ouvrières des genres *Atta* et *Acromyrmex* possèdent un glomérule hypertrophié dédié au traitement de cette phéromone ce qui a permis de démontrer pour la première fois l'existence de MG chez des individus non sexués (Kleineidam *et al*., 2005 ; Kelber *et al*., 2009 ; Kuebler *et al*., 2010).

Il est cependant important de noter que tous les MG ne sont pas forcément des adaptations spécifiques d'un sexe ou d'une caste. Un MG de forme et de position identiques entre les différentes castes (ouvrières, mâles, et reines) de *Melipona scutellaris* (Apidae, Meliponini), soulignant une probable analogie de fonction, a été récemment identifié (Roselino *et al*., 2015). De plus, bien que le traitement en ligne dédiée semble être assez répandu chez les insectes dans le cas des phéromones sexuelles, la présence de MG chez les ouvrières fait pour l'instant figure d'exception. L'étude de la représentation neuronale des phéromones, par la méthode d'imagerie calcique *in vivo*, dans le LA d'ouvrières, a pu mettre en évidence que les phéromones de piste et d'alarme induisent l'activation d'une combinaison de glomérules, non spatialement ségrégés, chez la fourmi *Camponotus floridanus* (Zube *et al*., 2008), au même titre qu'un grand nombre de phéromones testées chez *A. mellifera* (Sandoz *et al*., 2007 ; Wang *et al*., 2008 ; Carcaud *et al*., 2015).

Une autre particularité du lobe antennaire des Hyménoptères est l'organisation des glomérules en grappes (ou clusters) distinctes. Chez l'abeille *A. mellifera*, le nerf antennaire se divise à l'entrée du LA et forme 4 tractus olfactifs (T1-T4) (Suzuki, 1975 ; Kirschner *et al*., 2006 ; Nishino *et al*., 2009) alors qu'ils sont au nombre de 7 (T1-T7) chez les fourmis *Camponotus japonicus* et *C. floridanus* (Zube & Rössler, 2008 ; Nakanishi *et al*., 2010) et au nombre de 6 (T1-T6) chez *Atta vollenweideri* (Kelber *et al*., 2010). Actuellement, on ne sait pas si certaines de ces grappes de glomérules observées chez des espèces différentes sont homologues entre elles et si elles ont des fonctions similaires. Cependant, ces espèces possèdent toutes, dans la partie la plus dorsale du LA, des glomérules densément innervés en leurs cœurs par des axones relativement épais (glomérules du tractus T4 chez l'abeille et du tractus T7 chez les fourmis camponotes) et recevant exclusivement les afférences du nerf antennaire ventral (Nishino *et al*., 2009 ; Nakanishi *et al*., 2010). Par analogie morphologique avec les glomérules dorsaux de la blatte *Periplaneta americana* dont la fonction a été caractérisée grâce à des enregistrements électrophysiologiques (Nishikawa *et al*., 1995 ; Nishino *et al*., 2003), il a été suggéré (Zube *et al*., 2008 ; Nishino *et al*., 2009 ; Nakanishi *et al*., 2010) que ces glomérules recevraient les

NSO des sensilles ampullacea, coelocapitulaires et coeloconiques impliquées dans la détection du CO_2 (Kleineidam *et al*., 2000), de l'hygrométrie (Yokohari *et al*., 1982) et de la température (Ruchty *et al*., 2009 ; Nagel & Kleineidam, 2015). Au regard de l'importance de ces fonctions pour la survie des individus, certains groupes de glomérules impliqués dans des tâches vitales pourraient donc être conservés entre espèces. Cependant, aucunes données démontrant de telles homologies n'ont été produites à ce jour.

Une possible spécialisation de clusters glomérulaires pour la détection de composés particuliers est également étayée par la présence dimorphique d'une grappe de petits glomérules portée par un tractus sensoriel (le T6) chez *C. japonicus, C. floridanus* et *A. vollenweideri* (**Fig. 22**). Contrairement au reste du LA, ces glomérules reçoivent les afférences de NSO logés dans les sensilles basiconiques des femelles, et ne sont pas neuromodulés par la sérotonine (5-HT) (Zube & Rössler, 2008 ; Nakanishi *et al*., 2009 ; Kelber *et al*., 2010). Ces glomérules semblent former un sous-système olfactif relativement indépendant, puisqu'ils sont interconnectés par des interneurones confinés à ce cluster et leurs neurones de projection se projettent dans des zones ségrégées des aires supérieures (corne latérale et corps pédonculés) (Nishikawa *et al*., 2012).

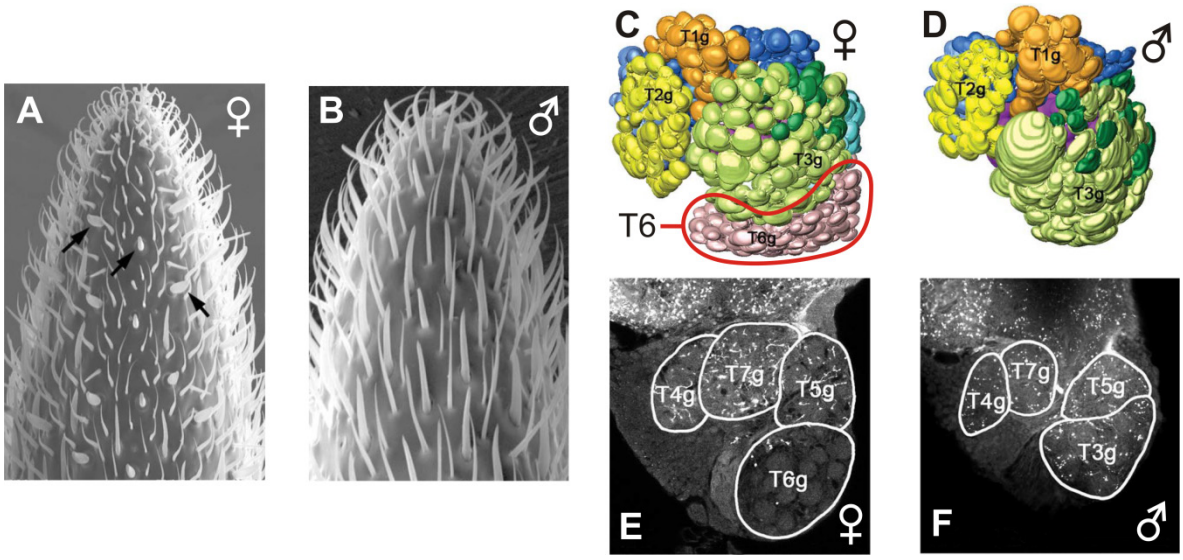


Figure 22 : Système spécifique des sensilles basiconiques
L'antenne des femelles de *Camponotus japonicus* porte des sensilles basiconiques (**A** : flèches noires) qui sont absentes sur l'antenne des mâles (**B**). Les NSO de ces sensilles se projettent spécifiquement dans un groupe de petits glomérules du lobe antennaire des femelles (**C** : T6) également absent dans le lobe antennaire des mâles (**D**). Des marquages immuno-histochimiques ont révélé que contrairement au reste du lobe antennaire, ce groupe de glomérules ne reçoit pas de fibres sérotoninergiques (5-HT) (**E, F**). (D'après Nakanishi *et al*., 2009 et 2010).

L'enregistrement électrophysiologique des sensilles basiconiques de fourmis componotes laisse supposer que ce complexe serait dédié au traitement des hydrocarbures cuticulaires, utilisés chez les insectes sociaux pour la reconnaissance des congénères (Ozaki *et al*., 2005 ; Sharma *et al*., 2015). Une étude montrant que les sensilles basiconiques des ouvrières d'abeille *A. mellifera* se

projettent préférentiellement dans les glomérules du T3, suggère qu'un tel sous-système existerait partiellement chez l'abeille (Kropf *et al*., 2014). Cependant, il n'est pas clairement démontré que ces clusters de glomérules, présents chez l'abeille et les fourmis, soient homologues.

Ces analogies dans l'organisation topographique du LA de différentes espèces suggèrent une histoire évolutive marquée par l'apparition et la perte de glomérules directement associées à un ajout ou à une perte de la capacité de détection et/ou de discrimination olfactive pour certains composés. Etant donné que le coût énergétique important lié au maintien des structures neuronales agit comme pression de sélection sur les systèmes sensoriels (Niven & Laughlin, 2008), l'organisation neuroanatomique du LA devrait coïncider avec la prévalence de certains composés odorants dans la biologie de chaque espèce.

c. Les neurones de projection

Comme mentionné précédemment (partie III, 3, d), chez les Hyménoptères les glomérules de la face ventrale et de la face dorsale du LA sont distinctement innervés par deux tractus de neurones de projection uniglomérulaires, qui se projettent vers les centres olfactifs supérieurs (la corne latérale et les corps pédonculés), formant ainsi deux sous-systèmes olfactifs (Abel *et al.*, 2001 ; Galizia & Rössler, 2010 ; Rössler & Zube, 2011). Des études fonctionnelles menées chez l'abeille *A. mellifera* ont révélé que les deux sous-systèmes traitent des informations principalement redondantes avec différentes propriétés de codage, ce qui suggère un traitement parallèle du message olfactif (Rössler & Brill, 2013 ; Carcaud, 2013). Les motifs d'activité glomérulaire observés en imagerie calcique ont montré que la voie médiane distingue mieux le groupe fonctionnel des odeurs alors que la voie latérale distingue mieux la longueur de chaîne carbonée (Carcaud *et al*., 2012). De plus, des enregistrements électrophysiologiques ont montré que les neurones du système m-ALT répondent plus lentement et plus spécifiquement aux odeurs que les neurones du système l-ALT (Müller *et al*., 2002 ; Brill *et al*., 2013).

Cette adaptation du système olfactif pour traiter en parallèle les différents attributs d'une odeur est présente chez la majorité des Hyménoptères (fourmis, abeilles et guêpes) à l'exception de certaines espèces de Symphytes (Dacks & Nighorn 2010 ; Rössler & Zube, 2011). Selon certains auteurs, ce double système olfactif serait donc apparu et aurait évolué chez des Hyménoptères ancestraux, et pourrait représenter une préadaptation à des exigences élevées en matière de discrimination olfactive, nécessaires pour certains styles de vie comme le parasitisme ou la socialité (Galizia & Rössler, 2010). Cette conjecture est soutenue par une étude récente qui témoigne d'un traitement différentiel des différentes catégories de phéromones impliquées dans la régulation sociale chez l'abeille. Ainsi les phéromones de couvain seraient traitées par le m-ALT alors que les phéromones de reine seraient traitées par le l-ALT (Carcaud *et al*., 2015).

d. Les centres supérieurs

Parmi les insectes, les Hyménoptères sociaux présentent des corps pédonculés particulièrement volumineux. De plus, contrairement aux autres ordres d'insectes, les calices sont morphologiquement et fonctionnellement subdivisés en trois régions, les lèvres, le collier et l'anneau basal (Mobbs, 1982 ; Ehmer & Gronenberg, 2002). Les lèvres et la partie interne de l'anneau basal reçoivent les informations olfactives, alors que le collier et la partie externe de l'anneau basal reçoivent les informations visuelles (Gronenberg, 2001). Au sein des lèvres, les NP du tractus latéral (l-ALT) se projettent uniquement dans la partie centrale, alors que ceux du tractus médian (m-ALT) se projettent dans la partie extérieure et, plus légèrement, dans la partie centrale (Kirschner *et al.*, 2006 ; Zube *et al.*, 2008). Cette ségrégation des terminaisons synaptiques des neurones de projection se retrouve également au niveau de la corne latérale.

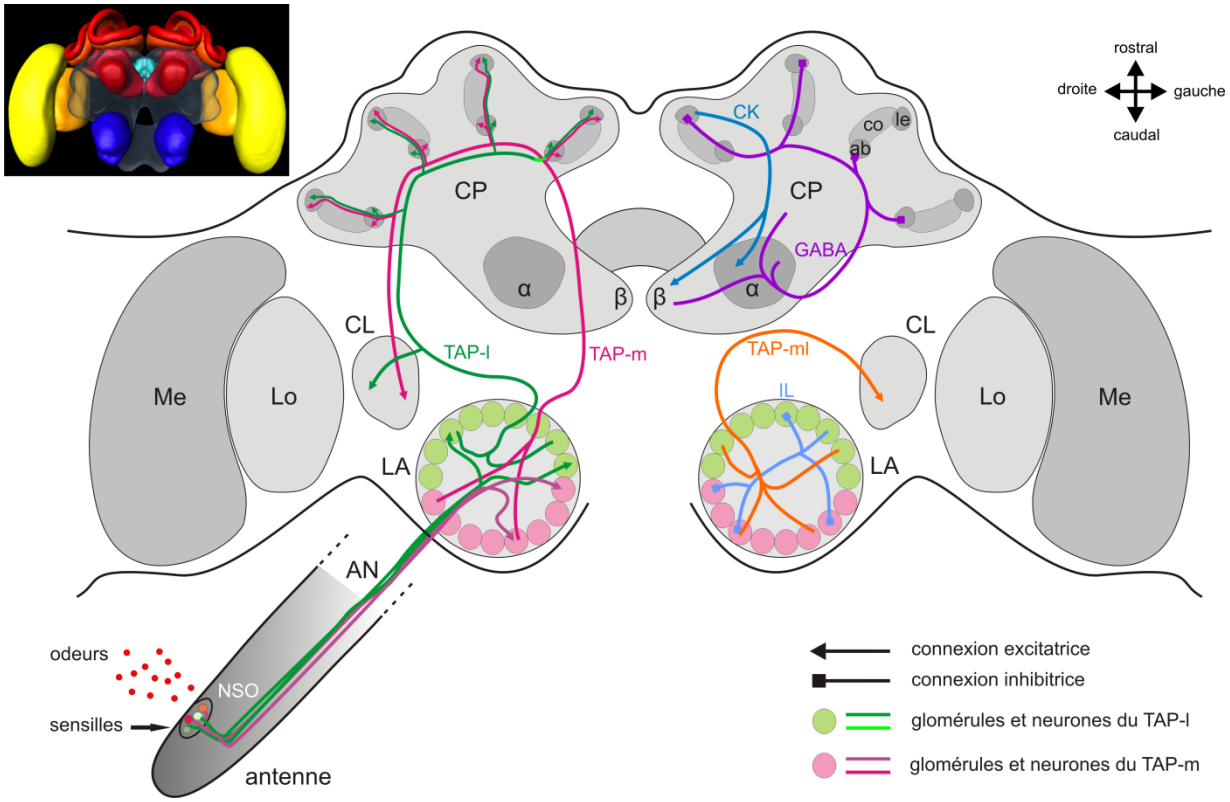


Figure 23 : Le système olfactif des Hyménoptères (l'exemple du cerveau de l'abeille)
Pour plus de clarté, différents types neuronaux sont présentés séparément dans les deux hémisphères cérébraux. Dans l'hémisphère gauche sont indiquées les principales voies neuronales impliquées dans la transmission de l'information olfactive jusqu'aux corps pédonculés. Dans l'hémisphère droit sont présents les circuits neuronaux des corps pédonculés et les principales connections inhibitrices. Le lobe antennaire (LA) reçoit les afférences des neurones sensoriels olfactifs (NSO) qui détectent les odeurs au niveau des sensilles de l'antenne. Au sein du LA, les glomérules sont interconnectés par les interneurones locaux (IL) qui permettent un premier traitement de l'information. Les neurones de projection arborisent le LA et acheminent l'information olfactive vers les centre supérieurs, les corps pédonculés (CP) et la corne latérale (CL). Les cellules de Kenyon (CK) formeent des contacts avec les terminaisons synaptiques des neurones de projection au sein des CP. Les axones des CK forment deux lobes α et β. Au sein des CP les neurones extrinsèques (GABAergiques) forment une boucle rétrograde en connectant le pédoncule et les lobes (α et β) avec les calices qu'ils inhibent (D'après Sandoz, 2011).

On pourra donc se demander quel degré de similarité existe-t-il entre le cerveau du frelon *V. velutina* et celui d'autres Hyménoptères sociaux tels que les abeilles et les fourmis. Notamment il serait intéressant d'évaluer la présence de dimorphisme entre les différentes castes d'une colonie et d'examiner les possibles adaptations le long de la voie olfactive, commune à ces Hyménoptères sociaux.

5) L'olfaction chez les frelons

a. Substances sémiochimiques dans le genre *Vespa*

L'observation du comportement des frelons laisse penser qu'ils disposeraient d'un système olfactif extrêmement performant. D'une part, ils exploitent un large éventail de ressources alimentaires aussi bien d'origine végétale (fruits, nectar, sève et miellat) que d'origine animale (prédation diversifiée) ou de synthèse (boissons sucrées), ce qui requiert la perception d'une gamme étendue d'odeurs différentes (Raveret Richter, 2000). D'autre part, leur niveau élevé d'organisation sociale (espèce eusociale) nécessite des moyens de communication efficaces pour coordonner l'activité des membres de la colonie. La communication chimique est la stratégie la plus répandue chez les insectes sociaux, y compris chez les frelons, qui doivent être capables de détecter et discriminer ces phéromones. De nombreuses glandes exocrines susceptibles de produire ces composées chimiques ont été décrites chez les guêpes sociales (**Fig. 21**) mais peu de molécules ayant un effet comportemental ont été identifiées à ce jour (Bruschini *et al.*, 2010).

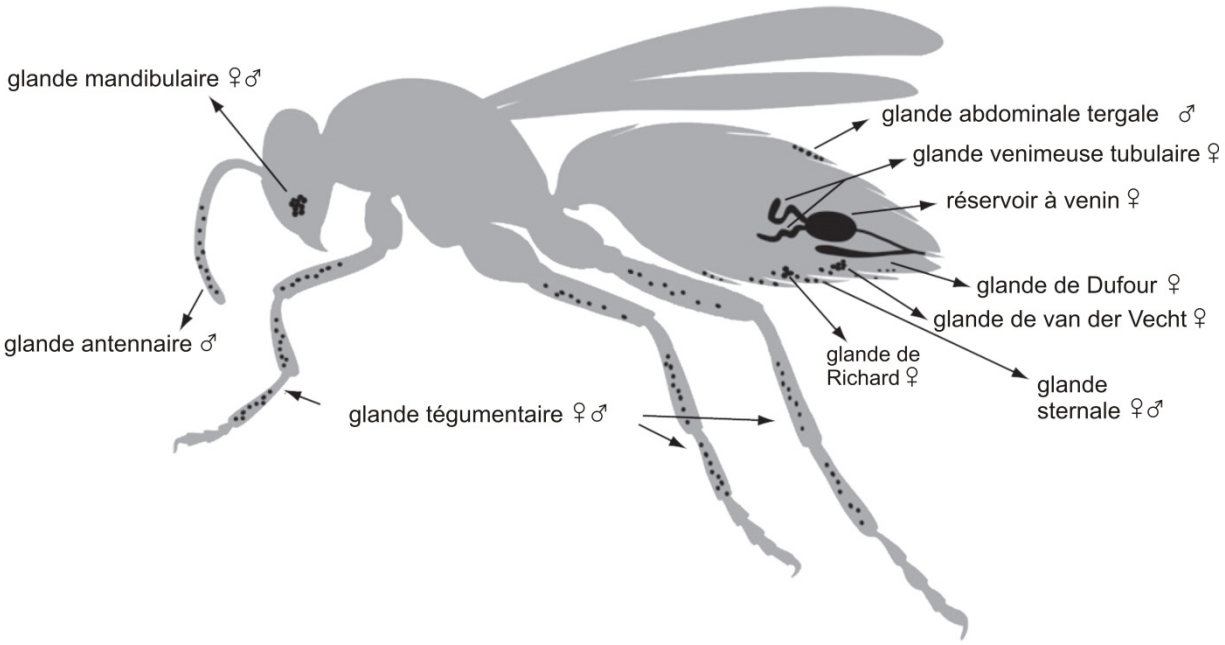


Figure 24 : Glandes exocrines des guêpes sociales
Représentation schématique des différentes glandes exocrines susceptibles d'être présentes chez une guêpe sociales générique. La taille relative et la localisation des glandes sont indiquées à titre approximatif. La présence de chaque glande chez les mâles et/ou chez les femelles est indiquée par des symboles (D'après Bruschini *et al.*, 2010).

Chez *V. velutina*, en particulier, aucun composé phéromonal n'est encore connu. La partie suivante résumera donc les composés déjà identifiés ainsi que les observations comportementales effectuées chez d'autres espèces du genre Vespa, suggérant l'existence de phéromones analogues chez *V. velutina*.

Le maintien de l'intégrité coloniale est un élément fondamental des groupes sociaux car il empêche des individus étrangers de s'intégrer à la colonie et d'en tirer avantage. Dans ce contexte, la capacité de reconnaître ses congénères permet d'éviter l'exploitation du comportement altruiste des membres d'une colonie par des individus non apparentés. Chez les frelons, comme chez la plupart des insectes, il existe de nombreux indices indiquant que la reconnaissance des congénères est sous-tendue par la détection d'hydrocarbures cuticulaires (Butts *et al*., 1991 ; Butts *et al*., 1995 ; Ruther *et al*., 1998). Ainsi, la modification artificielle de la signature cuticulaire d'un frelon *V. crabro* induit un comportement agonistique de la part de ses congénères lorsque celui-ci est réintroduit dans son propre nid (Ruther *et al*., 2002). Il existe même des cas de mimétisme chimique chez les frelons. Par exemple, le profil cuticulaire de *V. dybowskii*, frelon parasitant les nids des autres espèces, lui permet de faire accepter ses œufs dans les nids d'espèces phylogénétiquement proches (Martin *et al.*, 2008). Des observations faites à proximité des nids de plusieurs espèces signalent que des phéromones de marquage territorial, déposées à l'entrée des nids par les ouvrières, pourraient également exister (Batra, 1980 ; Tan, 2014). Ces phéromones auraient une fonction de marquage/localisation, comme les phéromones de piste des fourmis, mais seraient aussi bien utilisées par les ouvrières que par les mâles à la recherche de partenaires sexuels (Batra, 1980 ; Steinmetz *et al*., 2002).

Plusieurs molécules volatiles identifiées dans les venins de *V. crabro, V. orientalis* et *V. mandarinia* semblent induire une forte réaction comportementale chez les ouvrières de ces mêmes espèces, avec un probable effet synergique (Saslavasky *et al.*, 1973 ; Veith *et al*., 1984 ; MacLean & Schmolz, 2004 ; Ono *et al*., 2003). Dans des essais comportementaux, la présentation de ces substances induit un comportement de défense caractérisé par des attaques répétées et une augmentation de l'activité locomotrice autour de la cible. Ces composés ont donc été considérés comme une possible phéromone d'alarme qui permettrait, lors d'une attaque du nid, de recruter des congénères pour sa défense. Un comportement similaire peut être observé chez *V. bicolor* et *V. crabro* lors de la présentation des composés de la phéromone d'alarme de l'abeille *A. mellifera,* qui agirait ici comme signal pour la localisation de proies (kairomone) (Brodman *et al*., 2009 ; MacLean & Schmolz, 2004). De plus, il semblerait que le frelon *V. mandarinia* est capable de recruter ses congénères pour attaquer « *en masse* » les colonies d'autres Hyménoptères grâce à une molécule de la phéromone d'alarme, contenu dans la glande de Van der Vecht (Ono *et al*., 1995 ; Ono *et al*., 2003). Ainsi, plusieurs substances sémiochimiques (phéromone d'alarme, phéromone de recrutement ou kairomones produites par les abeilles) pourraient provoquer un comportement d'attraction chez les ouvrières *V. velutina*.

Au sein du nid, la cohésion sociale et la régulation des différentes activités effectuées par les ouvrières, passent également par une communication chimique. En élevant des colonies de *V. orientalis* en laboratoire, Jacob Ishay mit en évidence et identifia une phéromone présente dans la capsule céphalique des reines (Ishay, 1964 ; Ishay *et al.*, 1965 ; Ikan *et al.*, 1969). Lorsque la reine d'une colonie est enlevée, les ouvrières affichent un comportement erratique (absence de cohésion sociale) qui peut être restauré par l'introduction d'extraits de tête de fondatrices. Il put également mettre à jour l'existence de phéromones impliquées dans la régulation de la construction du nid (Ishay & Perna, 1979) et une autre phéromone produite par le couvain et signalant un besoin de thermorégulation (Ishay, 1973).

Les Hyménoptères sociaux utilisent des phéromones sexuelles produites par les femelle fertiles et attractives pour les mâles lors de la reproduction (Ayasse *et al.*, 2001). Il existe de nombreux indices de leur usage chez les frelons. Dans un premier temps, il fut démontré que les femelles fertiles de *V. crabro* possèdent sur leur thorax une phéromone de contact qui déclenche un comportement de copulation chez les mâles (Batra, 1980). Ensuite, en observant un groupe de mâles *V. mandarinia* rassemblés à l'entrée d'un nid, Masato Ono et ses collaborateurs émirent l'hypothèse que ces frelons pourraient être attirés par des phéromones émises par les fondatrices, à l'intèrieur du nid (Ono *et al.*, 1985). Par la suite, ils réussirent à induire en laboratoire l'accouplement de mâles avec des leurres chargés en extraits de fondatrices, démontrant ainsi l'existence d'une phéromone sexuelle chez les frelons (Ono & Sasaki, 1987). Lors de ces expériences il fut également observé que ces phéromones sexuelles ne sont pas complètement spécifiques puisque l'accouplement peut être provoqué avec le même extrait chez 5 différentes espèces sympatriques (Ono & Sasaki, 1987). Plus récemment, l'attraction à longue distance de mâles de *V. crabro* à l'aide d'extraits de reines semble confirmer la présence d'une phéromone sexuelle attractive chez cette espèce (Spiewok *et al.*, 2006). Cependant, aucune phéromone sexuelle n'a pour l'instant été chimiquement identifiée chez les frelons.

Ainsi, à l'instar des abeilles et des fourmis qui ont servi depuis plusieurs décennies comme modèles biologiques pour comprendre les bases nerveuses et comportementales de l'olfaction chez les insectes sociaux, les frelons évoluent dans un environnement olfactif extrêmement riche et montrent des comportements olfactifs nombreux et complexes. Par conséquent, on pourrait s'attendre à ce que leur système olfactif comporte aussi bien des adaptations spécifiques à la biologie particulière de ces espèces que des adaptations communes aux autres Hyménoptères sociaux.

b. Connaissances actuelles sur le système olfactif des frelons

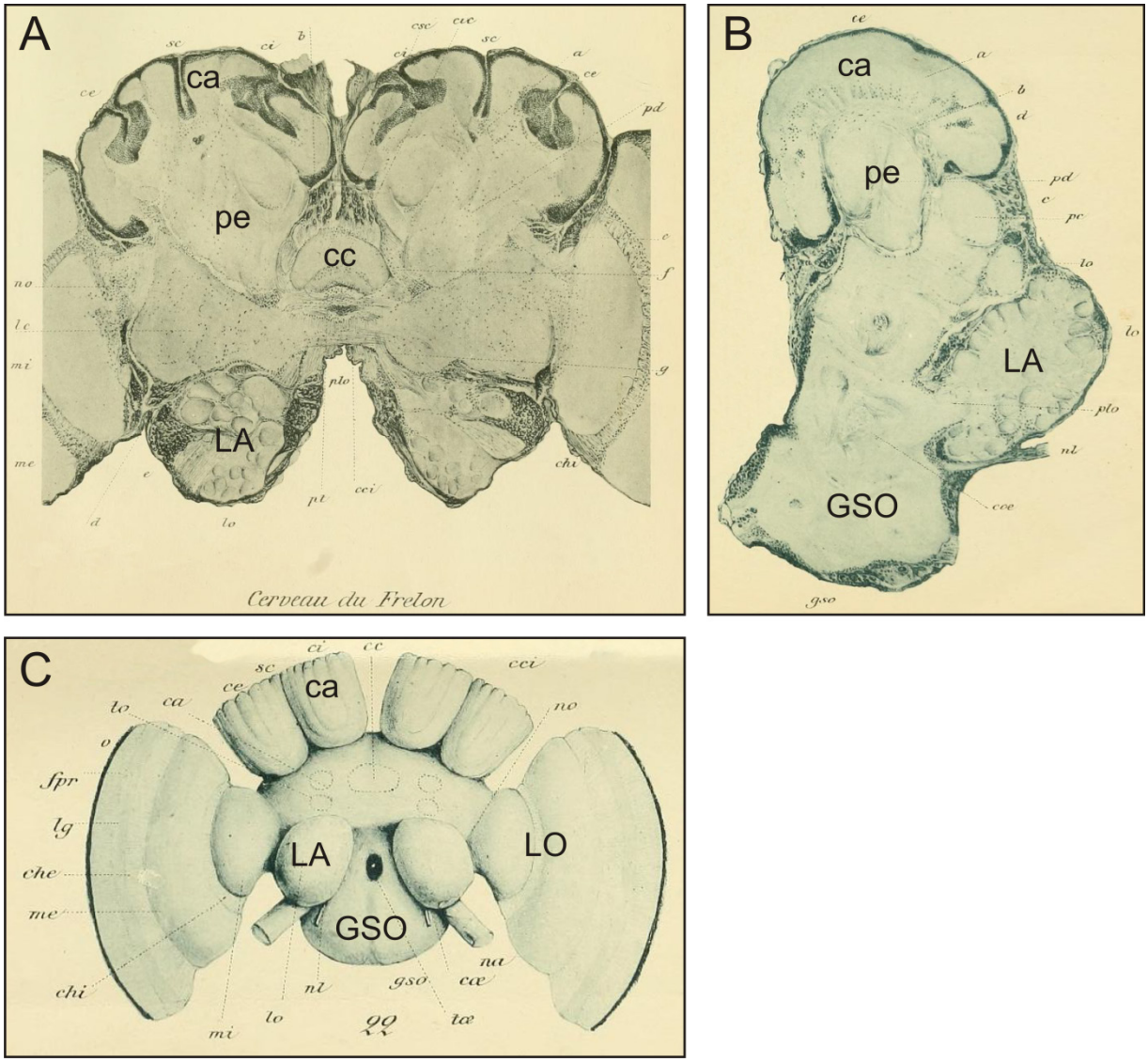
A l'heure actuelle, très peu de données sont disponibles concernant l'organisation des réseaux neuronaux dans le cerveau des frelons (ou même des guêpes sociales). Pourtant, les premières

descriptions de leur cerveau apparurent très tôt, puisque dès 1878, dans une étude comparée du cerveau des insectes, le naturaliste allemand Johann Heinrich Ludwig Flögel observa le système nerveux central de *V. crabro* grâce à des coupes histologiques dont voici les conclusions : « *Il est étonnant de voir que chez la guêpe [sic], le cerveau n'est pas construit sur le même type que le cerveau de l'abeille ; dans l'état actuel de nos connaissances, je considère comme très difficile de retrouver chez la guêpe les homologues des parties cérébrales de l'abeille*. » Plus loin Flögel ajoute : « *Le cerveau de la guêpe s'éloigne d'une manière si étonnante du type offert par la fourmi ou par l'abeille, qu'il diffère plus du cerveau de ces animaux qu'il ne diffère du cerveau de la blatte*. »

Ces conclusions étonnantes sont basées sur la morphologie singulière des corps pédonculés du frelon qui atteignent des dimensions particulièrement importantes chez cette espèce. De plus, chez les frelons, les fibres axonales des cellules de Kenyon se projettent moins distinctement en lobes verticaux et horizontaux (lobe α et β) que chez l'abeille ou les fourmis. En 1887, Henri Viallanes documenta spécifiquement ce sujet en reproduisant extrêmement précisément de nombreuses coupes histologiques du cerveau de *V. crabro* colorées au carmin aluné (**Fig. 25**). Il remarqua que la taille des deux lobes est fortement réduite par rapport à des calices et à des pédoncules relativement hypertrophiés. Bien plus tard, Jawlowski proposa que chez les guêpes les lobes α et β soient fusionnés en un seul et même neuropile (Jawlowski, 1959). Finalement ce n'est qu'en 2000 que Birgit Ehmer et Ron Hoy comparèrent les corps pédonculés d'un certain nombre de Vespidés pour en déduire que les deux lobes sont bien présents mais sont le fruit d'une lente évolution, avec une séparation progressive du lobe β en deux parties se projetant vers la face dorsale du cerveau.

A notre connaissance, une seule description, très fragmentaire, du lobe antennaire des frelons a été réalisée (Hanström, 1928). Dans son rapport sur l'anatomie comparée du système nerveux des invertébrés, Bertil Hanström affirma que similairement au LA des criquets, les frelons possèdent environ un millier de petits glomérules non individuellement identifiables et arrangés autour d'une fibre centrale (Hansson & Anton, 2000). Mais ces travaux ne furent, à ce jour, ni repris ni contestés.

Le cerveau du frelon a donc fait l'objet de certaines interrogations du fait de sa singularité morphologique par rapport à celui de tous les autres Hyménoptères, sociaux ou non, décrits à ce jour (O'Donnell *et al.*, 2004, 2011, 2015 ; Ehmer & Hoy, 2000 ; Kelber *et al.*, 2009). Ces particularités sont peut être dues à des adaptations liées à leur niveau d'organisation sociale, à leurs capacités cognitives et/ou à la complexité de leur environnement olfactif. Notamment on peut imaginer que le LA des frelons puisse contenir des glomérules hypertrophiés (MG) spécialisés dans la détection des nombreuses phéromones utiles à sa biologie. Cependant aucune étude récente ne s'est attelée à une exploration précise des structures et du fonctionnement des voies olfactives de ces espèces.



<u>Figure 25 :</u> Illustration d'Henri Viallanes sur l'anatomie du cerveau des frelons
Reproduction des coupes du cerveau de *V. crabro* observées par Henri Viallanes en 1887. La coupe frontale (**A**) permet d'observer la structure des corps pédonculés comprenant des calices (ca) dans la partie la plus rostrale du cerveau d'où partent les fibres des cellules de Kenyon. Ces dernières se projettent vers la partie caudale du cerveau et forment le pédoncule (pe). **B**) Ainsi les corps pédonculés entourent la partie rostrale du cerveau, observable en coupe orthogonale. **C**) Les lobes antennaires (LA) se situent à la surface ventro-caudale du cerveau, au dessus du ganglion sub-œsophagien (GSO), alors que les extrémités latérales sont occupées par les lobes optiques (LO). (cc : corps central).

IV. Objectifs de la thèse

Le but de cette thèse est d'acquérir des connaissances sur le système nerveux et le comportement olfactifs du frelon *Vespa velutina*. Une meilleure connaissance de la biologie des frelons pourrait permettre de proposer des pistes efficaces pour lutter contre cette espèce invasive, notamment en interférant avec son comportement olfactif.

- Dans un premier temps, **nous avons cherché à décrire avec précision les structures nerveuses impliquées dans le traitement de l'information olfactive chez les différents membres d'une colonie de *Vespa velutina,*** afin d'appréhender les possibles adaptations de la voie olfactive en relation avec le rôle de chaque caste. Nous avons également cherché à replacer nos observations dans un contexte évolutif, en comparant le système olfactif de *V. velutina* avec celui d'autres organismes modèles phylogénétiquement proches et dont la biologie comporte des similitudes avec celle des frelons : il s'agit d'Hyménoptères sociaux comme l'abeille domestique, *Apis mellifera,* et les fourmis charpentières, *Camponotus sp*. Nous avons donc procédé à une étude neuroanatomique comparative utilisant des marquages fluorescents de populations neuronales, l'imagerie confocale et des reconstructions en 3 dimensions.

- Compte tenu des similarités frappantes observées dans l'organisation du système olfactif de ces différentes espèces et notamment de leur lobe antennaire, nous **avons analysé l'existence probable de structures nerveuses homologues chez *V. velutina,*** en nous basant sur des singularités structurales et fonctionnelles d'un groupe de glomérules particulier. Chez certaines espèces de fourmis, les hydrocarbures cuticulaires sont détectés au niveau de l'antenne par les sensilles basiconiques. Celles-ci abritent des neurones sensoriels qui se projettent exclusivement dans un groupe remarquable de petits glomérules (Sharma *et al*., 2015 ; Nishikawa *et al*., 2012). Nous avons donc inspecté l'équipement sensillaire de *V. velutina* grâce à la microscopie électronique à balayage et avons cherché à révéler les projections des neurones sensoriels de ces sensilles, pouvant suggérer une homologie au sein du lobe antennaire des fourmis et des frelons. Enfin, nous avons recherché une possible similarité de fonction de ce sous-système olfactif chez ces deux modèles, en réalisant des enregistrements électrophysiologiques des sensilles basiconiques de *V. velutina*.
- Les Hyménoptères représentent un ordre d'insecte extrêmement diversifié dont les espèces occupent des niches écologiques et des modes de vie très variés (parasitisme, eusocialité, entomophagie, phytophagie, etc.). Une importante variabilité du nombre de glomérules et de l'organisation du lobe antennaire ont été observés dans cet ordre, jusqu'au sein d'une même famille. Parmi les 22 espèces de frelon actuellement identifiées, certaines vivent dans des biotopes éminemment différents. Les observations du comportement de prédation de ces

espèces suggèrent aussi des différences de stratégie, aussi bien dans le type de sources de nourritures préférentiellement exploitées que dans le niveau d'agrégation (et possiblement de recrutement) des ouvrières sur ces sources de nourritures**. Nous avons donc comparé la structure du lobe antennaire de 4 espèces allopatriques du genre Vespa**, afin de déterminer la variabilité du nombre de glomérules et de l'organisation du lobe antennaire chez ces frelons. En outre, nous avons mesuré le volume de quelques glomérules remarquables, qui pourraient jouer un rôle dans la détection de composés impliqués dans le recrutement des ouvrières sur les sources de nourritures.

- Alors que les ouvrières de certaines espèces de frelon possèdent un macroglomérule, les ouvrières de l'espèce *V. velutina* semblent en être dépourvues. Cependant cette espèce invasive présente un haut niveau d'agrégation sur les sources de nourriture abondantes comme les ruches d'abeille domestique, *Apis mellifera*, ou les chairs animales. Nous avons donc essayé d'évaluer quelles odeurs pouvaient être impliquées dans le comportement de recherche de nourriture et être potentiellement responsable de cette forte attraction sur les ruchers ou les étales des bouchers et poissonniers. Dans des tests comportementaux réalisés en laboratoire, **nous avons analysé l'attraction de *V. velutina* envers différentes sources de nourriture et envers des composés odorants émis par ces sources de nourriture**. Les résultats de ces tests pourraient ensuite être reproduits en conditions naturelles afin de mieux comprendre le comportement de prédation des ouvrières de *V. velutina*. Enfin, une fois les substances attractives isolées, celles-ci pourraient être utilisées dans une stratégie de lutte visant à perturber le comportement du frelon invasif.

Ces quatre objectifs ont été abordés au sein de quatre chapitres, pour lesquels nous listons ci-dessous les questions auxquelles nous avons essayé de répondre.

Chapitre 1 : Anatomie du système olfactif du frelon *Vespa velutina* : nouvelles perspectives sur l'évolution du lobe antennaire des hyménoptères.

- De quelle manière est organisé le système olfactif au sein du cerveau des différentes castes de *Vespa velutina* ?
- Existe-t-il des adaptions du système nerveux olfactif, spécifiques aux différentes castes de *V. velutina,* telles que la présence de glomérules hypertrophiés (macroglomérules) ?
- Le système olfactif des frelons est-il organisé suivant le même schéma que chez les autres hyménoptères sociaux ?

L'étude de ces questions a abouti à la publication d'un article :

The olfactory pathway of the hornet *Vespa velutina*: new insights into the evolution of the Hymenoptera antennal lobe

Antoine Couto, Benoit Lapeyre, Denis Thiéry, et Jean-Christophe Sandoz

Journal of Comparative Neurology (2016)

Chapitre 2 : Un sous-système olfactif conservé chez les hyménoptères, impliqué dans le traitement des hydrocarbures cuticulaires.

- De quel équipement sensillaire le frelon *Vespa velutina* dispose t-il au niveau des antennes pour la détection périphérique des odeurs ?
- Où se projettent les neurones sensoriels olfactifs provenant des sensilles basiconiques, au sein du système nerveux central de *Vespa velutina* ?
- Existe-t-il chez *Vespa velutina* une dichotomie de l'immunoréactivité sérotoninergique du lobe antennaire, comme celle observée chez les fourmis ?
- A quelle gamme de composés odorants ce système spécifique aux sensilles basiconiques est-il sensible ?

Ces questions sont abordées dans le manuscrit en préparation :

A conserved olfactory subsystem for cuticular hydrocarbon processing in Hymenoptera: the basiconic sensilla subsystem of the hornet *Vespa velutina*

Antoine Couto, Aniruddha Mitra, Denis Thiéry, Frédéric Marion-Poll et Jean-Christophe Sandoz

Chapitre 3 : Variabilité interspécifique dans l'organisation du lobe antennaire chez quatre espèces de frelon.

- Quelle est la variabilité de l'organisation du lobe antennaire entre différentes espèces du genre Vespa ?
- Existe-t-il des adaptations neuronales chez les ouvrières de différentes espèces de frelons en relation avec leurs différentes stratégies de prédation, comme la présence de macroglomérules dédiés au traitement de phéromones d'agrégation ?

Ces questions sont à l'origine d'un manuscrit en préparation :

Interspecific differences in antennal lobe organization among four hornet species with differing biotope, predation behavior and sympatry

Antoine Couto, Hiroyuki Ai, Gérard Arnold et Jean-Christophe Sandoz

Chapitre 4 : Attraction olfactive du frelon *Vespa velutina* envers les odeurs de colonie et les phéromones d'abeilles.

- Vers quelles sources de nourriture les ouvrières prédatrices *Vespa velutina* sont-elles préférentiellement attirées ?
- Quels composés odorants émis par ces différentes sources de nourriture sont susceptibles d'attirer les ouvrières *V. velutina* ?
- Les phéromones produites par les abeilles, peuvent-elles agir comme des kairomones et être responsable de l'attraction des frelons vers les ruches ?

Ces questions ont donné lieu à une publication :

Olfactory attraction of the hornet *Vespa velutina* to honeybee colony odors and pheromones

Antoine Couto, Karine Monceau, Olivier Bonnard, Denis Thiéry et Jean-Christophe Sandoz

PLoS ONE (2014)

CHAPITRE 1

The olfactory pathway of the hornet *Vespa velutina*: new insights into the evolution of the hymenopteran antennal lobe

Antoine Couto, Benoit Lapeyre, Denis Thiéry, Jean-Christophe Sandoz

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Abstract

In the course of evolution, eusociality has appeared several times independently in Hymenoptera, within different families such as Apidae (bees), Formicidae (ants) and Vespidae (wasps and hornets) among others. The complex social organization of eusocial Hymenoptera relies on sophisticated olfactory communication systems. Whereas the olfactory systems of several bee and ant species have been well characterized, very little information is yet available in Vespidae, although this family represents a highly successful insect group, displaying a wide range of lifestyles from solitary to eusocial. Using fluorescent labeling, confocal microscopy and 3D reconstructions, we investigated the organization of the olfactory pathway in queens, workers and males of the eusocial hornet *Vespa velutina*. First, we found that caste and sex dimorphism is weakly pronounced in hornets, with regards to both whole-brain morphology and antennal lobe organization, although several male-specific macroglomeruli are present. The *Vespa velutina* antennal lobe contains approximately 265 glomeruli (in females), grouped in 9 conspicuous clusters formed by afferent tract subdivisions. As in bees and ants, hornets display a dual olfactory pathway, with two major efferent tracts, the medial and the lateral antennal lobe tracts (m- and l-ALT), separately arborizing two antennal lobe hemilobes and projecting to partly different regions of higher-order olfactory centers. Finally, we found remarkable anatomical similarities between the glomerular cluster organizations among hornets, ants and bees, suggesting the possible existence of homologies in the olfactory pathways of these eusocial Hymenoptera. We propose a common framework for describing AL compartmentalization across Hymenoptera and discuss possible evolutionary scenarios.

Keywords: social insect, wasps, three-dimensional reconstruction, macroglomerulus

Introduction

Insects are influential models in the study of the evolution of olfactory processing and have revealed how selection pressures influence the organization and complexity of the olfactory system (Rospars, 1988; Kleineidam & Rössler, 2009; Ramdya & Benton, 2010; Hansson & Stensmyr, 2011). In insects, volatile molecules are detected by olfactory sensory neurons (OSNs) housed in cuticular sensilla on the antennae (Zacharuk, 1980). These neurons project to a primary olfactory center, the antennal lobe (AL), where they synapse onto local interneurons and projection neurons (PNs), within dense synaptic neuropils, the glomeruli (Hildebrand & Shepherd, 1997; Anton & Homberg, 1999; Vosshall *et al.*, 2000). PNs convey the olfactory message processed within AL networks to higher-order olfactory centers, the mushroom body (MB) and/or the lateral horn (LH).

Many previous works have revealed specific adaptations in AL organization, in relation with the particular biology and ecology of a wide range of insect species (Rospars, 1983; Martin *et al.*, 2011; Clifford & Riffell, 2013). For instance, whereas the number of AL glomeruli is almost invariant among individuals of a given species, it varies greatly between species, which is thought to be related to the complexity of their olfactory environments (Anton & Homberg, 1999; Hansson & Anton, 2000; Kelber et al, 2009). Another striking example of such neuroanatomical and physiological adaptations is the existence of enlarged glomeruli, called macroglomeruli, dedicated to pheromone detection and processing (Burrows *et al.*, 1982; Arnold *et al.*, 1985; Hanson *et al.*, 1992; Sandoz, 2006; Nishikawa *et al.*, 2008; Nishino *et al.*, 2012). In most cases, macroglomeruli are male dimorphic adaptations devoted to sex pheromone processing, but they have also been discovered in the females of some social Hymenoptera (Arnold *et al.*, 1988; Kleineidam *et al.*, 2005; Roselino *et al.*, 2015). In leaf cutting ants, for instance, a macroglomerulus found only in large workers is thought to process trail pheromone information (Kelber *et al.*, 2009; Kuebler *et al.*, 2010). Thus, the presence of hypertrophied glomeruli may inform about particular pheromonal communication channels in a species.

In some cases, neuronal adaptations of the olfactory pathway are found throughout a specific taxon. For instance, in Hymenoptera, uniglomerular PNs project to the MB and the LH through two parallel output tracts, the medial and the lateral antennal lobe tracts (m- and l-ALT). The AL is thus composed of two hemilobes which send their information separately to both higher-order centers (Abel *et al.*, 2001; Galizia & Rössler, 2010; Rössler & Zube, 2011; Rössler & Brill, 2013). Investigations in basal Hymenoptera showed that this neuronal organization is present in some sawfly (Symphyta) families but absent in others, leading to the hypothesis that it has evolved in basal Hymenoptera (Dacks & Nighorn, 2011; Rössler & Zube 2011). The benefit of this parallel processing system and the selective pressures that favored its evolution remain unknown. Some authors hypothesized that it could represent a preadaptation to high demands on olfactory discrimination needed for particular

life styles such as parasitism, central place foraging, or sociality (Galizia & Rössler, 2010; Rössler & Zube, 2011). These examples demonstrate that essential knowledge on the evolution of the insect olfactory system can be obtained from its comparative analysis in well-chosen taxa. Thus, comparing the organization of the AL and its specific adaptations in terms of glomerular number, size, and input-output connectivity may allow one to understand adaptations linked to changes in diet, host preference, or social lifestyle in Hymenoptera (Rospars, 1983; Zube & Rössler, 2008; Kelber *et al.*, 2009; Dacks *et al.*, 2010; Ibba *et al.*, 2010; Das & Fadamiro, 2013; Guidobaldi *et al.*, 2014).

Social insects ensure colony defense and cohesion, division of labor, and behavioral regulation through a highly elaborate communication system (Vander Meer, 1998). Specific adaptations of the olfactory pathway can be observed among the different members of an eusocial insect colony, which are usually revealed through sex and caste polymorphism (Zube & Rössler, 2008; Groh & Rössler, 2008; Kuebler *et al.*, 2010; Nakanishi *et al.*, 2010; Stieb *et al.*, 2011; Roselino *et al.*, 2015). Eusociality has appeared several times in Hymenoptera, in at least three different families, Apidae (bees), Formicidae (ants), and Vespidae (wasps and hornets) (Hines *et al.*, 2007; Hughes *et al.*, 2008; Cardinal & Danforth, 2011; Johnson *et al.*, 2013). Although possible adaptations of olfactory circuits related to the social lifestyle have been investigated in several Apidae (honey bees, stingless bees : Arnold *et al.*, 1985; Flanagan & Mercer, 1989; Galizia *et al.*, 1999; Kelber *et al.*, 2006; Nishino *et al.*, 2009; Streinzer *et al.*, 2013; Roselino *et al.*, 2015) and Formicidae (several ant species: Nishikawa *et al.*, 2008; Zube & Rössler, 2008; Mysore *et al.*, 2009; Nakanishi *et al.*, 2010; Stieb *et al.*, 2011; Kuebler *et al.*, 2010), Vespidae are poorly documented. This group, however, represents a key group for studying the evolution of eusociality it contains species with a wide range of social organizations, from solitary to eusocial (Hunt, 2007; Pickett & Carpenter, 2010).

In their highest level of sociality, Vespidae display a large repertoire of odor-guided behaviors, comparable to those of ants and bees, including kin recognition and cooperation for brood care, colony defense, and foraging (Spradbery, 1973; Edwards 1980; Matsuura & Yamane, 1990; Oi *et al.*, 2015). Hornets (*Vespa* sp.) are the largest eusocial wasps. They are generalist foragers that gather carbohydrates and proteins for their progeny, by exploiting flower nectar, tree sap, fruit pulp, and arthropod preys (Raveret Richter, 2000). For such task, they use odors emanating from the food sources but also intraspecific olfactory recruitment signals, which increase their foraging efficiency (Ono *et al.*, 1995, 2003; Brodmann *et al.*, 2009; Couto *et al.*, 2014). Females can release scent marks that are used by both workers and males to locate the nest (Batra, 1980; Steinmetz *et al.*, 2002; Tan, 2014). Within the nest, hornets live in organized colonies with a highly efficient task allocation system regulated through social pheromones acting on workers (Ikan *et al.*, 1969; Ishay, 1973; Veith *et al.*, 1984; Monceau *et al.*, 2013). To prevent exploitation of such altruistic behavior by alien individuals, hornets are also able to discriminate nestmates and nonnestmates by means of cuticular hydrocarbons (Ruther *et al.*, 1998; Ruther *et al.*, 2002). In addition, queens produce a sex pheromone

that triggers copulatory behavior in males (Batra, 1980; Ono & Sasaki, 1987; Spiewok *et al.*, 2006). Therefore, both hornet males and females develop and live in a rich olfactory environment and use a wide range of inter- and intraspecific chemical cues (Bruchini *et al.*, 2010).

To date very few works have described the central nervous system of Vespidae, and most studies date back more than a century (Flögel, 1878; Viallanes, 1887; von Alten, 1910). Flögel (1878) proposed the first anatomical description of the wasp brain and observed striking differences in the morphology of its MBs compared to the brains of other eusocial Hymenoptera, such as ants and honeybees. This was confirmed by Viallanes (1887), who reported that despite the huge size of their MBs, the vertical (α) and medial (β) lobes appear atrophied. More recently, Ehmer and Hoy (2000) highlighted a strong anatomical divergence of MBs within this family, which could, however, not be related to the social organization level of the investigated species. Since then, neuroanatomical studies on the wasp or hornet brain have mainly focused on higher order centers (O'Donnell *et al.*, 2004, 2011, 2015), but its olfactory pathway, and especially the AL, which may support important adaptations for foraging, pheromonal processing and/or sociality, has been the subject of little consideration. According to Hanström (1928), the AL of social wasps would contain ~1000 small glomeruli arranged in multiglomerular layers, as in locusts (Hansson & Anton 2000). In theory, such a high number of glomeruli would be consistent with the rich olfactory environment of these species. However, in the light of recent data, serious doubts can be raised concerning Hanström's (1928) description (Kelber *et al.*, 2009). First, none of the Hymenoptera studied so-far displays such a high number of glomeruli, the maximum being 630 glomeruli in the ant *Apterostigma cf. mayri* (Kelber *et al.*, 2009). Second, given current knowledge on the neural organization of the olfactory pathways of the different insect orders, it seems highly unlikely that the hornet AL presents a true locust-like organization, especially with regard to PNs arborization. Whereas PNs are multiglomerular in locusts (Ignell, 2001; Ignell *et al.*, 2001; Anton *et al.*, 2002), Hymenoptera typically display two major tracts of uniglomerular PNs, and three lesser tracts of multiglomerular PNs which form a broad network in the protocerebrum (medio-lateral ALT, ml-ALT; Abel *et al.*, 2001; Kirschner *et al.*, 2006; Galizia & Rössler, 2010; Rössler & Zube, 2011).

In the present study we performed a detailed neuroanatomical exploration of the olfactory pathway of a eusocial member of the Vespidae, the hornet *Vespa velutina.* This otherwise typical hornet species is particularly remarkable for its ecological success when introduced to new locations, such as in Europe, in which it has become an invasive species (Monceau *et al.*, 2014; Arca *et al.*, 2015). A nest founded by a single mated queen can produce up to 13,000 individuals, mostly sterile females (workers), which actively cooperate for the survival of the colony (Rome *et al.*, 2015). They are vigorous predators of insects and very actively prey on honey bees, representing a threat for both local biodiversity and beekeeping activities (Monceau *et al.*, 2014). Using fluorescent dye injection coupled with confocal microscopy, we compared AL features in queens, workers, and

males. We asked whether the hornet olfactory system displays caste- or sex-specific adaptations, potentially related to the roles of the different members of a hornet colony. In particular, we looked for differences in the organization, size and volume of AL glomeruli. Furthermore, in an evolutionary perspective, we investigated the input-output connectivity of the AL and compared it with the well-described olfactory pathways of ants and honeybees.

Materials and Methods

Animals

Queens, workers, and males of the yellow-legged hornet, *Vespa velutina,* were used in the experiments. Queens and workers were obtained from field trapping in the area of INRA-Bordeaux Aquitaine (France) between April and November. Males were collected from wild nests in September in the same area. Males were identified by their specific morphological features such as long antennae (11 antennal segments instead of 10 in females) and the lack of a sting. Females were assigned to the reproductive or sterile caste by measuring their wing area according to Perrard *et al.* (2012). Wings were carefully flattened and clamped between 2 glass slides before capturing images with a film scanner (OpticFilm 7400, Plustek, Taipei, Taiwan). Wing size was evaluated in ImageJ (RRID:SCR_003070). A proxy measure of wing size was defined as the area between nine conspicuous vein intersections of the forewing (**Fig. 1A**, corresponding to landmarks 1-4, 9, 10, 13, 15 and 16 of Perrard *et al.*, 2012). In hornets, queens are usually larger than workers (Matsuura & Yamane, 1990). However, in *V. velutina*, this difference is not so clear-cut as in other species (Perrard *et al.*, 2012). In particular, because of an increase in the size of emerging workers in the course of colony cycle, wing areas of the two female castes may overlap in autumn, whereas they are clearly different in early summer. Therefore, females captured from April to June with a large wing area could be unambiguously assigned to queens, and small females captured from July to November could likewise be assigned to workers (**Fig. 1B**). Only such clearly identifiable individuals were used in this study (**Fig. 1B**; dark grey: workers; light grey: queens). For all experiments, hornets were anesthetized on crushed ice for 10 minutes before being placed in individual Plexiglas holders (see Sandoz *et al.*, 2003, Fig. 1).

Whole brain neuropil staining

To visualize and reconstruct the hornet brain, Lucifer yellow was used as a neuropil background stain (Ai & Kanzaki, 2004; Rybak *et al.*, 2010). The head of each hornet was fixed to the chamber with low melting-point wax (Deiberit 502, Schöps and Dr Böhme, Goslar, Germany) and the head capsule was opened. The brain was dissected out under phosphate buffered saline (PBS) solution. It was then immediately transferred to 500 µL of fixative solution (4% paraformaldehyde in

PBS) containing 1 µL of 4% Lucifer yellow (Lucifer Yellow CH, Potassium Salt, L-1177; Invitrogen, Eugene, OR) and kept for 4 days at 4°C.

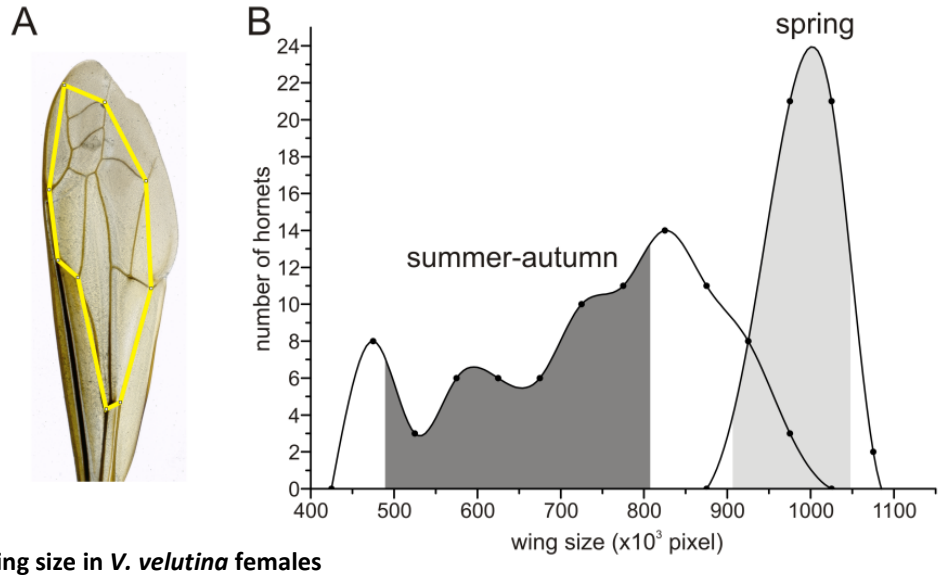

Figure 1: Wing size in *V. velutina* females
A) The area between nine vein intersections on the forewing (yellow polygon) was calculated as a proxy of wing size. **B**) Distribution of the wing sizes of all *V. velutina* females caught. Females caught in spring (April – May), at the start of the season, are necessarily founding queens. Females caught in summer-autumn (June to November) are mostly workers. Initially, workers are much smaller than queens. However, worker wing size increases throughout colony cycle and may eventually (at the end of autumn) overlap with that of queens. Therefore, small individuals caught during summer and autumn (dark grey area) and large individuals trapped in spring (light grey area), which could be unambiguously assigned to workers and queens, respectively, were used in the experiments.

Specific neuronal staining

To describe the glomerular organization of the antennal lobe, antennal sensory neurons were stained anterogradely. A window was cut into the scape of each antenna, and the antennal nerves were severed with a glass electrode loaded with crystals of fluoro-ruby (Tetramethylrhodamine dextran, 10 000 MW, D-1817; Invitrogen; in 2% Bovine Serum Albumin). The preparation was then covered with saline solution (130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, with PH = 6.7) and kept for 24 hours in a dark room to let the dye diffuse. On the next day, the brain was dissected out and plunged into 4% PFA solution for 24 hours at 4°C.

For visualization of projections of antennal lobe tracts (ALTs) through the protocebrum towards higher order centers, a massive dye injection in the AL was performed. The hornet's head cuticle was removed by cutting a rectangle between the antennae and the compound eyes to expose the AL neuropil. Subsequently, a glass electrode coated with fluoro-ruby was inserted into the AL and kept in this position until dissolution of the dye crystal, allowing uptake by injured AL neurons, including projection neurons (PNs). The electrode was then dislodged from the AL, and the brain was washed with saline solution to remove extra dye. As described above, preparations were kept for 24 hours in a dark room during dye migration before brains were dissected out and fixed in 4% PFA.

Double labeling

To assign AL glomeruli to one of the two main PN tracts (l-ALT, m-ALT or both), each tract was retrogradely labeled with different dyes in the same preparation. A piece of head cuticle was removed to expose the region between the ALs and the mushroom body calyces. The l-ALT was labeled by fluorescent tracer injection (Dextran, Alexa Fluor 488, 10 000 MW, D-22910; Invitrogen; in 2% BSA) into the caudolateral area of the protocerebrum where the l-ALT runs between the AL and the lateral horn. The m-ALT was labeled by fluoro-ruby injection into the mediocaudal area of the protocerebrum, where the m-ALT runs between the AL and the MB. To avoid nonspecific staining, extra dye was removed after each injection by washing the brain with saline solution. After 24 hours of dye migration, the brains were dissected out and fixated in 4% PFA.

To visualize olfactory and visual inputs to the MB calyces, AL projection neurons and visual output neurons were separately stained in the same preparation by mass tracer injections of Alexa Fluor into the AL and fluoro-ruby into the optic lobe (medulla and lobula).

Brain preparations

After being removed from the fixative solution, all brains were washed three times in PBS solution (10 minutes each), dehydrated in ethanol baths following an ascending series (50%, 70%, 90%, 95% and 3 x 100% for 10 minutes each), and clarified in methylsalicylate (Sigma-Aldrich, Steinheim, Germany) for at least three days at 4°C. Brains were then mounted on aluminum slides with a central hole filled with methylsalicylate and covered by thin coverslips on both sides.

Confocal microscopy

Clarified brains were scanned from the ventral side with a laser-scanning confocal microscope (LSM-700; Carl Zeis, Jena, Germany). Whole-brain optical sections were acquired at a resolution of 2.5 µm/pixels (x,y) with 3 µm intervals (z) using a water immersion objective (10x achroplan 0.3 NA). Antennal lobe optical sections were acquired at 0.52 µm/pixels (x,y) with 1 µm intervals (z) using a water immersion objective (20x plan-apochromat 1.0 NA). Given the large size of the hornet brain, complete scans were obtained by stitching adjacent “tiles” (512 x 512 pixels) of optical sections with the tile function of the Zen software (Carl Zeiss; RRID:SCR_013672). Fluoro-ruby-labeled neurons were visualized with a 555 nm solid-state laser, whereas Lucifer yellow and Alexa Fluor were visualized with a 488 nm laser. Doubly stained neuropils were scanned sequentially to avoid emission overlap from distinct fluorescent dyes.

3D reconstructions and olfactory tract affiliation

Serial optical sections were saved as LSM files before adjusting brightness and contrast with ImageJ software and the Bio-formats plugin (RRID:SCR_000450). Adjusted pictures were then converted into TIFF files and imported into three-dimensional (3D) analysis software (AMIRA 5.4.3;

FEI, Berlin, Germany; RRID:SCR_007353). Glomeruli were individually reconstructed by manual labeling in three planes (*xy*, *xz* and *yz*) and using the Wrap function to obtain its 3D model. Larger structures, such as brain neuropils and macroglomeruli, were outlined in several stacks along one focal plane (*xy*), and the Interpolate function was used to create the 3D model.

Each reconstructed glomerulus was assigned to a glomerular cluster by closely following OSN bundles in the original scan. Because similar features such as input tract trajectories, glomerular morphologies and output tract affiliations could be observed in different hymenopteran species, we attempted to unify olfactory tract nomenclature. Within each species, antennal sensory tracts had been previously named with numbers (T1-Tx), starting from the ventral and going to the dorsal surface of the antennal lobe (*Apis mellifera:* Suzuki, 1975; Flanagan & Mercer, 1989; Galizia *et al.*, 1999; Kirschner *et al.*, 2006; Nishino *et al.*, 2009; *Camponotus* sp.: Zube *et al.*, 2008; Nakanishi *et al.*, 2010; Mysore *et al.*, 2009; *Atta vollenweideri:* Kelber *et al.*, 2010; *Cataglyphis* sp.: Stieb *et al.*, 2011), resulting in a mismatch in tract designations between presumably homologous ones. The present study thus used a novel nomenclature, naming glomerular clusters with Roman letters (T_A - T_I), starting from the dorsal surface to the ventral surface because this arrangement better revealed similarities among different species. Among the species listed above, the hornet antennal lobe was especially compared with the honeybee and carpenter ant ALs because these species provide solid data on AL input-output connectivity. For honeybees, *Apis mellifera*, we used the description of AL compartmentalization made by Kirschner *et al.* (2006), Nishino *et al.* (2009) and our personal data. For carpenter ants, the descriptions by Zube *et al.* (2008) and Zube & Rössler (2008) for *Camponotus floridanus* and Nakanishi *et al.* (2010) for *C. japonicus* were used.

Spatial directions given in each figure are based on the neuraxis (Fig. 1H of Strausfeld, 2002). Consequently, rostral and caudal correspond, respectively, to anterior and posterior, according to the body axis. The “frontal” surface of the brain corresponds to its most ventral region in the neuraxis.

Data analysis and statistics

To assess caste dimorphism in the number of glomeruli, a Mann-Whitney U test was used on 6 reconstructed ALs from each hornet caste. Potential type I errors induced by multiple comparisons were reduced by using a Bonferroni correction ($\alpha_{corr} = 0.05/2 = 0.025$).

Glomerular volumes were calculated from 3D reconstructions using Amira 5.4.3. To overcome the problem of AL size variability in different individuals, the volume of each glomerulus was normalized with respect to the size of AL (calculated as the sum of all glomerular volumes; **Fig. 4D**). To identify macroglomeruli a quantitative threshold that defines outliers according to 80% of the distribution of glomerular volumes was used:

$$V_{outlier} > V_U + k\ (V_U - V_L)$$

Where V_U is the upper percentile (90%) and V_L is the lower percentile (10%) of glomerular volume distribution. We used k = 3 as a conservative value that successfully categorized macroglomeruli in several hymenopteran species (Kuebler *et al.*, 2010; Streinzer *et al.*, 2013; Roselino *et al.*, 2015). Thus, glomeruli whose volume was above this threshold were considered as macroglomeruli.

Results

General brain morphology

Vespa velutina males and females can be distinguished based on head and abdomen morphology, but there are no evident morphological differences between the two female castes (worker and queen; **Fig. 2**). Queens (**Fig. 2A**) are usually larger than workers (**Fig. 2C**), but the sizes of these two castes sometimes overlap because of the increase in the body dimensions of workers produced throughout the colony cycle. Males' heads (**Fig. 2E**) show a typical triangular shape that is more elongated than females' heads, with slightly more bulbous compound eyes. In addition, males' antennae are longer than those of females, with shorter scapes but 11 flagellum segments instead of 10 in females. Confocal image stacks acquired from whole brain staining in each caste allowed 3D reconstruction of selected neuropils (**Fig. 2B, D, F**). No clear differences in brain structure were observed between sexes or between female castes, the different neuropils occupying similar relative volumes in all these individuals (**Table 1**). In particular, both male and female hornets display voluminous visual sensory structures, with 30.6% of brain volume occupied by the medulla and the lobula in the queen, 32.3% in the worker and 34.7% in the male. Likewise, the primary olfactory centers, the antennal lobes (ALs) display only slight sexual differences in their relative sizes, with 7.2% and 7.1% of brain volume in queen and worker, respectively, and 9.2 % in the male.

Caste	Units	Protocerebrum	Me	Lo	AL	co	li	br	Peduncles	CB	∑
queen	$X10^6$ µm^3	522.03	292.71	103.88	92.63	86.18	59.55	23.49	109.64	3.36	1293.48
	%	40.36	22.63	8.03	7.16	6.66	4.60	1.82	8.48	0.26	100
worker	$X10^6$ µm^3	442.80	291.68	92.16	84.08	109.15	55.12	22.20	85.85	3.76	1186.82
	%	37.31	24.58	7.77	7.08	9.20	4.64	1.87	7.23	0.32	100
male	$X10^6$ µm^3	428.01	273.23	94.85	97.46	58.73	30.83	15.98	58.17	3.43	1060.70
	%	40.35	25.76	8.94	9.19	5.54	2.91	1.51	5.48	0.32	100

Table 1: Volumes of the *V. velutina* brain neuropils
The absolute volume (in cubic micrometers) and the relative volume (in percentage) of major brain structures are given for each caste. For multiple structures (such as the collar), the sum of all instances of this structure is given. AL, antennal lobe; Me, medulla; Lo lobula; CB, central body; li, lip; co, collar.

Hornets' mushroom bodies have a characteristic shape, with two highly voluminous calyces on each brain hemisphere and comparatively small peduncle and lobes (**Fig. 2B, D, F**). As in other Hymenoptera (bees, ants, etc.), the calyces are composed of three concentric and morphologically distinct areas: the collar, the lip and the basal ring. However, in each calyx, the lips are placed more

centrally compared with the collar, which bulges around the lip, resembling a double-lip from an external viewpoint (**Fig. 2B, D, F**).

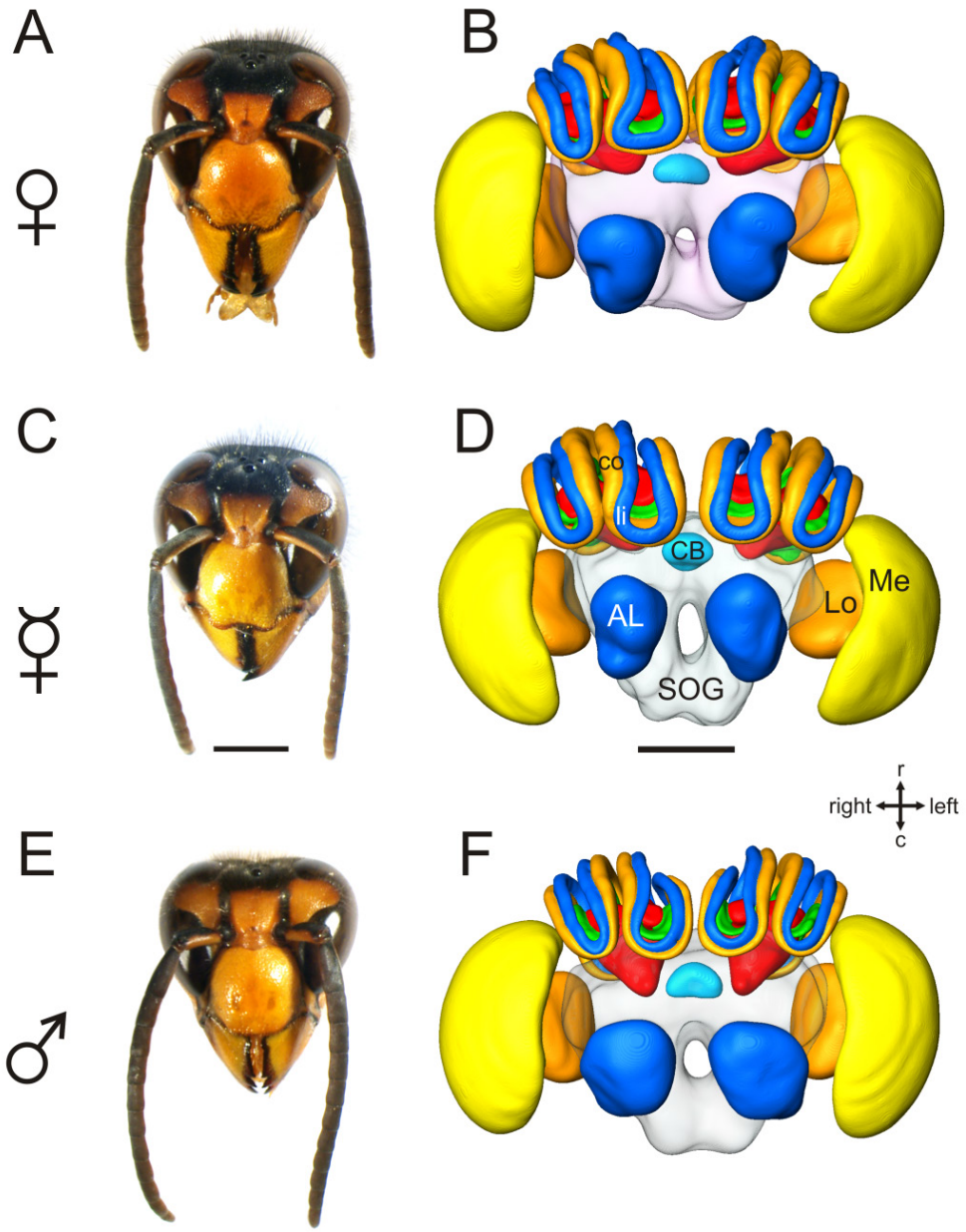

Figure 2: Head morphology and brain anatomy of the three main castes within a *V. velutina* colony
A, C, E) photographs of *V. velutina* heads (scale bar = 2 mm) showing no noticeable morphological differences between queen (**A**) and worker heads (**C**) but a triangular head with longer antennae in males (**E**). **B**, **D**, **F**) 3D reconstructions of hornet brains (scale bar = 500 µm). The brains of queen (**B**) and worker (**D**) do not show conspicuous differences in their organization but slightly smaller calyces and thinner peduncles were observed in males (**F**). Heads and brains are shown from the ventral surfaces and are oriented following the same axis. r, rostral; c, caudal; SOG, suboesophageal ganglion; AL, antennal lobe; Me, medulla; Lo lobula; CB, central body; li, lip; co, collar. Scale bars = 2 mm in **C** (applies to **A**, **C, E**); 500 µm in **D** (applies to **B**, **D**, **F**).

Differential mass staining of optic lobe and AL revealed that the collars are targeted by visual output neurons, whereas the lips receive only olfactory input (**Fig. 3A, B**). Our staining procedure revealed strong olfactory input in the hornet basal ring, but we could observe only sparse visual input in this area. Although this differential staining was not aimed at detailing visual sensory projections in

the hornet brain, it suggested an arrangement of these projections similar to that of honeybees (Mobbs, 1984; Ehmer & Gronenberg, 2002; Mota *et al.*, 2011). In brief, a prominent visual tract, the anterior optic tract (AOT), projects into the anterior optic tubercle, whereas in the rostral part of the brain a neuronal network composed of several subtracts connects the medulla and lobula with ipsi- and contra-lateral MB calyces. Direct inter-hemispheric connections between optic lobes were observed in more dorsal layers of the brain. At this level, diffuse ipsi-lateral projections into the dorsal protocerebrum are also found.

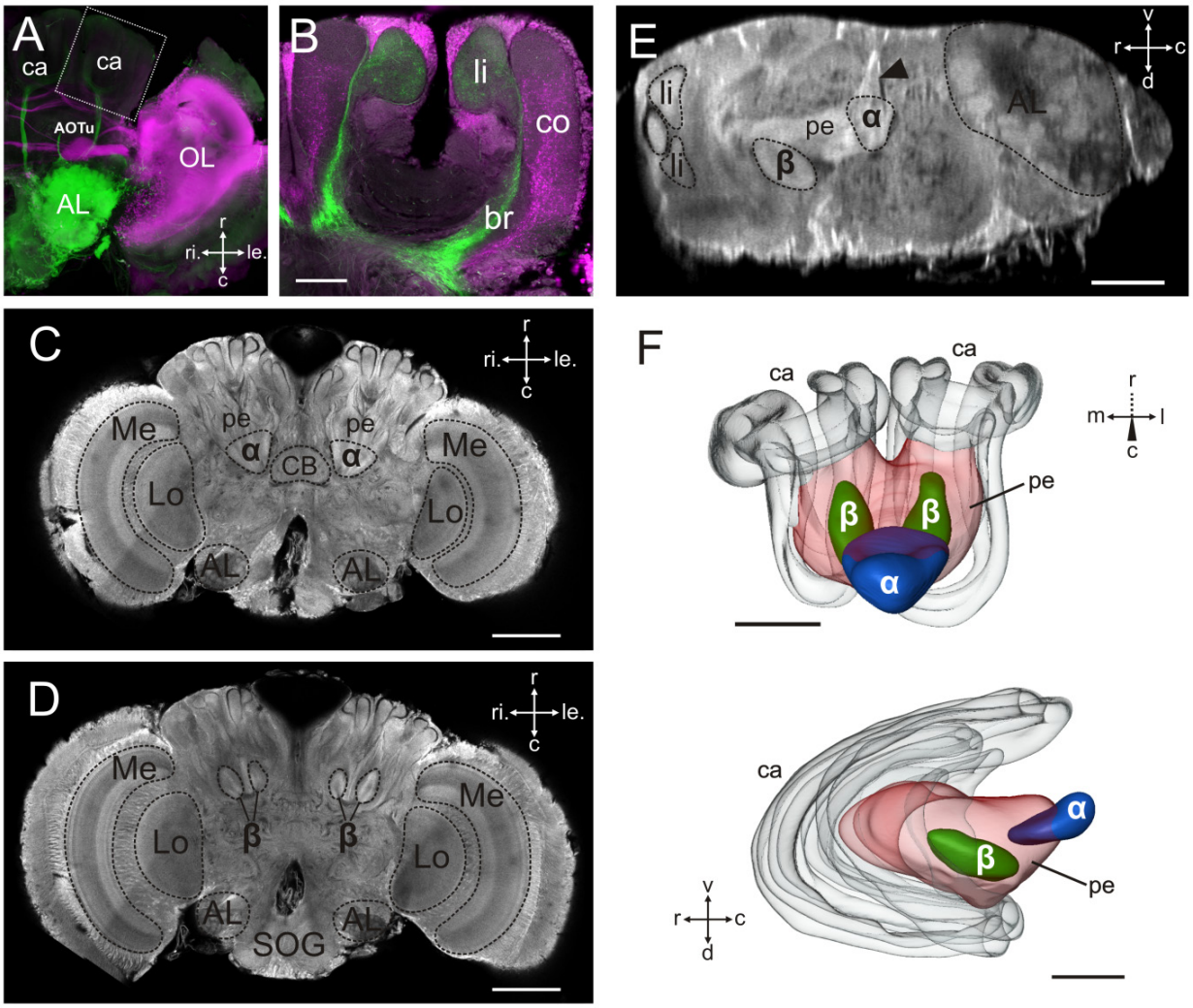

Figure 3: Mushroom body morphology in *V. velutina*
A, **B**) Frontal optical sections after mass staining in the optic lobe and in the AL with different fluorescent dyes. **B**) Section through the lateral mushroom body calyx (delimited in **A**). The collar receives visual input (magenta), whereas the lip receives olfactory input (green). **C, D**) Frontal sections through the brain at, respectively, 345 µm and 425 µm depths from the ventral surface. **E**) Sagital section of the brain. Each brain hemisphere contains a double β-lobe pointing dorsally and a single α-lobe projecting caudally, with only a fiber tract extending to the ventral surface (arrowhead). **F**) 3D model of a hornet mushroom body, showing the double β-lobe and the single α-lobe per hemisphere (left mushroom body). AL, antennal lobe; OL, optic lobe; Me, medulla; Lo, lobula; pe, peduncle; ca, calyces; li, lip; co, collar; CB, central body; r, rostral; c, caudal; v, ventral; d, dorsal; m, medial; l, lateral; ri, right; le, left. Scale bars = 80 µm in **B**; 400 µm in **C**, **D**; 200 µm in **E**, **F**.

Hornets' MB lobes are clearly different from those described for honeybees and ants (Mobbs, 1982; Mobbs, 1984; Ehmer & Gronenberg 2004; Gronenberg, 2008). In contrast to bees' or ants' MB

in which the α-lobe extends vertically to the ventral surface of the brain, the *V. velutina* α-lobe points horizontally in a caudal direction, and only a fine fiber tract attributed to MB extrinsic neurons (see Ehmer and Hoy, 2000) extends to the ventral surface (**Fig. 3C, E**). Moreover, whereas honeybees' or ants' β-lobes extend horizontally towards the brain midline, the hornet β-lobe is split in two subparts, which point dorsally (**Fig. 3D, E**), in much the same way as previously described in the wasp *Vespula germanica* (Ehmer and Hoy, 2000).

Overall, we did not observe any clear polymorphism in the hornet brain organization except for MB calyces and peduncles, which are slightly thinner in males than in females (**Fig. 2F**; 21.6% of brain volume in the queen, 23% in the worker, and 15.4% in the male). The brains of the two female castes are not visually distinguishable.

Antennal lobe organization

Confocal microscopic observations after mass staining of the antennal nerve allowed the segmentation of hornets' antennal lobes into glomerular units (n = 6 individuals per caste). We found no difference between female castes in the number of AL glomeruli, with approximately 269 ± 9 glomeruli in queens (mean ± SD, n = 6) and 264 ± 7 glomeruli in workers (n = 6; Mann-Whitney test, U = 10, nonsignificant). We observed, however, a slight sexual dimorphism; the male AL contains 247 ± 6 glomeruli (n = 6), i.e., significantly fewer than in both female castes (Mann-Whitney test, male vs. queen, U = 1, $p < \alpha_{corr} = 0.025$; male vs. worker, U = 1, $p < \alpha_{corr} = 0.025$).

From these 3D-reconstructions, we measured the volumes of glomeruli in the antennal lobes of each caste. Figure **4A-C** shows individual and average distributions of glomerular volumes in queens, workers and males (n = 6 in each group). In all castes, approximately 80% of glomerular volumes were distributed between 5 x 10^3 and 100 x 10^3 µm^3, most glomeruli displaying a volume of approximately 15 x 10^3 µm^3. In workers and queens, the largest glomeruli reached an absolute volume of approximately 225 x 10^3 µm^3. In males the distribution reached higher values, with the largest glomeruli approaching 1300 x 10^3 µm^3. Thus, the three castes seem to share a common pattern of glomerular organization with the exception of a few substantially enlarged glomeruli in males.

To determine whether these glomeruli are large enough to be termed macroglomeruli, we used a standard quantitative measure that defines outliers with regards to the distribution of glomerular volumes (**Fig. 4D**). In queens, as in workers, the four largest glomeruli are located in the most dorsal (two glomeruli) and in the ventrolateral regions (two glomeruli) of the antennal lobe (**Fig. 4B**, inset). They do not reach the statistical threshold and cannot be considered macroglomeruli. Similarly sized glomeruli are found at the same positions in males (see black dots, **Fig. 7**). By contrast, the male AL showed several enlarged glomeruli that reached the significance threshold (**Fig. 4D** and **Fig. 5**). They were grouped in two clusters, a rostral cluster containing the largest hornet

macroglomerulus (MG1) and two smaller macroglomeruli (MG2, MG3), and a ventrocaudal cluster, positioned close to the antennal nerve, containing a group of four macroglomeruli. Three of them (MG4, MG5 and MG6) pass the threshold, but the fourth is marginally below the threshold. However, these four glomeruli show exactly the same OSN innervation pattern, which is much denser than that of all the other normal-sized glomeruli in this area. We thus consider this enlarged glomerulus as a possible macroglomerulus, termed MG7. We conclude from these observations that queens and workers have similar antennal lobes without hypertrophied glomeruli, whereas males possess seven macroglomeruli distributed in two clusters.

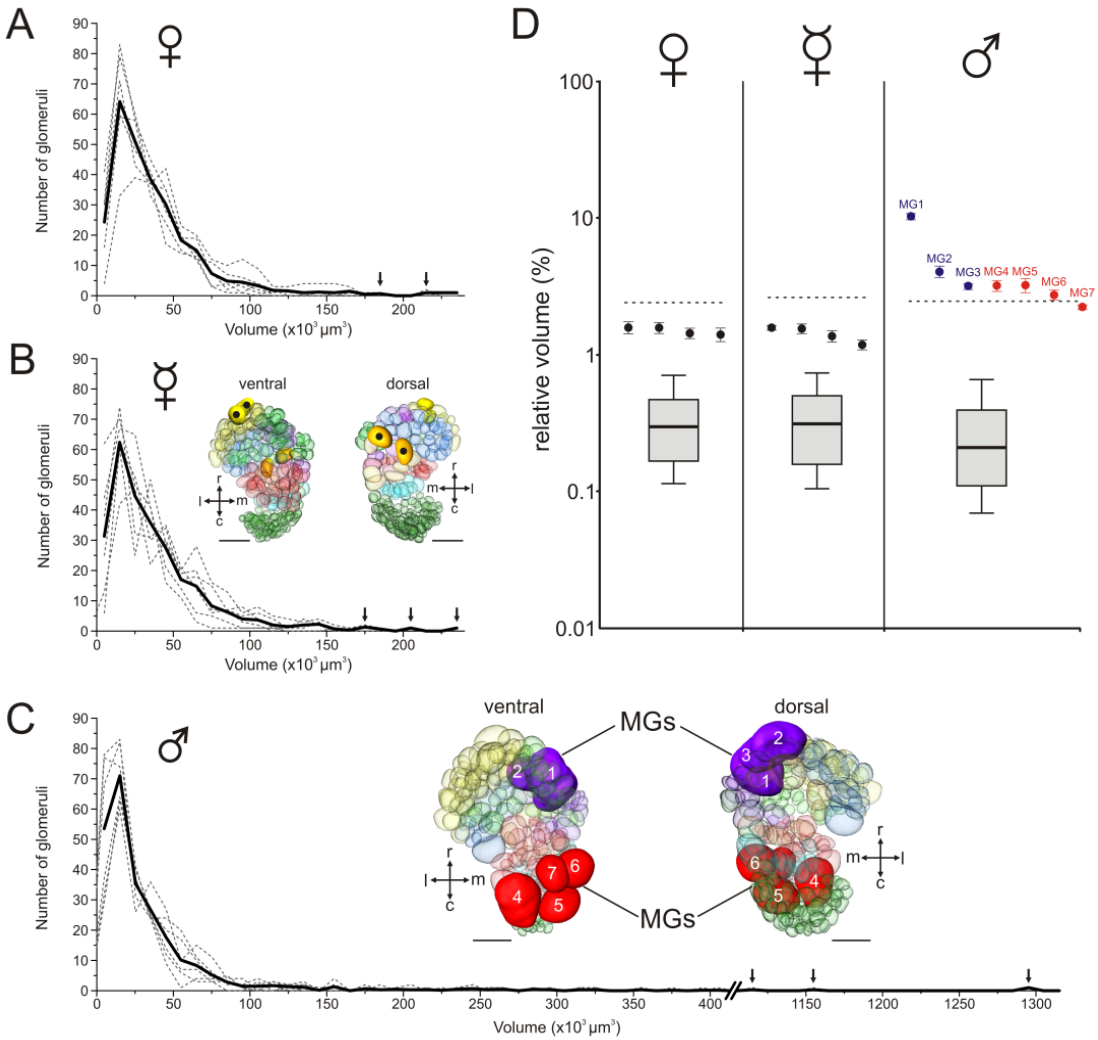

Figure 4: Volume distributions

A, **B, C**) Glomerular volume distributions in the AL of six individuals (dotted lines) of each caste and their average distributions (solid line). Queens (**A**) and workers (**B**) exhibit the same glomerular volume distribution. The largest glomeruli were identified at the same locations in the AL of workers and queens (black dots in **B**; see also in **Fig. 7** and **8D**, **E**). In males (**C**), the upper part of the distribution extends to much greater volumes than in female castes. The AL of males contains seven hypertrophied glomeruli arranged in 2 clusters (MG1-7). Coloring of glomeruli relates to glomerular clusters as in Figure 7. **D**) Relative glomerular volume distribution in the 3 three castes and positions of the largest glomeruli with regards to the statistical macroglomerulus threshold (dotted line). Dots Indicate the average volume of the four (workers and queens) or seven (males) largest AL glomeruli of each caste. Only males possess glomeruli exceeding the statistical threshold. Bold lines indicate the median of glomerular volumes, boxes the first and the third quartiles (25-75%), and whiskers the 10th to 90th percentiles of the distribution. MGs, macroglomeruli; r, rostral; c, caudal; m, medial; l, lateral. Scale bar = 100 µm.

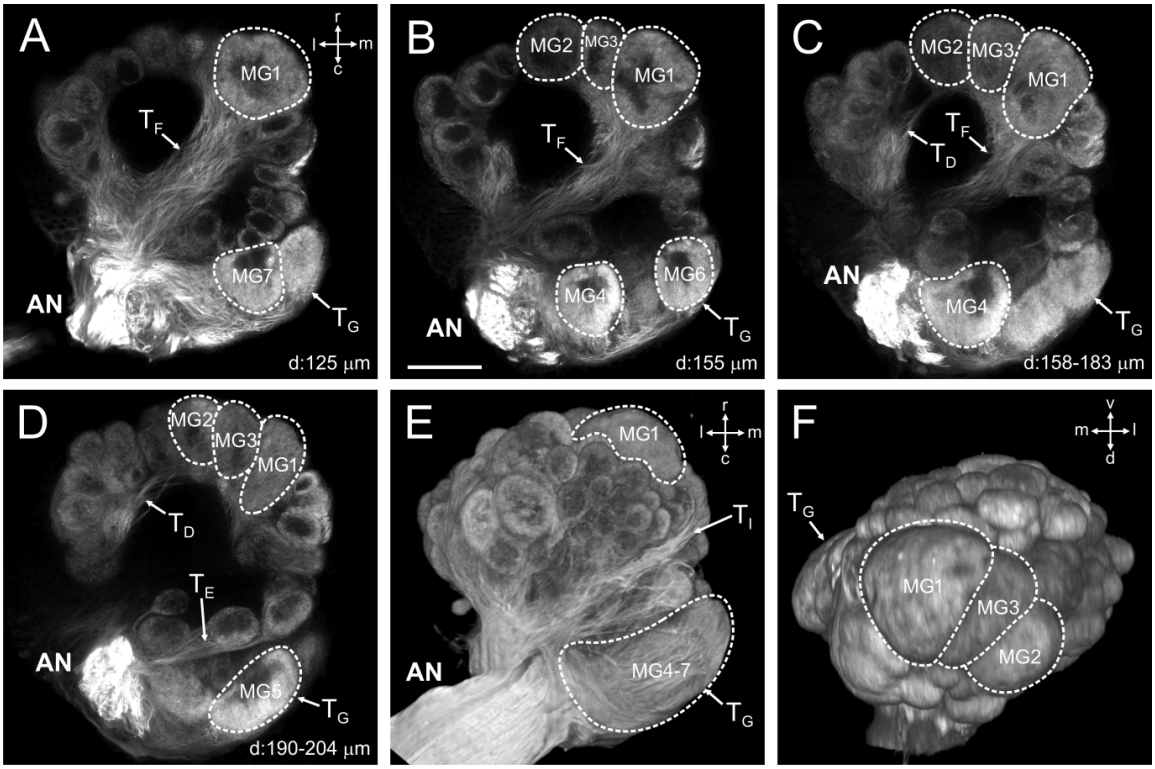

Figure 5: Macroglomeruli of the male antennal lobe
A-D) Confocal images (single section or z projection) at different depths from the ventral surface of a right antennal lobe in a *V. velutina* male. **E**, **F**) Ventral and rostral view, respectively, of a 3D volume rendering of the male AL. The macroglomeruli (dotted lines) are arranged in two clusters: the rostral cluster, receiving a thick olfactory tract through the AL, contains three macroglomeruli (MG 1-3). The ventrocaudal cluster, located close to the antennal nerve entrance, contains four macroglomeruli (MG 4-7). r, rostral; c, caudal; v, ventral; d, dorsal; m, medial; l, lateral; AN, antennal nerve. Scale bar = 100 µm.

Antennal lobe afferent connection pattern

We next examined the precise projection pattern of OSNs in the AL of each caste. Because close inspection of projection patterns showed that queen and worker ALs are indistinguishable, they are often described together as “female" antennal lobes hereafter. In hornets, the antennal nerve approaches the AL laterally from the ventral side. As in other Hymenoptera, part of the antennal nerve bypasses the AL and slopes down toward the antennal mechanosensory and motor center (AMMC). OSN axons separate from this main trunk at different depths with various orientations, forming nine distinct sensory tracts, which we termed $\mathbf{T_A}$ - $\mathbf{T_I}$, from the most dorsal to the most ventral (**Fig. 6**). These tracts give rise to nine clusters of glomeruli, which display distinctive morphological features. **Table 2** gives an overview of glomeruli numbers and volumes for each cluster.

- In the most dorsal part of the AL, eight glomeruli are innervated by several loose bundle tracts ($\mathbf{T_A}$) which were particularly brightly labeled in most preparations. These glomeruli are identifiable in the three castes by their typical shapes and positional arrangements (**Fig. 6M**-**O**). They are characterized by elongated or bent shapes and receive axon terminals penetrating the

entire glomerulus. Their volumes are relatively large, especially for 2 of them that correspond to the largest female glomeruli (black dots in **Fig. 7E**, **F**).

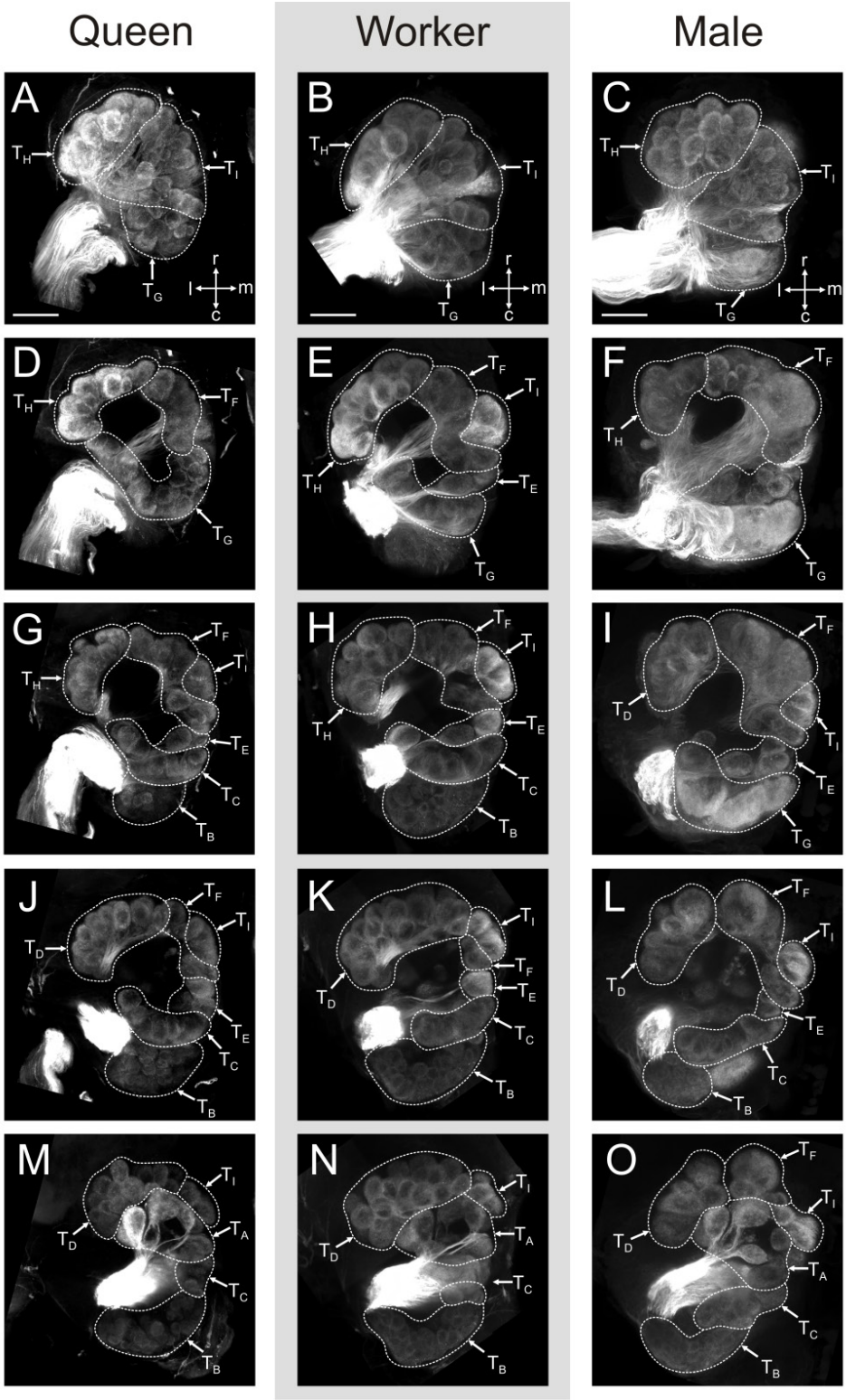

Figure 6: **Sensory innervation of the antennal lobe of each caste**

A-**O**) Projection views of approximately 50-µm-thick slices (~50 optical sections), starting from the ventral surface to the dorsal surface of a right AL after mass staining of the antennal nerve. The hornet AL contains nine sensory tracts (T_A-T_I) innervating distinct clusters of glomeruli. All antennal sensory tracts were observed in queens (**A**, **D**, **G**, **J**, **M**), workers (**B**, **E**, **H**, **K**, **N**), and males (**C**, **F**, **I**, **L**, **O**), but some tracts were thicker in males than in females (for instance, T_F in **F** compared to **D** and **E**). r, rostral; c, caudal; m, medial; l, lateral. Scale bar = 100 µm.

- The dorsocaudal region of the AL harbors a conspicuous cluster of numerous and comparatively smaller glomeruli than in the rest of the AL, rather lightly innervated in their cortex (**Fig. 6J-O**). This cluster is mostly responsible for the observed variability between sexes and among individuals in the total number of AL glomeruli. Because it was not brightly stained in some preparations, the variability observed in this cluster might be due in part to difficulties in discerning all glomeruli. We counted 93 ± 7 (mean ± SD) glomeruli in queens, 89 ± 7 glomeruli in workers and 71 ± 5 glomeruli in males. These glomeruli receive OSN axons from the $\mathbf{T_B}$, which originates from the ventral side and points dorsomedially (**Fig. 7E, F**).
- The $\mathbf{T_C}$ tract runs transversally through the AL, i.e., from the antennal trunk (on the lateral side) toward the dorsomedial side, determining a separation between the T_B cluster and the rest of the AL (**Fig. 6G, H, J-L**). It sparsely innervates approximately 15 glomeruli, mostly from their caudal periphery.
- Facing this cluster, the $\mathbf{T_D}$ proceeds transversally through the inner dorsorostral part of the AL and densely innervates approximately 30 glomeruli in females and 25 glomeruli in males (**Fig. 6I-O**). We observed there a slight sexual dimorphism, with a thicker tract and relatively larger glomeruli in males, although none of them reached the macroglomerulus volume threshold (**Table 2** and **Fig. 7E, F**).
- More ventrally, we found in both sexes a brightly labeled tract, $\mathbf{T_E}$, which runs in the inner part of the AL, parallel to the T_C. This tract projects mediodorsally to only three glomeruli (**Fig. 6E, G-I,** and **Fig. 7C, D**). The deepest T_E glomerulus shows a bent shape similar to that of T_A glomeruli and also receives axon terminals in its core region. It is however clearly innervated by the T_E tract.
- The $\mathbf{T_F}$ is a thick tract that crosses the AL from the caudolateral side to the rostromedial side and innervates approximately 20 glomeruli in both males and females (**Fig. 6D-F**). Despite similar numbers of glomeruli between the sexes, the T_F is much thicker in males. It contains numerous axons that densely project to three macroglomeruli MG1, MG2 and MG3, forming the rostral macroglomerular cluster (**Fig. 7C, D**).
- Running at the ventral surface of the AL, the $\mathbf{T_G}$ separates from the antennal nerve and takes a mediocaudal direction. It innervates approximately 30 glomeruli mostly from the caudal periphery (**Fig. 6A-F**). In males, this tract is also thicker than in females and projects into four macroglomeruli close to the antennal nerve (MG4-7), forming the ventrocaudal macroglomerular cluster (**Fig. 7A-B**).
- The $\mathbf{T_H}$ spreads from the antennal nerve to the rostral surface of the AL (**Fig. 6A-H**). This cluster consists of approximately 30 relatively large glomeruli and contains among the largest female glomeruli (black dots, **Fig. 7A, B**).

- Finally, the **T_I** extends its axons within approximately 40 glomeruli from the lateral to the medial side of the AL, forming a cluster sided by T_G and T_H glomeruli (**Fig. 6A-C**). It innervates glomeruli on the ventral surface and then continues medially toward the dorsal side of the AL (**Fig. 6G-I**).

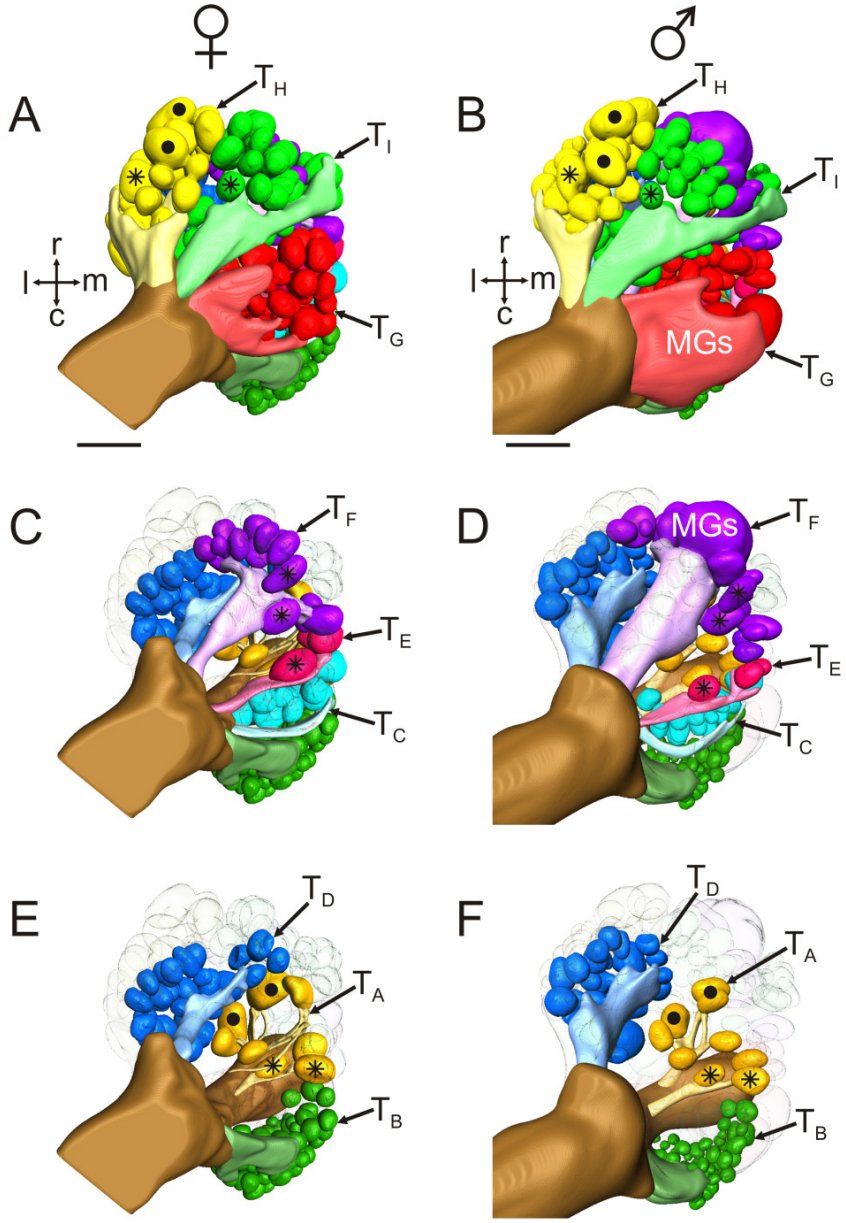

Figure 7: 3D reconstructions of the antennal lobes of *V. velutina*
A, **C**, **E**) female. **B**, **D**, **F**) male. The nine clusters of glomeruli and their afferent tracts (T_A-T_I) are color coded and are represented from the ventral to the dorsal surface (right AL). AL organization is highly similar in males and females, but sexually dimorphic clusters that contain macroglomeruli in males are innervated by thicker tracts (T_F and T_G). Dots indicate the four largest glomeruli in females and their homologues in males. Asterisks indicate landmark glomeruli. r, rostral; c, caudal, m, medial; l, lateral. Scale bar = 100 µm.

Antennal lobe efferent pathways

Mass staining of the AL with a fluorescent dye revealed that *V. velutina* has a typical hymenopteran olfactory pathway with 5 output PN tracts (**Fig. 8A**). They seem to correspond to the three multiglomerular (ml-ALT) and two uniglomerular (l- and m-ALT) PN tracts observed in other

hymenopteran species (Abel *et al.*, 2001; Kirschner *et al.*, 2006; Zube *et al.*, 2008). Among the ml-ALT tracts, two leave the AL medially and project transversally to the lateral protocerebrum and the LH. They contribute to a lateral network, and their characteristics are highly similar to those of the honeybee ml-ACT1 and 2 (Abel *et al.*, 2001; Kirschner *et al.*, 2006). A third ml-ALT tract projects rostrally and bends around the fiber tract extending from the α-lobe, sending collaterals towards the peduncle and the lateral network. This tract ramifies in several bundles, ending in the LH and in the caudal protocerebrum. Its trajectory and projections in the protocerebrum are therefore different from those of the honeybee's ml-ACT3 (Kirschner *et al.*, 2006).

Next to these ml-ALT PNs, two prominent tracts originate from the dorsal region of the AL and run through the protocerebrum along two widely differing routes, innervating both the LH and the MB calyces. Their course resembles greatly that observed in bees (Abel *et al.*, 2001; Kirschner *et al.*, 2006) and ants (Zube *et al.*, 2008). One median tract (m-ALT) projects rostrally, then follows a mediolateral route to send first collaterals to both MB calyces and then continues onward to end in the LH. The lateral tract (l-ALT) bends laterally from the exit of the AL, first sends collaterals to the LH and then continues rostrally, eventually forming multiple branches innervating the MBs calyces. We investigated the glomerular connectivity of these tracts in the AL by differentially labeling m-ALT (magenta) and l-ALT (green) PNs (n = 2 females, **Fig. 8B-H**). As in honeybees and ants, both tracts arborize in different hemilobes of the AL (**Fig. 8B**) and project to partly segregated areas in the lips (**Fig. 8B, C**) and the LH (not shown). The l-ALT arborizes in ~138 glomeruli in the ventral surface and the rostral region of the AL, whereas the m-ALT arborizes in ~127 glomeruli in the dorsal and the caudal regions (**Fig. 8D, E**). Thus, compared to the honeybee AL, the two hemispheres of the hornet AL seem to be rotated by approximately 45° along the rostrocaudal and mediolateral axes, similarly to what has been observed in the ant *C. floridanus* (Zube *et al.*, 2008). Somata of l-ALT neurons encircle the rostral side of the AL (lSC; **Fig. 8D, E, G**). By contrast, m-ALT somata are distributed mainly in two clusters, a dorsolateral cluster close to the antennal nerve (mSC1; **Fig. 8D, E, H**) and a rostromedian cluster located next to l-ALT somata (mSC2; **Fig. 8D, E, G**).

Antennal lobe input-output connectivity

To allocate glomerular clusters to either the m- or the l-ALT, we compared OSN and PN stainings of different individuals with the help of the remarkable tract architecture of the hornet AL and of landmark glomeruli (see asterisks and black dots in **Fig. 7** and **Fig. 8D, E**). **Table 2** presents the output tract affiliation of the different glomerular clusters. We observed that the $\mathbf{T_A}$ cluster is slightly innervated by m-ALT PNs, except for two large landmark glomeruli which were not stained by either of the uniglomerular tracts. Dorsocaudal clusters, $\mathbf{T_B}$ and $\mathbf{T_C}$, clearly belong to the m-subsystem whereas ventro-rostral clusters $\mathbf{T_D}$, $\mathbf{T_F}$, $\mathbf{T_H}$ and $\mathbf{T_I}$ clearly belong to the l-subsystem. The $\mathbf{T_E}$ cluster is innervated by m-ALT PNs except for one glomerulus that receives processes from both m- and l-ALT

neurons. Glomeruli of the T_G are divided in 2 groups; about 24 glomeruli belong to the m-subsystem and nine belong to the l-subsystem.

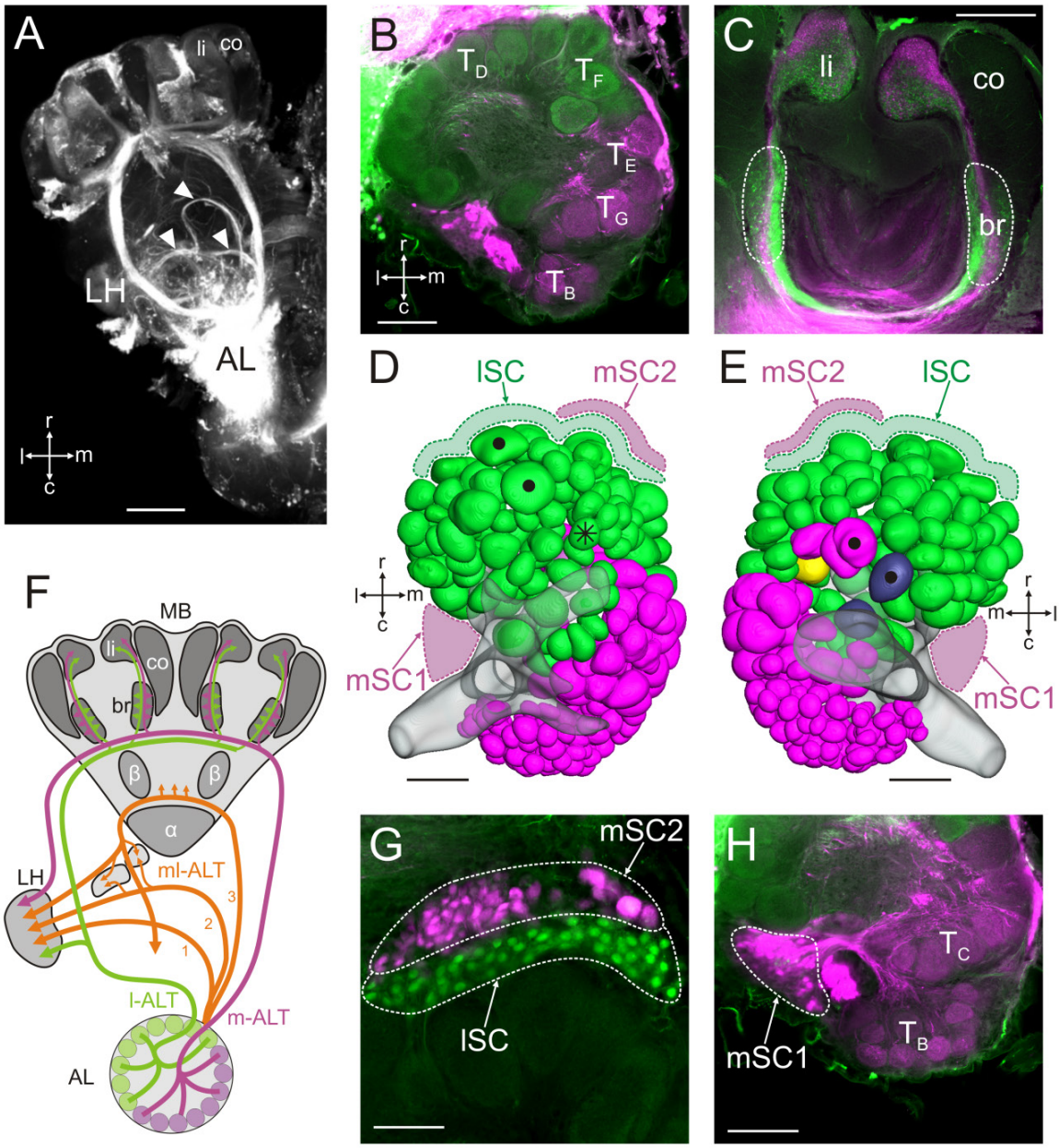

Figure 8: Efferent innervations of the antennal lobe of *V. velutina*

A) Projection view of an anterograde mass-fill of all AL output tracts (300 µm thick slice, scale bar = 200 µm). Two prominent tracts (m and l-ALT) project from the AL to the MB calyces and the LH, whereas three ml-ALT (arrowheads) project broadly to the lateral protocerebrum and the LH. **B**, **C**) Optical section through the AL (**B**) and MB calyces (**C**) after differential labeling of the l-ALT (green) and m-ALT (magenta) (scale bars = 100 µm). The two output tracts arborize in segregated parts of the AL and project differentially to the lips and the basal ring of the MB calyces. **D**, **E**) 3D reconstruction of the *V. velutina* antennal lobe showing the affiliation of glomeruli with the two uniglomerular AL output tracts (m- and l-ALT; **D** = ventral view and **E** = dorsal view, scale bar = 100 µm). Glomeruli innervated by the l-ALT (green) are in the rostroventral hemilobe of the AL whereas m-ALT innervated glomeruli (magenta) are located in the dorsocaudal hemilobe. Two glomeruli (dark blue) show no innervations from ALTs, whereas one glomerulus (yellow) receives processes from both m- and l-ALT. The somata of the l-ALT are spread along the rostral rim of the antennal lobe (lSC), whereas the somata of the m-ALT are distributed in a caudolateral cluster (mSC1) and a rostral cluster (mSC2). **F**) Schematic overview of the output network of the antennal lobe. **G**) Rostral somata clusters of l-ALT (lSC) and m-ALT neurons (mSC2). Scale bar = 50 µm. **H**) Caudo-lateral somata cluster of the l-ALT (m-SC1), Scale bar = 100 µm. All views correspond to the right brain hemisphere.

Caste	Measure	T_A	T_B	T_C	T_D	T_E	T_F	T_G	T_H	T_I	∑
♀	∑ glomeruli	8	90	15	29	3	22	33	29	37	266
	Min volume ($x10^3$ μm^3)	51.3	3.3	11.3	10.5	58.9	4.6	8.1	2.5	10.3	
	Max volume ($x10^3$ μm^3)	200.1	31.2	57.7	92.3	85.5	74.6	66.2	104.5	103.6	
	Mean volume ($x10^3$ μm^3)	91.1	12.6	33.0	35.7	72.8	35.4	33.5	37.6	32.1	
	output tract	m-ALT (6 out of 8)	m-ALT	m-ALT	l-ALT	m-ALT (2) / both-ALT (1)	l-ALT	l-ALT (9) / m-ALT (24)	l-ALT	l-ALT	m-ALT (138) / l-ALT (127)
♂	∑ glomeruli	8	74	17	24	3	21	32	32	38	249
	Min volume ($x10^3$ μm^3)	39.4	2.2	8.5	6.9	13.7	1.2	3.7	5.3	4.1	
	Max volume ($x10^3$ μm^3)	136.1	25.2	47.4	250.7	38.4	763.1	285.6	87.3	62.9	
	Mean volume ($x10^3$ μm^3)	67.1	8.9	21.8	44.1	25.8	90.9	51.3	30.8	22.7	

Table 2: Cluster characteristics in hornets
Number, volume and output tract affiliation of glomeruli belonging to the nine different clusters of the antennal lobe in female and male hornets.

Common features of the hymenopteran antennal lobe

When studying the input-output connectivity of the hornet AL, we noticed conspicuous similarities to the ALs of two well-described hymenopteran species, the honeybee *Apis mellifera* (Apidae) and the carpenter ants *Camponotus* sp. (Formicidae). Several glomerular clusters presented strikingly similar features in the different species, which led us to propose a common framework for describing these antennal lobes. **Table 3** presents the putative homologies between the antennal lobes of the three model species, and **Table 4** lists the typical features defining each cluster type.

A. mellifera [a]			*V. velutina*			C. floridanus[b] and C. japonicus[c]		
Input	∑ glomeruli	output tracts	Input	∑ glomeruli	output tracts	Input	∑ glomeruli	output tracts
T4	7	m-ALT / 5 out of 7	T_A	8	m-ALT / 6 out of 8	**T7**	6 or 10	m-ALT / 5 out of 6
T3b	12	m-ALT	T_B	90	m-ALT	**T6**	128 or 140	m-ALT
T3c	42	m-ALT	T_C	15	m-ALT	**T5**	36 or 35	m-ALT
			T_D	30	l-ALT	**T4**	78 or 95	l-ALT
T2	7	l-ALT / m-ALT / both	T_E	3	m-ALT / both			
T1	72	l-ALT	T_F	20	l-ALT			
T3a	24	l-ALT / m-ALT	T_G	30	l-ALT / m-ALT	**T3**	96 or 110	l-ALT / m-ALT
			T_H	30	l-ALT	**T2**	56 or 45	l-ALT
			T_I	40	l-ALT	**T1**	34 or 35	l-ALT

Table 3: Putative homologies between glomerular clusters in three well-described hymenopteran species
Each line contains corresponding antennal sensory tracts in honeybees (*A. mellifera*), hornets (*V. velutina*) and carpenter ants (*C. floridanus* and *C. japonicus*) with reported numbers of glomeruli and their output tract affiliations. Dark grey cells indicate missing tracts in the given species. Putatively homologous tracts are aligned according to the new denomination implemented in hornets, from most dorsal to most ventral.
[a] after Kirschner *et al.* (2006) and Nishino *et al.* (2009); [b] after Zube *et al.* (2008) and Zube and Rössler (2008); [c] after Nakanishi *et al.* (2010).

The most striking observation is that all glomerular clusters found in honeybees and in ants have an equivalent cluster in hornets, whereas some clusters found in ants are not found in bees and vice versa. For observing these homologies in the best conditions, the ALs should be oriented similarly in the three species, with the antennal nerve entrance on the same axis, and the separation plane between l-ALT and m-ALT hemilobes in the same orientation across species. We detail below the clues supporting the proposed homologies.

Glomerular cluster $\mathbf{T_A}$ is the most dorsal in hornets and presents very distinctive features, found in all three species. It contains a few large glomeruli with peculiar shapes and positional arrangements. It belongs mostly to the m-ALT subsystem, but, remarkably, two glomeruli are innervated neither by m-ALT nor by l-ALT PNs. In honey bees and carpenter ants, the most dorsal cluster (T4 in *Apis mellifera,* T7 in *Camponotus*) shows the same features, which led previous authors to suggest their homology (Zube *et al.*, 2008; Nakanishi *et al.*, 2010). The "necklace" organization of glomeruli $\mathbf{T_A}$-01, -03, -05, -06 and -08 in hornets greatly resembles that of glomeruli D01, D03, D05, D06, D08 in honeybees (**Fig. 9A**; compare Fig. 5B of Kirschner *et al.*, 2006). The homology is even more remarkable between hornet T_A-02 glomerulus (**Fig. 9A**) and honeybee D02 glomerulus (see Fig. 3H, I, of Kirschner *et al.*, 2006); both are egg-shaped, positioned close to the antennal nerve, and lack any uniglomerular PN arborization.

The $\mathbf{T_B}$ is a dorsomedial cluster, partly segregated from the main part of the AL, which flanks the antennal nerve and generally contains smaller glomeruli than the main AL, innervated by m-ALT PNs (**Fig. 9A**). These features clearly correspond to those of the T6 cluster of *Camponotus* workers (compare Fig. 2C of Zube *et al.*, 2008) and are also found in the T3b of *Apis mellifera*, although it contains much fewer glomeruli than in the other species (see Fig. 4A-C of Kirschner *et al.*, 2006).

Glomeruli belonging to the $\mathbf{T_C}$ also belong to the m-ALT subsystem. They occupy a caudomedial position within the AL of hornets and receive sensory innervations mostly from the caudal periphery of the AL. These features fit with those of the honeybee T3c and the ant T5 clusters, which have similar PNs arborization, locations, and afferent innervations (**Fig. 9B**; compare Fig. 3G of Kirschner *et al.*, 2006; see also contralateral view in Fig. 2J-O of Nakanishi *et al.*, 2010).

The $\mathbf{T_D}$ glomeruli belong to the l-ALT subsystem and receive axon terminals from a highly characteristic thick tract that proceeds along the inner lateral part of the AL (**Fig. 9B**). Similar features were observed in *Camponotus* ants with the T4 tract (compare Fig. 1C of Zube *et al.*, 2008), but we did not find any clear correspondence in *Apis mellifera*.

Conversely, two other clusters, $\mathbf{T_E}$ and $\mathbf{T_F}$, had clear correspondences in *A. mellifera* (with T2 and T1, respectively) but not in carpenter ants. The honeybee T2 was considered as homologous to the hornet $\mathbf{T_E}$ because in both species this sensory tract runs through the inner medial part of the AL and projects to very few glomeruli with singular shapes (**Fig. 9C**; compare Fig. 3E of Kirschner *et al.*, 2006). This homology is strongly supported by the features of a landmark glomerulus. The *Apis*

mellifera B02 glomerulus is innervated by both l- and m-ALT PNs and receives sensory axon terminals in both its cortex and its core (see Fig. 4F,H of Nishino *et al.*, 2009). The hornet **T_E**-01 glomerulus shows exactly the same features (**Fig. 9C**).

On the other hand, the putative homology between the hornet **T_F** and the *Apis mellifera* T1 is based on their similar affiliation to the l-ALT subsystem and to the conspicuous sensory tract projecting through the midline of the AL (**Fig. 9C**; compare Fig. 3E of Kirschner *et al.*, 2006). In both species, it exhibits a clear sex dimorphism, being much thicker in males than in females and containing several macroglomeruli (**Fig. 5B**; see Fig. 5D-F of Nishino *et al.*, 2009).

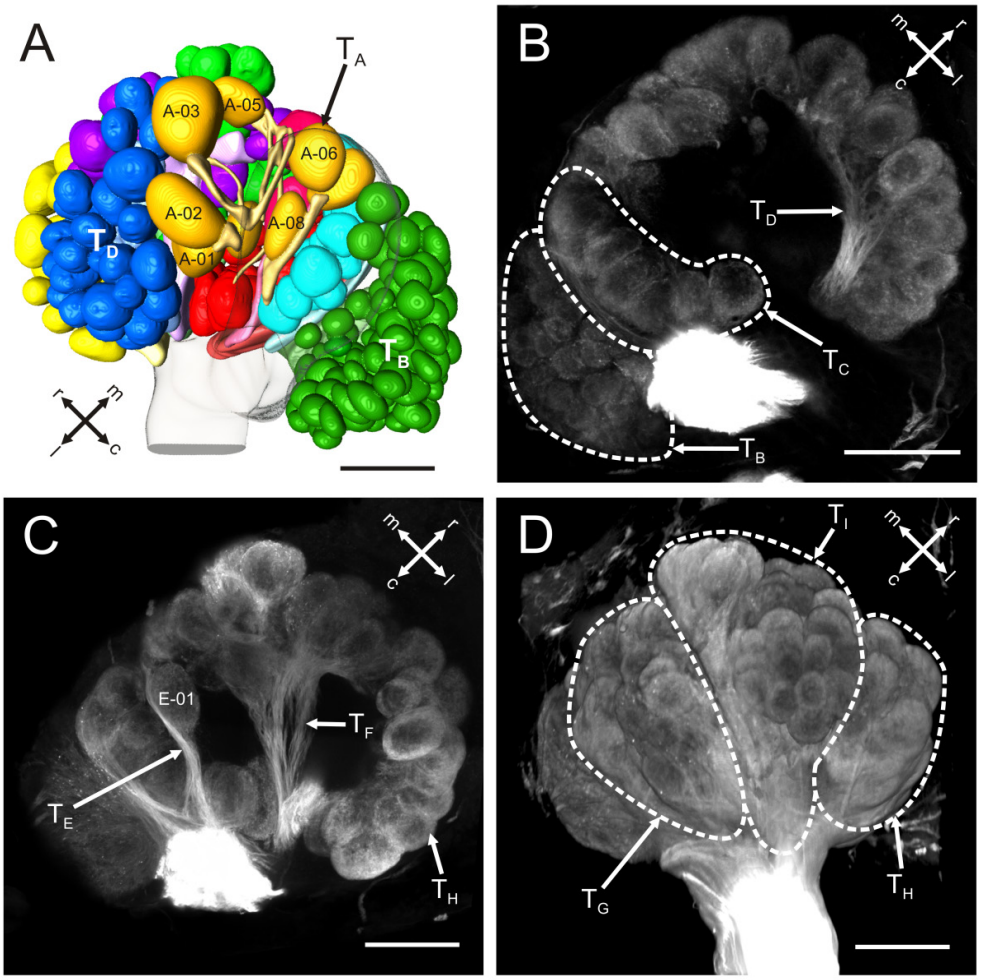

Figure 9: Morphological similarities between the AL of *V. velutina* and the ALs of other hymenopterans
A) Dorsal view of a 3D reconstruction of a *V. velutina* AL. Compare to Fig 2B, C of Zube *et al.* (2008) and Fig 4A-C of Kirschner *et al.* (2006). **B**) Projection view of a 30-µm-thick slice of a left *V. velutina* AL after mass staining of the antennal nerve (depth: 175-205 µm). Compare Fig 1C of Zube *et al.* (2008) and Fig 3G of Kirschner *et al.* (2006). **C**) Idem as **B**, but depth: 120-150 µm. Compare Fig 3E in Kirschner *et al.* (2006). **D**) Ventral view of a 3D volume rendering of a left female AL. Compare Fig 3A of Zube and Rössler (2008) and Fig 2A-F of Nakanishi *et al.* (2010). For each picture, the antennal nerve orientation was aligned with that of bees and ants as presented in the cited studies. For comparison with previous studies, all images show a left AL. Remarkable glomerular clusters and landmark glomeruli are labeled with dotted lines and arrows. Scale bars = 100 µm.

In *V. velutina* as well as in *A. mellifera* and carpenter ants, only one glomerular cluster is shared between the l- and the m-ALT subsystems, some of its glomeruli containing only l-ALT

processes, others only m-ALT processes (they are termed **T_G**, T3a, and T3, respectively in the three species). This cluster is located in the mediocaudal part of the AL in all species and contains glomeruli that are innervated mostly from their medial periphery (**Fig. 9D**; compare Fig. 1A of Zube *et al.*, 2008; see also Fig. 3C-F of Kirschner *et al.*, 2006).

Visually, the ventral surface of the hornet AL bears a strong resemblance to that of the carpenter ant, exhibiting similar clusters (**Fig. 9D**; compare Figs. 1A and 2B of Zube & Rössler, 2008). Glomeruli of the lateral surface receive sensory afferences from the T_H in *V. velutina* or the T2 in *Camponotus sp.* and belong to the l-ALT. Glomeruli from the hornet **T_I** or ant T1 cluster also belong to the l-ALT and are innervated by a sensory tract that first projects to the rostral part of the AL before sloping down dorsally (**Fig. 9D**; compare contralateral view Fig. 2A-F of Nakanishi *et al.*, 2010). No equivalent of these clusters is, however, present in honeybees. All in all, these observations suggest that the hornet antennal lobe presents both honeybee and ant features.

Tract	Depth	Cluster position	Uniglomerular PN output	OSN input pattern	Glomerular size	Glomerular features / OSN innervation
T_A	dorsal	necklace arrangement	m-ALT / none	several bundles	large	egg-shaped, dense innervation in both core and cortex
T_B	dorsal	Caudal / medial	m-ALT	from caudo-ventral surface	small	clumped glomeruli, innervation in cortex
T_C	relatively dorsal	Caudal / medial	m-ALT	from caudal to medial (running along T_B)	normal	innervation in cortex, thin cortex
T_D	relatively dorsal	Rostral / lateral	l-ALT	thick, inner lateral part of the AL	normal	innervation in cortex
T_E	medium	medial	l-ALT / m-ALT / both	through the inner medial part of the AL	relatively large	dense innervation in both core and cortex
T_F	medium	rostral	l-ALT	transversally through AL midline	normal (possible sex dimorphism)	innervation in cortex
T_G	relatively ventral	medial	l-ALT / m-ALT	from caudal periphery to medial	normal (possible sex dimorphism)	innervation in cortex
T_H	ventral	lateral	l-ALT	From lateral periphery to rostral	relatively large	innervation in cortex
T_I	ventral	rostral	l-ALT	caudal to rostral	normal	innervation in cortex

Table 4: Typical features of glomerular clusters across species
Each line gives the relative depth, the localization and the output tract affiliation of each cluster. The fifth column indicates the projection pattern (orientation) of the olfactory sensory tract. The sixth and the seventh columns specify typical glomerular features such as relative sizes, shapes and OSN innervation types (in the whole glomerulus or only in the glomerular cortex).

Discussion

We studied the neuronal olfactory pathway of the hornet brain and its sex and caste-specific adaptations. Whole-brain staining revealed that the overall organization of the *Vespa velutina* brain is highly similar in the two sexes and between female castes. Whereas the morphology of the hornet MBs appears distinct from that of other Hymenoptera, we found homologous structures and the same subdivision of olfactory and visual inputs (lips and collars) in the MB calyces. Our data also

show that the antennal lobe of *V. velutina* consists of ~265 glomeruli distributed in 9 clusters innervated by distinct antennal sensory tracts. The number of glomeruli and their volume distribution are similar between the female castes. The antennal lobe of males contains fewer glomeruli but harbors seven macroglomeruli organized in two clusters. We observed five output (PN) tracts that arborize in the AL and project to higher order centers; two prominent tracts arborize in distinct antennal lobe hemilobes and project toward partially segregated areas of MB calyces and LH, in a reverse order. Three relatively smaller output tracts project through the protocerebrum toward the LH. Finally, the input-output relationships of hornet ALs display striking similarities to those of honeybees and ants, allowing a common framework for describing the olfactory pathway in these species.

Low dimorphism in the hornet brain

Several observations that we made for hornets differ substantially from what is known in the other Hymenoptera described. First, a marked sex dimorphism within the central nervous system has been reported for many hymenopteran species and was assumed to be impelled by sexual selection (Ehmer & Gronenberg, 2004; Nishikawa *et al.*, 2008; Streinzer *et al.*, 2013; Roselino *et al.*, 2015). The competition between males could act as a selective pressure on the sensory system for improving the localization of fertile females (Anderson & Iwasa, 1996). To assess a potential differential investment of the different genders in particular sensory systems, the relative size of brain neuropils has been compared between males and females in several eusocial species. In these species, as in *Apis mellifera* (honey bee), *Camponotus japonicus* (carpenter ant), *Atta vollenweideri* (leaf-cutting ant) or *Melipona scutellaris* (stingless bee), males present larger optic lobes (medulla and/or lobula) than females, suggesting sex differences in visual processing (Gronenberg & Hölldobler, 1999; Ehmer & Gronenberg, 2004; Nishikawa *et al.*, 2008; Kuebler *et al.*, 2010; Streinzer *et al.*, 2013; Roselino *et al.*, 2015). It was suggested that this sex-specific sensory adaptation is related to the importance of vision to detect and pursue fast movements of fertile females during mating flights (Ehmer & Gronenberg, 2004). Our data on the hornet *V. velutina* show that enlarged optic lobes in males are not a general feature of social Hymenoptera, as we did not find conspicuous size differences between males and females. In fact, behavioral observations in hornets in general, and *V. velutina* in particular, indicate that mating occurs on the nests or other solid substrate but not in flight (Batra, 1980; Matsuura & Yamane, 1990; Monceau *et al.*, 2014). These observations are in accordance with a less crucial role of vision in hornet males for finding their sexual partners than in other Hymenoptera. Alternatively, the hornet brain could be generally preadapted for highly-demanding visual tasks (such as catching preys on the fly), so that the system is already sufficient for processing all the information relevant to mating.

Secondly, fewer antennal lobe glomeruli and smaller MBs in males are ubiquitous features in social Hymenoptera and were assumed to be linked to a smaller behavioral repertoire in males (Arnold *et al.*, 1985; Ehmer & Gronenberg, 2004; Kuebler *et al.*, 2010; Smith *et al.*, 2010; Streinzer *et al.*, 2013). Typically, a lower number of glomeruli suggests a lower number of expressed olfactory receptors and can accordingly be linked to reduced olfactory discrimination abilities compared with females. In addition, smaller MBs, brain centers crucial for multimodal sensory integration, memory, and complex learning (Heisenberg, 1998; Farris, 2005; Strausfeld *et al.*, 2009; Devaud *et al.*, 2015), suggest lower cognitive capabilities in males. A recent comparative study underscored a less pronounced sexual dimorphism in number of glomeruli and MB size in solitary than in social bees (Streinzer *et al.*, 2013). In contrast to males of eusocial species, which benefit from colonial altruism and leave the colony only for mating flights, males of solitary species require a higher behavioral plasticity, which might be responsible for such lower sex dimorphism (Stubblefield & Seger, 1994). Contrary to this general rule, we observed only ~20 (7%) less glomeruli in *V. velutina* males compared with females, which is clearly low compared with the approximately ~30-40% reduction found in honey bees and carpenter ants (Arnold *et al.*, 1985; Zube & Rössler, 2008; Nishino *et al.*, 2009; Nakanishi *et al.*, 2010). In the same way, the calyces and peduncle of hornet male MBs were only slightly thinner than those of females (see also Molina & O'Donnell, 2008). In hornets, males were observed to be rejected from their colony and to then to perform foraging by their own means (Monceau *et al.*, 2013, 2014). From these observations, a less pronounced sex dimorphism in the hornet brain could be attributed to a more elaborate male behavioral repertoire than in other species.

Hornet macroglomeruli

To estimate the presence of enlarged glomeruli in the antennal lobe of *V. velutina*, we measured glomerular volumes using confocal microscopy and 3D reconstruction. We did not find any macroglomeruli in hornet females, but the male AL presented two clusters with seven strongly enlarged glomeruli. These glomerular clusters were densely innervated by OSNs, resulting in thicker $\mathbf{T_F}$ and $\mathbf{T_G}$ tracts in males than in females. Whereas female macroglomeruli are adaptations related to the colony life style of eusocial insects, male macroglomeruli are found throughout insects because they are involved in mating (Arnold *et al.*, 1985; Hanson *et al.*, 1992; Nishikawa *et al.*, 2008; Nishino *et al.*, 2012). In many species, such as moths or cockroaches, male-specific macroglomeruli are located in the caudal region of the antennal lobe, close to the entrance of the antennal nerve (Rospars & Chambille, 1986; Skiri *et al.*, 2005; Nishino *et al.*, 2012; Das & Faddamiro, 2013). In the ant *Camponotus japonicus*, two macroglomeruli at similar locations were attributed to the T3 tract and were assumed to have a sex pheromone-receptive function (Nakanishi *et al.*, 2010). By contrast, in honey bee drones, three macroglomeruli attributed to the T1 tract are located rostrally, one of which

specifically responds to the sex pheromone compound 9-ODA (Arnold *et al.*, 1985; Sandoz, 2006; Nishino *et al.*, 2009). Compared to other species, hornet males present numerous macroglomeruli of both types, an ant-like cluster of four ventrocaudal macroglomeruli (**T_G**), located close to the entrance of the antennal nerve and a honeybee-like cluster of three rostral macroglomeruli (**T_F**). Such sex-specific investment in several hypertrophied glomerular structures should be connected to a crucial role played by several odorants in the mating behavior of *V. velutina* males. Observation of the mating behavior of a related species, *Vespa crabro*, highlighted the existence of at least two different pheromones (Ayasse *et al.*, 2001). Hornet females produce a long range pheromone that attracts males from long distances (Ono & Sasaki, 1987; Spiewok *et al.*, 2006). In addition, a contact pheromone on the queen's thorax elicits copulatory behavior in males (Batra, 1980). One possibility is that one of the macroglomerulus clusters of the male hornet AL is involved in long-range detection of the sex attractant pheromone, whereas the second would process contact recognition cues such as fertility signals. More extensive data on the mating behavior of *V. velutina* and on the implicated chemical signals are needed for substantiating these hypotheses.

A common reference for the hymenopteran antennal lobe

Despite the considerable variations observed in the number of glomeruli across hymenopteran species, previous studies already pointed out striking similarities, as well as important differences, in the organization of ant and honeybee ALs. The most remarkable similarity between these species resides in the subdivision of the AL in two hemilobes of almost equal size, separately innervated by two prominent output tracts (m- and l-ALT; Abel *et al.*, 2001; Galizia & Rössler, 2010; Rössler & Zube, 2011). Such a dual olfactory pathway has been found in diverse taxa among Hymenoptera, except in some sawflies, suggesting its initial appearance in basal Hymenoptera (Dacks & Nighorn, 2011; Rössler & Zube 2011). In accordance with these observations, we show that Vespidae also possess such a dual pathway, which appears, as in the ant *Camponotus floridanus,* rotated by 45° on the rostrocaudal and mediolateral axis compared with that of the honey bee *Apis mellifera.* However, we found other significant similarities among the antennal lobes of these three species, which we attempted to integrate within a common framework (**Table 3**). Generally, the homologies we propose fit with and expand those proposed in an earlier work comparing bees and carpenter ants (Zube *et al.*, 2008). We observed, Based on OSN projection patterns, that the hornet antennal lobe contains nine clusters, each receiving differentially oriented branches of the antennal nerve. With similar classification rules, the antennal lobe of honey bees has been subdivided into six glomerular clusters (T1-T4, taking into account the T3a-c subdivision; Galizia *et al.*, 1999; Kirschner *et al.*, 2006; Nishino *et al.*, 2009) whereas seven clusters have been identified in carpenter ants (Zube *et al.*, 2008; Nakanishi *et al.*, 2010). The possible homologies that we described are based mostly on neuroanatomical features related to glomerular output connectivity, OSN tract innervation patterns,

cluster localizations, and average glomerular size. These features alone may not be sufficient to demonstrate these homologies unambiguously, and further comparative studies including functional data from these clusters would certainly be helpful for proving their validity. However, for some of them, available functional and neuroanatomical data already strongly support homologies across species. For instance, the $\mathbf{T_A}$ cluster contains a few (six to ten) large glomeruli located on the most dorsal region of the AL and harbors typical whole-glomerulus OSN projections, features which fit perfectly with those of the honeybee T4 cluster (seven glomeruli; Kirschner *et al.*, 2006; Nishino *et al.*, 2009) and of the ant T7 cluster (six to ten glomeruli; Zube *et al.*, 2008; Nakanishi *et al.*, 2010). These glomeruli are often described as homologous to the T10 cluster of the cockroach, containing hygro-/thermo-sensitive neurons (Nishino *et al.*, 2009; Watanabe *et al.*, 2010), so homologies for this cluster may reach beyond Hymenoptera. Another strongly supported homology concerns the $\mathbf{T_B}$ cluster. It is characterized in hornets by many small glomeruli forming a tight group slightly segregated from the main AL. This cluster has already been observed in the wasp *Polistes gallicus,* in the form of a glomerular compartment with distinct enzymatic activity compared with the main AL (Masson & Strambi, 1977). This compartment corresponds to the T6 cluster of carpenter ants (Zube *et al.*, 2008; Nakanishi *et al.*, 2010). This cluster is female specific in ants and bees and noticeably receives olfactory neurons housed in basiconic sensilla (Zube & Rössler, 2008; Kelber *et al.*, 2010; Nakanishi *et al.*, 2010; Nishikawa *et al.*, 2012; Kropf *et al.*, 2014). It is currently thought that these glomeruli might be involved in the processing of cuticular hydrocarbons, which are crucial for nestmate recognition in social insect colonies (Ozaki *et al.*, 2005; Sharma *et al.*, 2015). We recently obtained neuroanatomical and functional data suggesting a similar role in hornets, which supports the $\mathbf{T_B}$ homology (Couto *et al.*, in prep).

For other clusters with less conspicuous features, homologies could be more difficult to discern and demonstrate. The present data in hornets, however, allows resolving some previous uncertainties in the comparison between honeybees and ants. Thus, the *Camponotus floridanus* T1, T2 and T4 clusters were all suggested as possible homologous clusters to the honey bee T1 cluster (Zube *et al.*, 2008). The honey bee T1 projects rostrally, through the middle of the AL and, in males, contains three macroglomeruli. These features are strikingly similar to those of the hornet $\mathbf{T_F}$ (Kirschner *et al.*, 2006; Nishino *et al.*, 2009). In addition, we found other clusters more similar to the ants' T1, T2 and T4 tracts in the hornet AL ($\mathbf{T_I}$, $\mathbf{T_H}$ and $\mathbf{T_D}$, respectively). Our current view is thus that none of the carpenter ant clusters is homologous to the T1 cluster of honeybees. Future work should strive to provide additional data allowing corroboration of the putative homologies that we proposed. In this prospect, a systematic investigation of sensillar equipment (morphological types of olfactory sensilla) on the antenna, and their antennal lobe afferences in the different species (see Kropf *et al.*, 2014 and Kelber *et al.*, 2006), along with systematic immunohistochemistry for the main

neurotransmitters and neuropeptides (Masson & Strambi, 1977; Zube & Rössler, 2008; Dacks *et al.*, 2010; Siju *et al.*, 2014), could provide pivotal clues for testing our model.

New insights into the evolution of the hymenopteran antennal lobe

Because the olfactory system is at the interface between the animal and its environment, it receives strong selection pressures, which induce the gain or loss of subcircuits depending on changes in the relative importance of particular chemical stimuli in the biology of the species (Ramdya & Benton, 2010). Glomerular clusters are conspicuous entities in the hymenopteran antennal lobe but it is still unclear whether different clusters are systematically involved in the detection of different odorant classes. As detailed above, for some remarkable clusters like the $\mathbf{T_A}$ or $\mathbf{T_B}$, current data points in this direction. For most other clusters, however, this may not be so clear-cut, because even different AL hemilobes show strongly overlapping odor response spectra (Carcaud *et al.*, 2012; Brill *et al.*, 2013; but see Carcaud *et al.*, 2015). It is nevertheless possible that within each cluster, OSNs express groups of closely related olfactory receptor (OR) genes, as suggested by data in fruit flies. In this insect, OR genes with highest sequence similarity tend to be expressed in OSNs targeting neighbor glomeruli, suggesting such lineage constraints on OSN projection patterns (Couto *et al.*, 2005; Vosshall *et al.*, 2005). This may appear through the action of specific transcription factor genes controlling OSN axon targeting (Komiyama *et al.*, 2004; Tichy *et al.*, 2008). Thus, the antennal sensory tract ramifications observed in Hymenoptera might be the result of genetically encoded axonal-guidance factors, which could participate in a coarse odotopic representation of odors in the antennal lobe.

The evolution of the antennal lobe is thought to entail a continuous 'birth-and-death' process of ORs, inducing the budding/pruning of glomeruli within each cluster, and, eventually, of whole clusters (Ramdya & Benton, 2010). The comparative analysis of AL neuroanatomy, and the homologies that we observed, could help in retracing the evolution of the AL in Hymenoptera. At this stage, the phylogeny of Hymenoptera is not totally resolved and remains controversial (Brothers, 1999; Pilgrim *et al.*, 2008; Heraty *et al.*, 2011; Sharkey *et al.*, 2012). However, according to a recent phylogeny of stinging Hymenoptera, supported by extensive genomic and transcriptomic data sets (Johnson *et al.*, 2013), Vespidae would be more basal among Aculeates than Formicidae and Apoidae, which would be sister groups. If this phylogeny is confirmed, then one could speculate that the common ancestor of Vespidae, Formicidae and Apoidea harbored a relatively complex AL with many sensory tract subdivisions and that different secondary losses took place in Formicidae ($\mathbf{T_E}$ and $\mathbf{T_F}$) and Apoidea ($\mathbf{T_D}$, $\mathbf{T_H}$ and $\mathbf{T_I}$). Clearly, these stimulating hypotheses can be substantiated only through the long-term study of AL neuroanatomical features and input-output relationships in a wider range of hymenopteran taxa. Interestingly, we note that the most strongly conserved clusters ($\mathbf{T_A}$, $\mathbf{T_B}$ and $\mathbf{T_C}$) are related to the m-ALT PN tract, whereas the clusters that are related to the l-ALT

output tract greatly vary among species (see **Table 3**). It seems remarkable that this output tract is the one that diverged more recently, probably in some basal Hymenoptera, whereas the m-ALT is the ancestral tract (Rössler & Galizia, 2010). If confirmed in other hymenopteran taxa, this could indicate that the invention of the l-ALT tract has provided Hymenoptera with a highly adaptable and plastic olfactory subsystem, potentially instrumental for their adaptation to a wide range of ecological niches and their ecological success. On the other hand, basic biological functions would be secured by the other, ancestral tract.

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CHAPITRE 2

A conserved olfactory subsystem for cuticular hydrocarbon processing in Hymenoptera: the basiconic sensilla subsystem of the hornet *Vespa velutina*.

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Abstract

In insects, cuticular hydrocarbons (CHCs) are critical cues for intra- and interspecific interactions. They provide information about species membership, fertility status and, in eusocial insects, colony membership. Although the chemical and behavioral aspects of nestmate discrimination have been well documented in social Hymenoptera, much less is known about its neural basis, especially the detection and processing of such olfactory signals. In carpenter ants, previous work identified a female-specific olfactory subsystem potentially in charge of processing CHC information, through antennal detection by basiconic sensilla. It is still unclear however whether this specialized olfactory subsystem exists in other eusocial hymenopteran families which also actively apply nestmate discrimination. We thus assessed the existence of a possible homologous subsystem in a Vespidae, the hornet, *Vespa velutina*. First, we analyzed the distribution of basiconic sensilla on the antenna of males and females and explored the projection pattern of its sensory neurons into the first olfactory processing center, the antennal lobe (AL). The neurons from these sensilla project to a conspicuous cluster of small glomeruli with anatomical and immunoreactive features reminiscent of the ants CHC olfactory subsystem. Extracellular electrophysiological recordings further show that neurons within these basiconic sensilla respond preferentially to CHCs. These observations suggest that the CHC subsystem is conserved across distinct eusocial Hymenoptera families. However, contrary to carpenter ants, the CHC subsystem is not restricted to females and shows exactly the same features in hornet males. Our results suggest that an ancestral olfactory structure has favored an elaborate intraspecific communication through CHCs potentially facilitating the multiple emergence of eusociality among Hymenoptera.

Keywords: social insect, wasps, basiconic sensilla, cuticular hydrocarbon

Introduction

Insects strongly rely on chemical cues for adapting to their environment and interacting with other individuals from the same or different species. Volatile molecules provide crucial information regarding the long range localization of food sources as well as of potential mates (sex pheromones) (Touhara & Vosshall, 2009). In addition, low volatile molecules also play an important role at short distance. For instance, numerous insect species use a range of long chain cuticular hydrocarbons (CHCs) as recognition signals, providing essential information about species membership and fertility status (Hadley, 1978; Butts *et al.*, 1991; Ferveur, 2005; Howard & Blomquist, 2005; Everaerts *et al.*, 2010). In the case of eusocial insects, which display outstanding cooperative behaviors for vital tasks such as brood care, foraging or nest defense, the ability to discriminate nestmates from non-nestmates through CHCs is crucial for preventing exploitation of such altruistic behaviors by alien individuals (Lahav *et al.*, 1999; Ruther *et al.*, 2002; Greene & Gordon 2003; Dani *et al.*, 2005; Smith *et al.*, 2009; Johnson *et al.*, 2011). In general, whereas members of the same species share similar CHC compositions, the relative proportions of individual components vary across colonies, enabling the discrimination of colony-specific identities (Lorenzi *et al.*, 1997; Akino *et al.*, 2004; Foitzik *et al.*, 2007; Martin *et al.*, 2008; D'Ettorre & Lenoir, 2010). Moreover, several times within Hymenoptera, CHCs have also evolved as queen pheromones, advertising fecundity and/or suppressing worker reproduction (Oi *et al.*, 2015; Van Oystaeyen *et al.*, 2014). Thus, CHCs might be ancestral signals required for the emergence of colony-specific altruism and reproductive division of labor (Kather & Martin, 2015).

Insects detect low volatile compounds at a very short range (0 to ~1cm) when approaching another individual with their antennae (Anton & Gnatzy, 1998; Brandstaetter *et al.*, 2008; Lang & Menzel, 2011). For instance, females of the parasitoid wasp *Liris niger*, recognize their preys by probing the cuticle of potential candidates with the dorso-apical side of their antennae (Anton & Gnatzy, 1998). Gradual ablation of antennal flagellomers showed a remarkable correlation between the wasps' ability to recognize a prey and the distribution of one type of sensillum on the antenna, the sensillum basiconicum (Anton & Gnatzy, 1998). This sensillum is typically characterized by a pegged base, smooth walls, a blunt tip and a non-flexible socket (Keil, 1999). In Hymenoptera, *sensilla basiconica* have been found on the antennae of females from both solitary and social species, but appear to be generally absent in males (Esslen & Kaissling, 1976; Ågren, 1977; Ågren, 1978; Ågren & Hallberg, 1996; Amornsak *et al.*, 1998; Renthal *et al.*, 2003; Nakanishi *et al.*, 2009; Mysore *et al.*, 2010; Streinzer *et al.*, 2013). Electrophysiological recordings performed in carpenter ants demonstrated that basiconic sensilla significantly respond to CHCs, suggesting that they play a major role in hydrocarbon detection (Ozaki *et al.*, 2005; Sharma *et al.*, 2015). These sensilla usually house numerous sensory neurons (more than 130 in *C. japonicus*; Nakanishi *et al.*, 2009), which

project to a recognizable group of small glomeruli in the antennal lobe (AL), the primary olfactory processing center of the brain, in several ant species (Kelber *et al.*, 2010; Nakanishi *et al.*, 2010). Remarkably, this female-specific cluster of glomeruli differs from other AL clusters in that it lacks any serotonin-immunoreactive fibers and its local interneurons are isolated from the rest of the AL (Zube & Rössler 2008; Nakanishi *et al.*, 2010; Nishikawa *et al.*, 2012). Lastly, uniglomerular projection neurons from this glomerular cluster innervate segregated areas within higher-order centers, the lateral horn and the mushroom bodies (Nishikawa *et al.*, 2012). All these observations suggest the existence of an olfactory subsystem involved in the processing of social information related to female-specific tasks in ants (Ozaki *et al.*, 2005; Nishikawa *et al.*, 2012; Sharma *et al.*, 2015).

Based on the observation of a sexually dimorphic cluster of glomeruli and the (non-exclusive) projection of sensory neurons from basiconic sensilla, this female-specific subsystem was also assumed to be present, although greatly diminished in size, in the honey bee Apis mellifera (Kropf *et al.*, 2014). To date, it remains unknown if this basiconic sensilla-specific subsystem is ubiquitous among social Hymenoptera and whether it could have played a role in the advent of eusociality in these insect groups. Although phylogenetic relationships among Hymenoptera are still controversial, it is widely agreed that eusociality has arisen several times independently within this order and more specifically within Aculeata, in the Apidae, Formicidae and Vespidae families, among others (Hines *et al.*, 2007; Hughes *et al.*, 2008; Sharkey *et al.*, 2012; Johnson *et al.*, 2013). According to these studies, a basiconic sensilla-specific subsystem exists in ants (Formicidae) and has been suggested in honeybees (Apidae). No data were yet available in Vespidae, although these insects represent a key group for studying the evolution of eusociality, as they present a wide range of social organizations, including solitary life, nest sharing, reproductive dominance and eusociality (Hunt, 2007; Pickett & Carpenter, 2010). Investigating a basiconic-sensillum specific subsystem, involved in CHC processing, in Vespidae could thus be instrumental for understanding its possible role in the evolution of eusociality.

Several studies have already shown the importance of CHCs as recognition cues in social wasps (Gamboa *et al.*, 1986; Mitra *et al.*, 2014; Oi *et al.*, 2015). For example, hornets are eusocial insects which use long chain hydrocarbons in several different contexts such as nestmate discrimination, worker policing and trail following behaviors (Butts *et al.*, 1995; Ruther *et al.*, 1998; Ruther *et al.*, 2002; Steinmetz *et al.*, 2002; Steinmetz *et al.*, 2003). Interestingly, a recent neuroanatomical description of the olfactory pathway in the hornet *Vespa velutina* showed strong similarities with the organization of the AL of ants and honeybees (Couto *et al.*, 2016). In all these species, AL glomeruli are distributed in 6-10 different clusters innervated by distinct antennal sensory tracts (Pareto, 1972; Kirschner *et al.*, 2006; Zube *et al.*, 2008; Nakanishi *et al.*, 2010). One glomerular cluster in the hornet, termed T_B, showed anatomical features (numerous small glomeruli, located on the dorso-caudal side of the AL) that were strongly reminiscent of the basiconic sensillum-specific glomeruli of carpenter

ants (Couto *et al.*, 2016). However, clear structural and functional homologies between these systems remain to be demonstrated.

In this study we investigated the sensillar equipment of *V. velutina* females and males, in order to assess the presence of basiconic sensilla on the hornet antenna, and its sex-specificity. We used fluorescent tracers to explore the projections in the antennal lobe of the sensory neurons housed in basiconic sensilla. Then, we studied the serotonin-like immunoreactivity of the related glomerular cluster. Finally, we performed single sensillum electrophysiological recordings to test whether hornet basiconic sensilla sensory neurons respond to long-chain alkanes, which belong to their CHC profile (Martin *et al.*, 2009). Our results demonstrate the presence in hornets of an olfactory subsystem involved in the processing of long-chain hydrocarbon. This subsystem is highly similar to that found in ants, suggesting that it could be ancestral and more widespread among Hymenoptera than initially thought. If so, this subsystem might have favored elaborate intraspecific communication through CHCs in Hymenoptera and played a central role in the advent of eusociality.

Materials and Methods

Animals

Hornets (*Vespa velutina*) were collected in the wild, on the campus of INRA-Bordeaux Aquitaine from July to November or were obtained at emergence from a comb artificially maintained in an incubator. They were thus obtained from an important natural population with high nest densities (Monceau & Thiéry, 2016). Males and females (workers) were sorted by observing the presence of an aedeagus or a sting, respectively, at the end of the last abdominal segment. For each experiment, hornets were cold anesthetized on ice for 10 minutes before further handling.

Scanning electron microscopy

Antennae were obtained by cutting off the base of the scape. Samples were then fixed with 2.5% glutaraldehyde solution in 0.1 M phosphate buffered saline (PBS) at 4°C for 24 hours. After three washes with PBS (10 minutes each), samples were dehydrated with increasing concentrations of ethanol (from 50% to 3x 100%) at room temperature (10 minutes each). Samples were then dried at ambient air temperature under a hood and mounted on aluminum stubs with double-sided sticky tape. The antennae were sputter coated in argon plasma with platinum (approximately 30 nm thickness) in a Polaron SC 7640 device (Elecience, Verrières-le-Buisson, France) at 10 mA and 0.8 kV for 200 s. Observations were performed in an FE-SEM Hitachi S4500 (Hitachi, Tokyo, Japan), with a low secondary electron detector, at 2 kV and 18 mm working distance, at the MIMA2 microscopy platform (http://www6.jouy.inra.fr/mima2).

Selective staining of OSNs from a single basiconic sensillum

To investigate axonal innervation patterns of OSNs housed in basiconic sensilla, staining of a single sensillum was performed in several individuals of each sex (n = 10 / gender). Hornets were placed in Plexiglas holders (see Sandoz *et al.*, 2003, Fig. 1) and their antennae were fixated horizontally with low melting point wax (Deiberit 502, Schöps and Dr. Böhme, Goslar, Germany). Preparations were placed under a macroscope (Z16 APO A, Leica Microsystems, Wetzlar, Germany) to visualize and identify a basiconic sensillum from the fourth to the eighth antennal segment. Using a micromanipulator, sensilla were approached with a glass electrode filled with 2% micro-ruby (Dextran, Tetramethylrhodamine and biotin, 3 000 MW, D-7162; Invitrogen, Eugene, OR) in distilled water. A single basiconic sensillum on each antenna was perforated with the electrode's sharp tip, and then remained in contact to let the dye diffuse for 3 hours. The electrode was then removed and the hornets were released in a breeding box with available food and water, for 48 hours in the dark. After dye migration, the brains were dissected out in 0.1 M PBS solution and plunged into a fixative solution (4% paraformaldehyde in PBS) for 24 hours at 4°C.

Serotonin immunohistochemistry

As described above, hornet brains were dissected out in PBS and fixed for 24 hours at 4°C in 4% paraformaldehyde. The brains were washed 3 times (10 minutes each) in PBS solution containing 0.2% of Triton X-100 (PBST) and preincubated for 3 hours at room temperature in PBST with 10 % normal goat serum (G9023, Sigma-Aldrich, Steinheim, Germany), henceforth NGS/PBST, to avoid unspecific staining. Tissues were probed with rabbit anti-serotonin primary antibody (S5545, Sigma-Aldrich, Steinheim, Germany) diluted (1:250) in NGS/PBST for 7 days at 4°C. Then, the brains were washed 3 times (10 minutes each) in PBST and incubated in Alexa-fluor 488-conjugated goat anti-rabbit secondary antibody (A-11008, life technologies; diluted 1:200 in NGS/PBST) for 7 days at 4°C. According to manufacturer data, pre-incubation of the primary antibody with 500 µM serotonin inhibits specific staining. This antibody has been used successfully for revealing serotoninergic processes in moths (Sinakevitch *et al.*, 2008) and honeybees (French *et al.*, 2014). We did not observe any staining when the primary antibody was withdrawn from the process, indicating that the secondary antibodies did not bind with anything in the tissue.

Brain preparations and confocal imaging

After selective staining of basiconic sensilla or after the immunostaining procedure, brains were washed in PBS (3 times 10 minutes), dehydrated in series of increasing ethanol concentrations (50%, 70%, 90%, 95% and 3 times 100%, for 10 minutes each) and clarified in methylsalicylate (Sigma-Aldrich, Steinheim, Germany) for at least 3 days at 4°C. Brains were then mounted in the wells of aluminum slides filled with methylsalicylate and covered from both sides with cover slips.

Antennal lobes were scanned with a laser-scanning confocal microscope (LSM-700; Carl Zeis, Jena, Germany) equipped with a water immersion objective (20x plan-apochromat 1.0 NA). To visualize micro-ruby labeled neurons, the brain was scanned at 1 µm intervals (z axis) creating confocal stacks of 1024 x 1024 (x,y) pixels, at a resolution of 0.45 µm/pixel, using a 555 nm solid-state laser. Autofluorescence obtained from the excitation of a 488 nm laser was used as anatomical background. Both wavelengths were scanned sequentially. For immunohistochemical preparations, the Alexa fluor 488-conjugated secondary antibody was visualized using the 488 nm laser.

Image processing and 3D reconstructions

Serial optical sections were saved as LSM files and opened using ImageJ software with the Bio-Formats library plugin. Brightness and contrast of images were adjusted before being saved as TIFF files. For basiconic sensilla stainings, the 488 nm (autofluorescence) and the 555 nm (micro-ruby) signals were split and adjusted independently. Then, both TIFF files were imported in three-dimensional analysis software (AMIRA 5.4.3, VSG, Berlin, Germany). Glomeruli were individually reconstructed by manual labeling in three planes (*xy*, *xz* and *yz*) and using the Wrap function to obtain their 3D models. The number of stained glomeruli was visually assessed by overlapping the 555 nm wavelength image stacks with the background staining and corresponding 3D reconstruction.

Electrophysiological recordings from basiconic sensilla

Single sensillum extracellular electrophysiological recordings (SSR) were obtained from basiconic sensilla (type bs2) on the antenna flagellum. Hornets were held in Plexiglas holders and their antennae were fixed horizontally with low melting point wax (Deiberit 502, Schöps and Dr Böhme, Goslar, Germany). The body of the hornet was electrically connected to the ground by inserting a silver wire at the anterior part of the head (on or near the clypeus), and covering the crack made by the wire in the cuticle with a drop of electrocardiogram gel (Redux electrolyte Gel, Parker Laboratories, Fairfield, USA). One antenna was placed in a humidified constant air stream (15 mL/s) and examined with a stereomicroscope (Leica Z16 APO, Germany) to localize basiconic sensilla, focusing on flagellomeres 6 to 10. When a sensillum with the proper orientation was located, the end of an electrolytically sharpened tungsten wire was carefully inserted at the base of the sensillum using a micromanipulator (Microstar, Scientifica UK). The tungsten electrode was connected to a custom-built preamplifier (x10), and further amplified (x100) and bandpass filtered at 10–2800 Hz by a CyberAmp 320 amplifier (Axon Instruments, USA). The filtered signal was digitally sampled at 10 kHz (DT9816; Data Translation) and analyzed using a custom software to observe and detect spikes (dbWave, Marion-Poll, 1996).

Odorant stimulations were performed with a stimulus controller (CS05, Syntech, Germany) by blowing air during 5 s through a Pasteur pipette held ~2 cm from the tip of the sensillum being

recorded. The Pasteur pipette contained a filter paper (1 cm²) loaded with 5 µL of odorant solution. We tested low-volatility long-chain hydrocarbons (alkanes) and more volatile aliphatic compounds (short chain alcohols, ketones and aldehydes). Alkanes (docosane, C22; pentacosane, C25 and heptacosane, C27) were dissolved in hexane (50 µg/µL) and the filter papers soaked with the solutions was maintained for 10 min under a constant air stream, to let the solvent evaporate. For stimulations, the pipettes were first heated at 60°C into an incubator, for a few minutes to volatilize the chemicals, and used immediately (see Carcaud *et al.*, 2015). Control stimulations were performed with solvent alone (5 µL hexane) heated at 60°C, to rule out mechanosensory or thermosensory responses. Volatile aliphatic compounds (1-hexanol, C6ol; 2-nonanone, C9one and nonanal, C9al) were presented pure at ambient temperature.

In each sensillum we could record action potential of different amplitudes in a continuous distribution, reflecting the large number of OSNs housed in this sensillar type (about 35 stained glomeruli). We adjusted a spike detection threshold by zooming on portions of the data, in order to discriminate spiking activity from random baseline fluctuations. The events detected were considered as an estimate of the total spiking activity and used as a measure of sensillum activity (Sharma *et al.*, 2015). For each stimulus presentation, we measured the spike frequency within 100 ms bins for 15 s, 5 s before, 5 s during and 5 s after the stimulus. To decide whether a stimulus induced a significant activity, we compared the spike frequency during the stimulus to the activity recorded for 5 s before the stimulus. If activity during the stimulus presentation was above 2 SD of the spike frequency before the stimulus, it was considered as a response. For visual evaluation, we also represented spike frequency as a number of SD above baseline at each 100 ms bin. The result is indicated for all odor presentations and all responding sensilla following a color code from dark blue to red in **Suppl. Fig. 1**.

Results

Sensilla types and their distribution on the hornet antenna

The antenna of *V. velutina* is classically composed of a scape, a short pedicel and a flagellum, which contains 10 flagellomeres in females and 11 in males (**Fig. 1A**). Female flagellomeres are completely covered by cuticular sensory structures called sensilla (**Fig. 1B** and **Fig. 2A**). Male flagellomeres are also covered with sensilla, except for pairs of bulging oval cuticular structures, localized on the ventral side of each segment (**Fig. 1C**, **D** and **Fig. 2B**). These male-specific structures, the tyloids (Romani *et al.*, 2005; Steiner *et al.*, 2010), contain large pores considered as excretory ducts and a few hair-like sensilla (**Fig. 1D**). Sensilla can be classified in several different types based on their morphology (Zacharuk, 1980). We did not observe any marked sexual dimorphism with regards to sensillar types and their distribution on antennae (**Fig. 2**).

Four types of sensilla are ubiquitous on all flagellomeres (see grey areas in figure 2) and will be described first. **Trichoid sensilla** (**Fig. 1E**) are the most abundant, and are characterized by a long, curved hair shape with grooved walls and presumably having an olfactory and/or a mechanosensory function (Esslen & Kaissling, 1976; Lacher, 1964). Two types of trichoid sensilla can be defined, based on their socket size: trichoid 1 sensilla have a wide socket and are highly abundant over the whole antenna, while trichoid 2 sensilla have a narrow socket and are much less abundant.

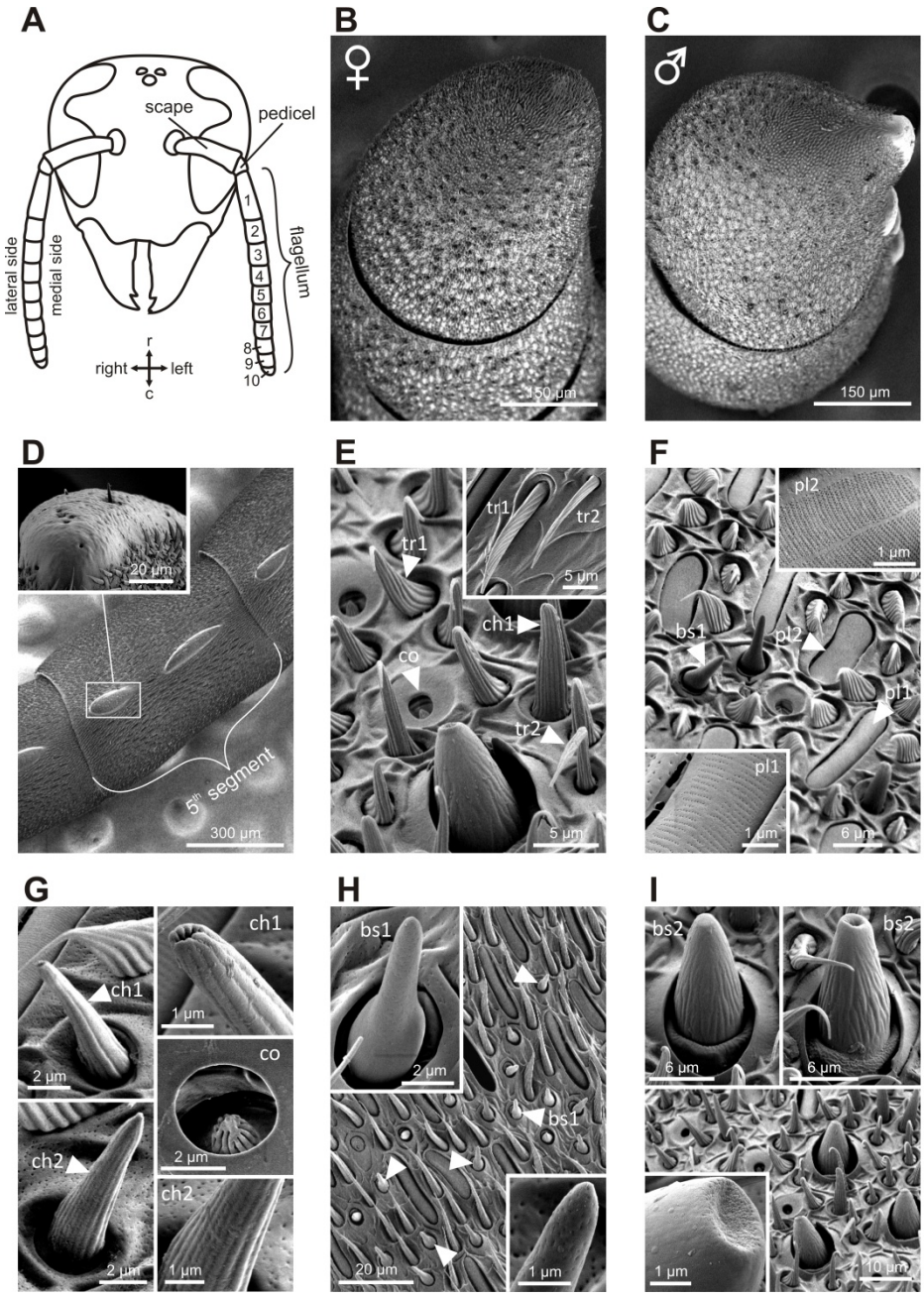

Figure 1: Structure of the antenna in *V. velutina* and its sensillar equipment
A) Schematic representation of a hornet head. The bending angle between the scape and the pedicel defines the medial side of the antenna. The antenna comprises 10 flagellomeres in workers and 11 in males. Abbreviations: r, rostral; c, caudal. **B**, **C**) scanning electron micrographs of the last flagellomeres in female and male, respectively. The male antenna harbors 2 tyloid structures on each flagellum. **D**) Tyloids are cuticular bumps on which only rare trichoid and chaetic sensilla were observed. **E-I**) scanning electron micrograph of antennal sensilla in *V. velutina.* We identified nine distinct morphological types of antennal sensilla: tr: trichoid; co: coeloconic; pl: placode; ch: chaetic, bs: basiconic.

The hornet antenna is also covered with two types of **poreplate sensilla** (*sensilla placodea*), formed by oval cuticular plates punctured by numerous minute pores (**Fig. 1F**). Sensilla placodea 1 are long and narrow and display on their surface two elongated regions with multiple pores, organized in single stripes. Sensilla placodea 2 are also elongated, but wider than the aforementioned type. They also carry two regions of multiple pores, but these are organized in double stripes (**Fig. 1F**). Sensilla placodea are widely known as olfactory sensilla (Lacher 1964; Leal & Mochizuki, 1993; Akers & Getz, 1992).

The other sensilla types are less abundant on the antenna and their general localizations are indicated by different symbols in figure 2. The hornet antenna thus carries two types of **sensilla chaetica**, which are straight hair-shaped sensilla with grooved walls. Chaetic sensilla 1 (with a possibly gustatory function; Whitehead & Larsen, 1976; Bénédet *et al.*, 2002; Haupt, 2004; de Brito Sanchez, 2011) are mostly found on the distal segments, and become gradually sparser towards proximal segments (**Fig. 2**). Chaetic sensilla 1 possess a terminal pore and wide grooves, while chaetic sensilla 2, with a possible mechanosensory function (Dumpert, 1972), have a sharp, closed tip, and tighter grooves (**Fig. 1G**). This last type is found throughout the flagellum.

Another sensillum type that is found on all segments is the **coeloconic sensillum** (**Fig. 1G**). These sensilla form a pit at the antennal surface with a short cone-shaped and grooved sensillar apparatus in its lumen. This sensillar type was mostly found in groups on the ventro-lateral side of the antennae and on apical segments (**Fig. 2**). They may be involved in hygro- and thermo-reception (Yokohari *et al.*, 1982; Ruchty *et al.*, 2009).

Lastly, we also identified two types of **basiconic sensilla**, which always consist of a peg on a socket (**Fig. 1H**, **I**). Such sensilla are known for their chemosensory function (Ozaki *et al.*, 2005; Sharma *et al.*, 2015). Basiconic sensilla 1 are long and slender with a smooth surface perforated by numerous minute pores especially in the tip region (**Fig. 1H**). This sensillar type was found on the dorsal surface of the antenna, mostly in the distal region of antennal segments but it seems to be lacking on the ventral surface and on proximal segments (**Fig. 2**). Basiconic sensilla 2 have a much larger base, exhibiting ridges (**Fig. 1I**). The sensillum tip has a smooth, possibly porous surface (Martini, 1986), which was alternatively observed with a flat or inwardly bent shape, and often displayed a large terminal hole (**Fig. 1I**). These sensilla are particularly densely represented on the dorsal surface of the last flagelomers and become progressively sparser towards proximal segments in both sexes (**Fig. 2**). They are completely absent on the ventral surface of the antenna. Given their dorso-apical distribution and their singular morphological features, we hypothesized that basiconic sensilla 2 may have a contact chemosensory function and be homologous to the CHC-sensitive basiconic sensilla of ants (Ozaki *et al.*, 2005; Nakanishi *et al.*, 2009; Nishikawa *et al.*, 2012; Sharma *et al.*, 2015).

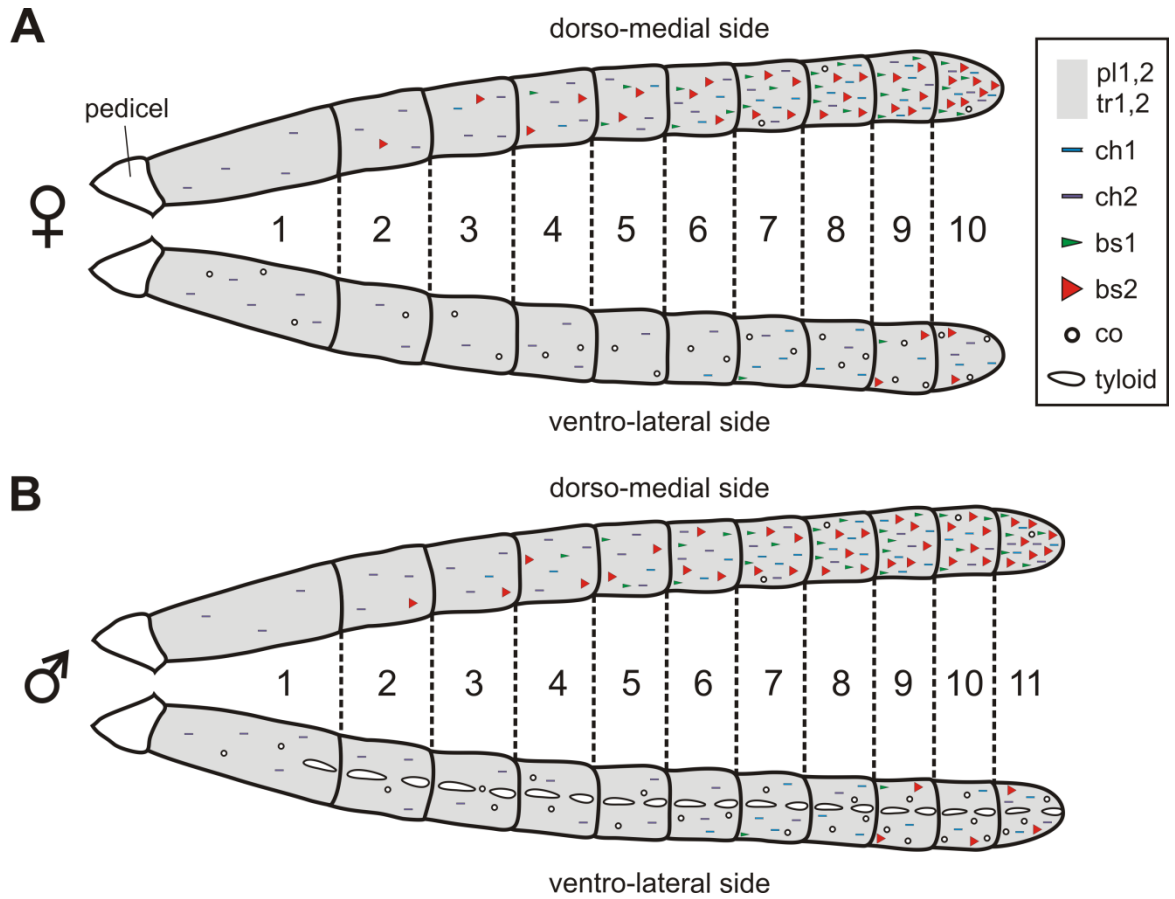

Figure 2: Distribution of antennal sensilla on the antennae of female and male *V. velutina*
Schematic representation of sensilla distribution along the antenna of female (**A**) and male (**B**) *V. velutina.* Trichoid and placode sensilla are homogeneously distributed on the whole flagellum and are indicated by a grey background. The general locations of other sensilla types, which are not homogeneously distributed, are indicated by symbols. For example, basiconic sensilla (bs, red and green triangles) are densely present on the dorso-medial side of the antenna especially in the distal part but become sparser on the ventro-lateral side. Abbreviations: tr, trichoid; pl, placode; ch, chaetic; bs, basiconic; co, coeloconic.

Central projections of sensory neurons from basiconic sensilla 2

Mass staining of the antennal nerve revealed nine axon bundles which project to ~265 olfactory glomeruli in the *V. velutina* antennal lobe. These olfactory sensory tracts, termed $\mathbf{T_A}$- $\mathbf{T_I}$, innervate nine distinct clusters of glomeruli, with the same innervations pattern in males and females (**Fig. 3**). We investigated the projection pattern of sensory neurons from basiconic sensilla 2, using specific single sensillum staining. Basiconic sensilla 2 were easily recognizable under the microscope, so that a glass electrode filled with fluorescent dye could be inserted into a single sensillum (inset in **Fig. 4A**). We obtained 5 successfully stained antennal lobes of each sex, exhibiting a clear innervation of AL glomeruli. When sensory neurons were particularly brightly labeled, OSNs could be traced from the antennal sensory tract to their glomerular termination (**Fig. 4A**). Typically, the stained axons run roughly in parallel within the antenna, until they all suddenly intermingle and eventually split up, thereby forming a conspicuous axon sorting-zone at the AL entrance (SZ in **Fig. 4A**). Within each glomerulus, each axon formed a claw-like innervation, penetrating the glomerulus' outer rim (cortex, **Fig. 4A**, **B**).

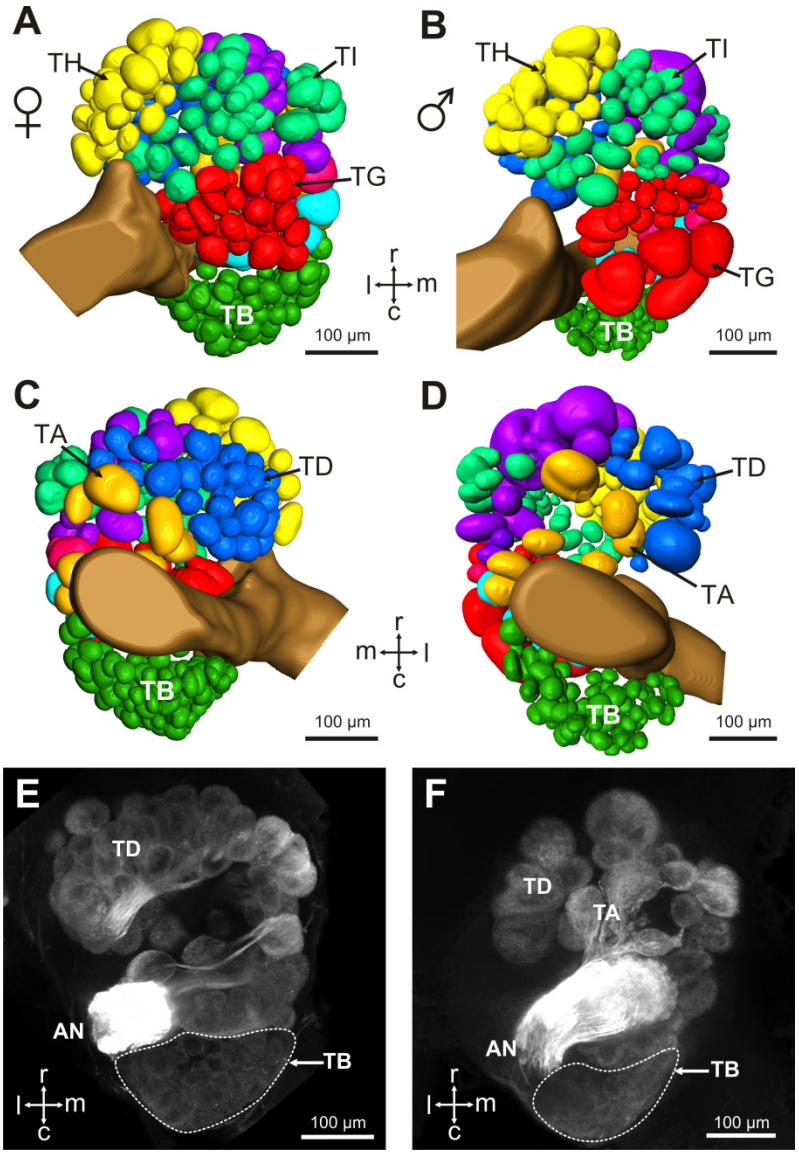

Figure 3: Antennal lobe organization in female and male *V. velutina*
A-**D**) 3 dimensional reconstructions of the antennal lobe of female (**A**, **C**) and male (**B**, **D**) *V. velutina*. The antennal lobe is represented as seen from the ventral side (**A**, **B**) or the dorsal side (**C**, **D**). **E**) projection view (60 µm thickness) starting at 160 µm from the ventral surface of a female antennal lobe. **F**) projection view (60 µm thickness) starting at 240 µm from the ventral surface of a male antennal lobe. The *V. velutina* antennal lobe contains nine glomerular clusters termed $\mathbf{T_A}$ – $\mathbf{T_I}$ (color coded in **A**-**D**) in both females and males. The $\mathbf{T_B}$ cluster is formed by a tight group of small glomeruli in the dorso-caudal region of the antennal lobe. Abbreviations: r, rostral; c, caudal; l, lateral; m, medial; AN: antennal nerve.

In all preparations (n = 10), the stained OSNs projected to a restricted region on the dorso-caudal side of the AL, innervating many small glomeruli of the $\mathbf{T_B}$ cluster. We used the most brightly stained preparations to assess the number of glomeruli innervated by the OSNs contained in a single basiconic sensillum (n = 1 in each sex). At least 36 glomeruli were stained in the female, corresponding to ~37.5% of the 96 $\mathbf{T_B}$ glomeruli (**Fig. 4C**-**E**). Similarly in the male, OSNs from a single basiconic sensillum projected to at least 29 glomeruli out of 80, corresponding to 36.2% of the $\mathbf{T_B}$ cluster (**Fig. 4F**-**H**). We did not observe any regionalization of sensory projections within the $\mathbf{T_B}$ cluster depending on the location of the basiconic sensillum on the antenna (**Fig. 4E**, **H**). These data

suggest that basiconic sensilla 2 contain approximately 35 sensory neurons, which project exclusively into the T_B glomerular cluster.

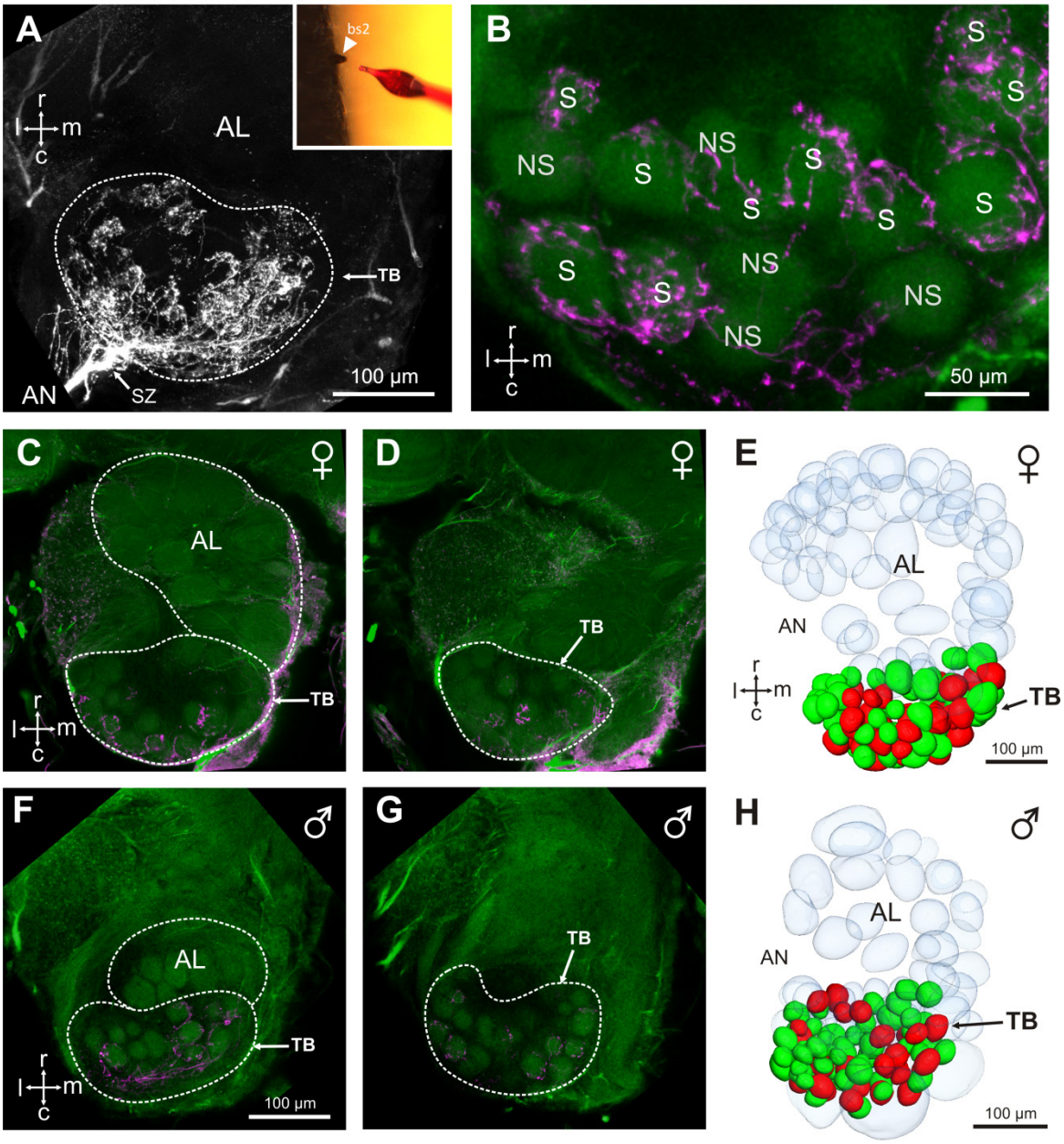

Figure 4: Central projection of sensory neurons from basiconic sensilla 2

A) Projection view (210 µm thickness) showing the central projections of the sensory neurons from a single basiconic sensillum 2 on the 4th flagellum segment. Note the presence of a sorting zone (SZ) at the AL entrance. Location of the T_B cluster is indicated by a dashed line. Inset: basiconic sensilla 2 were easily recognizable under optical control, thanks to their large size and conic shape. **B**) Projection view (5 µm thickness) showing stained (S) and not stained (NS) antennal lobe glomeruli from the T_B cluster (autofluorescence in green) after staining of a basiconic sensillum 2 (magenta). (**C**-**G**) Confocal optical sections through a female (**C**, **D**) and a male antennal lobe (**F**, **G**) after staining of a single basiconic sensillum. Each optical section is separated from the other by ~50 µm. The dorso caudal region of the antennal lobe corresponding to the T_B cluster receives projections from basiconic sensilla sensory neurons (in magenta) in both sexes, whereas we did not observe any staining in the other regions of the AL. (**E**, **H**) 3D reconstructions of the female and male antennal lobes shown in (**C**, **D**) and (**F**, **G**) respectively. Stained glomeruli are represented in red and unstained glomeruli are shown in green. Glomeruli from other regions of the AL are transparent. Note that because the scans in (**C**, **D**) and (**F**, **G**) were performed at high resolution on the T_B cluster, they did not include glomeruli from other AL clusters above or below this cluster. Hence the 3D reconstructions present only a portion of non-T_B glomeruli. Abbreviations: r, rostral; c, caudal; l, lateral; m, medial; AN: antennal nerve.

Serotonin-immunoreactivity in the antennal lobe

The restricted innervation of a group of dorso-caudal glomeruli by sensory neurons from basiconic sensilla demonstrated here in hornets is reminiscent of a similar subsystem described in carpenter ant workers (Zube & Rössler 2008; Kelber *et al.*, 2010; Nakanishi *et al.*, 2010). In these insects, the T6 cluster receiving input from basiconic sensilla also contrasts with other glomerular clusters by a lack of serotonin immunoreactive fibers (Dacks *et al.*, 2006; Zube & Rössler 2008; Nakanishi *et al.*, 2010). We thus aimed to check whether this feature is also present in the hornet antennal lobe and if it is, as in ants, a female specific trait. Using immunohistochemistry and an antibody against serotonin, we observed widespread serotonin-like immunoreactivity throughout the hornet brain, and in the antennal lobe (**Fig. 5**).

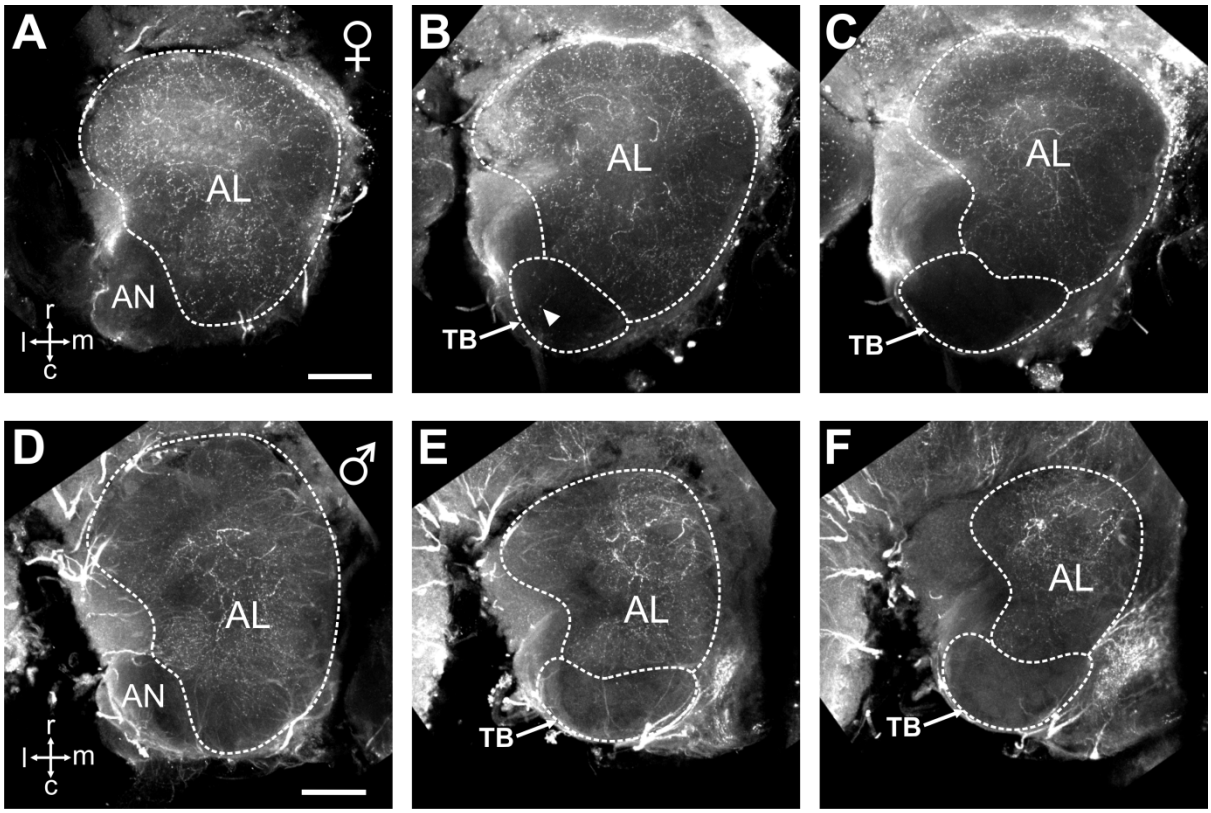

Figure 5: Serotonin-like immunoreactivity in the *V. velutina* antennal lobe
Projection views (80 µm thickness) of a female (**A**, **B**, **C**) and a male antennal lobe (**D**, **E**, **F**) after immunohistochemistry with an antibody against serotonin. Glomeruli from the dorso-caudal region of the antennal lobe corresponding to the $\mathbf{T_B}$ cluster do not show any immunoreactivity compared to other regions of the AL, which present a dense mesh of immunoreactive processes in both female and male. Abbreviations: r: rostral; c: caudal; l: lateral; m: medial; AN: antennal nerve.

All our stainings, both in females (n = 10) and males (n = 5), showed a clear dichotomy in the serotonin-like immunoreactivity of the *V. velutina* antennal lobe. Ventral and dorso-rostral glomeruli corresponding to $\mathbf{T_A}$, and $\mathbf{T_C}$ to $\mathbf{T_I}$ exhibited a clear and homogenous serotonin innervation while no labeling was observed in the glomeruli situated in the dorso-caudal area corresponding to the $\mathbf{T_B}$ cluster. As in carpenter ants (Nakanishi *et al.*, 2010), a few small fluorescent elements were

sometimes observed in the inter-glomerular space within the T_B cluster (arrow in **Fig. 5B**). However, they never entered the glomeruli. No difference appeared between males and females (compare **Fig. 5A, B, C** with **D, E, F**).

Electrophysiology

Next, we assessed the responses of basiconic sensilla 2 to odorant stimulations, using the single sensillum extracellular recording (SSR) technique (Sharma *et al.*, 2015). A total of 45 basiconic sensilla 2 located between the 6th and the 10th flagellum segments were recorded in 15 workers. A panel of three cuticular hydrocarbons (docosane, pentacosane and heptacosane), three volatile aliphatic odorants (1-hexanol, 2-nonanone and nonanal) and a control were presented. In most cases (n = 37 sensilla), the sensillum did not respond to any of the tested stimuli. Because of the hard cuticle of the sensillum, electrode insertion was difficult and may sometimes have damaged the sensory neurons. Alternately, our odor panel may not contain odorants activating this sensillum. In the remaining cases (n = 8 sensilla), clear responses to one or a few stimuli were recorded (**Suppl. Fig. 1**, **Table 1**). These responses appeared mostly for the long-chain alkanes, with three sensilla responding to docosane, two sensilla responding to pentacosane and four to heptacosane (two sensilla responded to both C25 and C27). Basiconic sensilla 2 also responded to volatile compounds, with two sensilla responding to 2-nonanone, one to 1-hexanol and two to nonanal (**Table 1**). In the same conditions, only one sensillum responded once to the control stimulation with a heated pipette containing the solvent (**Table 1**). Thus, the observed responses to alkanes were not mechano- or thermosensory responses, and were not due to the hexane solvent.

sensilla	Ctrl	C9one	C6ol	C9al	C22	C25	C27
bs #1	0/6	**2/4**	0/3	0/5	0/3	**2/2**	**1/7**
bs #2	0/5	0/2	0/0	0/3	**2/3**	0/0	0/0
bs #3	0/2	0/0	0/0	0/0	**1/2**	0/2	0/0
bs #4	0/2	0/0	0/0	**1/4**	**1/3**	0/3	0/0
bs #5	0/5	0/2	0/0	0/2	0/2	0/2	**5/5**
bs #6	0/2	0/2	0/2	**1/2**	0/2	**1/2**	**1/2**
bs #7	0/3	**1/2**	**1/3**	0/2	0/2	0/2	0/2
bs #8	**1/3**	0/0	0/0	0/2	0/0	0/1	**2/4**

Table 1: Electrophysiological responses of individual basiconic sensilla 2 to olfactory stimuli
The first digit indicates the number of significant responses (spike frequency > baseline + 2 SD) obtained for each responsive sensillum to each stimulus. The second digit indicates the number of presentations of each odorant to this sensillum.

In most cases, after a few clear responses, the sensillum stopped responding to the stimuli, sometimes remaining in a tonic spiking state. For two sensilla (#1 and #5), the recording was longer-lasting, and robust and reproducible responses could be monitored (**Fig. 6A, B, C**). Sensillum #1, located on the 7th flagellomere responded to C25, C27 and 2-nonanone but not to the other odorants

or to the control (**Fig. 6A**). On the other hand, sensillum #5, located on the 8th segment showed clear and reproducible, responses to C27 but not to the other odorants or to the control (**Fig. 6B, D**).

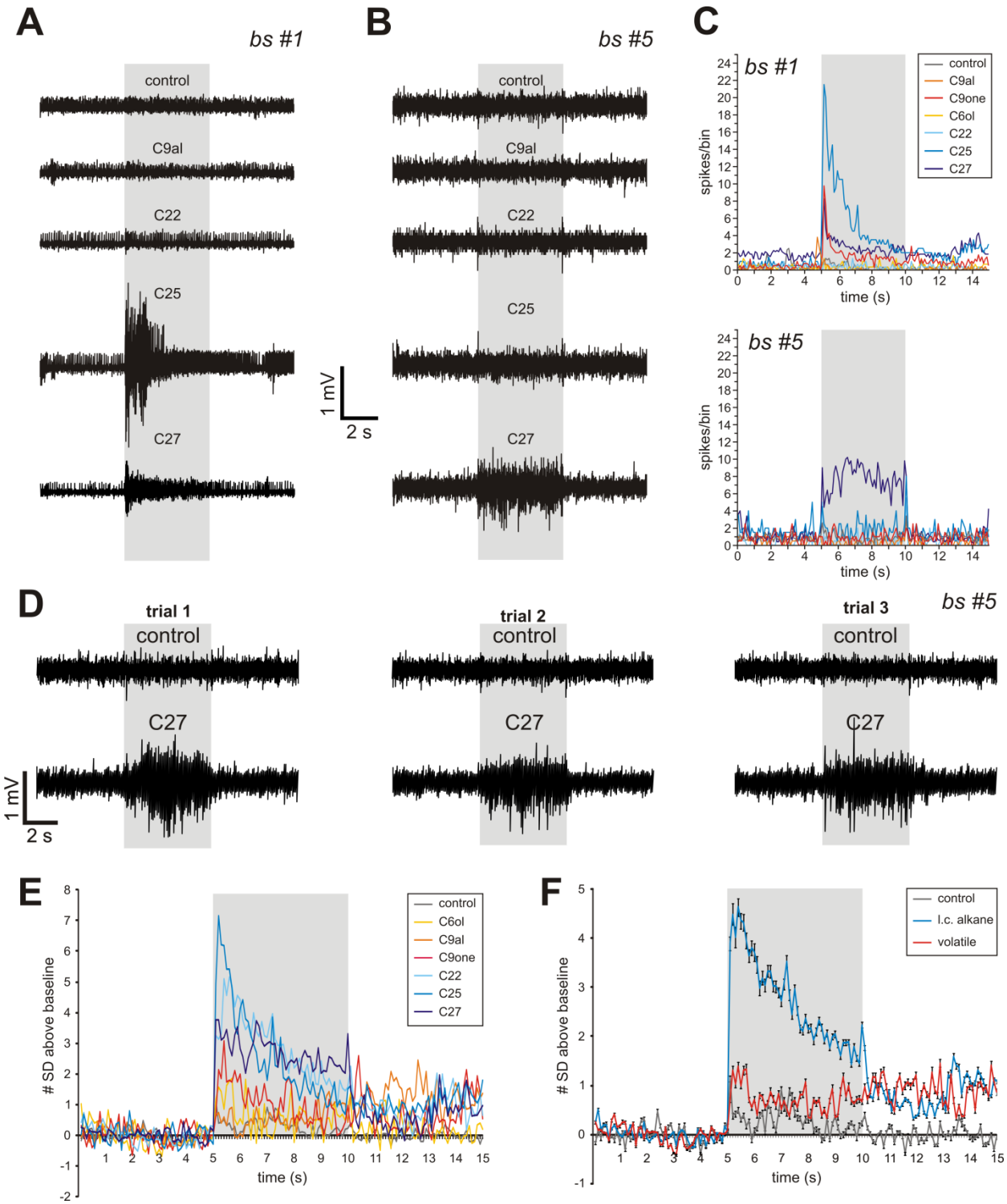

Figure 6: Single sensillum recording from basiconic sensilla 2

A) Responses of sensilla #1 (**A**) and #5 (**B**) to 5 sec presentations of 3 long-chain alkanes (docosane, C22; pentacosane, C25; heptacosane, C27), one volatile aliphatic odorant (nonanal, C9al) and a hot hexane control. **C**) Reproducibility of the response of sensillum #5 to heptacosane, shown at 3 different trials with this odorant and with the control. **D**) Average spiking activity to the different stimuli throughout a trial, showing a phasic reponse for sensillum #1 and a tonic response for sensillum #5. **E**, **F**) Average response of basiconic sensilla (n=8) represented as the number of SD above baseline, to the different odorants stimulation. Long chain alkane (l. c. alkane) stimulation induces stronger responses than volatile odorants (**F**).

We represented in **figure 6E** the response recorded to each stimulus and to the control in SD units (number of SD of the signal relative to baseline, n = 8 sensilla). While the three tested long-chain alkanes induced peak responses above 3 SD, the three volatiles induced responses between 2 and 3 SD (**Fig. 6E**). Consequently, the average response to alkanes during the stimulus was statistically higher than the responses to volatiles and to the control (**Fig. 6F**; Kruskal-Wallis test, $H = 13.55$, $p < 0.01$; multiple comparisons (Dunn test): alkane vs. control, $p < 0.01$; alkane vs. volatile, $p < 0.01$; volatile vs. control, $p = 1.0$, NS).

We conclude from these observations that OSNs contained in basiconic sensilla 2 respond preferentially to long-chain alkanes that are typically found in the cuticular hydrocarbon profiles of hornets (Martin *et al.*, 2009). They respond more strongly to these stimuli than to the volatile aliphatic odorants that were tested.

Discussion

The aim of this study was to investigate whether Vespidae exhibit an olfactory subsystem present in other families of social Hymenoptera. Describing the sensillar equipment on the antennae of the hornet *Vespa velutina,* we found clear correspondences with the major classes of sensilla described in ants and bees, and identified two basiconic sensilla types. While basiconic sensilla are lacking in the males of many hymenopteran species, we found this sensillar type on the antennae of both sexes. The sensory neurons housed in basiconic sensilla type 2 exclusively project to a cluster of olfactory glomeruli in the AL, both in males and females. Also in both sexes, this glomerular cluster contrasts with other regions of the AL by a lack of serotonin immunoreactive fibers, as observed in ant workers. Finally, electrophysiological recordings of single basiconic sensilla suggest that they preferentially respond to low volatile hydrocarbons. These results demonstrate the presence of a conserved olfactory subsystem in vespid wasps that could process CHC information, as it had been found earlier in ants. However, contrary to ants and bees, this olfactory subsystem is not sexually dimorphic in *V. velutina,* suggesting that it also plays a crucial role in the biology and/or mating behavior of males in Vespidae.

Specificity of hornets' sensillar equipment

Strong sexual dimorphisms in antenna size and/or in the distribution of cuticular sensilla have been reported in many hymenopteran species (Esslen & Kaissling, 1976; Ågren, 1977; Ågren, 1978; Ågren & Hallberg, 1996; Amornsak *et al.*, 1998; Nakanishi *et al.*, 2009; Mysore *et al.*, 2010; Streinzer *et al.*, 2013). In *V. velutina*, the general structure of the antenna, comprising a scape, a pedicel and ten flagellomeres in females but eleven in males, shows a similar sex dimorphism (one more antennal segment in males) as in other social Hymenoptera such as the honey bee and carpenter ants (Esslen & Kaissling 1976; Nakanishi *et al.*, 2009). This structural sex dimorphism also included

the presence of two tyloids on each male antennal segment. The multiple large pores observed on the surface of this male-specific structure were reported to be connected to secretory cells (Romani *et al.*, 2005). They have been observed in several paper and parasitoid wasp species, including the hornet *V. crabro* (Romani *et al.*, 2005; Steiner *et al.*, 2010), and are assumed to produce a contact pheromone, which is transferred to the female's antennae by males' antennation during courtship. Apart from these structural differences between sexes, the overall sensillar equipment of hornets is not sexually dimorphic, whereas it is strongly dimorphic in most other studied social Hymenoptera (Esslen & Kaissling, 1976; Ågren, 1977; Ågren, 1978; Ågren & Hallberg, 1996; Amornsak *et al.*, 1998; Nakanishi *et al.*, 2009; Renthal *et al.*, 2003; Gao *et al.*, 2007; Streinzer *et al.*, 2013). The low sexual dimorphism observed in hornets' sensillar equipment correlates with the much lower dimorphism we also observed in the organization of their AL compared to other social Hymenoptera (Couto *et al.*, 2016). Such a low sexual dimorphism could be related to a more elaborate male behavioral repertoire in hornets than in other species, since hornet males have been observed foraging on their own (Monceau *et al.*, 2014).

Homology of glomerular clusters

Neuroanatomical studies have pointed out striking similarities in AL organization across different hymenopteran species, especially concerning the division of the antennal nerve in different tracts projecting into distinct clusters of glomeruli (Zube *et al.*, 2008; Nishino *et al.*, 2009; Couto *et al.*, 2016). A currently debated question is whether morphologically similar AL clusters across different species are indicative of conserved olfactory structures (homologous clusters), or if they are simply the result of convergent developmental processes. Concerning the hornet T_B cluster, it bears strong homology with the female-specific cluster in the AL of ants (Nakanishi *et al.*, 2010; Kelber *et al.*, 2010; Nishikawa *et al.*, 2012). We carefully analyzed input-output relationships in the hornet $\mathbf{T_B}$ cluster and compared them to the situation in ants. In ants, the absence of basiconic sensilla in males is associated with the absence of a specific dorso-caudal cluster of small glomeruli, termed T6 (Nakanishi *et al.*, 2010; Nishikawa *et al.*, 2012). OSNs from basiconic sensilla exclusively project to this glomerular cluster, and information from this cluster is conveyed to higher-order centers by the median tract of uniglomerular projection neurons, m-ALT (Kelber *et al.*, 2010; Zube *et al.*, 2008; Zube & Rössler, 2008). We found exactly the same pattern of input-output connectivity in the hornet $\mathbf{T_B}$ cluster. OSNs from basiconic sensilla 2 exclusively project to the $\mathbf{T_B}$ cluster (**Fig. 4**) and this cluster is innervated exclusively by m-ALT projections neurons (Couto *et al.*, 2016). In our investigations, we were not able to assess the number of sensory neurons housed within a basiconic sensillum, but a range of 30 to 40 was previously reported (Lacher, 1964). This fits well with our counts of labeled glomeruli after staining of a single sensillum (29 and 36, **Fig. 4E, H**). Thus, input-output relationships of the hornet $\mathbf{T_B}$ cluster are coherent with those observed in the ant T6 cluster.

In addition to input-output relationships, a rare immunoreactive profile in a glomerular cluster also suggests a homology. In ant workers, the T6 cluster is the only subdivision of the AL that lacks serotonin immunoreactive fibers (Nakanishi *et al.*, 2010; Zube & Rössler 2008). We found a similar feature for the $\mathbf{T_B}$ cluster of both male and female hornets, as it was devoid of serotoninergic fibers whereas the rest of the AL was homogeneously stained. The similar input (basiconic sensilla OSN) and output (m-ALT PNs) connectivity as well as of a similar immunoreactivity (lack of serotonin) between the hornet $\mathbf{T_B}$ and the ant T6 clusters suggest that they may be homologous structures, sharing the same ancestral origin. This homology is also substantiated by previous histochemical studies, although the authors did not discuss this possible homology. Masson and Strambi (1977) identified two main subdivisions in the antennal lobes of an ant, *Camponotus vagus,* and a vespid wasp, *Polistes gallicus*. In both species, the ventro-rostral region of the antennal lobe strongly reacted to succinate dehydrogenase labeling while the staining was much weaker in the dorso-caudal region. An opposite staining pattern was found with respect to acetycholinesterase activity (Masson & Strambi, 1977). Careful observation of the figures suggests that the dorso-caudal region called "sensory lobule 2" by the authors actually corresponds to our $\mathbf{T_B}$. Likewise, this compartmentalization also seems to correlate with a sparser innervation by histamine immunoreactive processes found in a close vespid wasp species, *Polistes fuscatus* in the same AL region (Dacks *et al.*, 2010). These data, together with the observations of segregated LNs in ants (Nishikawa *et al.*, 2012), suggest that the vespid wasp and the ant AL contain a homologous, pharmacologically segregated, olfactory subsystem. The next important question was whether these subsystems are involved in similar functions in ants and hornets.

Function of the T_B glomerular cluster

Electrophysiological recordings by tip-capping of basiconic sensilla in *Camponotus japonicus* highlighted exclusive responses to non-nestmates CHC profiles, suggesting that ant workers were anosmic to nestmate colony odors (Ozaki *et al.*, 2005). Although further investigations contradicted this hypothesis by showing peripheral as well as central neuronal activity in response to nestmate colony odors (Brandstaetter *et al.*, 2011; Brandstaetter & Kleinedam, 2011), there is accumulating evidence that this system is involved in CHCs processing in ants (Ozaki *et al.*, 2005; Sharma *et al.*, 2015). Using single sensillum electrophysiological recordings, we showed that hornet basiconic sensilla 2 predominantly respond to long-chain alkanes. This suggests that this homologous subsystem in hornets and ants may have conserved a similar function, namely to detect and process CHC information. Interestingly, hornet basiconic sensilla also displayed responses to volatile aliphatic compounds (**Fig. 6**, **table 1**). This pattern is similar to that observed in ants, although these insects' basiconic sensilla appear more broadly tuned than hornets' (Sharma *et al.*, 2015). This difference may be explained by discrepancies between species in the number of sensory neurons housed within

basiconic sensilla. In carpenter ants, basiconic sensilla contain as many as 130 OSNs (Nakanishi *et al.*, 2009) while about 35 OSNs are housed in wasps' basiconic sensilla (see above, Lacher, 1964; **Fig. 4E**, **H**). It seems reasonable to think that more sensory neurons potentially participating in the recorded activity may broaden the apparent response profile of the sensillum.

What could be the function of a CHC-sensitive olfactory subsystem in the biology of hornets? Social wasps are known to discriminate nestmates from non-nestmates at the nest entrance (Gamboa, 1986; van Zweden & d'Ettorre, 2010). The central role played by CHCs in this recognition has already been demonstrated in hornets (*Vespa crabro,* Ruther *et al.*, 1998; 2002). In a given colony, the different hornet castes (queen *vs* worker) display different CHC profiles (Butts *et al.*, 1995; Tokoro & Makino, 2011; Spiewok *et al.*, 2006). In the common wasp, *Vespula vulgaris*, some CHCs have been shown to act as queen pheromones, advertising the queen's fertility status (van Oystaeyen *et al.*, 2014; Oi *et al.*, 2015). Some data also suggest that hornets perform worker policing, by removing the eggs that were not laid by the queen but by other workers (Foster *et al.*, 2002; Takahashi *et al.*, 2003, 2004). In *V. vulgaris*, the signals involved in this policing have recently been shown to be CHCs, involving the same compounds as the queen pheromone (Oi *et al.*, 2015). Thus, possessing an efficient CHC-processing system should be crucial for hornets' social biology, as a basis of nestmate discrimination, caste differentiation and worker policing.

A CHC-processing subsystem in males

One feature differed markedly between the CHC subsystems of hornets and ants: male hornets present numerous basiconic sensilla, whereas they are ubiquitously absent in the males of numerous solitary and social Hymenoptera (Formicidae: *carpenter ants*, Nakanishi *et al.*, 2009; Mysore *et al.*, 2010; Apidae: honey bees, Esslen & Kaissling, 1976; bumble bees, Ågren & Hallberg, 1996; Eucera, Streinzer *et al.*, 2013; Colletidae: Ågren, 1977; Andrenidae: Ågren, 1978; Trichogrammatidae: Amornsak *et al.*, 1998). Accordingly, male hornets display a $\mathbf{T_B}$ cluster that was identical to that of females (**Fig. 3**, **4**). In ants, the lack of a CHC-specific system in males was explained by their low involvement in social tasks, the males being utterly focused on mating (Nakanishi *et al.*, 2009; Nishikawa *et al.*, 2012). The exact role of males in the social life of a hornet colony is still unclear (Matsuura & Yamane, 1990), but as mentioned above, they may have a more elaborate behavioral repertoire than in most eusocial hymenopteran species (Monceau *et al.*, 2014). The mate finding strategy of hornet males consists in a nonaggressive patrolling behavior in the vicinity of nesting sites (Batra 1980; Spiewok *et al.*, 2006). Mating itself probably does not take place in flight, but rather on a hard substrate, possibly on the nest envelope (Batra, 1980, but see Spiewok *et al.*, 2006). While the existence of volatile queen-emitted sex pheromones is well demonstrated for attracting the males to females (Ono & Sasaki, 1987; Spiewok *et al.*, 2006), copulation occurs after the male has had the opportunity to touch the female with its antennae (Batra, 1980). This suggests that low volatile

compounds, possibly CHCs, are involved in the decision by the male to copulate with a given female. The presence of a CHC-processing subsystem in hornet males may thus be relevant for the recognition of fertile females (over workers) but also to avoid inbreeding when close to their own nest. In addition, different hornet species share the same sex pheromones, since cross-reactivity of queens onto males was demonstrated among six sympatric Japanese species (Ono & Sasaki, 1987). Since different species display different CHC profiles (Martin *et al.*, 2009), a CHC-processing subsystem in hornet males may also participate in pre-mating reproductive isolation in natural conditions, allowing males to avoid mating with females from other species.

The evolution of CHC detection and eusociality

Many aspects of the intricate organization of a social insect colony are regulated through sophisticated pheromonal communication (Vander Meer, 1998). Since eusociality emerged several times independently within different Hymenoptera families, it is currently thought that ancestral Hymenoptera may have presented a set of olfactory preadaptations to the high demands related to this social lifestyle (Galizia & Rössler, 2010; Nishikawa *et al.*, 2012; Streinzer *et al.*, 2013). Our study revealed a conserved olfactory subsystem between ants (Formicidae) and hornets (Vespidae), which appears to be involved in CHC processing. Since CHCs are critical cues for recognizing colony and caste membership, two crucial questions are how widespread this olfactory subsystem is among Hymenoptera, and whether or not it favored the multiple emergence of kin-specific altruism in this insect order. Among Hymenoptera, the primitive parasitoids use complex blends of CHCs for mate- and host-recognition (Singer, 1998; Sullivan, 2002). Therefore, elaborate CHC recognition and discrimination capacities are thought to precede all cases of eusociality within Hymenoptera (Kather & Martin, 2015). Since eusociality appeared independently in ants and hornets, one can speculate that the basiconic sensilla-specific olfactory system present in these two families was inherited from a common solitary ancestor. In addition, since, an Apoid solitary wasp (*Liris Niger,* Crabronidae) was suggested to recognize its prey by means of cuticular CHCs through basiconic sensilla (Anton & Gnatzy, 1998), this subsystem could have been already present in the common ancestor of Formicidae, Vespidae and Apoidea.

Interestingly, CHC recognition ability is highly developed in eusocial species, which seems to correlate with their harboring of a more complex chemoreceptor repertoire (Zhou *et al.*, 2015). The 9-exon olfactory receptor subfamily has been proposed as CHC-receptor candidates and hence might be expressed in olfactory sensory neurons housed in basiconic sensilla (Engsontia *et al.*, 2015). If this hypothesis holds true, we expect a general correspondence between the number of 9-exon olfactory receptors expressed in eusocial species and the number of glomeruli in their T_B cluster. Available data are however too sparse to prove this relationship yet. Future work should now provide more examples of the existence of this CHC-processing system among different hymenopteran families,

aiming to understand its evolution. Our working hypothesis is that this subsystem is ancestral and more widespread among Hymenoptera than initially thought. We favor the idea that complex CHC recognition/discrimination abilities, possibly involving the basiconic sensilla/T_B subsystem, may have been a "spring-loaded preadaptation" playing a central role in the advent of eusociality (Kather & Martin, 2015).

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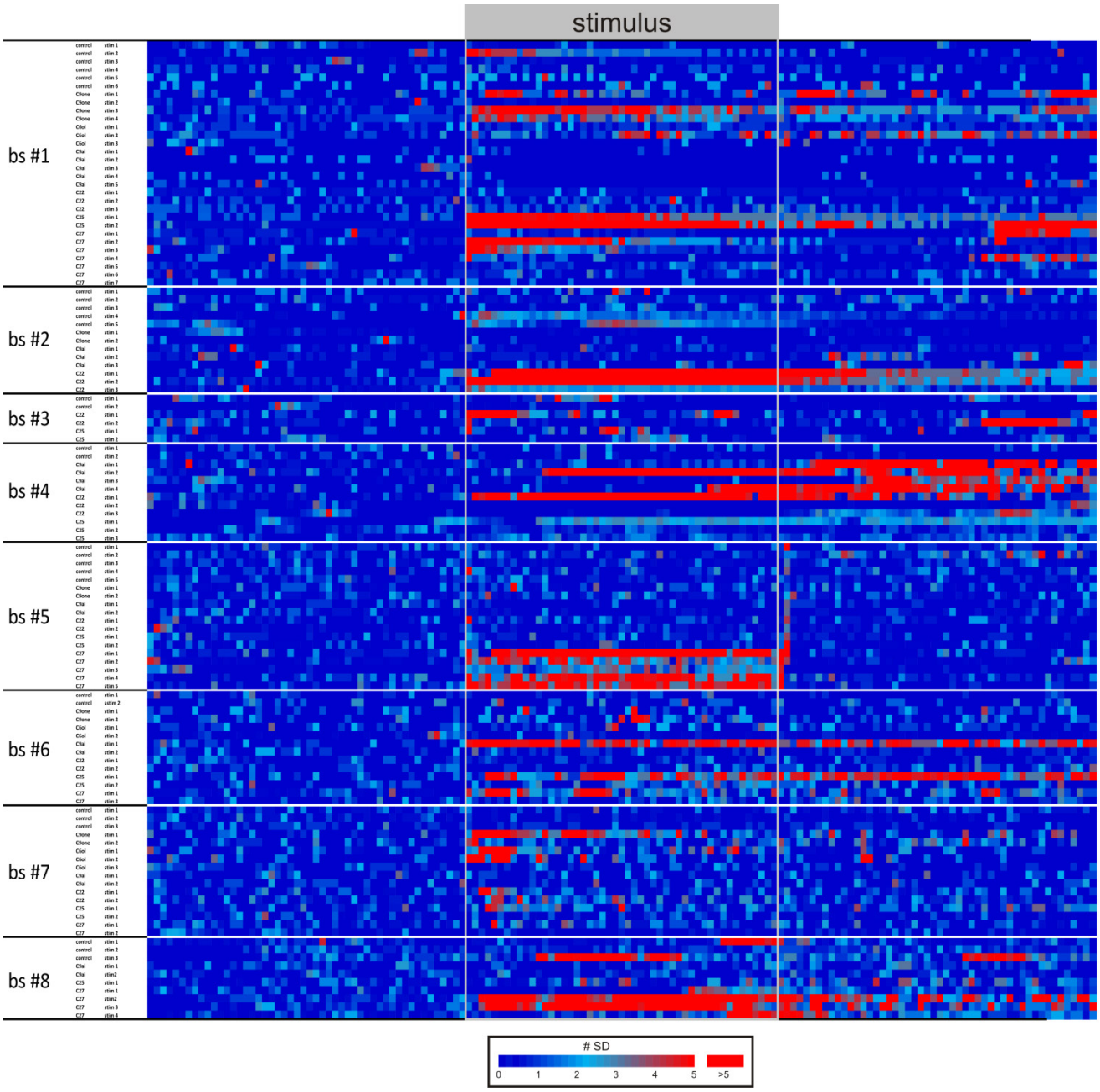
Supplementary figure



<u>Supplementary figure 1:</u> Electrophysiological responses of basiconic sensilla 2

For each responding sensillum and all stimulus presentations the spike frequency as a number of SD above baseline (baseline, average spike frequency during 5 s before stimulus) is color coded from dark blue to red.

CHAPITRE 3

Interspecific differences in antennal lobe organization among four hornet species with differing biotope, predation behavior and sympatry

Antoine Couto, Gérard Arnold, Hiroyuki Ai, Jean-Christophe Sandoz

Manuscript in preparation

Abstract

Olfaction is a crucial sensory modality for most insect species. Since the olfactory system is at the interface between the animal and its environment, it receives strong selection pressures which give rise to neuronal adaptations leading to interspecific phenotypic variability. Hornets are large eusocial predatory wasps which require high olfactory performance for foraging and intra-specific communication. In their natural range, these species often live with a high level of sympatry, hence evolving with strong interspecific competition. Consequently, different hornet species display outstanding differences in olfactory-based behaviors, which might correlate with specific adaptations of their olfactory system. The first olfactory processing center of the insect brain, the antennal lobe, is made of morphological and functional units called glomeruli. Using fluorescent staining, confocal microscopy and 3D reconstructions, we compared the antennal lobe structure, the glomerular numbers and volumes in four hornet species (*Vespa crabro*, *V. velutina*, *V. mandarinia* and *V. orientalis*), which exhibit differences in their nesting site preferences and predatory behavior. We found that despite a conserved organization of antennal lobe compartments, the numbers of glomeruli varied between species, even in a glomerular subsystem thought to process intraspecific cuticular signals. Moreover, specific adaptations corresponding to enlarged glomeruli were found in two species, *Vespa crabro* and *V. mandarinia*, but not in the others. We discuss the possible function of these adaptations based on species-specific behavioral differences.

Keywords: olfaction, hornet, antennal lobe, glomerulus, adaptations

Introduction

The genus Vespa (Hymenoptera, Vespidae) comprises 22 species of large eusocial wasps commonly called hornets (Archer, 1991; Perrard *et al.*, 2013). These species are mainly distributed from temperate to tropical regions of South East Asia, often displaying a high level of sympatry (Matsuura & Yamane, 1990; Carpenter & Kojima, 1997; Nguyen *et al.*, 2006). Only two hornet species present a natural range that extends outside of the Asian continent, which consequently is considered to be their diversification center. The hornet *Vespa crabro* is widely spread in temperate areas of Europe and Asia, while *V. orientalis* is found in subtropical regions of North Africa, around the Mediterranean Sea and in the Middle East (Carpenter & Kojima, 1997). In addition, two hornet species, *V. crabro* and *V. velutina,* have been introduced to novel locations and are now well established in North America and Europe respectively, illustrating the great ecological success of Vespa species (Shaw & Weidhaas, 1956; Monceau *et al.*, 2014; Arca *et al.*, 2015).

All hornets live according to an annual life cycle. Usually, a single mated queen founds a paper nest for laying the eggs that will produce the first workers (Matsuura & Yamane, 1990). As in other social Hymenoptera, all individuals of the colony cooperate by performing different tasks according to their caste, thanks to a complex communication system involving pheromones (Ikan *et al.*, 1969; Ishay & Perna, 1979; Bruschini *et al.*, 2010). To feed their larvae with proteins, hornets are usually generalist predators which prey on numerous insect species and play a key role in the regulation of insect populations (Edwards, 1980; Spradbery, 1973; Raveret Richter, 2000).

Because many hornet species are sympatric, interspecific competition can be severe, inducing strong selection pressures on these species' biology and behavior. Accordingly, acute differences have been observed in the biology and behavior across hornet species (Matsuura, 1984; Matsuura & Yamane, 1990). For instance, in tropical regions in which temperature is constant, hornet colonies could in principle be founded throughout the year. However, it has been observed that sympatric species display an asynchronous development of their colonies, reducing competition for resources (Matsuura & Yamane, 1990). Furthermore, strong differences are also observed among species concerning nesting site preferences. For instance, *V. velutina* hornets build their nests preferentially in the trees, under treetop branches (Nakamura & Sonthichai, 2004; Monceau *et al.*, 2014), whereas *V. crabro* build theirs in tree hollow cavities (Matsuura & Yamane, 1990; Steinmetz *et al.*, 2002). Some species have also adopted remarkable strategies allowing to overcome sympatric competition. *V. dybowskii* queens are thus able to perform social parasitism by usurping the nests of *V. crabro* and *V. simillima* using chemical camouflage (Sakagami & Fukushima, 1957; Martin *et al.*, 2008). Different species also colonize different biotopes, in which feeding resources and the competition to obtain them vary, exerting in turn different selective pressures on predatory behavior. Different strategies, such as specialization on a particular prey type or the recruitment of nestmates to food sources have

been reported (Matsuura, 1984). For instance, *V. tropica* hunt almost exclusively Polistes wasps, and conduct massive raids on the nests of these wasps (Matsuura, 1984).

Cooperative hunting is however more or less pronounced depending on the hornet species, ranging from *en masse* predation (e.g. *Vespa mandarinia*), in which scouts actively recruit nestmates with pheromones to attack insect colonies (Ono *et al.*, 1995; Ono *et al.*, 2003), to solitary hunters finding food sources independently of other nestmates (e.g. *Vespa crabro:* Baracchi *et al.*, 2010), with intermediate cases of group predation (e.g. *Vespa velutina;* Monceau *et al.*, 2013*, Vespa orientalis*; Glaiim, 2009). All these examples show that phylogenetically close species, different Vespa species here, can undergo strong selective pressures for avoiding inter-specific competition and adapting to different ecological niches. These selective pressures may act on a number of levels, including the central nervous system, which is critically involved in insects' behavior. Especially, because olfaction plays a crucial role in the intraspecific communication, mating and foraging behaviors of hornets (Bruschini *et al.*, 2010), their olfactory system could present a number of species-specific adaptations.

In insects, odorant molecules are detected by olfactory sensory neurons (OSN) on the antennae, which project to a primary olfactory structure, the antennal lobe (AL) (Anton & Homberg, 1999). This structure is made of morphological and functional units called 'glomeruli' which each gather input from a given type of OSN (Gao *et al.*, 2000; Hansson & Anton, 2000; Couto *et al.*, 2005). The AL of social Hymenoptera is particularly complex as it contains a high number of glomeruli, compartmentalized in clusters formed by distinct bundles of OSN axons (Suzuki, 1975; Kirschner *et al.*, 2006; Zube *et al.*, 2008; Nakanishi *et al.*, 2010; Couto *et al.*, 2016). A differential investment in terms of numbers of glomeruli and glomerular clusters has already been observed across different Hymenoptera, possibly reflecting diverse demands on olfactory performance in this insect order (Galizia *et al.*, 1999; Smid *et al.*, 2003; Kelber *et al.*, 2009; Mysore *et al.*, 2009; Das & Fadamiro, 2013; van der Woude & Smid, 2015; Couto *et al.*, 2016). Moreover, in males, but also in workers of some hymenopteran species, strongly enlarged glomeruli called 'macroglomeruli' are involved in the detection and processing of pheromonal signals (Arnold *et al.*, 1985; Sandoz, 2006; Kleineidam *et al.*, 2005; Kelber *et al.*, 2009; Kuebler *et al.*, 2010). An increased glomerular volume is thought to correlate with an expanded number of sensory neurons targeting the corresponding glomerulus (Hansson *et al.*, 1995; Berg *et al.*, 1998; Bressel *et al.*, 2016). Thus, in addition to increased glomerulus and/or cluster numbers, the presence of macroglomeruli might indicate specific neuronal adaptations for the enhanced detection of particularly relevant odorants in a given species. A number of previous studies compared AL organization and glomerular numbers among closely related species, in Diptera (Kondoh *et al.*, 2003; Ignell *et al.*, 2005; Ghaninia *et al.*, 2007), Lepidoptera (Rospars, 1983; Skiri *et al.*, 2005; Berg *et al.*, 2002) and Hymenoptera (Smid *et al.*, 2003; Kleineidam *et al.*, 2005; Kelber *et al.*, 2009; Mysore *et al.*, 2009; Das & Fadamiro, 2013). A general observation of

these studies was that glomerular numbers were much more variable among closely-related hymenopteran species than in other insect orders. However, in Hymenoptera, all the studies performed until now addressed only one family, the Formicidae.

In the present work, we addressed this question in the Vespidae family, by comparing the antennal lobe organization of four hornet species of the Vespa genus. These species differ in their their native range, nesting site preference and predatory behavior, which may have given rise to specific olfactory adaptations. We first assessed the variability in AL compartments across species and evaluated the number of glomeruli. Then, we measured and compared across species the volumes of AL glomeruli, asking if macroglomeruli exist in the workers of some of these species. Our results show that although the global organization of the AL is clearly conserved across hornet species, the numbers of glomeruli and their volumes vary strongly. In particular, some species present a worker macroglomerulus, while others do not.

Materials and Methods

Animals

Four species belonging to the Vespa genus were chosen (**Table 1**). Two species were collected in France: European hornets, *Vespa crabro*, were trapped on the CNRS campus in Gif-sur-Yvette whereas yellow-legged hornets *Vespa velutina* were caught on the site of INRA Bordeaux-Aquitaine (Couto *et al.*, 2016). Giant hornets, *Vespa mandarinia* were trapped in Fukuoka, Japan, while the oriental hornets, *Vespa orientalis*, were captured around Porto-Heli, Greece. All species were easily distinguishable based on their distinct sizes and body colorations.

Species	Collection	Nesting	Predation	Sympatry
V. mandarinia	Fukuoka, Japan	underground cavity	*en masse*	high
V. velutina	Bordeaux, France	open space (treetops)	group	high
V. crabro	Gif-sur-Yvette, France	tree hollow cavity	solitary	low
V. orientalis	Porto Heli, Greece	underground cavity	small group	low

Table 1: Hornet species (Vespa sp.) used in this study
The table shows the locations where the samples were collected, as well as important aspects of each species' biology. They include preferential nesting sites, typical predation behavior and the sympatry level (corresponding to low or high numbers of sympatric Vespa species).

Staining procedure

To investigate the glomerular organization of the AL, antennal sensory neurons were stained anterogradely in the two species collected in France, *Vespa crabro* and *Vespa velutina*. The antennal nerve was exposed by removing a piece of cuticle at the scape. Then, the antennal nerves were severed with a glass electrode loaded with crystals of fluoro-ruby (Tetramethylrhodamine dextran, 10 000 MW, D-1817; Invitrogen, Eugene, OR; in 2% Bovine Serum Albumin). The preparation was

then covered with saline solution (130 mM NaCl, 6 mM KCl, 4 mM MgCl2, 5 mM CaCl2, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, with PH = 6.7) and kept in a dark room to let the dye diffuse. The next day, brains were dissected out and immediately fixed in 4% PFA solution for 24 hours.

After the capture of *V. mandarinia* and *V. orientalis*, the head of each insect was immediately separated from the body and plunged into 500 µL of fixative solution (4% paraformaldehyde in PBS) containing 1 µL of 4% Lucifer yellow (Lucifer Yellow CH, Potassium Salt, L-1177; Invitrogen, Eugene, OR). Samples were kept in this solution for 5 to 7 days. The brains were then dissected out with special attention to completely remove the retina, as failure to do so may result in unclear staining.

Thus, staining varied among species but the fixation and dehydration steps were the same (see below). To evaluate a potential bias of the different staining applied, we performed both approaches in *V. velutina* workers. Since both techniques provided good contrast, we obtained the same 3D reconstruction and glomerular distribution. In addition, we did not observe any systematic bias in glomerular numbers or relative sizes depending on staining type.

Brain preparation and confocal microscopy

Brains were removed from the fixative solution and washed 3 times in 0.01 M of PBS (10 minutes each). Brains were then dehydrated in ascending concentrations of ethanol (50, 70, 90, 95 and 3x 100% for 10 minutes each) and clarified in methylsalicylate (Sigma-Aldrich, Steinheim, Germany) for at least 3 days. The samples were then mounted on aluminum slides with a central hole filled with methylsalicylate and covered by thin coverslips on both sides.

Antennal lobes were scanned with a laser-scanning confocal microscope (LSM-700; Carl Zeis, Jena, Germany). Using a water immersion objective (20x plan-apochromat 1.0 NA), optical sections were acquired at 1 µm intervals (z), with a resolution ranging from 0.52 µm/pixels to 69 µm/pixels (x,y), depending on the size of the AL of each species. Given the large size of the hornet AL, complete scans were obtained by stitching adjacent “tiles” (512 x 512 pixels) of optical sections with the tile function of the ZEN software (Carl Zeiss, Jena, Germany). Fluoro-ruby labeled neurons were visualized using a 555 nm solid-state laser, while Lucifer yellow was excited at 488 nm.

Image processing and 3D reconstruction

Confocal image stacks were saved as LSM files and processed using ImageJ software and the bio-formats plugin (LOCI). Contrast and brightness were adjusted for each series of optical sections, which were then imported into a three-dimensional analysis software (AMIRA 5.4.3, VSG, Berlin, Germany). Glomeruli were reconstructed by manual labeling in three orthogonal plans (xy, xz and yz) and the 3D model of each glomerulus was obtained using the *wrap* function of AMIRA. When the antennal nerve was stained, it was possible to assign each glomerulus to a glomerular cluster by closely following OSN bundles. In contrast, global staining with Lucifer yellow allows estimation of

antennal lobe compartmentalization on the basis of glomerular position, shape and relative size. However, irrespective of the staining method, the cluster termed T_B could be unambiguously delineated thanks to its typical features (slightly detached from main AL, with smaller, tightly packed glomeruli).

Volume measurement and statistical analysis

The volume of glomeruli was measured on the basis of their 3D reconstruction with AMIRA software. To overcome the problem of antennal lobe size variability in different individuals or across species, the volume of each glomerulus was normalized with respect to the size of the AL (calculated as the sum of all glomerular volumes, **Fig. 4**). For interspecific comparisons of glomerular numbers and volumes, non parametric Kruskal Wallis tests were used. When significant, they were followed by a Dunn pairwise test, which includes a correction for multiple comparisons.

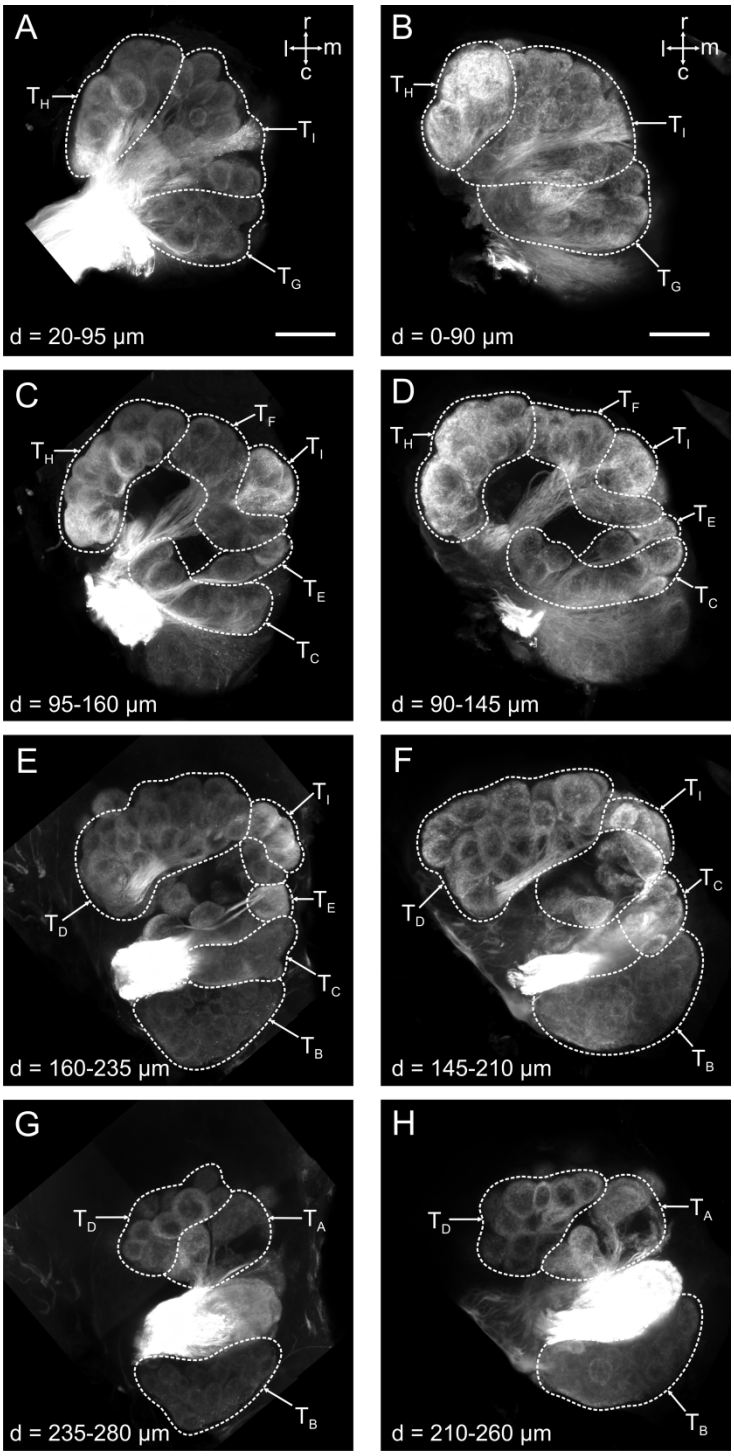
In order to determine the presence of macroglomeruli, the four largest glomeruli of each species were identified in all individuals and the mean relative volume was calculated for each homologous glomerulus. Then, a quantitative threshold that defines outliers according to 80% of the distribution of glomerular volumes was used: $V_{outlier} > V_U + k (V_U - V_L)$, where V_U is the upper percentile (90%) and V_L is the lower percentile (10%) of glomerular volume distribution. We used k = 3 as a conservative value that successfully categorized macroglomeruli in previous work (Kuebler *et al.*, 2010; Streinzer *et al.*, 2013; Roselino *et al.*, 2015; Couto *et al.*, 2016). Thus, glomeruli whose volume was above this threshold were considered as macroglomeruli.

Results

Antennal lobe organization

In previous work, we extensively described the olfactory pathway of the hornet *V. velutina* and showed that the antennal lobe is compartmentalized in a number of conspiscuous glomerular clusters subtended by OSN axon bundles (Couto *et al.*, 2016: Fig. 6). To evaluate the similarity of this organization in other Vespa species, we injected a fluorescent tracer into the antennal nerve of *V. crabro* workers and observed the antennal lobe using confocal microscopy. This procedure revealed a very similar organization of the AL in *V. crabro* (n=4) and in *V. velutina* (n=6). In both species, the antennal nerve separates at the entrance of the AL into 9 OSN bundles (**Fig. 1**). At the dorsal surface of the AL, a few large glomeruli receive several bundles of sensory neurons (T_A) and, located at the caudal side close to the antennal nerve, a remarkable cluster of numerous small and tightly grouped glomeruli (T_B) is also present in both species (**Fig. 1E-H**). The cluster termed T_C groups glomeruli along a sensory tract which proceeds in a medial direction and innervates glomeruli from their caudal periphery (**Fig. 1C-F**). Facing this cluster, the T_D tract projects in the inner dorsorostral region of the AL (**Fig. 1E**, **F**). More ventrally, in *V. velutina* as well as in *V. crabro*, a conspicuous sensory tract (T_F)

crosses the AL midline and forms a rostral cluster of glomeruli, whereas another often brightly labeled sensory tract (T_E) proceeds in parallel to the inner caudal part of the AL (**Fig. 1C**, **D**). Again in both species, glomeruli of the ventral AL surface are innervated by three tracts with distinct orientations. One sensory tract projects rostrally in relatively large glomeruli (T_H), a second tract projects in a caudomedial direction (T_G) and a third tract (T_I), flanked by the two previous ones, first points in a rostromedial direction and then slopes down dorsally (**Fig. 1A**-**D**). Thus, the 9 olfactory sensory tracts show similar orientations and innervate glomeruli with relatively similar shapes and positions in the ALs of *V. crabro* as in *V. velutina*.



<u>Figure 1:</u> Antennal lobe organization in hornets
Projection view starting from the ventral surface to the dorsal surface of a right antennal lobe after mass staining of the antennal nerve in *V. velutina* (**A**, **C**, **E**, **G**) and *V. crabro* (**B**, **D**, **F**, **H**). The antennal lobes are similarly organized as both species exhibit 9 olfactory sensory tracts (T_A-T_I) which innervate similar clusters of glomeruli in both species. r, rostral; c, caudal; m, medial; l, lateral. Depth (d) from the ventral surface is indicated on each panel. The scale bars in **A** (applies to **C**, **E**, **G**) and **B** (applies to **D**, **F**, **H**) correspond to 100 µm.

All our next observations with other species confirm this finding, leading us to use a common nomenclature for presumably homologous sensory tract in all these species (T_A-T_I; Couto *et al.*, 2016). Although global staining with bath-applied Lucifer yellow does not reveal OSN bundles as clearly as with tracer injection, we could still compartmentalize the AL of *V. orientalis* and *V. mandarinia*, using recognizable glomerular features such as their relative size, shape and location. For instance, the large glomeruli in the most dorsal region of the AL (T_A) were easily observed in all species (orange glomeruli in **Fig. 2.E-H**). Also, the T_B cluster was easily identifiable as a dorsal cluster, partly segregated from the main AL (dark green in **Fig. 2.E-H**). We conclude from these observations that the AL of four different hornet species shares the same organization.

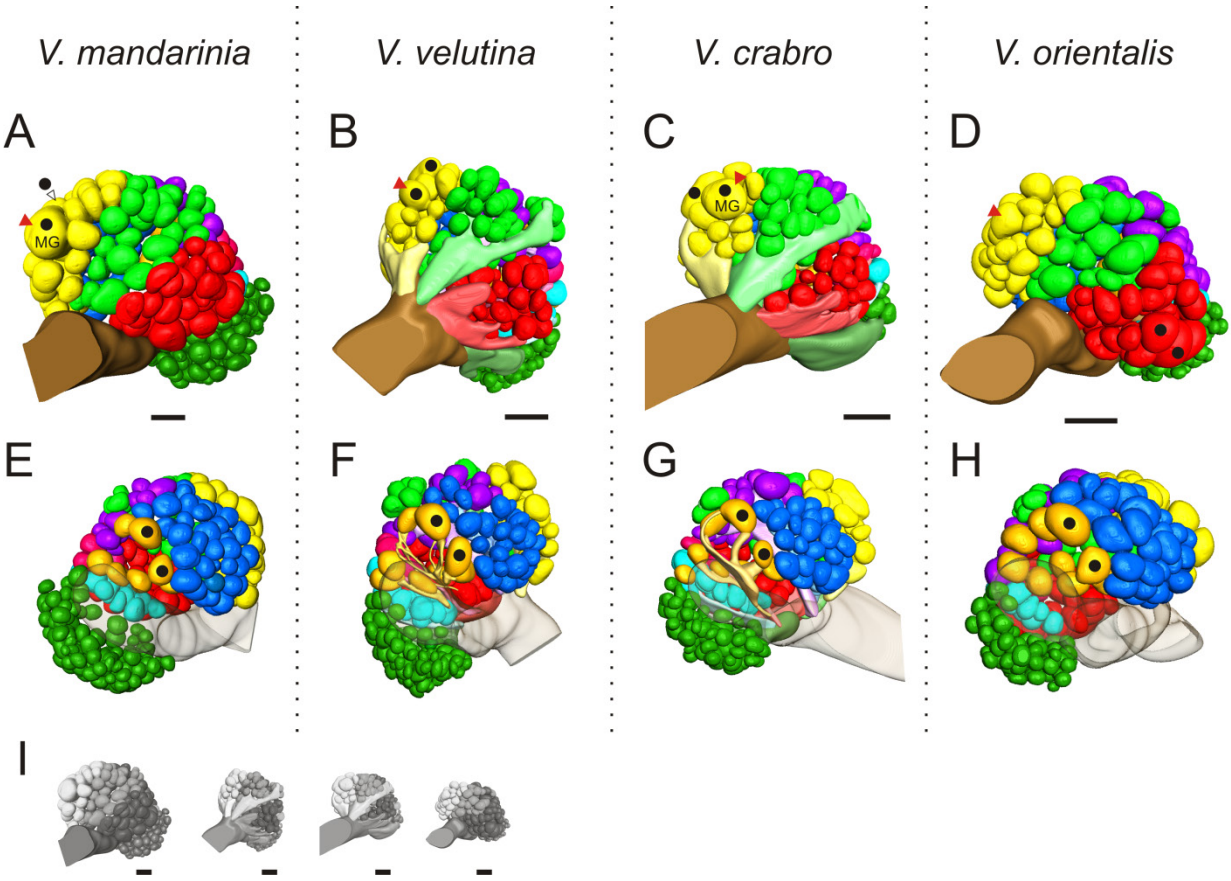


Figure 2: 3D reconstructions of the antennal lobes of four hornet species
3D models of the antennal lobes of *V. mandarinia* (**A**, **E**), *V. velutina* (**B**, **F**), *V. crabro* (**C**, **G**) and *V. orientalis* (**D**, **H**) are represented in a ventral view (**A**, **B**, **C**, **D**) or in a dorsal view (**E**, **F**, **G**, **H**). All lobes are scaled to the same size in **A-H** for easier comparison (scale bar = 100 µm). Black dots indicate the four largest glomeruli in the antennal lobe of each species. The red triangle shows a possibly homologous glomerulus in the 4 species. MG stands for macroglomerulus and relates to the volumetric measures shown in **Fig. 4**. For reference, all lobes are represented according to their true relative sizes in **I** (scale bar = 100 µm).

Number of glomeruli

We assessed the number of glomeruli found in the ALs of the four hornet species. It ranged from 231 ± 3.6 (mean ± SD, n=3) glomeruli in *V. orientalis* to 281 ± 2.9 (n=3) glomeruli in *V. mandarinia* with intermediate cases at 265 ± 7.2 glomeruli in *V. velutina* and 240 ± 2.9 glomeruli in *V.*

crabro (**Fig. 3A**). The number of glomeruli was heterogeneous among the 4 hornet species (Kruskal-Wallis test, H = 14.6, $p < 0.01$). More precisely, the number of glomeruli in *V. crabro* and *V. orientalis* was significantly lower than that in the giant hornet *V. mandarinia* (Dunn test, Z = 2.722, $p < 0.05$ and Z = 3.395, $p < 0.01$, respectively).

We next focused on one particular subsystem of the hornet AL. As mentioned above, the T_B forms a conspicuous cluster, which is partly segregated from the main part of the AL and is assumed to process cuticular hydrocarbon information for intra- and interspecific discrimination (hornets: Chapter 2; ants: Ozaki *et al.*, 2005; Nishikawa *et al.*, 2012; Sharma *et al.*, 2015). We thus analyzed separately the number of glomeruli contained by the T_B cluster and by the main AL for each species (**Fig. 3A**). We found that both AL compartments contained significantly different numbers of glomeruli across species (Main AL: Kruskal-Wallis test, H = 12.2, $p < 0.01$; T_B: H = 14.2, $p < 0.01$). On average, the main AL contains about 162 glomeruli in *V. crabro* (± 2.7) and *V. orientalis* (± 3.0) whereas about 176 ± 3 glomeruli where counted in *V. velutina* and 178 ± 2.3 in *V. mandarinia*. A significant difference appeared between *V. crabro* and *V. mandarinia* (Dunn test, Z = 2.733, $p < 0.05$). The T_B cluster contains about 68 ± 0.6 glomeruli in *V. orientalis*, 79 ± 1 glomeruli in *V. crabro*, 90 ± 6.9 glomeruli in *V. velutina* and 103 ± 2.6 glomeruli in *V. mandarinia*, with a significant difference in *V. crabro* and *V. orientalis* compare to *V. mandarinia* (Dunn test, Z = 2.658, $p < 0.05$ and Z = 3.395, $p < 0.01$, respectively). Thus, both in the CHC subsystem and in the main AL, different hornet species present different numbers of glomeruli.

To evaluate the hornets' investment in the CHC subsystem in terms of its number of functional units (glomeruli), we compared the percentage of the number of glomeruli in this system relative to the whole AL. The relative investment in the T_B cluster varied significantly across species (Kruskal-Wallis test, H = 11.9, $p < 0.01$). It represented from 29.6% of the total number of glomeruli in *V. orientalis* to 36.5 % in *V. mandarinia*. We found a significant difference between these two species (Dunn test, Z = 3.395, $p < 0.01$). We conclude that the relative investment in the CHC subsystem in terms of functional units differs across species within the genus Vespa.

Volume of glomeruli

Based on 3D reconstructions, we measured the volume of all glomeruli in the ALs of the four Vespa species. In hornets, the size of individuals varies strongly between species, affecting the absolute volume of neural structures. Here, the AL of the giant hornet *V. mandarinia* was on average twice as large (21.9×10^6 µm^3) as those of the three other species (ranging from 82.9×10^5 to 12.2×10^6 µm^3; **Fig. 2I**). To overcome such size variability, we calculated the relative volume of each glomerulus relative to the sum of all AL glomeruli, as a measure of hornets' investment in this particular functional unit. This first allowed us to compare across species the relative volume of the T_B subsystem. In all species, the relative volume of T_B glomeruli was smaller (in average from 0.14 to

0.23%, **Fig. 3B**) than the relative volume of glomeruli from the main AL (in average from 0.45 to 0.54%; Wilcoxon test, Z = 3.621, $p < 0.001$). The whole T_B system represented ~20 % of the whole AL volume in *V. mandarinia*, 18 % in *V. crabro*, but only about 12.5 % in *V. velutina* and *V. orientalis* (**Fig. 3C**). These proportions were statistically heterogeneous among species (Kruskal-Wallis test, H = 12.41, $p < 0.01$), with a significant difference between *V. orientalis* and *V. mandarinia* (Dunn test, Z = 2.748, $p < 0.05$).

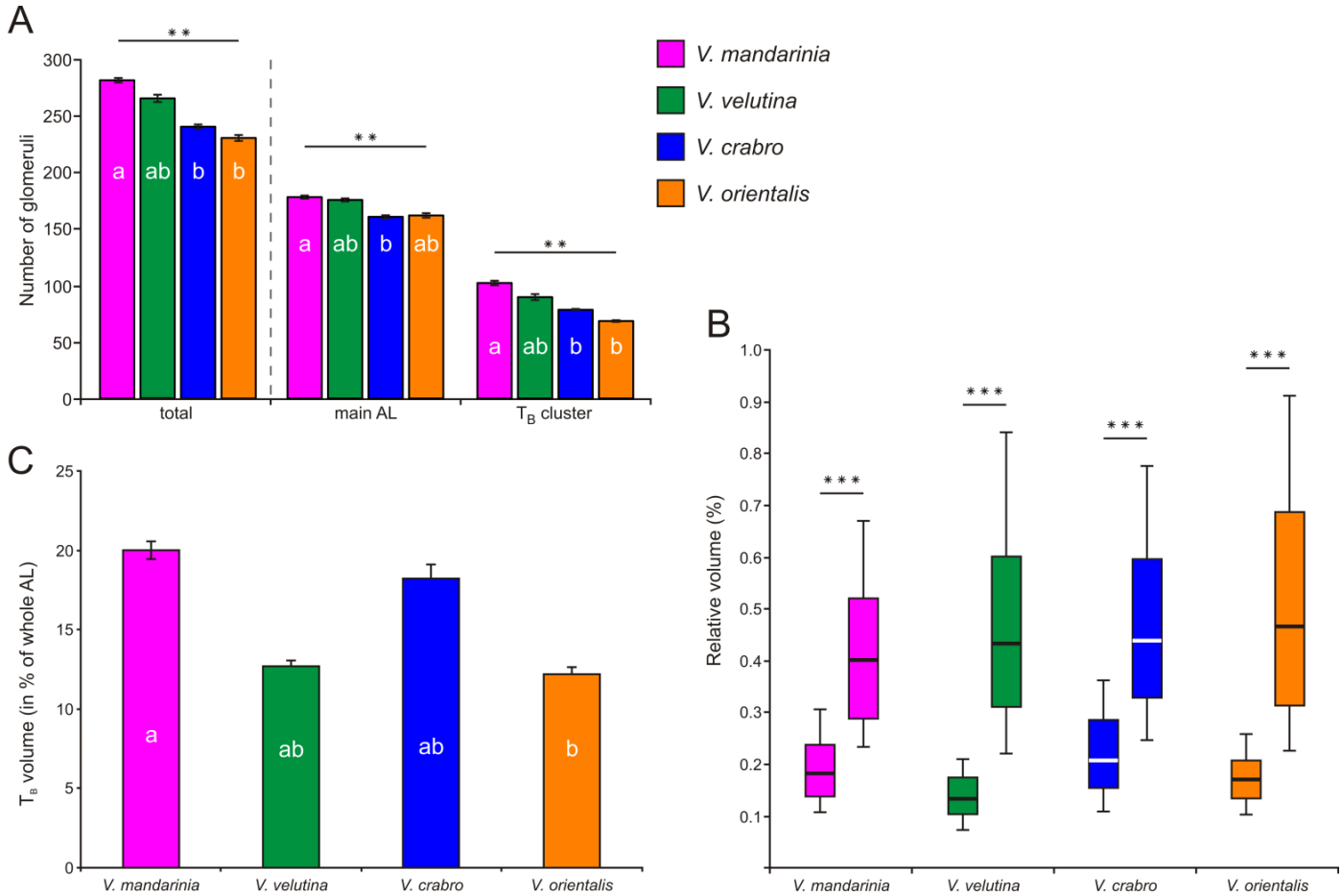


Figure 3: Number and volume of olfactory glomeruli in four hornet species
A) Number of glomeruli the whole antennal lobe (total) or its two main compartments (main AL and T_B cluster) in *V. mandarinia* (magenta), *V. velutina* (green), *V. crabro* (blue) and *V. orientalis* (orange). Significant differences are found within both compartements (Kruskal-Wallis test, ** $p < 0.01$). **B**) Box plot showing the relative volume of the T_B glomeruli compared to glomeruli from the main AL in the four hornet species. In all cases, T_B glomeruli are smaller than other glomeruli. **C**) Occupancy rate (in terms of total glomerular volume) of the T_B cluster relative to the whole antennal lobe. The T_B cluster occupies between 12.5 and 20% of the whole antennal lobe in the different species, showing inter-specific differences (Kruskal-Wallis test, ** $p < 0.01$). Different letters indicate significant differences in post hoc Dunn tests ($p < 0.05$).

The calculation of the relative glomerular volumes also allowed us to evaluate the possible presence of macroglomeruli in the hornet AL. In the four species, most of the glomeruli displayed a relatively similar size, each representing less than 1 % of the total AL (**Fig. 4**). Nonetheless, within each lobe, a few conspicuously large glomeruli were found. We focused on the four largest glomeruli found in the AL of each species (black dots in **Fig. 2A-H**). In all species, two of these were located in

the dorsal cluster T_A (black dots in **Fig. 2E-H**). In three of the species, *V. mandarinia*, *V. velutina* and *V. crabro*, the next two large glomeruli were found on the ventral side, in the T_H cluster (black dots in **Fig. 2A-C**). All these glomeruli were found at similar locations across different species and might be homologous. *V. orientalis* differed from the other species because two of the four largest glomeruli were rather found in the T_G cluster, on the ventral side (black dots in **Fig. 2D**). We then evaluated whether any of these four largest glomeruli per species can be considered as macroglomeruli, based on a volumetric threshold which defines outliers according to the distribution of glomerular volumes (**Fig. 4**). We found that only the largest glomerulus located on the ventral AL surface of *V. mandarinia* and *V. crabro* passes the threshold and can be classified as a macroglomerulus ('MG' in **Fig. 2A**, **C** and outliers in **Fig. 4.A**, **C**). We conclude that only the ALs of *V. mandarinia* and *V. crabro* workers contain a macroglomerulus.

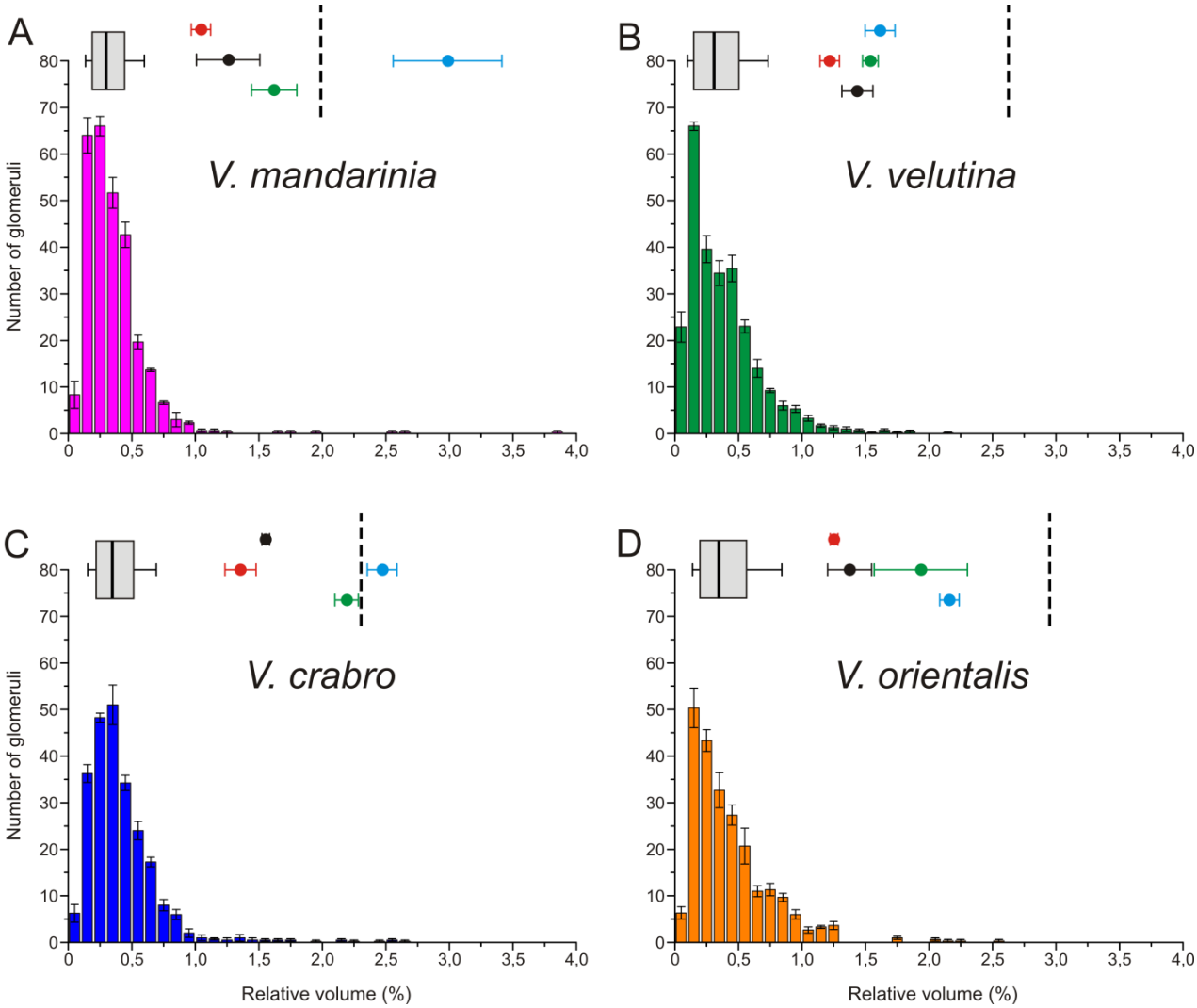


Figure 4: Distribution of glomerular volumes in four hornet species

Distribution of all glomerular volumes in the reconstructed antennal lobes of *V. mandarina* (**A**, n = 3), *V. velutina* (**B**, n = 7), *V. crabro* (**C**, n = 4) and *V. orientalis* (**D**, n = 3). The box plots above each distribution show the median and interquartile range (25-75%) of glomerular volumes whereas whiskers show the 10th and 90th percentiles. The average volumes of the four largest glomeruli of each species are represented as colored circles and the statistical macroglomerulus threshold is represented by a dotted line. Only the antennal lobes of *V. mandarinia* (**A**) and *V. crabro* (**C**) contain a glomerulus that exceeds this threshold (labeled MG in **Fig. 2**).

Discussion

The goal of this study was to investigate possible neuronal adaptations within the antennal lobes of different hornet species in relation with differences in their biology and behavior. We found that the general organization of the AL, such as its compartmentation in nine recognizable clusters of glomeruli, is conserved across species. However, two phenotypical traits were found to be variable. First, the number of glomeruli varied across species, and in particular within a peculiar cluster of glomeruli thought to be involved in nestmate discrimination. Second, although the relative distribution of glomerular volumes was relatively stable across species, a macroglomerulus (MG) was found in only two of the investigated species.

Number of glomeruli

The primary olfactory processing center, the AL, is made of dense synaptic neuropil units which each gather all OSNs expressing a given olfactory receptor (Gao *et al*, 2000; Vosshall *et al.*, 2000; Couto *et al.*, 2005). Consequently, the number of glomeruli closely correlates with the number of olfactory receptor genes expressed within a species and is thought to reflect the general olfactory discrimination power. For instance, consistently with the one-receptor/one-glomerulus, 163 functional olfactory receptor genes were annotated in the honeybee, which has about 165 olfactory glomeruli in the antennal lobe (Robertson & Wanner, 2006). Social insects deal with a highly complex olfactory environment in which they must be able to discriminate numerous semiochemical signals. Interestingly, important expansions of chemoreceptor gene repertoires have been observed in Hymenoptera, correlating with an expansion of the number of AL glomeruli found throughout this insect order (Zhou *et al.*, 2015). We found that hornets follow this rule, since all investigated species displayed more than 230 glomeruli. We also found that the variance of glomerular numbers across species was relatively high (50 glomeruli difference between *V. mandarinia* and *V. orientalis*). Such variance within a given genus is much higher than that observed for instance in Lepidoptera (Heliothis moths ~4 glom: Berg *et al.*, 2002; Skiri *et al.*, 2005) or Diptera (anopheles mosquitoes ~10 glom in: Ghaninia *et al.*, 2007; Ignell *et al.*, 2005; and no variation in drosophila: Kondoh *et al.*, 2002), but still lower than that demonstrated in other social Hymenoptera, such as leaf cutting ants (*Atta* sp. 116 glom; *Acromyrmex* sp. 108 glom: Kelber *et al.*, 2009). It thus suggests a significant level of species-variability of the antennal lobe in hornets.

The hymenopteran antennal lobe presents a remarkable organization with multiple compartments with possible homologies in different families (Masson & Strambi 1977; Zube *et al.*, 2008; Couto *et al.*, 2016). The most conspicuous subdivision of the hornet AL is the T_B cluster, which is clearly a homologous structure in ants and vespid wasps (Chapiter 2). This cluster, which is partly segregated from the main AL, is thought to process cuticular hydrocarbon information detected by basiconic sensilla (ants : Ozaki *et al.*, 2005; Nishikawa *et al.*, 2012; Sharma *et al.*, 2015; hornets:

Chapter 2). One could speculate that high demands on inter- and intraspecific discrimination in hornets could act as selection pressures, thereby increasing the number of glomeruli in the T_B cluster in some species. We therefore assessed separately the numbers of glomeruli in the T_B cluster and in the main AL. We found that although the number of glomeruli in the main AL slightly (but significantly) varied across species, much larger differences among species were found for the T_B cluster. This cluster showed a high diversification, with *V. mandarinia* having 51% more numerous functional units in its T_B subsystem (103 glomeruli) than *V. orientalis* (68 glomeruli). Interestingly, hornet species which have their natural range in Asia (*V. mandarinia and V. velutina*), which implies a high level of sympatry (Matsuura, 1984; Nguyen *et al.*, 2006) also have larger numbers of T_B glomeruli than the European species *V. crabro* and *V. orientalis,* which experience low sympatry levels in this region. This finding fits with the idea that selection pressures on intra- and interspecific discrimination might act on the evolution of particular clusters of olfactory glomeruli. Interestingly, a separate subspecies of *V. crabro* (*V. crabro flavofasciata*) which forms large colonies is found in Japan, with accordingly high sympatry levels (Takahashi *et al.*, 2004). It would be especially interesting to compare the numbers of T_B glomeruli in both *V. crabro* subspecies.

Glomerular volume of T_B glomeruli

Many studies have shown sex and caste polymophism in glomerular volume within the same species (bees: Arnold *et al.*, 1985; Arnold *et al.*, 1988; Streinzer *et al.*, 2013; Roselino *et al.*, 2015; ants: Kleineidam *et al.*, 2005; Nishikawa *et al.*, 2008; Mysore *et al.*, 2009; Kelber *et al.*, 2009; Stieb *et al.*, 2011). The size of a glomerulus generally correlates with the number of OSNs terminating in this glomerulus, and a large number of OSNs is thought to enhance the detection sensitivity of its related compounds (Hansson *et al.*, 1995; Berg *et al.*, 1998; Bressel *et al.*, 2016). Thus the presence of hypertrophied glomeruli is generally an indication for the prevalence of some compounds in the biology of a given species. There is however a possible exception to this rule. We measured the relative volume of olfactory glomeruli across hornet species and found that T_B glomeruli are always smaller than glomeruli within the main AL. Following the rule stated above, this finding could be interpreted to mean that cuticular hydrocarbons detection is not a predominant function in the biology of hornets. It must be noted however that these low volatile chemicals are detected through direct antennal contact (Anton & Gnatzy, 1998; Ozaki *et al.*, 2005; Sharma *et al.*, 2015) or at very close range (< 1cm: Brandstaetter *et al.*, 2008). Thus, the detection of such compounds might not depend as much on the sensitivity of the system (i.e. the number of OSNs) as the detection at long range of volatile odorants. Therefore, concerning CHC detection, glomerular volume may not be the best criterion for measuring adaptations, and we believe that the number of functional units (glomeruli or expressed OR genes), as discussed above, may represent a more useful criterion. All

studied hornet species dedicated a high part of their AL (~29-36% of their functional units) to the T_B subsystem.

Worker macroglomeruli

For some odorants like volatile pheromones, glomerular volume is a relevant and well established measure of the system's sensitivity. The best known illustration of hypertrophied glomeruli are the macroglomeruli found in the male antennal lobe of numerous insects such as moths, cockroaches, honeybees and ants (Arnold *et al.*, 1985; Hanson *et al.*, 1992; Nishikawa *et al.*, 2008; Nishino *et al.*, 2012; Couto *et al.*, 2016). In these species, the male-specific macroglomeruli are related to the detection and processing of sex pheromones. Within Hymenoptera, macroglomeruli have also been found in females but their function is often unclear (Arnold *et al.*, 1988; Kleineidam *et al.*, 2005; Roselino *et al.*, 2015). The best described example is the macroglomerulus found in the large workers of some leaf cutting ants (*Atta* sp. and *Acromyrmex* sp.), which is involved in the processing of a trail pheromone component (Kelber *et al.*, 2009; Kuebler *et al.*, 2010). In the present study, we found a ventral glomerulus with widely differing volumes in the four hornet species (red triangles in **Fig. 2A-D**). Due to its highly-increased size in two species (*V. mandarinia* and *V. crabro*), it is considered as a worker macroglomerulus in these species following the use of a standard statistical threshold. This suggests a progressive size increase in some of these species and a putative adaptation for the detection of a particularly relevant compound in their biology.

At this stage, without functional data, we can only speculate about the possible function of such worker macroglomerulus. *V. crabro* and *V. mandarinia* are both typical cavity-nesting species which could use trail pheromone for advertising the nest entrance (Matsuura & Yamane, 1990; Steinmetz *et al.*, 2002). However, *V. orientalis,* which does not feature such a macroglomerulus, also nests within cavities (Plotkin *et al.*, 2010). Thus, except if one supposes different processing strategies in different hornet species (for instance, including a strategy based on a combinatorial code without the use of macroglomeruli, such as in the ant *Componotus floridanus,* Zube *et al.*, 2008), the nesting behavior of hornets cannot explain the presence of a macroglomerulus in some species and not in others.

An alternative hypothesis relates to pheromones involved in hornets' recruitment behavior. *V. mandarinia* performs *en masse* predation and is able to recruit nestmates to food sources by means of recruitment pheromones (Ono *et al.*, 1995; Ono *et al.*, 2003). However, *V. crabro,* which has a widely differing, mostly solitary, predation behavior also displays a macroglomerulus. Consequently, we are currently not able to provide a solid hypothesis based on present knowledge of these hornets' behavior. Further functional data aiming to find the receptive range of OSNs targeting the hypertrophied glomeruli in the two species might help understand its function.

Since the macroglomeruli were found at the same location in these species, we considered them as potentially homologous. We do not know however which olfactory receptor genes are expressed in the related OSNs. Treating them as possibly homologous glomeruli, we thereby supposed that orthologous (or closely related) olfactory receptors are expressed in these OSNs of the two species, and macroglomeruli being possibly involved in the same behavioral function. It is however possible that from the same olfactory receptor in a common ancestor, these olfactory receptor genes have drifted in the two species and now display differing olfactory receptive ranges. To clarify this point, future work should attempt to identify ligands for the ORs corresponding to these macroglomeruli, which could be performed using *in vivo* optical imaging of the antennal lobe. Indeed, since these macroglomeruli are placed on the ventral brain surface, they are relatively easy to access with optical techniques. Finally, our results suggest that the volume of glomeruli is a highly variable phenotypic trait in hornets. This suggests that selection pressures might strongly act on the number of OSN targeting a given glomerulus, finely tuning the detection sensitivity of the hornets' olfactory system to particular compounds.

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CHAPITRE 4

Olfactory attraction of the hornet *Vespa velutina* to honeybee colony odors and pheromones

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Abstract

Since the beginning of the last century, the number of biological invasions has continuously increased worldwide. Due to their environmental and economical consequences, invasive species are now a major concern. Social wasps are particularly efficient invaders because of their distinctive biology and behavior. Among them, the yellow-legged hornet, *Vespa velutina*, is a keen hunter of domestic honeybees. Its recent introduction to Europe may induce important beekeeping, pollination, and biodiversity problems. Hornets use olfactory cues for the long-range detection of food sources, in this case the location of honeybee colonies, but the exact nature of these cues remains unknown. Here, we studied the orientation behavior of *V. velutina* workers towards a range of hive products and protein sources, as well as towards prominent chemical substances emitted by these food sources. In a multiple choice test performed under controlled laboratory conditions, we found that hornets are strongly attracted to the odor of some hive products, especially pollen and honey. When testing specific compounds, the honeybee aggregation pheromone, geraniol, proved highly attractive. Pheromones produced by honeybee larvae or by the queen were also of interest to hornet workers, albeit to a lesser extent. Our results indicate that *V. velutina* workers are selectively attracted towards olfactory cues from hives (stored food, brood, and queen), which may signal a high prey density. This study opens new perspectives for understanding hornets' hunting behavior and paves the way for developing efficient trapping strategies against this invasive species.

Keywords: hornet, honeybee, olfactory cues, predation

Introduction

Since the beginning of the 20th century, as a result of globalization and the development of international exchanges, biological invasions have critically increased (Elton, 1958; Vitousek *et al.*, 1996; Vitousek *et al.*, 1997; Hochberg & Gotelli, 2005). Invasive species frequently disturb biodiversity by competing with indigenous species for habitat and food resources and/or by exerting a strong predation pressure on local species. Due to the ecological and economical problems that ensue from biological invasions, they have become a major concern worldwide (Park, 2004; Pimentel *et al.*, 2005). Introduced species do not however all become invasive and only ~1% actually lead to permanent invasions (Williamson & Fitter, 1996). Social insects, like social wasps, are particularly efficient invaders. They live in populous colonies organized according to highly efficient task allocation systems, which confers them outstanding abilities for exploiting local food resources and outcompeting local species (Moller, 1996). Social wasps gather food resources from the surroundings of the nest, mostly gathering carbohydrates and proteins (Raveret Richter, 2000). These generalist hunters typically find protein resources by preying on numerous insect species including many pollinators. Because of the current decline of pollinator populations (Brown & Paxton, 2009; Potts *et al.*, 2010) and of the severe ecological consequences of the introduction of such generalist hunters in new ecosystems (Snyder & Evans, 2006), acquiring a better knowledge of the predation behavior of social wasps has become a crucial endeavor.

Predation site selection by social wasps is strongly influenced by prey density (Stamp, 2001; Armstrong & Stamp, 2003), as a high number of preys at a particular location gives rise to conspicuous visual and olfactory cues. Social wasps are keen learners which are able to associate color or odor cues with food reward (Shafir, 1996; Mc Pheron & Mills, 2007). Thus, after several hunting bouts on a particular hunting site, these insects learn the cues that allow them to localize their prey (Raveret Richter, 2000; D'Adamo & Lozada, 2003; Toh & Okamura, 2003; Weiss *et al.*, 2004; Lozada & D'Adamo, 2006; Moreyra *et al.*, 2012). This high behavioral plasticity supported by excellent cognitive abilities has been proposed as another explanation for the invasive success of social wasps (Lozada & D'Adamo, 2009, 2011; D'Adamo & Lozada 2011).

The yellow-legged hornet, *Vespa velutina*, is an attractive species for studying hornets' hunting behavior. Recently, this hornet originating from China has been accidently introduced to new locations in Western Europe and in Korea (Jung *et al.*, 2008; Choi *et al.*, 2011; Monceau *et al.*, 2014a). In Europe, this species was first observed in 2004 (Haxaire *et al.*, 2006; Monceau *et al.*, 2014a). Like other wasps of the Vespinae subfamily, hornets prey on various arthropod species but also scavenge on dead vertebrate flesh (fish and meat) (Spradbery, 1973; Akre *et al.*, 1980; Edwards, 1980; Matsuura & Yamane, 1990; Raveret Richter, 2000). Above all, however, *V. velutina* hunts domestic honeybees, including the Asian honeybee, *Apis cerana* (in their natural range) (Li *et al.*, 2008) and

the European honeybee, *Apis mellifera* (both in their natural and introduction ranges) (Perrard *et al.*, 2009). Because of the strong and immediate impact of this hornet's hunting pressure on honeybee colonies, several studies have been dedicated to honeybees' defense behavior against it. These studies essentially showed that most European honeybees are not able to display efficient anti-predator behaviors contrary to Asian honeybees which coevolved with several bee-hunting hornet species (Ono *et al.*, 1995; Ken *et al.*, 2005; Papachristoforou *et al.*, 2007; Tan *et al.*, 2007; Tan *et al.*, 2010; Tan *et al.*, 2012; Tan *et al.*, 2013; Arca *et al.*, 2014). In invaded areas, hornets' feeding sites are thus primarily apiaries, because they present an abundant and defenseless prey source. However, little is known about the hunting behavior of *V. velutina* (Monceau *et al.*, 2013a; Monceau *et al.*, 2014b) and how they locate and choose these feeding sites.

At the peak of predation, as many as 20 hornets can be seen hovering simultaneously in front of a given hive, catching honeybee foragers on the fly. This hunting strategy strongly impairs the foraging efficiency of bees (Monceau *et al.*, 2013a). Two mechanisms may be involved in such high numbers of preying hornets. First, *V. velutina* workers could learn the characteristics and/or location of feeding sites, as the same foragers can be seen several times hunting on the same apiary (Monceau *et al.*, 2014b). Second, like other Vespine wasps, hornets may recruit nestmates to feeding sites (Ono *et al.*, 1995; D'Adamo *et al.*, 2004). In any case, honeybee colonies are especially attractive to hornets, but the exact cues they may use to locate honeybee colonies in the first place remain unclear. Recently, a laboratory study showed that *V. velutina* workers can use both visual and olfactory cues to locate bees (Wang *et al.*, 2014). In the wild, visual cues are critical at short-range for catching flying honeybee foragers, while olfactory cues may be more relevant for long-range attraction to honeybees hives. To this day such odorants remain unknown despite their considerable interest for understanding *V. velutina* foraging tactic.

In theory, predators can take advantage of different categories of olfactory cues (Vet & Dicke, 1992). The most immediate and accurate way to locate a prey is to use olfactory cues emanating directly from the prey (kairomones) (Hendrichs *et al.*, 1994; Day & Jeanne, 2001; Brodmann *et al.*, 2009). However, if such odorants are not sufficient for guiding the predator, other strategies using indirect olfactory cues which emanate from the habitat or from the food of the prey have been reported (Turlings *et al.*, 1990; Mattiacci *et al.*, 1995; Paré & Tumlinson, 1999; Hilker *et al.*, 2002). Hence, hornets may be attracted to volatiles emitted by honeybees, to volatiles emitted by the bee hives as well as to odorants emitted by the flesh they sometimes scavenge on.

In the present study, we tested the attraction behavior of *V. velutina* workers to olfactory stimuli in a multiple choice test in controlled conditions. We first asked which elements (hive products, honeybees or fish or meat samples) may attract hornets. Then, we tested hornets' relative attraction to a range of different molecules that are typical honeybee- or flesh-emitted volatiles. In

particular, we tested a number of known honeybee pheromone that may act as kairomones for hornets.

Materials and Methods

Animal collection and caging procedure

Vespa velutina workers preying on honeybee hives were individually caught with a butterfly net between August and November 2013, at the experimental apiary of INRA Bordeaux-Aquitaine, France (GPS: N44°47'27.05" W0°34'38.35"). Animals were then brought to a dark and temperature-controlled (22°C) experimental room in the laboratory containing a cubic cage (60×60×60 cm), described in (Monceau *et al.*, 2013b). Groups of 10 hornets were transferred to the cage and were allowed to move freely for 3 hours during a period of acclimatization prior to the experiments. The base of the cage contained a mobile drawer with a stainless steel plate allowing to securely deliver stimuli into the cage (**Fig. 1A**).

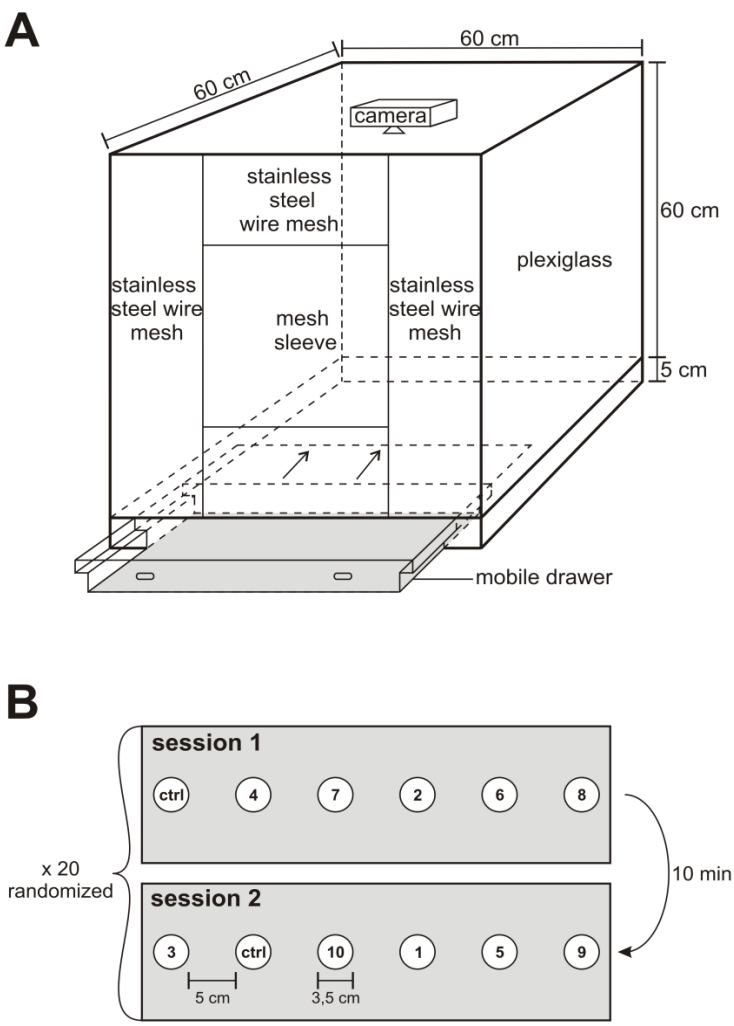

Figure 1: Experimental setup
A) Schematic drawing of the cubic wooden cage (60×60×60 cm) used for the experiments. The front part was equipped with a mesh sleeve for introducing hornets into the cage. The base contained a 5 cm deep mobile drawer (in grey) and a stainless steel plate used to securely deliver tested stimuli. Inside the cage and attached to the roof, a digital camcorder allowed the recording of hornets' behavior. **B**) Experimental procedure used in the first experiment. Each of the 20 replications was divided in 2 consecutive sessions separated by a 10 minute interval. Within a session, 5 stimuli among the 10 tested (numbered circles) were placed 5 cm apart in the mobile drawer, and delivered together with a control (ctrl circle). The sequence of stimuli presentation and exact location in the drawer were fully randomized. The second experiment was similarly designed but contained 4 sessions of 5 stimuli among the 20 tested.

A digital camcorder (HD pro webcam C920; Logitech, Lausanne, Switzerland) was placed inside the cage and attached to its roof to record *V. velutina* behavior during the tests. The cage was

illuminated with red light by means of an incandescent light bulb. Red light allowed filming the hornets while precluding their use of visual cues during the experiments, since, as shown for the closely related species *V. crabro*, hornets lack red-light sensitive photoreceptors (Peitsch *et al.*, 1992). The Virtualdub software (http://www.virtualdub.org) was used for recording high quality video at a frame rate of 22 fps.

Experiment 1

In order to screen attractive food sources or hive products, 10 different stimuli were used. For all stimuli, the same mass of substance was presented, i.e. 80 mg±2 mg, as it equates to the average weight of a honeybee worker. The stimuli were:

- *Honey*: organic raw mixed-flower honey (not warmed), obtained from a local beekeeper
- *Pollen*: organic, bee-collected, fresh pollen (not dried), obtained from a local beekeeper
- *Wax*: new honeybee comb foundation wax obtained from a local beekeeping shop
- *Propolis*: purified in ethanol (ethanol was evaporated after filtration in liquid phase)
- Bee boost: commercial synthetic honeybee queen mandibular pheromone (QMP) on a plastic strip (Contech, Victoria, BC, Canada). QMP is a blend of five constituents, 9-oxo-(E)-2-decenoic acid (9-ODA), two enantiomers of its biosynthetic precursor, (R)- and (S)-9-hydroxy-(E)-2-decenoic acid (9-HDA) and two other compounds, methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) (Slessor *et al.*, 1988).
- *Honeybee*: worker honeybee taken from a hornet-predated hive
- *Larva*: honeybee larva taken from a hornet-predated hive
- *Meat*: minced beef meat, obtained from a local supermarket
- *Fish*: salmon flesh, obtained from a local supermarket
- *Wet paper*: white cellulose wadding moisturized with distilled water

Stimuli were individually placed in Ø 35 mm Petri dishes (Petri dishes, EL46.1; Roth, Lauterbourg, France) and delivered into the cage using the mobile drawer. Six Petri dishes were placed at a time in the drawer, and were spaced 5 cm apart. Hornets were subjected to 2 consecutive sessions separated by a 10 minute interval (**Fig. 1B**). Each session presented 5 of the 10 stimuli plus a joint control (empty Petri dish). The order of presentation of the stimuli (session 1 or 2), as well as their location in the drawer, were fully randomized using Research Randomizer (http://www.randomizer.org). Before each session, the mobile drawer was thoroughly washed with ethanol to remove any stimulus residues. Following introduction of the Petri dishes, hornets' behavior in the drawer area was recorded for 15 minutes with the digital camcorder. This experiment was repeated 20 times (20×2 sessions) with different groups of 10 hornets. New stimuli were used for each test.

Experiment 2

Food odors are complex blends of odorant molecules present in variable ratios. To attempt to isolate attractive odorants for hornets, we selected 20 volatile compounds (Sigma-Aldrich, Steinheim, Germany) based on their occurrence at high concentration in the different food sources of *V. velutina*. **Table 1** shows the list of tested compounds. For commodity, the tested stimuli were categorized in 3 main groups (honeybee, seafood and meat odors), although each stimulus can also be emitted by a variety of other sources.

Chemical name	CAS	MW ($g.mol^{-1}$)	Purity	Sources	References
isopentyl acetate	123-92-2	130.18	99%	**bee alarm pheromone**	[1; 2]
2-heptanone	110-43-0	114.18	99%	**bee alarm pheromone**	[1; 3; 4]
geraniol	106-24-1	154.24	98%	**bee aggregation pheromone**	[5; 1; 6]
citral	5392-40-5	152.23	95%	**bee aggregation pheromone**	[7; 8]
homovanillyl alcohol	2380-78-1	168.18	99%	**bee queen pheromone**	[9; 10]
10-hydroxydecanoic acid	1679-53-4	188.26	99%	**bee queen pheromone**	[11; 12]
methyl 4-hydroxybenzoate	99-76-3	152.14	99%	**bee queen pheromone**	[13]
methyl palmitate	112-39-0	270.45	99%	**bee brood pheromone**	[14]
ethyl oleate	111-62-6	310.51	98%	**bee brood pheromone**	[15]
β-ocimene (E, Z mixture)	13877-91-3	136.23	90%	**bee brood pheromone**	[16]
heptanal	111-71-7	114.18	95%	**seafood** / plants / meat	[17; 18; 19; 20]
(E)-2-nonenal	18829-56-6	140.22	95%	**seafood** / aged beer	[17; 18; 21; 22]
(E,E)-2,4-octadienal	5577-44-6	124.18	95%	**seafood**	[17; 23]
(E,Z)-2,6-nonadienal	557-48-2	138.20	96%	**seafood** / meat / plants	[17; 18; 20; 21; 24; 25]
p-xylene	106-42-3	106.16	99%	**seafood** / meat / plants	[17; 20; 21; 26]
1-octen-3ol	3391-86-4	128.21	98%	**meat** / honey	[17; 18; 20; 21; 27; 28]
benzaldehyde	100-52-7	106.12	98%	**meat** / plant	[19; 20; 27; 29; 30]
octanoic acid	124-07-2	144.21	98%	**meat** / plant	[19; 30]
4-ethyloctanoic acid	16493-80-4	172.26	98%	**meat**	[31]
(E,E)-2,4-decadienal	25152-84-5	152.23	89%	**meat** / plant /seafood	[17; 18; 24; 27; 29; 32]

Table 1: List of molecules tested in Experiment 2
The table presents the chemical names of the 20 molecules tested in Experiment 2. CAS number, molecular weight and purity are indicated, respectively, in the second, third and fourth column. The fifth column shows a non exhaustive list of sources that emit these molecules, as mentioned in the literature cited in the last column. 1, Free, 1987; 2, Boch & Shearer, 1962a; 3, Shearer & Boch, 1965; 4, Collins & Blum, 1982; 5, Boch & Shearer, 1962b; 6, Slessor *et al.*, 2005; 7, Winston, 1987; 8, Schmidt, 1994; 9, Slessor *et al.*, 1988; 10, Keeling *et al.*, 2003; 11, Brockmann *et al.*, 2006; 12, Le Conte & Hefetz, 2008; 13, Pankiw *et al.*, 2000; 14, Mohammedi *et al.*, 1996; 15, Le Conte *et al.*, 1990; 16, Maisonnasse *et al.*, 2010; 17, Varlet *et al.*, 2006; 18, Selli *et al.*, 2006; 19, Formisano *et al.*, 2009; 20, Vasta *et al.*, 2012; 21, Piveteau *et al.*, 2000; 22, Santos *et al.*, 2003; 23, D'Ippolito *et al.*, 2003; 24, Faraji & Lindsay, 2004; 25, Zhang *et al.*, 2009; 26, Grigorakis *et al.*, 2003; 27, Vasta *et al.*, 2007; 28, Agila & Barringer, 2013; 29, Janes *et al.*, 2009; 30, Acevedo, 2011; 31, Rota & Schieberle, 2005; 32, Christlbauer & Schieberle, 2011.

For each odorant, a filter paper piece (~1 cm^2; Whatman, Maidstone, England) was soaked with 5 µL of the odor solution and placed in a Ø 35 mm Petri dish. Liquid substances were presented pure, while odorants in solid form at room temperature (homovanillyl alcohol, 10-hydroxydecanoic acid, methyl 4-hydroxybenzoate, methyl palmitate and ethyl oleate) were dissolved in 2-propanol

and presented at a dose of 50 µg/µL. 2-propanol was allowed to evaporate for 1 min before presenting the stimuli. As in experiment 1, 5 stimuli and a control (1 cm^2 filter paper without a stimulus) were delivered at a time into the cage using the mobile drawer. The 20 stimuli were thus delivered in 4 consecutive sessions lasting 15 minutes each and separated by a 10 minute interval. To remove residual odors before each session, the drawer was thoroughly washed with ethanol. Sequence of stimulus presentation and location in the drawer were randomized as above. The experiment was repeated 20 times (20×4 sessions) on different groups of 10 hornets. New stimuli were used for each test.

Videos analysis

Preliminary observations of hornet's behavior showed that in Experiment 1, some food sources could be consumed within 10 minutes of the presentation of the stimuli. Thus, in this experiment, hornet behavior was only analyzed during the first 10 minutes of each recording. In Experiment 2, none of the odorant sources were consumed, so hornet behavior was analyzed during the full 15 min of the experiment. Videos were visualized using Movie Maker V. 2011 software (Microsoft Corporation, Redmond, USA) allowing us to precisely record the number of visits and the amount of time hornets spent on each stimulus. At each separate occasion a hornet entered a Petri dish (at least with its entire head), times of entry and of exit were recorded. Hornet attraction to the different stimuli was measured according to two variables: 1) *Visit number*: the total number of hornet visits during each session; and 2) *Total visit duration*: the sum of all individual visit durations performed by hornets during this session.

Statistics

A Friedman test was used to compare visit frequencies and durations on the control stimuli during different sessions. To evaluate the attractiveness of the different stimuli, we compared the number of visits to each test stimulus with its joint control (i.e. with all control stimuli presented in the same sessions), using a Wilcoxon paired sample test. Likewise, the amount of time that hornets spent on each stimulus was compared to its joint control with a Wilcoxon paired sample test. As multiple comparisons were carried out with the same control (always 5 stimuli with 1 control), we used a Bonferroni correction to reduce type I errors ($\alpha_{corrected} = 0.05/5 = 0.01$). A p value below $\alpha_{corrected}$ was considered as statistically significant, while a value between $\alpha_{corrected}$ and the standard 0.05 threshold was only regarded as near-significant (but potentially biologically relevant). Significance levels are presented in the figures as follows: ***: $p<0.0001$; **: $p<0.001$; *: $p<0.01$; (*) $0.01<p<0.05$).

Results

The behavior of 400 hornets was observed in two experiments aiming to test their olfactory attraction towards food sources (Experiment 1) or individual odorants (Experiment 2).

Experiment 1

In the first experiment, *V. velutina* workers were presented with several food sources and different elements of a honeybee colony. The hornets could explore the drawer at the base of the cage, containing 6 Petri dishes presenting 5 stimuli and a control. Because hornets were free to move around the cage, visits to the stimuli may occasionally occur through random exploration. Visits to control stimuli therefore allow us to evaluate the intensity of hornets' exploration behavior. Stimulus exploration was similar between the first and second session, as quantified through the number of visits to the control (Wilcoxon test, $Z = 1.24$, $p = 0.21$, NS).

Visits to the control stimuli presented concomitantly with each stimulus were highly similar (**Fig. 2A**; median visit number between 2.5 and 3.0) and indicated that exploration behavior was homogenous in the whole experiment (Friedman test, $Chi^2 = 6.40$, $p = 0.70$, NS, 9 df). When evaluating the attractiveness of the different stimuli, we observed that hornets visited *honey* and *pollen* significantly more often than the control stimulus (Wilcoxon test, $Z = 3.33$, $p<0.001$ and $Z = 3.57$, $p<0.001$). Although hornets showed no significant preference for other stimuli, some showed near-significant attraction ($\alpha_{corrected} = 0.01<p<0.05$): *propolis* (Wilcoxon test, $Z = 2.37$, $p = 0.017$), *bee boost* (Wilcoxon test, $Z = 2.48$, $p = 0.013$) and *honeybee* (Wilcoxon test, $Z = 2.07$, $p = 0.038$).

Experiment 2

In the second experiment, we asked which food odorants or honeybee pheromones may attract *V. velutina* (**Table 1**). As in experiment 1, hornets explored the newly accessible drawer and visited the stimuli (**Fig. 3A**). When comparing visits to the control stimuli, we did not find any significant variation among the four sessions, although a trend for increased exploration between sessions 1 and 4 appeared (Friedman test, $Chi^2 = 7.77$, $p = 0.051$, NS, 3 df). In any case, full randomization of stimulus sequence presentation allowed avoiding any influence of exploration changes on the results. Visits to control stimuli presented concomitantly with each stimulus were homogeneous (**Fig. 3A**, Median visit number between 3.0 and 5.0, Friedman test, $Chi^2 = 14.27$, $p = 0.077$, NS, 19 df).

When evaluating the attractiveness of single odorant stimuli, we observed more visits on *geraniol* and *p-xylene* than on the control (Wilcoxon test, $Z = 3.64$, $p<0.001$ and $Z = 3.07$, $p<0.01$). Although hornets showed no significant preference for other stimuli, *homovanillyl alcohol* (Wilcoxon test, $Z = 2.22$, $p = 0.026$) and *β-ocimene* (Wilcoxon test, $Z = 2.12$, $p = 0.034$) showed near-significant attraction ($\alpha_{corrected} <p<0.05$).

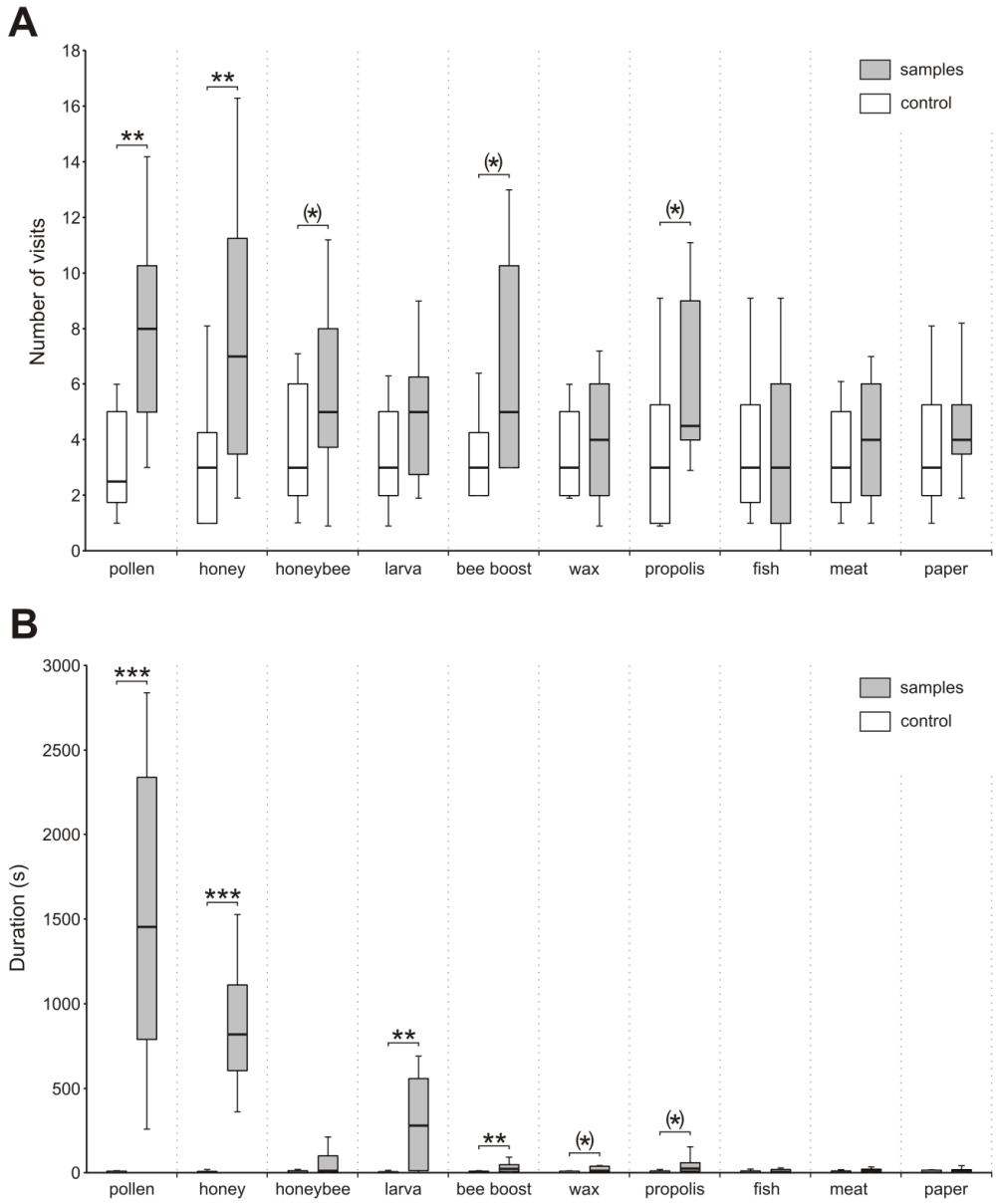

Figure 2: Hornet attraction towards hive products and other food samples
A) Distribution of the number of visits on each sample for groups of 10 hornets. Bold lines show the median number of visits per session (20 replications, N = 200 hornets). The boxes represent the first and third quartiles (25–75%), while whiskers represent the distribution from 10 to 90%. The thick vertical dashed lines separate the different sources emitting these molecules (from left to right: honeybee, fish, meat). **B**) Distribution of the cumulative duration that groups of 10 hornets spent on each sample. Bold lines show the median duration by session (20 replications, N = 200 hornets). Grey bars: samples; white bars: control. Comparison between samples and control were done with Wilcoxon tests (***: p<0.0001; **: p<0.001; *: p<0.01; (*) 0.01<p<0.05).

Consistently with visit numbers, visit duration measurements showed that hornets spent more time on *geraniol* and *p-xylene* (**Fig. 3B**) than on the control (Wilcoxon test, Z = 3.81, p<0.001 and Z = 3.92, p<0.0001). Furthermore, hornets also spent longer periods of time on*homovanillyl alcohol* (Wilcoxon test, Z = 2.69, p<0.01), *methyl 4-hydroxybenzoate* (Wilcoxon test, Z = 2.87, p<0.01) and *β-ocimene* (Wilcoxon test, Z = 2.95, p<0.01) than on the control. Lastly, the period of time hornets spent visiting *4-ethyloctanoic acid* (Wilcoxon test, Z = 2.54, p = 0.011) and *isopentyl acetate* (Wilcoxon test, Z = 2.13, p = 0.033) was near-significantly longer than on the control ($\alpha_{corrected}$ <p<0.05).

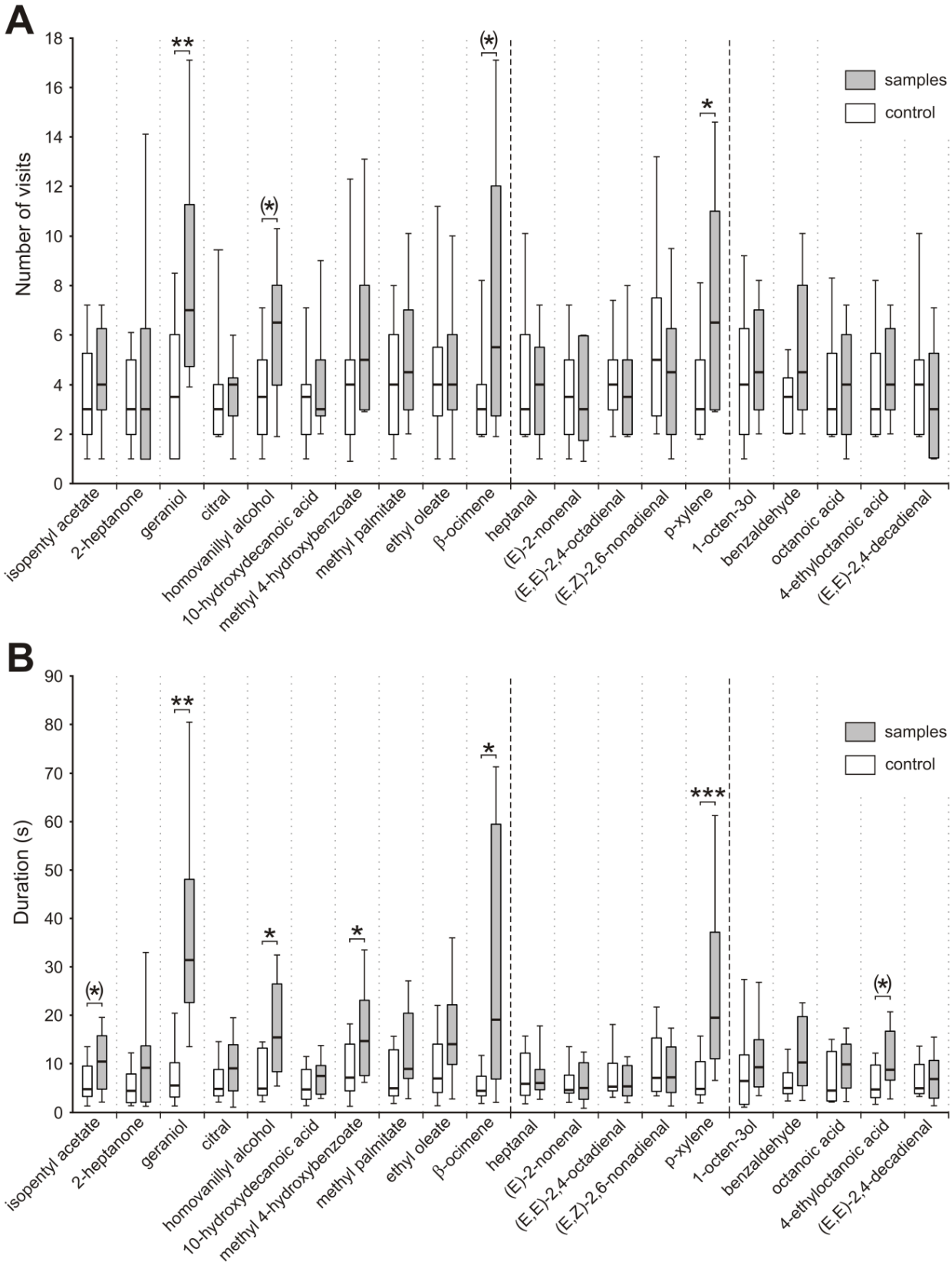

Figure 3: Hornet attraction towards chemical substances

A) Distribution of the number of visits on each stimulus exhibited by groups of 10 hornets. Bold lines show the median number of visits per session (20 replications, N = 200 hornets). The boxes represent the first and third quartiles, while whiskers represent the distribution from 10 to 90%. **B**) Distribution of the cumulative duration that groups of 10 hornets spent on each stimulus. Bold lines show the median duration by session (20 replications, N = 200 hornets). Grey bars: stimuli; white bars: control. Comparison between odor stimuli and control were done with Wilcoxon tests (***: $p<0.0001$; **: $p<0.001$; *: $p<0.01$; (*) $0.01<p<0.05$).

Discussion

Using a controlled laboratory assay to measure hornets' olfactory attraction, we found that they are primarily attracted to (and spent more time on) two products from honeybee hives, *pollen* and *honey*. By contrast, the *honeybees* themselves were only near-significantly attractive. Among individual odorants, however, one honeybee aggregation pheromone, *geraniol*, induced significant attraction and longer visit durations than the control. Our experiments also allowed observing a few more subtle effects. Honeybee *larva* and *bee boost* induced longer visit durations than the control, and similar results were observed when presenting some of their compounds alone such as *β-ocimene* (larva pheromone), *homovanillyl alcohol* (HVA) or *methyl-4-hydroxybenzoate* (HOB) (queen pheromones present in the bee boost). Stimuli associated with protein food sources, such as fish and meat samples or typical compounds from these protein sources did not induce any particular attraction, with the exception of one compound, *p-xylene*. Thus some stimuli were attractive: *honey* and *pollen* in experiment 1 and *geraniol* and *p-xylene* in experiment 2 whereas some stimuli only induced longer visit durations: *larvae* and *bee boost* in experiment 1 and *homovanillyl alcohol* and *methyl 4-hydroxybenzoate* in experiment 2.

Removal of potential visual cues

Hornets are known to use both visual and olfactory cues to locate food sources (Wang *et al.*, 2014). In the present study, we aimed to compare the attractiveness of a range of olfactory cues without any influence from visual information provided by the samples or the experimental cage. Because of the need to film the hornets, our assays were conducted under low levels of red light. Intracellular recordings of photoreceptors have shown that the visible spectrum of hornets (in this case *V. crabro*) starts around 300 nm and quickly decreases around 600 nm (Peitsch *et al.*, 1992) due to a lack of red photoreceptors as in many hymenopterans (Briscoe & Chittka, 2001). Thus, in this context, visual cues were, if not totally absent, at least very strongly reduced. In addition, to avoid any potential bias of each samples' location with respect to the red light, sample positions were placed randomly at each session. We are therefore confident that hornets' orientation behavior in our experiments was influenced by olfactory cues and not by visual cues.

The prey itself or its products?

As many hornet species, and especially *V. velutina*, are keen honeybee hunters (Ono *et al.*, 1995; Papachristoforou *et al.*, 2007; Tan *et al.*, 2007; Li *et al.*, 2008) and the hornet workers we tested were caught while hunting on honeybee hives, we expected them to be primarily attracted to and to feed on their prey. This was not the case as the honeybee samples were only mildly attractive (**Fig. 2A**) and hornets did not remain on them when encountering them (**Fig. 2B**). By contrast, honeybee products, *pollen* and *honey*, were highly attractive (**Fig. 2A**). They strongly attracted

hornets, sometimes even in groups and the hornets stopped for long periods of time on these samples, eventually feeding on them (**Fig. 2B**). Previous studies showed that the foraging behavior of some parasitic wasps (Turlings *et al.*, 1990; Paré & Tumlinson, 1999) and some social wasps (Cornelius, 1993; McPheron & Mills, 2007), are influenced by cues associated with their prey but not emanating directly from the prey. For instance, the social wasp *Mischocyttarus flavitarsis* uses olfactory cues originating from a plant damaged by the feeding activity of its prey (McPheron & Mills, 2007). One important activity of honeybees during summer is to store high quantities of food within their colony and such stores may become a conspicuous signal for the predator. Taking advantage of cues emitted by hive stores could be more efficient for the hornet than searching for individual flying honeybees. We therefore hypothesize that *pollen* and *honey* odors emanating from the hives' food stores represent prominent distant cues helping *V. velutina* foragers to find their prey.

Hornets gather proteins to feed their larvae but also need carbohydrates (and possibly proteins) for their own needs. As V. velutina workers were sometimes seen feeding on *pollen* and *honey* in our experiment (**Fig. 2B**), we cannot exclude the possibility that the long time hornets spent on these stimuli may be related to their nutritional quality. However, the significant numbers of visits recorded to these stimuli demonstrate their olfactory attractiveness for hornets. In addition, during hunting, *V. velutina* workers usually hover at the hive entrance and catch flying honeybee foragers, some of which are loaded with pollen and/or nectar (Monceau *et al.*, 2013a). Hornets have never been observed feeding directly on pollen pellets from caught workers. Therefore, we rather favor the idea that hornets' attraction to pollen and honey odors was related to a potential role of these odors as long-distance signals for the presence of hives.

Could honeybee pheromones be used as kairomonal signals?

There is increasing literature reporting how prey pheromone can be counterused as a kairomonal signal by predators or parasitoids (Vet & Dicke, 1992; Hendrichs *et al.*, 1994; Day & Jeanne, 2001; Brodmann *et al.*, 2009). In addition to the observation that hive products strongly attract *V. velutina*, our experiments revealed that hornets can also be attracted to some honeybee pheromones. The only other honeybee-related stimulus that significantly attracted hornets was the honeybee worker aggregation pheromone component, *geraniol*. Hornets chose to orient towards *geraniol* (**Fig. 3A**) and remained on this stimulus for long periods of time (**Fig. 3B**). Geraniol is a Nasanov gland secretion, which acts as an aggregation pheromone, attracting honeybee workers during swarming or at the colony entrance (Boch & Shearer, 1962b; Free, 1987; Slessor *et al.*, 2005). Because of its role in aggregating honeybees, geraniol may represent an honest signal for hornets, indicating high densities of honeybee workers. It may thus work as a kairomone, possibly together with hive products to help hornets finding honeybee colonies.

The other bee pheromone constituents tested in our work (**Table 1**) did not induce any significant increase in the number of visits, but some (*β-ocimene*, *homovanillyl alcohol* and*methyl-4-hydroxybenzoate*) were found to act as arrestants (**Fig. 3B**). Indeed, in this experiment, we noticed that visit duration increased when hornets exhibited typical arrestment responses (Kennedy, 1978) with klinotaxis behavior around the stimuli. *β-ocimene*, a volatile pheromone produced by honeybee larvae (Maisonnasse *et al.*, 2010) showed only near-significant attraction but significant arrestment (**Fig. 3A, B**). Consistently, *larvae*, when presented in the first experiment, did not induce any significant attraction but induced longer visit durations once hornets had found them (**Fig. 2A, B**). These results suggest the existence of arrestment responses by hornets towards*β-ocimene*, which could also act as a kairomone, giving relevant cues for locating larvae.

A similar situation was observed for queen pheromones. While in the first experiment, the *bee boost*, an artificial blend of 5 components composing the honeybee Queen Mandibular Pheromones (QMP) showed near-significant attraction and significantly longer durations, two QMP components, *homovanillyl alcohol* (HVA) and *methyl-4-hydroxybenzoate* (HOB), showed the same pattern in experiment 2, with a significant arrestment to both stimuli. Here again, some honeybee queen pheromones may act as kairomones for hornets.

Such a result is consistent with previous studies on another hornet species, *V. bicolor*, which was shown to be attracted to (Z)-11-eicosen-1-ol, a major component in the alarm pheromone of both Asian and European honeybees (Brodmann *et al.*, 2009). Another prominent example is found in the wasp*Vespula germanica* which uses the pheromone produced by sexually active Mediterranean fruit fly males as olfactory cues to locate its prey (Hendrichs *et al.*, 1994). Taken together, our results may indicate that hornets use a combination of honeybee-related odorants to locate hives from a distance (attraction to *honey*, *pollen* or *geraniol*). In addition, when a honeybee colony is weak, hornets sometimes enter the hive to plunder its resources. When in the hive, they may use particular honeybee pheromones as suggested by the significant arrestment observed to larvae, worker and queen pheromones.

Lack of attraction to generic animal protein sources

Vespine wasps are generalist foragers that hunt arthropod preys but also collect meat from vertebrates to feed their larvae (Edwards, 1980; Matsuura & Yamane, 1990). They are notorious pests in butchers' and fishmongers' stalls where they cut small pieces of meat and fish and fly away (Spradbery, 1973; Raveret Richter, 2000). In this study, unlike hive product, neither *meat* nor *fish* attracted hornets significantly (**Fig. 2A**). As also shown by the low time spent on these stimuli (**Fig. 2B**), we did not observe any hornets feeding on *meat* or *fish* samples. Thus, contrary to our expectations, hornets fed on only two stimuli considered as protein sources, mainly *pollen* and to a lesser extend *larva*, but neglected other animal proteins. Although carbonyl

compounds resulting from lipid oxidation contribute strongly to the typical fishy odor (Refsgaard *et al.*, 1999; Varlet *et al.*, 2006), hornets exhibited significant attraction and arrestment responses only towards one fishy odor, *p-xylene* (**Fig. 3A, B**). This compound can be a product of carotenoid degradation in fish flesh and has been reported as an active component in many seafood products (Grigorakis *et al.*, 2003; Varlet *et al.*, 2006; Piveteau *et al.*, 2000). But, since *fish* flesh was not attractive in our experiment, the reason why *p-xylene* was of interest to *V. velutina* remains unclear. This chemical may also emanate from many different sources such as some fruit trees and some orchids (Borg-Karlson *et al.*, 1985; Baraldi *et al.*, 1999).

Recruitement and learning as potential mechanisms

Hornets' attraction to particular stimuli in our experimental setup may be the product of both innate attraction and appetitive learning processes. Social insects are able to recruit nestmates to valuable food sources through active communication. For instance, honeybees can actively promote food sources to nestmates with a sophisticated dance (Von Frisch, 1967; Dyer, 2002). Several foraging recruitment forms by means of scent marks have been reported in Vespoidea (Ono *et al.*, 1995; D'Adamo *et al.*, 2004; David Morgan, 2009). When the predation activity of *V. velutina* reaches its peak, up to 20 individuals can hunt on a single hive at the same time (Monceau *et al.*, 2013a). Therefore, the high number of hornets present at the hive entrance might be due to recruitment by conspecifics. Some of our hornets may have been caught during their first visits to the location and may have displayed innate attraction to the stimuli. However, taking into account the high learning abilities of Vespine wasps (Shafir, 1996; Toh & Okamura, 2003; Lozada & D'Adamo, 2006; McPheron & Mills, 2007), tested individuals may also have learned hive cues in the course of preying visits to the hive. Indeed in a previous study the same individuals were observed several times hunting at the same site (Monceau *et al.*, 2014b). In the framework of associative learning, hive and honeybee odors may correspond to conditioned stimuli (CS) while the successful catch of a prey may act as reinforcement. If so, then our experiments reflect the attraction of honeybee-specialist hunters, which would explain the lack of attraction to meat/fish stimuli. Similar experiments comparing the behavior of *V. velutina* workers caught on different food sources should clarify how foraging specialization influences olfactory attraction in hornets.

Outlook

In our experiments, *V. velutina* hornets mainly exhibited interest to hive products and to honeybee emitted pheromones but not to honeybees themselves. All these attractive stimuli seem to share a common feature: they might signal not only the presence of honeybees, but their presence at a high density. Interestingly, *pollen* was strongly attractive and the prey spectrum of *V. velutina* includes a large variety of pollinators such as social Hymenoptera and hoverflies (Rome *et*

al., 2011). Preliminary trapping experiments in the field gave promising results for using *pollen's* attractiveness for protecting hives from hornet aggression. Such field experiments will now be performed on a larger scale, testing the trapping properties of the stimuli identified in the present study, alone or in combination.

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DISCUSSION

Depuis son introduction dans le Sud-ouest de la France vers 2004, le frelon *Vespa velutina* a su coloniser une grande partie du territoire français et une portion des pays frontaliers (Espagne, Belgique, Italie). Cette progression rapide suggère que cette espèce est bien adaptée aux écosystèmes Ouest-Européens où il y trouve d'abondantes sources de nourriture. Les premières observations de *V. velutina* ont rapidement relevé un comportement de prédation particulièrement efficace contre les insectes pollinisateurs, et en particulier contre les colonies d'abeilles domestiques. Son expansion a donc fait craindre de lourdes conséquences aussi bien sur la biodiversité locale que sur les activités humaines (apiculture). Puisque l'olfaction joue un rôle central dans la biologie et le comportement des insectes, et notamment dans celui des Hyménoptères sociaux, cette thèse avait pour objectif d'étudier le comportement et le système olfactif du frelon *V. velutina* afin de pouvoir proposer des stratégies de lutte, spécifiques et durables, contre cette espèce invasive.

Les données disponibles sur ce sujet étant datées et lacunaires, il convenait tout d'abord de s'intéresser à l'organisation centrale du système olfactif afin d'établir une cartographie des structures nerveuses impliquées dans le traitement de l'information olfactive. Dans le **premier chapitre** nous avons étudié la neuroanatomie des voies olfactives chez les différentes castes qui composent une colonie de frelon. Nous avons ainsi pu montrer que le lobe antennaire des fondatrices et des ouvrières est strictement identique alors que les mâles présentent des adaptations, vraisemblablement en relation avec une communication pheromonale complexe en ce qui concerne le comportement de reproduction. Ce travail nous a également permis de mettre en évidence des similarités étonnantes dans l'organisation du lobe antennaire de différentes familles d'Hyménoptères sociaux. Notamment, des grappes de glomérules formées par les subdivisions des voies nerveuses entrant et sortant du lobe antennaire, présentent de fortes homologies structurales entre l'abeille *Apis mellifera*, les fourmis charpentières, *Camponotus sp*. et le frelon *V. velutina*. Ces résultats suggèrent que des groupes de glomérules, possiblement liés à des fonctions particulières, auraient été conservés au cours de l'évolution des Hyménoptères.

Dans le **deuxième chapitre** nous avons cherché à vérifier que les similarités morphologiques observées entre ces espèces n'étaient pas simplement le résultat de contraintes développementales mais correspondaient, de fait, à des homologies évolutives. Des études récentes chez les fourmis ont mis au jour des singularités neuroanatomiques d'une grappe de glomérules qui serait associée aux sensilles de type basiconique et serait impliquée dans la reconnaissance intra- et interspécifique (Ozaki *et al.*, 2005 ; Nishikawa *et al.*, 2012 ; Sharma *et al.*, 2015). Nous avons donc inspecté l'équipement sensillaire de *V. velutina* à la recherche de sensilles basiconiques. Nous avons ensuite remonté la voie neuronale partant des sensilles basiconiques des frelons et avons pu montrer l'existence des mêmes singularités structurales et fonctionnelles que chez les fourmis. Ces observations démontrent l'existence d'une homologie au sein des lobes antennaires des fourmis et du frelon *V. velutina*.

Nos recherches s'étant limitées jusque là à l'étude du système olfactif de *V. velutina*, nous tenions à évaluer la variabilité de l'organisation du lobe antennaire des frelons et les possibles adaptations liées à des différences de comportement de prédation des ouvrières de différentes espèces. Dans le **troisième chapitre**, nous avons donc comparé le lobe antennaire de 4 espèces du genre *Vespa* (*V. crabro*, *V. velutina*, *V. orientalis* et *V. mandarinia*) et montré que malgré des différences concernant le nombre de glomérules, le lobe antennaire de ces espèces semble être organisé suivant un schéma commun. Nous avons également observé une hypertrophie différentielle d'un glomérule de la face ventrale du lobe antennaire chez les différentes espèces. Cette hypertrophie glomérulaire qui pourrait être liée au traitement de phéromones de recrutement, n'est remarquable que chez les ouvrières des espèces *V. crabro* et *V. mandarinia*, mais n'est pas significative chez *V. velutina.*

Le système olfactif des ouvrières de l'espèce *V. velutina* ne semblant pas porter d'adaptation en relation avec leur comportement de prédation coopératif, nous avons donc cherché à comprendre quels peuvent être les signaux olfactifs responsables de la forte pression de prédation qu'elles exercent sur les colonies d'abeilles. Dans le **quatrième chapitre**, nous avons effectué des tests comportementaux et montré que les ouvrières de l'espèce *V. velutina* sont principalement attirées par des signaux olfactifs émis par les ruches (stocks de nourriture, phéromone royale, phéromones de couvain et d'agrégation). L'attraction sélective des ouvrières envers ces signaux souligne une stratégie de prédation efficace pour trouver des proies présentes en forte densité. La compréhension de la stratégie de prédation de *V. velutina,* ainsi que des signaux olfactifs impliqués, pourrait conduire à la mise en œuvre de méthodes de lutte pour perturber le comportement des ouvrières.

Dans la section suivante, nous apporterons un éclairage plus général sur ces travaux que celui qui a été développé au sein de chaque chapitre. Dans un premier temps nous intégrerons les résultats des différents chapitres afin de mettre en évidence ce qu'ils apportent à la compréhension de la biologie de *V. velutina,* ainsi que leurs contributions du point de vue du contrôle de l'espèce invasive. Ensuite, nous passerons en revue l'apport de ce modèle dans l'étude du système olfactif et de son évolution chez les Hyménoptères. Enfin, dans une dernière partie, nous discuterons des possibles mécanismes évolutifs et des pressions de sélection agissant sur l'organisation du système olfactif de ces espèces.

I. Biologie et contrôle de *V. velutina*

Les colonies de *Vespa velutina* sont fondées par une reine (colonie monogyne) qui sort d'hibernation et pond ses premiers œufs au printemps. Il a été estimé qu'entre le printemps et l'automne une fondatrice produit en moyenne 6000 individus et jusqu'à 13000 dans les colonies les plus développées (Rome *et al.*, 2015). Parmi eux, les ouvrières sont majoritairement produites en début de saison (juillet-août) alors que les autres individus, environ 300 fondatrices et 900 mâles en moyenne par nid, sont produits en automne (Monceau *et al.*, 2014 ; Rome *et al.*, 2015). Chaque caste/sexe présente un comportement particulier, lié à son rôle au sein de la colonie et aux différentes tâches qu'elle effectue. Ainsi, plusieurs stratégies différentes cherchant à interférer avec le comportement olfactif des différentes castes peuvent être envisagées pour lutter contre *Vespa velutina*. Les stratégies actuelles s'appuient principalement sur des pièges contenant des substances alimentaires attractives comme des carbohydrates ou des protéines (Monceau *et al.*, 2012 ; 2014). Cependant ces pièges sont peu sélectifs et attirent une large gamme d'insectes, ce qui peu avoir un effet délétère sur la biodiversité locale (Rome *et al.*, 2011 ; Decante, 2015). L'un des aspects centraux de cette thèse était donc de comprendre les spécificités olfactives de l'espèce *V. velutina* mais aussi des différentes castes de l'espèce.

Les Hyménoptères sociaux disposent d'une communication phéromonale extrêmement élaborée. Chaque caste est susceptible d'adopter un comportement spécifique en réponse à l'émission d'une phéromone par un conspécifique. Ainsi l'utilisation de phéromones attractives pourrait représenter une piste pour augmenter l'efficacité et la spécificité des pièges. On peut alors envisager le piégeage des fondatrices au printemps, celui des ouvrières en été, mais aussi des reproducteurs (mâles et fondatrices) en automne, à l'aide d'appâts distincts pour chaque période de l'année.

1) Les ouvrières

a. *Existe-t-il une phéromone de recrutement ?*

Les ouvrières représentent la grande majorité des individus au sein des colonies de frelons. Elles effectuent des tâches variées telles que l'approvisionnement en nourriture de la colonie, le soin au couvain ou la défense du nid. En France, les premières ouvrières émergent généralement au mois de Juin et commencent rapidement à collecter des proies pour nourrir les larves de la colonie (Monceau *et al.*, 2014). Au cours de l'été, leur nombre augmente considérablement aboutissant à une forte pression de prédation sur l'entomofaune. L'une des particularités du frelon *V. velutina* par rapport à l'espèce européenne *V. crabro* réside dans son comportement de prédation. En effet, les observations effectuées sur les ruches d'abeilles *Apis mellifera* ont compté jusqu'à 20 ouvrières *V.*

velutina chassant simultanément sur la même ruche, alors que *V. crabro* semble chasser de manière plus solitaire (Baracchi *et al.*, 2010 ; Monceau *et al.*, 2013b). Cette observation suggère la possible existence d'une phéromone de recrutement chez l'espèce invasive.

Plusieurs études indiquent que de telles phéromones de recrutement pourraient exister chez diverses espèces de Vespidés. Par exemple, le frelon japonais *Vespa mandarinia* recrute ses congénères pour attaquer « *en masse* » les colonies d'autres Hyménoptères sociaux (Ono *et al.*, 1995). D'autre part, des tests comportementaux ont permis de mettre en évidence que la guêpe *V. germanica* est attirée par une substance contenue dans la tête de ses conspécifiques (D'Adamo *et al.*, 2000 ; 2004 ; Lozada *et al.*, 2016). Enfin, des tests de piégeage réalisés en France par différentes équipes de recherche suggèrent l'existence d'une telle attraction conspécifique chez *V. velutina,* car le nombre de frelons capturés dans un piège augmente considérablement lorsque le piège est amorcé par la capture des premiers individus (Olivier Bonnard et Eric Darrouzet, communications personnelles). Ainsi, l'étude précise du comportement d'orientation de *V. velutina* face à la présentation d'odeurs de congénères pourrait nous renseigner sur la réelle existence de phéromones de recrutement chez cette espèce.

L'exemple le plus documenté de recrutement des congénères à l'aide de substances odorantes est celui observé chez certaines espèces de fourmis. Ces dernières disposent d'éclaireurs capables de créer des pistes phéromonales afin de signaler aux congénères le chemin à suivre pour atteindre une ressource alimentaire (Tumlinson *et al.*, 1971 ; Morgan, 2009). Chez les fourmis coupeuses de feuilles du genre Atta, le lobe antennaire des grandes ouvrières porte une adaptation pour la détection de cette phéromone, consistant en l'hypertrophie d'un glomérule olfactif (Kleineidam *et al.*, 2005 ; Kelber *et al.*, 2009 ; Kuebler *et al.*, 2010). Nous nous sommes donc demandé si le lobe antennaire des ouvrières du genre *Vespa* pouvait porter ce type d'adaptation. Dans les **chapitres 1 et 3** nous avons mesuré le volume de tous les glomérules du lobe antennaire de différentes espèces de frelons. Nos résultats suggèrent que malgré un comportement de prédation qui semble relativement coopératif, les ouvrières de l'espèce *V. velutina* ne possèdent pas de macroglomérule. En revanche, un glomérule de la surface ventrale du lobe antennaire, apparaissant comme homologue entre les différentes espèces, est hypertrophié chez *V. crabro* et *V. mandarinia* (**chap. 3 : fig. 2** et **4**). Ce résultat est assez étonnant car ces deux espèces montrent des comportements de prédation très différents (solitaire et recrutement « en masse », respectivement).

Pour l'instant, nous ne pouvons qu'émettre des hypothèses sur la fonction d'un tel macroglomérule chez ces espèces. Pour ce qui est de *V. crabro*, Steinmetz et ses collaborateurs (2002) ont émis l'hypothèse que *V. crabro* utiliserait une phéromone de piste pour s'orienter vers l'entrée du nid, souvent bâti au sein de cavités naturelles. Dans le cas de *V. mandarinia,* le macroglomérule des ouvrières pourrait être impliqué dans la détection du 1-methylbutyl 3-methylbutanoate, molécule possiblement impliquée comme phéromone de recrutement chez cette

espèce (Ono *et al.*, 2003). Puisque ce glomérule est situé à la surface du lobe antennaire, il est accessible aux enregistrements optophysiologiques. Des expériences d'imagerie calcique *in vivo* mesurant l'activité du lobe antennaire en réponse à des stimulations olfactives, pourraient permettre d'élucider la fonction du macroglomérule de *V. crabro* et *V. mandarinia*. Néanmoins, il est important de noter que l'absence de macroglomérule dans le lobe antennaire des ouvrières de l'espèce *V. velutina* ne signifie pas nécessairement qu'il n'existe pas de communication intraspécifique en relation avec leur comportement de prédation coopératif. Ces phéromones pourraient être traitées de manière combinatoire dans des glomérules de taille ordinaire, comme cela a été démontré pour de nombreuses phéromones chez l'abeille *Apis mellifera* et la fourmi *Camponatus floridanus* (Sandoz *et al*., 2007 ; Wang *et al*., 2008 ; Zube *et al*., 2008 ; Carcaud *et al*., 2015).

b. Quels signaux olfactifs attirent V. velutina sur les ruches d'abeilles ?

Le frelon *V. velutina* est une espèce prédatrice généraliste pouvant aussi bien obtenir des protéines en chassant des insectes qu'en prélevant de la chaire sur des cadavres ou sur les étals des marchés (Raveret Richter, 2000). Cependant, les ouvrières semblent particulièrement attirées par les ruchers d'abeilles domestiques qui représentent souvent une grande proportion des proies capturées (Rome *et al*., 2011). Dans le **chapitre 4** nous avons comparé l'attractivité de différentes sources de nourriture susceptibles d'attirer des frelons capturés alors qu'ils chassaient à proximité des ruches. De manière étonnante, les individus testés ont affiché une plus forte attraction pour les produits de la ruche (pollen, miel) que pour la viande, le poisson ou les abeilles elles-mêmes (**chap. 4 : fig. 2**). Il faut rappeler que ces expériences ont été réalisées sous un éclairage rouge, donc non visible pour les frelons. Leurs choix ont donc été effectués uniquement sur la base d'indices olfactifs. Notre hypothèse est que ces stimuli attractifs étaient indicatifs pour les frelons de la présence d'une *colonie* d'abeilles. En effet, il a été montré que de nombreuses espèces d'insectes, prédateurs ou parasitoïdes, sont capables de s'orienter grâce à des odeurs émises indirectement par les activités ou par l'habitat de leurs proies/hôtes, plutôt que grâce aux odeurs émanant directement de ceux-ci. Par exemple, la guêpe sociale *Mischocyttarus flavitarsis* localise ses proies (des chenilles) grâce à des signaux olfactifs émis par les plantes endommagées dont ses proies se nourrissent (Cornelius, 1993 ; McPheron & Mills, 2007a).

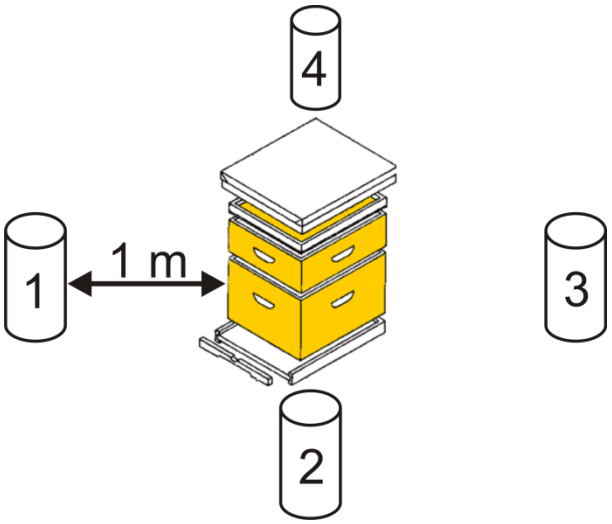
Dans un second temps, nous avons testé l'attraction des ouvrières de l'espèce *V. velutina* envers des molécules odorantes émises par les abeilles (phéromones) ou par d'autres sources protéiques comme le poisson ou la viande. Nous avons ainsi pu observer que les individus testés étaient particulièrement attirés par un composé de la phéromone d'agrégation de l'abeille, le géraniol, et dans une moindre mesure par des composés de la phéromone de reine, l'homovanillyl alcohol, et de couvain, le β-ocimène (**chap. 4 : fig. 3**). Ces résultats indiquent que *V. velutina* serait aussi capable d'utiliser les phéromones émises par les d'abeilles pour les localiser. Ces signaux

sémiochimiques agiraient donc au bénéfice de l'espèce réceptrice (kairomone). Ce type de stratégie semble être relativement répandu chez les Vespidés puisqu'il a été observé que le frelon *Vespa bicolor* est attiré par un composé de la phéromone d'alarme de l'abeille, le (Z)-11-eicosen-1-ol (Brodmann *et al.*, 2009) et que la guêpe *Vespula germanica* utilise la phéromone sexuelle émise par les mâles de *Ceratitis capitata* (mouche méditerranéenne des fruits) pour les localiser (Hendrichs *et al.*, 1994).

Ainsi, pris dans leur ensemble, nos résultats indiquent que les ouvrières *V. velutina* sont sélectivement attirés par des odeurs signalant la présence de colonies d'abeilles (miel, pollen, phéromones d'agrégation, de reine et de couvain) plutôt que par des odeurs d'abeilles isolées. Nos observations pourraient être la conséquence d'une stratégie efficace adoptée par *V. velutina* pour localiser les ruches qui contiennent des proies en grande quantité. Cependant, puisque les individus testés étaient capturés dans un contexte particulier (prédation sur des ruches), il est possible que ces frelons aient associé des signaux olfactifs avec la récompense que représente la capture d'une proie. Un argument en faveur de cette piste est que les ouvrières que nous avons testées n'étaient que très peu attirées par la viande ou le poisson, alors que cette espèce est connue pour être attirée par ces ressources alimentaires (Monceau *et al.*, 2014 ; Spradbery, 1973 ; Raveret Richter, 2000). De plus, plusieurs études indiquent que les guêpes sociales sont capables d'apprendre et de mémoriser des indices visuels et olfactifs (Shafir, 1996 ; McPheron & Mills, 2007b) qu'elles utiliseraient dans un contexte de recherche de nourriture (Toh & Okamura, 2003 ; D'Adamo & Lozada, 2009 ; Lozada & D'Adamo, 2009). A ce jour nous ne savons donc pas si les ouvrières de l'espèce *V. velutina* sont attirées de manière *innée* ou *acquise* vers ces signaux olfactifs. Il sera donc important pour la suite de ces travaux de comparer l'attractivité de ces signaux olfactifs chez des individus collectant différentes sources de nourriture, comme des abeilles (chap. 4) ou de la chair animale (viande ou poisson) afin de déterminer s'il existe une spécialisation des ouvrières pour la collecte d'un type de nourriture. Dans un second temps, nous pourrions essayer de changer les préférences olfactives des frelons dans un protocole de conditionnement qui associerait d'autres odeurs avec une récompense pour tester les capacités d'apprentissage de *V. velutina*.

Bien que les résultats que nous avons obtenus ne représentent qu'une première étape dans la compréhension du comportement de prédation de *V. velutina*, l'élaboration d'une stratégie de lutte pour détourner les frelons des ruchers d'abeille domestique pourrait alors reposer sur des odeurs attractives mimant la présence d'une colonie d'abeille. Dans cette optique, nous avons effectué quelques tests préliminaires de piégeage à proximité du rucher expérimental de l'INRA Bordeaux-Aquitaine. Ces tests ayant été réalisés en fin de saison alors que la pression de prédation sur le rucher avait fortement diminué à cause de mauvaises conditions météorologiques, nous ne pouvons conclure définitivement quant à l'efficacité d'une telle stratégie de lutte. De plus, faute de temps, nous n'avons pas pu tester tous les attractifs aux différentes périodes de l'année, comme nous le

souhaitions. Cependant nos essais ont donné des résultats prometteurs sur l'attractivité du pollen, ce qui semble confirmer nos résultats obtenus en laboratoire (**fig. 1**). Ces expériences pourraient donc être répétées en mettant en compétition l'attractivité des différents attractifs isolés en laboratoire afin de trouver des odeurs perturbant fortement et durablement le comportement de prédation des ouvrières de *V. velutina*.



posé le	contrôle	miel	pollen	Pollen + abeilles	relevé le
29/10	0	0	11	33	30/10
30/10	0	0	0	17	31/10
06/11	0	0	0	3	07/11
07/11	0	0	0	0	08/11
11/11	0	0	0	0	12/11
12/11	0	0	1	0	13/11
13/11	0	0	0	0	14/11
14/11	0	0	0	0	15/11
15/11	0	0	0	0	16/11

Figure 1: Bioessais de terrain
Les tests de piégeage ont été réalisés en positionnant des pièges à environ 1 mètre des quatre coins d'une ruche. Trois pièges contenaient des substances potentiellement attractives et un piège témoin était vide. A chaque essai, les substances testées et le contrôle étaient distribués aléatoirement autour de la ruche. Les résultats obtenus sont présentés dans le tableau de droite. Sont indiquées : les dates de réalisation du piégeage et le nombre de frelons capturés dans chaque piège. Seuls les pièges contenant du pollen, ou du pollen avec des abeilles mortes semblent avoir été attractifs. Hormis le frelon *V. velutina*, aucune autre espèce d'insecte n'a été retrouvée dans les pièges lorsque ceux-ci ont été relevés.

2) Les castes sexuées

a. *Existe-t-il des phéromones attractives pour les fondatrices ?*

Les reines fondatrices ont un rôle central dans la multiplication et la dispersion de cette espèce invasive. Chaque fondatrice initiant un nid est capable de produire des milliers d'ouvrières et plusieurs centaines de sexués. Les seuls moments de l'année où l'on peut agir contre ces individus sont au printemps, à l'initiation des nids, et en automne, pendant la période de reproduction. Une stratégie de lutte proposée notamment par les associations apicoles, consiste à se concentrer sur l'élimination des fondatrices afin de limiter le nombre de colonies qui se développeront au cours de la saison (Blot, 2009). Une phéromone induisant une forte attraction des fondatrices pourrait donc améliorer l'efficacité de cette méthode. Puisque les fondatrices semblent se regrouper dans des abris pendant la période d'hibernation (Spradbery, 1973 ; Edwards, 1980 ; Juliette Poidatz, communication personnelle), il est envisageable que des phéromones d'agrégation attirent ces individus. Dans le **premier chapitre** nous avons cherché la présence de particularités au sein de la voie olfactive des

fondatrices, comme la présence de glomérules hypertrophiés, qui pourraient correspondre à une adaptation pour la détection de phéromones.

Chez les frelons, les fondatrices sont généralement plus grosses que les ouvrières mais cette différence n'est que faiblement marquée chez l'espèce *V. velutina*. Nous avons donc prêté une attention toute particulière à la détermination de ces deux castes en notant scrupuleusement les dates de capture des individus et en mesurant leur surface alaire (**chap. 1 : fig. 1**). Pourtant, lors de nos investigations neuroanatomiques, nous n'avons observé aucune différence dans l'organisation du cerveau et du lobe antennaire entre les ouvrières et les fondatrices (**chap. 1 : fig.2**, **4** et **6**). De plus, nous n'avons trouvé aucune différence de volume relatif occupé par les différentes structures neuronales (glomérules et aires cérébrales). Ces résultats suggèrent que les systèmes nerveux centraux des ouvrières et des fondatrices sont identiques d'un point de vue neuroanatomique. Ceci est particulièrement surprenant car chez les guêpes sociales, les fondatrices présentent généralement des corps pédonculés plus volumineux que les ouvrières alors que ces dernières possèdent des lobes antennaires et des lobes optiques plus développés que ceux des fondatrices (O'Donnell *et al.*, 2011 ; O'Donnell *et al.*, 2013). Ces différences d'investissement relatif dans des structures cérébrales particulières pourraient être dues à des différences d'expérience au cours de la vie de l'adulte. Ainsi, les fondatrices, plus souvent exposées au contexte social de la colonie, investiraient plus dans les corps pédonculés alors que les ouvrières cherchant des ressources à l'extérieur du nid investiraient plus dans les structures sensorielles (lobe antennaire et lobe optique). Cependant, Chez les frelons, les fondatrices doivent également collecter des ressources à l'extérieur du nid, avant l'émergence des premières ouvrières, ce qui pourrait expliquer le faible dimorphisme cérébral que nous avons observé. Quoi qu'il en soit, ces deux castes affichent des comportements bien différents, correspondant à des rôles distincts au sein de la colonie. Les différences comportementales entre les ouvrières et les fondatrices pourraient donc reposer principalement sur des différences fonctionnelles au sein du système nerveux. Pour comprendre ces différences, il sera nécessaire de comparer non plus la structure, mais le fonctionnement des réseaux neuronaux chez ces deux castes, grâce à des enregistrements neurophysiologiques.

Du point de vue des stratégies de lutte contre l'espèce invasive, l'absence de différences mesurables entre les voies olfactives d'ouvrières et de fondatrices n'ouvre que peu de perspectives pour l'élimination spécifique des fondatrices de *V. velutina*. En effet, l'absence de macroglomérule dans le lobe antennaire de cette caste restreint les possibilités de trouver une phéromone spécifiquement attractive pour les fondatrices. Cependant une approche d'écologie chimique cherchant à mesurer les émissions différentielles de composés odorants entre les ouvrières et les fondatrices pourrait toujours permettre d'isoler des phéromones. Il sera alors important d'évaluer la réponse comportementale associée à leur présentation et leur spécificité. Toutefois, un certain nombre de chercheurs considèrent que le très grand nombre de fondatrices produites en fin

de saison et les fortes capacités de dispersion de celles-ci indiquent qu'une stratégie d'élimination de fondatrices par piégeage n'aurait que peu d'effet sur la densité de l'espèce (Monceau *et al.*, 2012). Pour être efficace, une telle stratégie devrait être appliquée de manière systématique et à très grande échelle, ce qui ne semble pas réalisable sans altérer la biodiversité locale tant que des attractifs spécifiques des fondatrices n'auront pas été isolés.

b. *Existe-t-il des phéromones attractives pour les mâles ?*

Chaque colonie de *V. velutina* produit environ 900 mâles dont l'unique fonction connue est de féconder les fondatrices. Généralement, les mâles sont haploïdes et produits en fin de saison (automne) en même temps que les fondatrices. Cependant, des mâles diploïdes, incapables de se reproduire, peuvent être produits précocement au sein des populations invasives (Arca, 2012 ; Darrouzet *et al.*, 2015). A notre connaissance, il n'existe que peu d'études ayant analysé le comportement de reproduction des frelons. Les études existantes suggèrent tout de même que des phéromones d'attraction sexuelle émises par les femelles fertiles existeraient (Batra, 1980 ; Ono & Sasaki, 1987 ; Spiewok *et al.*, 2006). Ainsi une stratégie de lutte envisageable consiste à perturber le comportement des mâles grâce à l'utilisation de phéromones sexuelles afin de les empêcher de féconder les futures fondatrices. Chez de nombreux insectes, le lobe antennaire des mâles contient des glomérules hypertrophiés (macroglomérules) répondant spécifiquement aux composés de la phéromone sexuelle (Galizia *et al.*, 2000 ; Sandoz, 2006). Dans le **chapitre 1** nous avons donc cherché la présence de tels macroglomérules au sein du lobe antennaire des mâles de *V. velutina*. Nos investigations neuroanatomiques nous ont permis d'observer que le lobe antennaire des mâles de l'espèce *V. velutina* est organisé suivant le même modèle que celui des femelles (**chap. 1 : fig. 6** et **7**). Cependant, les mâles possèdent sensiblement moins de glomérules que les femelles, mais présentent sept macroglomérules distribués dans deux régions (ou clusters) distinctes du lobe antennaire (**chap. 1 : fig. 4** et **5**).

Les observations du comportement de reproduction des frelons suggèrent que les femelles fertiles émettraient deux types de phéromones sexuelles (Ayasse *et al.*, 2001) : d'une part une phéromone d'attraction sur de longues distances et d'autre part une phéromone de contact qui déclencherait les tentatives d'accouplement des mâles (Batra, 1980 ; Ono & Sasaki, 1987 ; Spiewok *et al.*, 2006). Compte tenu des mécanismes à l'origine de l'organisation du lobe antennaire dont nous parlerons plus tard (Couto *et al.*, 2005 ; Ramdya & Benton, 2010), nous pensons que chaque cluster contenant des macroglomérules (clusters rostral et caudal) pourrait être impliqué dans le traitement séparé de ces deux types de phéromones. A l'aide d'une étude fonctionnelle visant à élucider quelles molécules odorantes activent les différents macroglomérules, nous pourrions donc considérablement améliorer notre compréhension de la reproduction chez les frelons. Dans cette optique, nous avons réalisés des enregistrements optophysiologiques (imagerie calcique *in vivo*) afin

d'observer l'activité du lobe antennaire des mâles en réponse à la présentation de différentes odeurs. A défaut de disposer de composés phéromonaux préalablement isolés, nous avons réalisé ces expériences en présentant différentes catégories d'odorants et des extraits de fondatrices dans l'éther. Bien que la plupart des odorants que nous avons présentés aient induit un motif d'activité pluriglomérulaire (**fig. 2**), nous n'avons pas observé d'activité notable en réponse à la présentation des extraits de fondatrices. Cette expérience n'est cependant que préliminaire et des expériences complémentaires au cours desquelles des extraits de fondatrices réalisés avec différents solvants seraient présentés aux mâles, pourraient permettre de progressivement isoler les composés qui activent spécifiquement les macroglomérules.

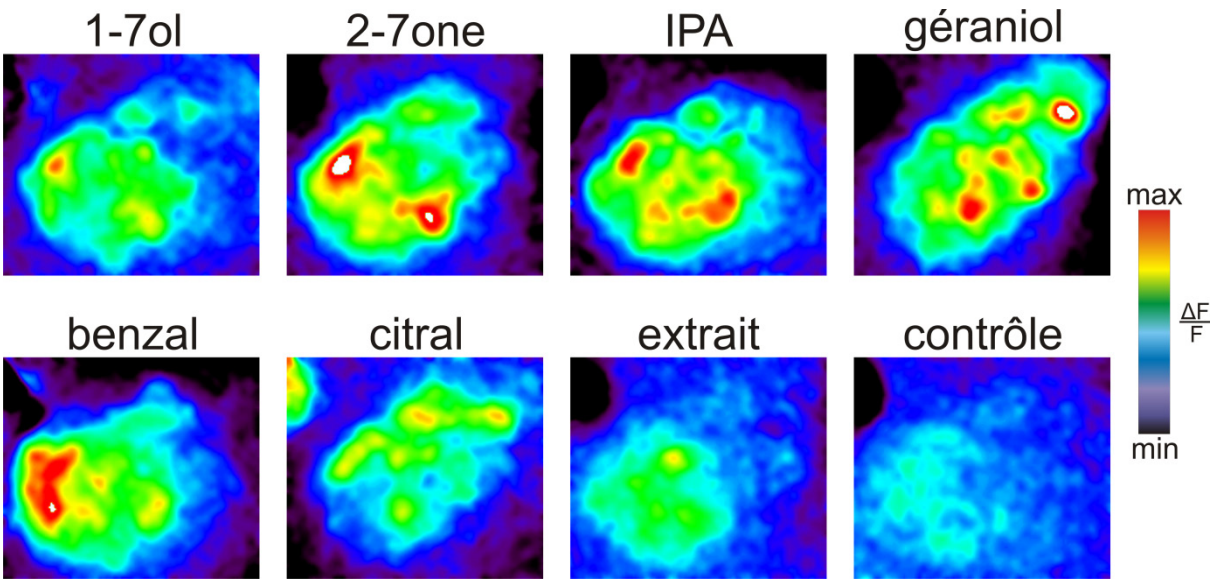


Figure 2: Enregistrement optophysiologique du lobe antennaire de mâle *Vespa velutina*
La présentation de différentes molécules odorantes induit des motifs d'activité différents au sein du lobe antennaire des mâles de *V. velutina*. Les extraits de fondatrices dans l'éther n'induisent pas d'activité spécifiquement localisée dans des macroglomérules. Il est donc possible d'enregistrer l'activité du lobe antennaire de *V. velutina* en utilisant la technique d'imagerie calcique. (1-7ol : 1-heptanol, 2-7one : 2-heptanone, IPA : isopentyl acetate, benzal : benzaldéhyde, extrait : extrait de fondatrice de V. velutina dans l'éther).

De manière plus indirecte, par une approche transcriptomique nous pourrions également chercher les gènes de récepteurs olfactifs surexprimés chez les mâles. Nous pourrions ensuite exprimer ces gènes en système hétérologue afin d'effectuer des enregistrements électrophysiologiques et tenter de trouver les ligands (odorants) qui activent ces récepteurs olfactifs. Les phéromones sexuelles isolées pourraient alors être utilisées dans une stratégie de piégeage ou de confusion sexuelle visant à perturber la reproduction de *V. velutina* (Howse *et al.*, 1998 ; Witzgall *et al.*, 2010). Cependant, au même titre que le piégeage des fondatrices, l'élimination d'une partie des mâles pourrait ne pas suffire pour diminuer le taux de fécondation et l'effectif des populations invasives. De plus, dans le cas d'une stratégie de confusion sexuelle, les phéromones pourraient ne pas être complètement spécifiques de *V. velutina* et potentiellement perturber la reproduction des

espèces proches comme le frelon européen *V. crabro*. En effet, chez les abeilles du genre Apis, il a été montré que le 9-ODA, composé majoritaire de la phéromone sexuelle qui induit une forte attraction des mâles, a un effet similaire chez plusieurs espèces telle que *Apis mellifera*, *Apis cerana*, *Apis dorsata* et *Apis florea* (Gary, 1962 ; Plettner *et al.*, 1997 ; Koeniger & Koeniger, 2000 ; Nagaraja & Brockmannn, 2009). Ce type d'activité croisée des phéromones sexuelles semble également exister chez les frelons, puisqu'il est possible d'attirer les mâles de 6 espèces différentes à l'aide d'extraits de fondatrice d'une seule de ces espèces (Ono & Sasaki, 1987). Cette stratégie de lutte doit donc être abordée avec prudence.

3) Conclusion sur les stratégies de lutte contre *V. velutina*

A ce jour aucune méthode de lutte ne semble vraiment efficace pour éradiquer le frelon à pattes jaunes des territoires européens. Que ce soit avec le piégeage des ouvrières, celui des fondatrices ou bien des mâles, il semble difficile de réellement faire diminuer la densité des populations de cette espèce sans altérer la biodiversité locale. En effet, les attractifs utilisés actuellement sont très peu spécifiques. Par exemple, dans une étude récente comparant différentes modalités de piégeage, même les pièges et appâts commercialisés par une société pharmaceutique (Véto-pharma) se sont révélés très peu sélectifs vis-à-vis du frelon européen et des abeilles domestiques (Decante, 2015). De plus, la forte densité de frelon *V. velutina* dans les territoires envahis, ainsi que ses fortes capacités de dispersion, ont amené certains analystes à estimer qu'il était tard pour éradiquer l'espèce. Toutefois, des actions de contrôle ayant pour but de diminuer l'impact de l'invasion sur les activités humaines (apiculture) et les écosystèmes locaux peuvent être envisagées. Une stratégie intégrée utilisant différents composés sémiochimiques pour attirer les différentes castes d'une colonie de frelon pourrait par exemple s'avérer plus spécifique et efficace.

Dans ce sens, notre travail a permis de cartographier chez chaque caste de *V. velutina* les structures nerveuses impliquées dans le traitement de l'information olfactive. Seuls les mâles possèdent des structures qui semblent spécialisées dans la détection de phéromones. Ceci représente une première étape dans la compréhension de la communication olfactive chez cette espèce qui nécessiterait d'être complétée par des données fonctionnelles (imagerie calcique, électrophysiologie). Ainsi, il apparaît nécessaire d'identifier les composés impliqués dans la communication intraspécifique de cette espèce. D'autre part, il semble envisageable de perturber le comportement de prédation des ouvrières en les détournant des ruches à l'aide de leurres olfactifs. Cette technique pourrait être appliquée pour limiter l'impact de cette prédation sur les activités apicoles, bien que son efficacité reste à démontrer.

II. Le système olfactif des Hyménoptères

Les Hyménoptères constituent un ordre d'insectes extrêmement diversifié qui est présent sur tous les continents à l'exception de l'Antarctique (Grimaldi & Engel, 2005). Ces espèces possèdent une vaste diversité de régimes alimentaires (phytophage, entomophage, etc.) et de modes de vie (parasitaire, grégaire, eusocial, etc.). Cela en fait un groupe clé pour déchiffrer les corrélats neuronaux des comportements naturels en relation avec la niche écologique des espèces (Kleineidam & Rössler, 2009 ; Hansson & Stensmyr, 2011). L'analyse comparative au sein des Hyménoptères permet ainsi d'appréhender les tendances adaptatives et évolutives qui façonnent la fonction et l'anatomie du système nerveux. Les multiples apparitions indépendantes de l'eusocialité (le plus haut niveau d'organisation sociale) au sein de cet ordre ont notamment amené la communauté scientifique à s'interroger sur les éventuelles préadaptations du système olfactif des Hyménoptères qui auraient pu favoriser l'émergence des comportements altruistes au travers d'une communication intraspécifique hautement élaborée (Galizia & Rössler, 2010 ; LeBoeuf *et al.*, 2013 ; Tsutsui, 2013). Dans cette perspective, l'anatomie et le fonctionnement du système olfactif de quelques espèces eusociales ont été étudiés, mais nos connaissances sont encore très parcellaires en ce qui concerne l'organisation de la voie olfactive dans son ensemble, son potentiel dimorphisme sexuel (ou de caste) et son évolution. L'étude de *V. velutina* fut donc l'occasion de caractériser le système olfactif d'une nouvelle espèce eusociale appartenant à une famille (les Vespidae) jusque là peu étudiée au plan neuroanatomique, puis de comparer son organisation aux descriptions existantes dans d'autres familles d'Hyménoptères dans lesquelles l'eusocialité est apparue indépendamment. Ces comparaisons nous ont permis d'extraire des principes organisationnels généraux du système olfactif des Hyménoptères, mais aussi de proposer l'existence de préadaptations à l'eusocialité au sein de cet ordre.

1) Evolution du système olfactif des Hyménoptères

a. *Existe-t-il des structures conservées au sein de la voie olfactive des Hyménoptères ?*

En comparaison avec d'autres ordres d'insectes, le système olfactif des Hyménoptères présente plusieurs singularités suggérant une complexification des voies neuronales impliquées dans le traitement de l'information olfactive au sein de cet ordre (Galizia & Rössler, 2010). Typiquement, le lobe antennaire des Hyménoptères contient de nombreux glomérules innervés soit par un tractus médian de neurones de projection uniglomérulaires (m-ALT) soit par un tractus latéral de neurones également uniglomérulaires (l-ALT) qui se projettent tous deux dans les centres supérieurs, la corne latérale et les corps pédonculés, suivant deux routes inversées (Rössler & Zube, 2011). De plus, l'étude approfondie des voies afférentes au lobe antennaire chez des espèces modèles comme

l'abeille *Apis mellifera* ou certaines fourmis (*Camponotus sp., Atta sp., Cataglyphis sp.*) ont montré que les glomérules sont arrangés en grappes (ou clusters) suivant des subdivisions du nerf sensoriel olfactif (Suzuki, 1975 ; Kirschner *et al.*, 2006 ; Zube *et al.*, 2008 ; Nakanishi *et al.*, 2010 ; Mysore *et al.*, 2009 ; Kelber et *al.*, 2010 ; Stieb *et al.*, 2011). Ainsi, le lobe antennaire de ces espèces présente de multiples compartimentations formées par les ramifications des faisceaux de fibres nerveuses entrantes (tractus sensoriels) et sortantes (tractus de neurones de projection) du lobe antennaire. A ce jour, les descriptions complètes des compartimentations du lobe antennaire, prenant en compte à la fois les tractus d'entrée et de sortie ainsi que le potentiel dimorphisme sexuel, ne sont disponibles que chez deux espèces, *Apis mellifera* et *Camponotus floridanus*, en intégrant plusieurs études (*A. mellifera* : Kirschner *et al.*, 2006 ; Nishino *et al.*, 2009 ; *C. floridanus* : Zube *et al.*, 2008 ; Zube & Rössler, 2008). Nous avons donc utilisé ces deux Hyménoptères en tant que modèles de référence, représentatifs de leur famille respective, les Apidae et les Formicidae.

Dans l'exploration anatomique du système olfactif de *V. velutina* nous avons d'abord marqué les neurones du nerf antennaire (**chapitre 1**). Nous avons ainsi pu observer neuf ramifications majeures des neurones sensoriels olfactifs donnant naissances à neuf grappes de glomérules (**chap. 1 : fig. 6** et **7**). Malgré des variations quant au nombre de glomérules au sein de chaque grappe, cette organisation du lobe antennaire semble être conservée chez les différentes espèces de frelon que nous avons étudiées dans le **chapitre 3**. De plus, nous avons observé des similarités morphologiques frappantes dans l'organisation de cette compartimentation entre les lobes antennaires du frelon et d'autres Hyménoptères sociaux (**chap. 1 : fig. 9**). En effet, l'orientation des différents tractus sensoriels du frelon ainsi que la taille, la forme et la position des clusters de glomérules qu'ils innervent, semblaient toujours trouver une correspondance dans le lobe antennaire de l'abeille *Apis mellifera* et/ou des fourmis charpentières (*Camponotus sp,* les mieux décrites). Ensuite, l'exploration des voies efférentes au lobe antennaire de *V. velutina,* et plus précisément l'affiliation de chaque cluster glomérulaire à l'un ou l'autre des tractus de neurones de projection uniglomérulaires (m- ou l-ALT) a révélé une cohérence avec les similarités morphologiques préalablement observées (**chap. 1 : fig. 8**). Ainsi, les groupes de glomérules recevant les neurones de tractus sensoriels morphologiquement similaires chez ces espèces de familles différentes sont également innervés par des voies de sortie similaires chez ces mêmes espèces (**chap. 1 : tabl. 3**). Ces observations suggèrent que l'organisation du lobe antennaire des Hyménoptères sociaux présenterait des synapomorphies, c'est-à-dire des traits hérités d'un ancêtre commun, mais n'excluent pas complètement la possibilité que ces similarités soient issues de convergences évolutives.

Dans le **chapitre 2,** nous avons utilisé les propriétés intrinsèques d'un groupe de glomérules déjà bien caractérisé chez plusieurs espèces de fourmis, afin de corroborer l'existence d'une structure homologue chez *V. velutina.* Nous avons pu montrer que les neurones sensoriels des sensilles basiconiques des frelons se projettent spécifiquement dans un groupe de petits glomérules,

et que ces derniers ne sont pas innervés par des fibres sérotoninergiques, contrairement au reste des glomérules du lobe antennaire (**chap. 2 : fig. 4** et **5**). Avec l'affiliation de ce cluster au tractus médian de neurones de projection (m-ALT ; **chap. 1 : fig. 8** et **tal. 2** et **3**), toutes ces propriétés correspondent rigoureusement entre fourmis et frelons (Zube & Rössler, 2008 ; Kelber *et al.*, 2010 ; Nishikawa *et al.*, 2012). De plus, nos enregistrements électrophysiologiques de l'activité des sensilles basiconiques suggèrent une implication de ce type sensillaire dans la détection des hydrocarbures à chaîne longue (**chap. 2 : fig. 6**), de manière similaire chez *V. velutina* et chez les fourmis charpentières (*Camponotus* sp. *;* Ozaki *et al.*, 2005 ; Sharma *et al.*, 2015). A mes yeux, les similarités observées au niveau de trois populations neuronales différentes (NSO, interneurones sérotoninergiques et neurones de projection) et leur implication dans des fonctions similaires (détection des hydrocarbures à chaîne longue) chez différentes espèces, réduit grandement la probabilité que les ressemblances morphologiques observées pour ce groupe de glomérules soient issues de convergences évolutives.

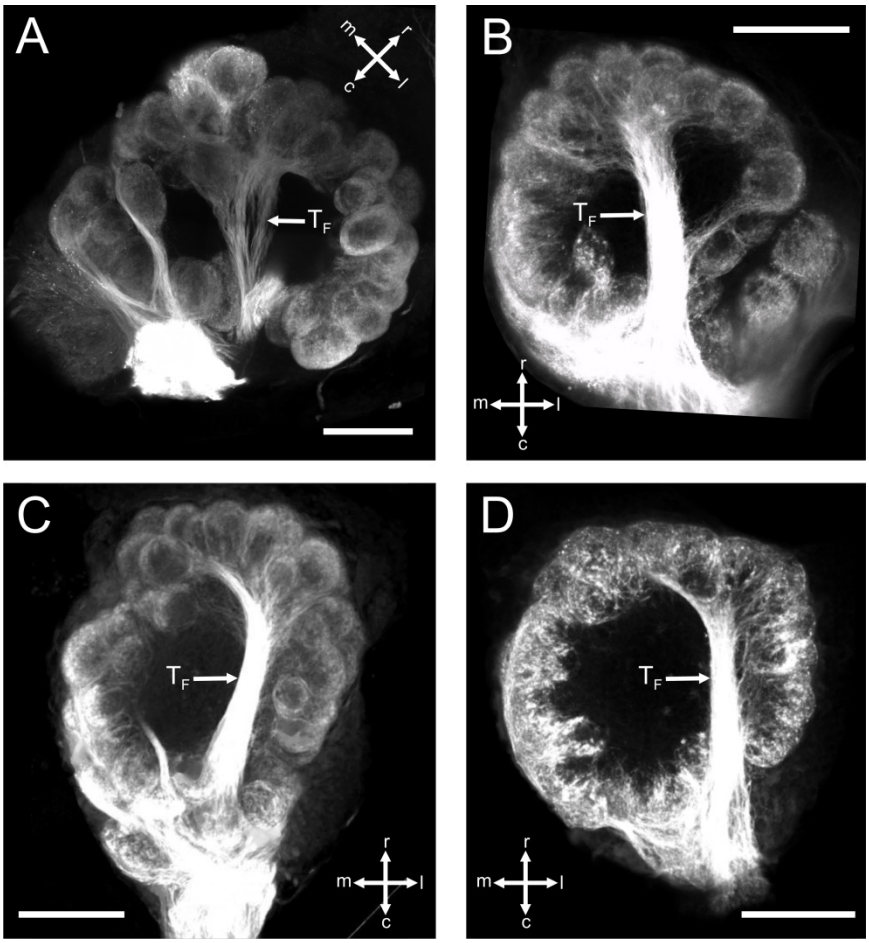
b. *Comment prouver l'homologie entre clusters glomérulaires de différentes espèces ?*

Dans le **chapitre 2**, nous avons donc apporté des arguments supplémentaires soutenant l'homologie d'un cluster glomérulaire en exploitant les caractéristiques spécifiques de ce cluster. Mais d'autres propriétés neuroanatomiques, moléculaires et fonctionnelles pourraient être utilisées pour déceler des correspondances sur l'ensemble du lobe antennaire de différentes espèces. En admettant que des ressemblances morphologiques entre les populations neuronales d'entrée et de sortie d'un cluster aient été observées, dans cette partie, je vais présenter l'ensemble des caractéristiques supplémentaires qui me semblent pertinentes pour étayer les potentielles homologies entre espèces.

D'abord, d'un point de vue neuroanatomique, les neurones sensoriels de certaines sensilles se projettent plus ou moins spécifiquement dans des groupes de glomérules, ce qui pourrait servir d'outil pour corroborer les homologies en examinant systématiquement les liens entre les clusters de glomérules et le(s) type(s) sensillaire(s) associé(s) (Kelber *et al.*, 2006 ; Kelber *et al.*, 2010 ; Watanabe *et al.*, 2012 ; Kropf *et al.*, 2014). Ceci correspond à ce que nous avons en partie réalisé dans le chapitre 2 en nous concentrant sur les neurones des sensilles basiconiques. Par ailleurs, les sous-divisions (ventrale et dorsale) du nerf antennaire qui innervent distinctement certains groupes de glomérules chez *Apis mellifera* ou *Camponotus japonicus* pourraient présenter les mêmes propriétés chez les frelons et apporter un argument supplémentaire (Nishino *et al.*, 2009 ; Nakanishi *et al.*, 2010). Ensuite, Les différentes populations d'interneurones locaux pourraient connecter spécifiquement les glomérules d'une ou plusieurs grappes de façon similaire chez les différentes espèces (Masson & Strambi, 1977 ; Dacks *et al.*, 2010). Nous avons par exemple exploité l'immunoréactivité à la sérotonine d'une population d'interneurones (**chap. 2 : fig. 5**) ce qui pourrait

être étoffé par l'étude de l'immunoréactivité à d'autres neurotransmetteurs tels que l'histamine, le GABA et différents neuropeptides. Enfin, d'un point de vue fonctionnel et moléculaire, les neurones sensoriels olfactifs se projetant dans une grappe de glomérules pourraient spécifiquement exprimer une famille de récepteurs olfactifs possédant un spectre de réponse particulier (Engsontia *et al.*, 2015 ; Zhou *et al.*, 2015). L'homologie pourrait donc être soutenue soit en démontrant une implication de clusters glomérulaires de différentes espèces dans la détection de composés similaires, soit en montrant une correspondance des récepteurs olfactifs exprimés dans les neurones sensoriels qui se projettent dans ces glomérules. Donc, en décelant des similarités structurales, moléculaires et fonctionnelles chez de nombreuses espèces, nous pourrions appuyer l'homologie de ces structures neuronales ou bien déceler de possibles cas de convergence.

Au cours de cette thèse, j'ai eu aussi l'opportunité d'observer les lobes antennaires de plusieurs espèces de Vespidés (plusieurs frelons et une espèce de poliste) mais également de plusieurs espèces d'Apidae, telles que l'abeille domestique *Apis mellifera*, l'abeille sans dard *Melipona scutellaris* et le bourdon *Bombus terrestris*. Bien que ces investigations n'aient pas été poussées jusqu'à une description complète des voies afférentes et efférentes du lobe antennaire, nous avons pu observer des ressemblances morphologiques qui m'ont persuadé du caractère synapomorphique de certains clusters glomérulaires (**fig. 3**).



<u>Figure 3:</u> Exemple de similarités morphologiques des tractus sensoriels au sein du lobe antennaire de différents Hyménoptères.
Projection de sections optiques obtenues en microscopie confocale après marquage fluorescent du nerf antennaire chez *Vespa velutina* (**A**), *Bombus terrestris* (**B,** obtenue au laboratoire par Marcel Mertes), *Apis mellifera* (**C,** d'après Kirschner *et al.*, 2006) et *Melipona sculpturalis* (**D,** obtenue au laboratoire par Ana Carolina Roselino). Toutes ces espèces présentent un tractus sensoriel (noté T_F) se projetant vers les glomérules rostraux en suivant la ligne médiane du lobe antennaire. Cette homologie de structure indique une possible origine commune. NB : le lobe antennaire de *V. velutina* (**A**) a subi une rotation pour aligner l'axe de projection du nerf antennaire avec celui des autres espèces. Les axes d'orientation sont indiqués pour chaque lobe antennaire (r : rostral, c : caudal, m : médian, l : latéral). L'échelle est indiquée par un trait représentant 100 µm.

Bien entendu, la démonstration formelle que ces similarités morphologiques sont bien héritées, demanderait l'observation directe de ces structures dans des fossiles de leur ancêtre commun (Ma *et al.*, 2012). Bien que cela semble difficilement imaginable actuellement, dans l'hypothèse où les traces de ces structures neuronales seraient conservées dans des fossiles emprisonnés dans l'ambre, la tomographie à rayon X pourrait se révéler un outil adéquat pour les observer (Sombke *et al.*, 2015). En outre, l'étude comparative du développement de ces structures et des facteurs moléculaires qui induisent le guidage axonal des neurones sensoriels pourrait apporter des preuves plus concrètes que des clusters de glomérules ont été conservés au cours de l'évolution des Hyménoptères.

c. *Quels scénarios évolutifs ?*

Les homologies proposées au sein du lobe antennaire des Hyménoptères suggèrent une histoire évolutive comprenant des pertes et des ajouts de clusters glomérulaires, sans doute en lien avec l'augmentation ou la diminution des capacités de discrimination des insectes pour certaines gammes de composés odorants à certains moments de leur évolution. Ainsi, des pressions de sélection différentes ont pu favoriser la conservation, l'émergence ou la perte de structures neuronales chez les différentes espèces.

Certaines pressions de sélection agiront de concert chez beaucoup d'espèces. Pour illustrer cette idée, je prendrai l'exemple d'une capacité largement répandue chez les animaux, celle de détecter le CO_2 ou les variations de température, ce qui permet d'éviter l'asphyxie et l'hypo- ou l'hyperthermie. Les pressions de sélection devraient donc tendre à favoriser la conservation d'un système permettant de détecter efficacement ces stimuli d'importance vitale. Chez la drosophile, les neurones sensoriels responsables de la détection du CO_2 ou de la température se projettent dans des glomérules de la face dorsale du lobe antennaire (glomérules V, VL2a et VL2p ; Suh *et al.*, 2004 ; Jones *et al.*, 2007 ; Kwon *et al.*, 2007 ; Hamada *et al.*, 2008 ; Silbering *et al.*, 2011). Ces neurones sensoriels expriment des récepteurs gustatifs ou ionotropiques (Gr21a et GR63a pour le glomérule V ; IR31a pour VL2p et IR84a pour VL2a) considérés comme ancestraux par rapport aux récepteurs olfactifs classiques (OR) (Benton *et al.*, 2009). Ces données suggèrent que les glomérules de la face dorsale procurant la capacité de détecter le CO_2 et la température sont apparus relativement tôt au cours de l'évolution. De plus, les glomérules de la face dorsale du cafard (cluster T10), *Periplaneta americana,* semblent posséder des fonctions similaires à ceux de la drosophile (Nishino *et al.*, 2003 ; Watanabe *et al.*, 2010 ; Watanabe *et al.*, 2012), ce qui indique qu'ils auraient pu être conservés chez différents ordres d'insectes. En effet, les fortes similarités anatomiques de ces glomérules avec ceux des tractus T4 et T7 (également dorsaux) de l'abeille ou des fourmis, respectivement, ont amené certains chercheurs à proposer leur homologie (Zube *et al.*, 2008 ; Nishino *et al.*, 2009 ; Nakanishi *et al.*, 2010). L'ensemble de ces observations nous a incités à implémenter, dans le **chapitre 1**, une

nouvelle nomenclature des clusters glomérulaires débutant par la face dorsale du lobe antennaire, afin de prendre en compte les correspondances observées entre différentes espèces. Ainsi, nous nous attendons à ce que ces glomérules de la face dorsale du lobe antennaire, nommés T_A dans notre étude, et que l'on retrouve aussi chez le frelon, soient présents dans le lobe antennaire de la plupart des insectes.

D'un autre coté, certaines pressions de sélection agissant sur les systèmes sensoriels pourraient varier en fonction de la niche écologique des espèces. En effet, les espèces évoluant dans des environnements différents devraient avoir des besoins variables en ce qui concerne la discrimination de composés odorants. Ainsi, il est fort probable que des différences dans l'organisation du lobe antennaire soient observées chez des espèces dont la biologie est dissimilaire. Par exemple, nous avons pu observer chez les Apidés, *Bombus terrestris* et *Mellipona scutellaris* un tractus traversant le centre du lobe antennaire qui ressemble fortement au tractus T1 décrit chez *Apis mellifera* (**fig. 3**). Nous avons trouvé dans le lobe antennaire du frelon un tractus (T_F) qui semble correspondre mais ce tractus était clairement absent chez les fourmis charpentières (*Camponotus* sp). Les pressions de sélections auraient donc pu, dans ce cas, favoriser l'apparition ou la conservation de ce cluster glomérulaire chez les Apidae et les Vespidae alors qu'elles auraient au contraire contre-sélectionné ce même cluster chez les Formicidae. Il est intéressant de noter que chez l'abeille, ce tractus (T1 ou T_F) a un lien étroit avec les sensilles placodées (Kelber *et al.*, 2006 ; Sandoz, 2006 ; Wanner *et al.*, 2007 ; Nishino *et al.*, 2009) que nous avons également identifiées chez le frelon (**chap. 2 : fig.1**) mais qui sont absentes chez les fourmis charpentières (Nakanishi *et al.*, 2009). L'absence de ce tractus olfactif chez les fourmis paraît donc être corrélée avec l'absence du type sensillaire associé. Ceci suggère que les pressions de sélection agiraient aussi bien sur les réseaux neuronaux (organisation du lobe antennaire) que sur l'équipement de détection périphérique (sensilles). Pour l'instant, il est difficile d'expliquer de telles différences entre ces espèces mais élucider le rôle fonctionnel des glomérules associés au cluster T_F, pourrait nous permettre de comprendre quelles pressions de sélection ont pu agir sur ce système.

Il est donc envisageable de retracer l'histoire évolutive du lobe antennaire des Hyménoptères par l'ajout ou la perte de clusters glomérulaires. Malheureusement, la phylogénie et les relations de parenté existant entre les familles/espèces dont le système olfactif a été décrit, restent sujets à controverse (Brothers, 1999 ; Pilgrim *et al.*, 2008 ; Heraty *et al.*, 2011 ; Sharkey *et al.*, 2012). Il paraît donc difficile en l'état actuel des connaissances d'affirmer avec certitude quelle espèce/famille présenterait des caractères ancestraux. Nous avons donc pris le parti de présenter deux scénarios évolutifs basés sur deux phylogénies différentes (**fig. 4**) : il s'agit de la phylogénie de Brother (1999), la plus communément admise, basée principalement sur des caractères morphologiques, et une phylogénie plus récente basée sur des données moléculaires de grande ampleur (Johnson *et al.*, 2013). L'évolution des systèmes sensoriels est souvent conçue comme aboutissant forcément à une

complexification des structures nerveuses (Ghysen, 2003 ; Farris, 2015 ; Strausfeld & Hirth, 2015). Pourtant, le maintien des structures neuronales est extrêmement couteux énergétiquement ce qui tendrait à faire disparaître les populations neuronales superflues (Niven & Laughlin, 2008). Dans le premier scénario, les Formicidae et les Vespidae font partie d'une même super-famille, les Vespoidea, alors que les Apidae seraient phylogénétiquement plus distants. Cette phylogénie suggère l'apparition de trois tractus glomérulaires situés à la surface ventrale du lobe antennaire chez les ancêtres communs aux Vespidae et Formicidae, puis la perte de deux clusters chez les Formicidae (**fig. 4, A**). Dans le second scénario, les Formicidae et les Apidae sont phylogénétiquement proches alors que les Vespidae sont plus distants. Le lobe antennaire de l'ancêtre commun à toutes ces familles serait donc complexe et arborerait au moins les neuf clusters observés chez les frelons, puis des pertes de 2 clusters chez les Formicidae et de trois clusters chez les Apidae se seraient produites à postériori (**fig. 4, B**). Ces deux scénarios indiquent que le lobe antennaire des aculéates ancestraux (Hyménoptères possédant un dard) devait déjà présenter une complexité élevée, caractérisée par un grand nombre de compartimentations.

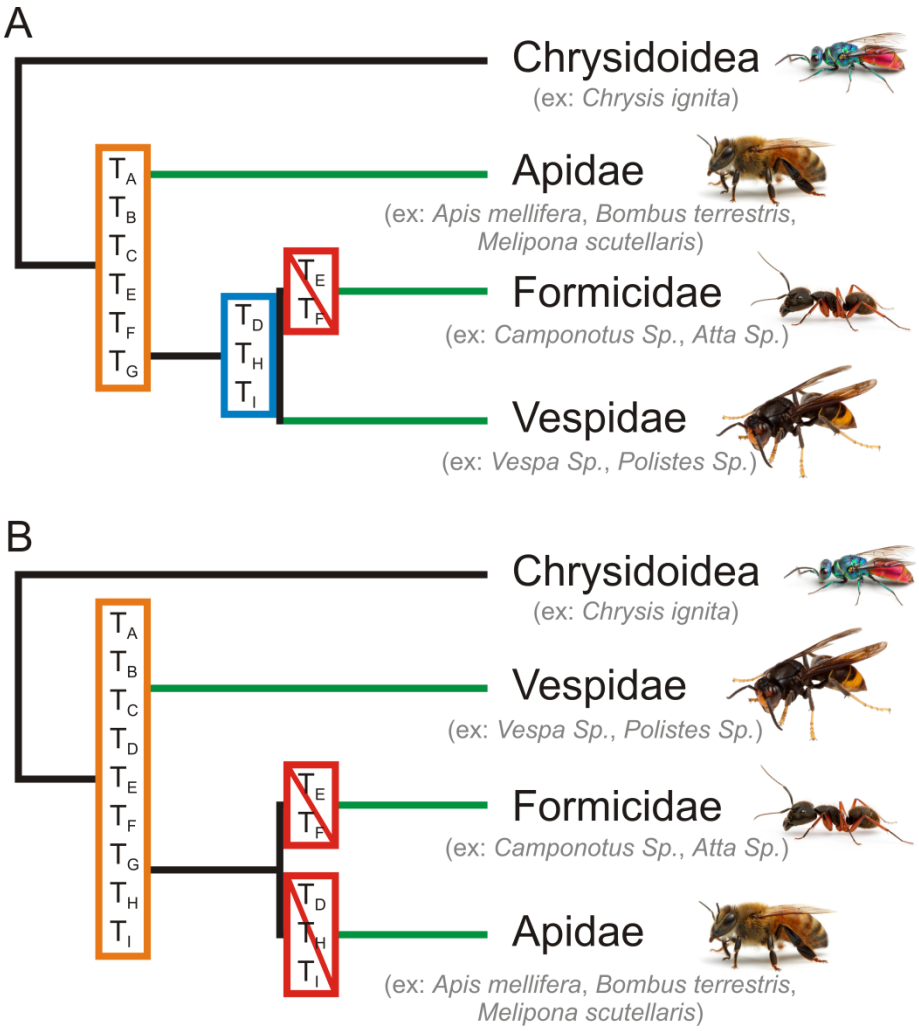


Figure 4: Relations phylogénétiques entre diverses familles d'Aculéates. **A**) Relations phylogénétiques selon Brothers (1999) entre les Apidae, les Formicidae et les Vespidae. **B**) Relations phylogénétiques selon Johnson *et al.* (2013). Les branches vertes représentent les familles au sein desquelles l'eusocialité est apparue. La super famille des Chrysidoidae est indiquée en tant que groupe externe. Pour chaque phylogénie, les clusters glomérulaires potentiellement présents chez l'ancêtre commun aux 3 familles eusociales (Apidae, Formicidae et Vespidae) sont encadrés en orange. L'hypothétique apparition de 3 clusters est encadrée en bleu (**A**) alors que les pertes sont encadrées en rouge. Ces pertes et ajouts ont été estimés selon l'explication la plus parcimonieuse aboutissant à nos observations.

d. *Le système olfactif des Hyménoptères a-t-il favorisé l'émergence de l'eusocialité ?*

L'eusocialité est le plus haut niveau d'organisation sociale dans lequel l'ensemble des membres d'une colonie coopèrent à l'exécution des tâches nécessaires pour la survie de cette même colonie. Dans *l'Origine des espèces*, Charles Darwin identifia l'évolution de l'eusocialité comme un potentiel contre-argument à sa théorie de la sélection naturelle (Darwin, 1859). En observant la structure sociale des colonies de fourmis, il s'interrogea notamment sur les facteurs qui auraient pu favoriser l'apparition de castes stériles. En effet, l'apparition d'individus altruistes, allant jusqu'à réduire leur propre potentiel reproductif pour s'occuper de la progéniture des autres, représentait un paradoxe à la sélection naturelle si eux-mêmes ne produisaient pas de descendance (Herbers, 2009 ; Nowak *et al.*, 2010). En d'autres termes, comment des traits désavantageux pour son porteur mais avantageux pour ses congénères ont pu se transmettre dans un processus de sélection naturelle ? Darwin proposa que dans des contextes particuliers, l'unité d'action de la sélection naturelle pouvait passer de l'individu à la colonie (Darwin, 1859). Près d'un siècle plus tard, William D. Hamilton avança une théorie basée sur des principes génétiques pour expliquer l'évolution des comportements altruistes propres à l'eusocialité (Hamilton, 1964a). Il mit en avant que puisque les individus apparentés partagent les mêmes gènes, les comportements altruistes peuvent apparaître et se transmettre s'ils bénéficient aux congénères, via le processus de sélection de parentèle (ou « kin selection » en anglais) (Lehmann *et al.*, 2007 ; Nowak *et al.*, 2010 ; Strassman *et al.*, 2011).

Cette théorie semble particulièrement bien adaptée pour expliquer l'évolution de l'eusocialité chez les Hyménoptères car ceux-ci sont haplodiploïdes (Hamilton, 1964b ; Trivers & Hare, 1976). Ainsi, dans le cas le plus simple d'une espèce monoandre, le coefficient de parenté entre sœurs est généralement de 0,75 alors qu'il n'est que de 0,5 entre un individu et sa descendance (**fig. 5**). De ce fait, une femelle d'Hyménoptère a plutôt intérêt à favoriser la survie de ses sœurs avec lesquelles elle partage une grande partie de son génome, plutôt que la survie de sa propre progéniture. Ce système de détermination sexuelle donne donc à l'altruisme une forte pertinence pour voir les gènes d'un individu être transmis de manière indirecte aux générations suivantes, en favorisant la progéniture des individus apparentés. Par conséquent, si cette théorie est exacte, les Hyménoptères auraient des prédispositions génétiques (haplodiploïdie) favorisant la transmission des comportements altruistes, ce qui pourrait expliquer les multiples apparitions indépendantes de l'eusocialité au sein de cet ordre (jusqu'à neuf fois ; Hughes *et al.*, 2008).

D'un point de vue neuroéthologique, ce modèle nécessite que les individus altruistes soient capables de distinguer les membres de leur parentèle des potentiels intrus, afin de favoriser la transmission de leurs propres gènes plutôt que ceux d'un tiers (Tsutsui, 2013). Il a été démontré chez de nombreuses espèces d'Hyménopètres aussi bien sociaux que solitaires que la reconnaissance intra- et interspécifique se fait grâce aux profils d'hydrocarbures cuticulaires (Gamboa & Reeve, 1986 ; Howard & Blomquist, 2005 ; D'Ettore & Lenoir, 2010). Ces substances permettent, entre

autres, chez les insectes eusociaux, de maintenir l'intégrité coloniale par le rejet des individus étrangers (Smith *et al.*, 2009 ; Richard & Hunt, 2013). Chez les fourmis charpentières, la détection à faible distance de ces composés peu volatiles semble se faire grâce à un sous-système olfactif spécifique, impliquant les sensilles basiconiques (Ozaki *et al.*, 2005 ; Brandstaetter *et al.*, 2008 ; Nishikawa *et al.*, 2012 ; Sharma *et al.*, 2015). Comme mentionné précédemment, nous avons démontré la présence d'un sous-système homologue chez *V. velutina* (**chapitre 2**) et par extension chez les frelons en général (**chapitre 3**), correspondant aux glomérules du cluster T_B. Puisque l'eusocialité est apparue de manière indépendante chez les Vespidés et les Formicidés (Hines *et al.*, 2007 ; Pickett & Carpenter, 2010), ces résultats suggèrent que ce sous-système olfactif aurait été hérité d'un ancêtre commun solitaire et pourrait représenter une préadaptation à l'eusocialité (voir aussi **fig. 4**). De manière intéressante, l'étude des projections des neurones sensoriels provenant des sensilles basiconiques chez l'abeille *Apis mellifera*, indique qu'une version très réduite d'un tel sous-système pourrait être également présente chez les Apidés (Kropf *et al.*, 2014). Ce constat nous a amenés à émettre l'hypothèse que ce système neuronal pourrait être plus largement représenté chez les Hyménoptères que ce qu'on pensait jusqu'à présent.

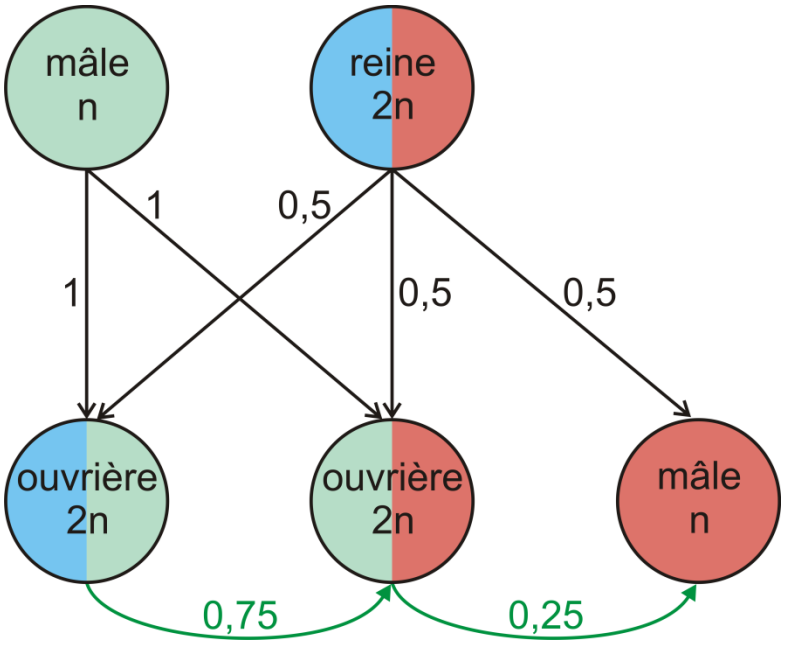


Figure 5: Système de détermination sexuelle haplodiploïde.
Chez les Hyménoptères, les femelles (diploïdes, 2n) sont issues d'œufs fécondés alors que les œufs non-fécondés donneront des mâles (haploïdes, n). Par conséquent, le coefficient d'apparentement moyen (en vert) entre deux ouvrières issues des mêmes parents est de 0,75 puisqu'elles ont en commun la moitié de leur génome provenant du père haploïde (1) et une probabilité de 0,25 (0,5 x 0,5) de partager l'autre moitié de leur génome, provenant de la mère. Par contre, le coefficient d'apparentement moyen entre un individu et son hypothétique descendance n'est que de 0,5. Le coefficient d'apparentement moyen entre une ouvrière et un mâle issus de la même mère est quant à lui de 0,25.

Chez les Hyménoptères solitaires, la détection des hydrocarbures cuticulaires par l'intermédiaire des sensilles basiconiques pourrait jouer rôle important dans la reconnaissance interspécifique, notamment chez les espèces prédatrices spécialistes (Anton & Gnatzy, 1998) ou chez les parasitoïdes (reconnaissance de l'hôte). Cette discrimination fine des profils cuticulaires, déjà présente chez des espèces solitaires ancestrales (Kather & Martin, 2015), aurait pu permettre l'émergence de l'altruisme et la formation de groupes sociaux suivant le modèle d'Hamilton (Hamilton, 1964a). Il a été montré que la monoandrie, maximisant le coefficient de parenté au sein

des colonies, est aussi un caractère ancestral chez les Hyménoptères sociaux (Hughes *et al.*, 2008). Ainsi, les pressions de sélection s'exerçant sur les capacités de discrimination des congénères auraient pu par la suite se relaxer chez les espèces sociales polyandres comme *Apis mellifera*, aboutissant à une atrophie de ce système neuronal et à une fermeture coloniale moins stricte.

Pour l'instant, ce scénario demeure extrêmement spéculatif et une étude comparative à large échelle ayant pour but de chercher les traces d'un sous-système homologue au T_B chez de nombreux Hyménoptères, aussi bien sociaux que solitaires, sera nécessaire pour étayer ou réfuter ces hypothèses. Puisque ce sous-système semble avoir divergé chez les Apidae (abeilles) par rapport à celui qui peut être observé chez les Vespidae et les Formicidae, il serait par exemple intéressant de chercher la présence d'un cluster correspondant au T_B dans le lobe antennaire des Sphecidae ou des Crabronidae. En effet, ces familles appartiennent à la même super famille (Apoidea) que les Apidae et il est supposé que les espèces représentantes de ces familles porteraient des caractères ancestraux aux Apidae (Lomholdt, 1982 ; Brothers, 1999 ; Debevec *et al.*, 2012). Si nos hypothèses se confirment, le sous-système T_B serait présent dans le lobe antennaire de ces familles et pourrait représenter une préadaptation importante du système olfactif des Hyménoptères, potentiellement impliquée dans le processus évolutif de sélection de parentèle.

D'un point de moléculaire, le répertoire de gènes codants pour les récepteurs olfactifs semble beaucoup plus étendu chez les Hyménoptères sociaux que chez les espèces solitaires (Zhou *et al.*, 2015). Ces expansions du nombre de récepteurs olfactifs ont logiquement été associées à une augmentation des capacités de discrimination olfactive nécessaires pour distinguer les nombreuses phéromones présentes au sein des colonies (LeBoeuf *et al.*, 2013 ; Zhou *et al.*, 2015). Parmi ces gènes, la famille des récepteurs à 9 exons semble particulièrement étendue chez les fourmis et il a été proposé qu'elle puisse correspondre aux récepteurs aux hydrocarbures cuticulaires (Smith *et al.*, 2011a ; 2011b ; Zhou *et al.*, 2012 ; Engsontia *et al.*, 2015). Il est intéressant de constater que chez l'espèce dont le système olfactif a été caractérisé à la fois du point de vue anatomique et moléculaire (*Camponotus floridanus*), le nombre de glomérules du T_B (env. 128) est en adéquation avec le nombre de gènes de récepteurs olfactifs à 9 exons (env. 115) (Zube *et al.*, 2008 ; Engsontia *et al.*, 2015). A l'inverse, chez *Apis mellifera,* le nombre de ces gènes (env. 34) est bien plus élevé que le nombre de glomérules (env. 12) du cluster que nous avons supposé être homologue au T_B (Kirschner *et al.*, 2006 ; Nishino *et al.*, 2009 ; Kropf *et al.*, 2014 ; Engsontia *et al.*, 2015). Le lien entre cette famille de récepteurs avec la détection des hydrocarbures cuticulaires reste donc encore à démontrer. En utilisant des techniques d'expression hétérologue pour exprimer ces gènes dans des ovocytes de xénope ou dans le système de « neurone olfactif vide » de drosophile, nous pourrions alors déterminer le spectre de réponse aux odorants de ces récepteurs par enregistrement électrophysiologique (Wetzel *et al.*, 2001 ; Dobrista *et al.*, 2003 ; de Fouchier *et al.*, 2015). De plus, les progrès récents en matière de transgénèse grâce à l'implémentation du système CRISPR-Cas 9

pourraient permettre de cartographier l'expression des récepteurs, à l'aide de constructions « OR-protéine fluorescentes », afin de vérifier le lien entre glomérules du T_B et la famille des gènes à 9 exons (Heidenreich & Zhang, 2016).

D'autres spécificités du système olfactif des Hyménoptères ont également été mises en relation avec l'émergence de l'eusocialité. D'une part, la double voie de sortie du lobe antennaire (m- et l-ALT) présente chez ces espèces pourrait représenter une préadaptation augmentant les capacités de discrimination des composés sémiochimiques (Galizia & Rössler, 2010 ; Rössler & Zube, 2011 ; Carcaud *et al.*, 2015). D'autre part, les premières études anatomiques du cerveau des insectes notèrent une forte variabilité de la taille des corps pédonculés chez les Hyménoptères, notamment entre les espèces sociales et solitaires, ce qui donna naissance à l'hypothèse d'un « cerveau social » chez les insectes (Dujardin, 1850 ; Farris, 2016). Cependant, cette idée est peu soutenue à ce jour, car des corps pédonculés complexes et volumineux peuvent également être trouvés chez des espèces non-sociales comme les blattes, les Lépidoptères ou certains Hyménoptères parasitoïdes (Newton, 1879 ; Sivinski, 1989 ; Mizunami *et al.*, 1998 ; Sjöholm *et al.*, 2005 ; Farris, 2016). L'augmentation de la taille des corps pédonculés chez les Hyménoptères semble être survenue chez ces parasitoïdes avec l'apparition de connections directes entre les lobes optiques et les calices (Farris & Schulmeister, 2011). Ainsi, les corps pédonculés n'ont pas évolué de façon concomitante avec l'eusocialité mais plutôt avec des capacités de traitement visuel. Cependant les corps pédonculés sont impliqués dans les processus de perception multimodale, de mémorisation et dans la résolution des tâches cognitives complexes. Des corps pédonculés volumineux pourraient donc tout de même être avantageux pour la prise de décision dans un contexte social particulier. En effet le mode de vie social nécessite l'intégration d'un ensemble complexe d'information provenant des différents individus de la colonie. Ainsi, la complexification des réseaux neuronaux au sein des corps pédonculés pourrait aussi représenter une préadaptation à l'émergence de l'eusocialité (Farris, 2016).

L'ordre des Hyménoptères semble donc être un groupe taxonomique particulièrement intéressant au sein duquel des études comparatives, tant du point de vue comportemental que neuroanatomique, fonctionnel et moléculaire, pourraient apporter des réponses pertinentes sur de grandes questions telles que l'évolution des comportements altruistes. A ce jour ces études sont menées de façon relativement parcellaire en ce qui concerne les espèces étudiés et les approches expérimentales. Il paraît donc important de définir des espèces modèles, en fonction de leur position taxonomique stratégique, dont les frelons pourraient faire partie en tant que représentants des Vespidés eusociaux.

2) Mécanismes évolutifs

Etant donné que le système olfactif est à l'interface entre l'animal et son environnement, il est sujet à de fortes pressions de sélection qui sont dépendantes de l'importance des différents composés chimiques pour la biologie de l'espèce (Hansson & Stensmyr, 2011). Ces pressions de sélection pourraient s'exercer à la périphérie du système olfactif en favorisant la conservation ou la diversification des récepteurs olfactifs, mais également au niveau central, en favorisant la conservation ou le réarrangement des réseaux neuronaux (Ramdya & Benton, 2010). L'évolution du lobe antennaire est généralement décrite comme suivant un processus continu de naissance et mort (processus de « birth-and-death » en anglais) de nouveaux récepteurs olfactifs, directement associés à l'émergence ou à la disparition de glomérules. Dans ce modèle, de nouveaux gènes codant pour des récepteurs olfactifs émergent par duplication d'un gène préexistant, due par exemple à des erreurs de recombinaison homologue (**fig. 6, A**). La diversification de ces gènes, du fait de mutations le long des séquences codantes et des séquences régulatrices (**fig. 6**, **B**), aboutirait ensuite au bourgeonnement de nouveaux glomérules par ségrégation des terminaisons axonales originaires d'un glomérule parental (Ramdya & Benton, 2010).

Si cette nouvelle entrée sensorielle ne présente aucune pertinence pour la biologie de l'espèce, celle-ci pourrait être rapidement contre-sélectionnée du fait du coût énergétique que représente le maintien des structures neuronales (Niven & Laughlin, 2008). Ce modèle évolutif d'apparition de nouveaux glomérules à partir d'un glomérule parental expliquerait pourquoi les gènes de récepteurs olfactifs possédant de fortes similarités de séquence tendent à être exprimés dans des neurones sensoriels ciblant des glomérules voisins (Couto *et al.*, 2005). Appliqué au lobe antennaire des Hyménoptères qui présente de multiples compartimentations, ce modèle suggère que les glomérules au sein d'une grappe (d'un cluster) pourraient être plus ou moins spécialisés dans la détection et le traitement d'une famille de composés odorants (Sachse *et al.*, 1999 ; mais voir Brandstaetter & Kleineidam, 2011). Nos résultats du **chapitre 2** vont dans ce sens car ils ont montré que les neurones sensoriels qui se projettent dans le cluster T_B répondent préférentiellement aux hydrocarbures à chaîne longue. Cependant, les champs récepteurs des glomérules de différents clusters doivent largement se chevaucher puisqu'il a été montré que l'information olfactive est traitée de façon fortement redondante dans les deux principaux hémilobes des abeilles (l- et m-ALT) (Brill *et al.*, 2013 ; Carcaud *et al.*, 2012).

Un résultat surprenant de cette thèse a été l'observation que malgré de larges différences dans le nombre de glomérules, le lobe antennaire de différentes espèces (**chapitre 1 et 3**) présentait tout de même des grappes morphologiquement similaires, formées par les faisceaux d'axones des OSN. Il est donc envisageable que les pressions de sélection n'agissent pas avec la même force ou

dans le même sens sur les différents clusters (ajout ou perte de glomérules) en fonction de la relative importance des odorants correspondants à ces clusters pour la biologie de chaque espèce.

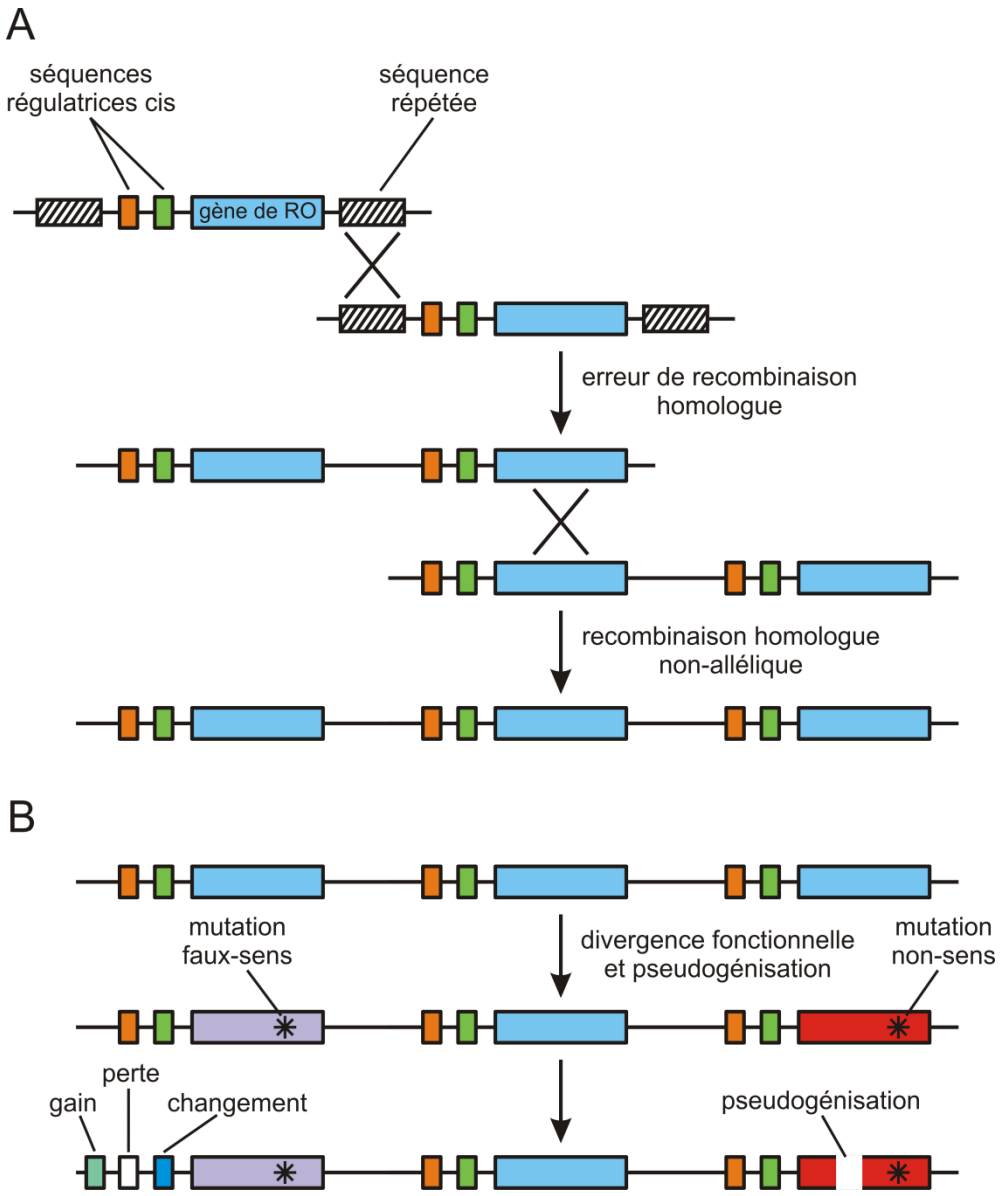


Figure 6: Expansion et diversification du répertoire de gènes codants pour les récepteurs olfactifs.
A) Représentation schématique d'événements amenant à la duplication d'un gène codant pour un récepteur olfactif (OR). Un gène de récepteur olfactif (rectangle bleu) dont l'expression est régulée par des séquences régulatrices cis, pourrait initialement être dupliqué par erreur de recombinaison en raison, par exemple, d'un mauvais alignement des séquences d'ADN répétées. Le nombre de copies pourrait encore s'étendre d'avantage par recombinaison homologue non-allélique. **B**) Conjointement avec les événements de duplication, les gènes d'OR peuvent se diversifier. Des mutations faux-sens peuvent changer la fonction du récepteur (rectangle mauve) alors que des mutations non-sens aboutissent à la pseudogénisation du gène d'OR (rectangle rouge). La divergence des séquences régulatrices peut aussi modifier l'expression du récepteur. L'acquisition d'un nouveau profil d'expression pourrait donc conduire à l'émergence d'une population d'OSNs exprimant le gène nouvellement dupliqué et se projetant dans un nouveau glomérule (D'après Ramdya & Benton, 2010).

Nos observations indiquent que de manière générale l'organisation des réseaux neuronaux au sein du lobe antennaire est un caractère phénotypique relativement conservé chez ces Hyménoptères (Kirschner *et al.*, 2006 ; Zube *et al.*, 2008 ; Kelber *et al.*, 2009 ; Nishino *et al.*, 2009 ;

Mysore *et al.*, 2009 ; Nakanishi *et al.*, 2010). Ce constat nous a amené à penser que cette organisation pourrait être relativement évolutivement stable à cause de la haute probabilité d'obtenir un comportement erratique des individus dès lors que des changements surviennent au sein des réseaux neuronaux. Par exemple, si l'on admet que la valence innée, attractive ou répulsive, d'un odorant est déterminée par les connections des neurones de projection au sein de la corne latérale (Knaden *et al.*, 2012 ; Fisek & Wilson, 2014 ; Knaden & Hansson, 2014 ; Roussel *et al.*, 2014), une réorganisation de ces projections pourrait vite être contre-sélectionnée si elle aboutissait à une attraction vers un stimulus toxique ou létal.

Bien entendu, dans un système dynamique, des modifications moins extrêmes du réseau neuronal apportant un avantage sélectif peuvent progressivement apparaître et se transmettre. Si nous prenons le cas particulier des macroglomérules, ceux-ci représentent une adaptation du réseau présente chez de nombreuses espèces pour une détection plus sensible des phéromones (Hansson & Stensmyr, 2011). Une fois la structure établie, l'avantage sélectif que procure cette adaptation pourrait favoriser sa conservation au cours de l'évolution. Ainsi, la présence d'un complexe macroglomérulaire à l'entrée du lobe antennaire semble être une caractéristique partagée par de nombreuses espèces de papillons de nuits, suggérant qu'il s'agisse d'un caractère ancestral dans cet Ordre (Rospars, 1983 ; Berg *et al.*, 2002 ; Vickers & Christensen, 2003 ; Skiri *et al.*, 2005 ; Lee *et al.*, 2006). De manière similaire, les trois macroglomérules en position rostrale (à l'opposé de l'entrée du nerf antennaire) innervés par le tractus T_F chez les mâles de *V. velutina* et d'*Apis mellifera* (**chap. 1 : fig. 5**) pourraient potentiellement avoir une origine commune (Arnold *et al.*, 1985 ; Nishino *et al.*, 2009). Cependant, même si ces structures dimorphiques (macroglomérules) ont une origine commune, il est peu probable que les composés phéromonaux utilisés par les abeilles et les frelons soient les mêmes. Les structures et le comportement d'attraction sexuelle auraient donc été conservés mais, du fait de mutations sur les gènes des récepteurs phéromonaux, ces macroglomérules seraient impliqués dans la détection d'odorants différents en fonction de l'espèce (Roelofs & Rooney, 2003 ; Vickers & Christensen, 2003 ; Groot *et al.*, 2016).

Ce processus évolutif expliquerait notamment qu'une phéromone sexuelle émise par une espèce puisse provoquer un comportement d'attraction chez les membres d'une autre espèce relativement proche comme cela a été observé chez les abeilles du genre Apis ou chez les frelons (Gary, 1962 ; Ono & Sasaki, 1987 ; Plettner *et al.*, 1997 ; Koeniger & Koeniger, 2000 ; Nagaraja & Brockmann, 2009). Des mécanismes d'isolement physique tel qu'un décalage des périodes de reproduction auraient alors pu relaxer les pressions de sélection sur la conservation du récepteur olfactif et permis sa dérive. A une échelle de temps plus large, impliquant une plus grande diversification des récepteurs phéromonaux tout en conservant les structures macroglomérulaires, ce processus pourrait, avec la séparation physique des populations, participer à l'isolement reproductif et la spéciation (Smadja & Butlin, 2008 ; Wicker-Thomas, 2011). Pour conclure, ces

observations et les résultats de notre travail suggèrent que l'organisation des réseaux neuronaux serait en général plus stable que les aspects moléculaires (récepteurs olfactifs) et que des structures homologues peuvent être observées dans le lobe antennaire d'espèces relativement éloignées phylogénétiquement.

III. Conclusion générale

Ce travail de thèse a permis de faire avancer notre connaissance du comportement olfactif et du système nerveux d'une espèce invasive en France, le frelon *Vespa velutina*. L'étude du comportement d'orientation des ouvrières en prédation a permis de montrer que cette espèce est capable de s'orienter sélectivement vers des signaux olfactifs indiquant la présence d'abeilles *Apis mellifera* en forte densité. Bien que ce travail ne représente qu'une première étape dans la compréhension du comportement de prédation de *V. velutina,* cette stratégie pourrait expliquer le nombre important de frelons observés devant les ruchers d'abeilles domestiques. Nos résultats semblent indiquer que cette attraction envers les odeurs de ruche pourrait être d'origine acquise et non innée. Ainsi, nos travaux ouvrent de nouvelles perspectives quant à l'étude des capacités d'apprentissage chez cette espèce. L'identification des signaux olfactifs qu'utilise *V. velutina* afin de s'orienter vers des sources de nourriture constitue une étape importante, non seulement pour la compréhension de son comportement de prédation, mais aussi pour la lutte contre cette espèce. Ces études pourraient à terme aboutir à la mise en œuvre de stratégie de protection des ruchers d'abeille domestique, à l'aide de leurres olfactifs perturbant les ouvrières *V. velutina* en prédation.

Ensuite, l'étude des voies neuronales impliquées dans le traitement de l'information olfactive chez les différentes castes de *V. velutina* a permis d'identifier des glomérules hypertrophiés dans le lobe antennaire des mâles. Ces macroglomérules représentent une adaptation typique du lobe antennaire des mâles de nombreuses espèces d'insectes et sont généralement impliqués dans le traitement des composés des phéromones sexuelles. L'identification des signaux sémiochimiques émis par les femelles fertiles, conjuguée à une étude fonctionnelle mesurant l'activité des macroglomérules, pourrait permettre d'isoler des composés phéromonaux attractifs pour les mâles de l'espèce *V. velutina*. Ces composés pourraient alors être utilisés pour perturber l'accouplement des frelons afin de diminuer l'effectif des populations invasives.

Par ailleurs, la caractérisation des voies nerveuses olfactives des frelons nous a également permis d'observer les similarités morphologiques existant avec les systèmes olfactifs d'autres familles d'Hyménoptère, nous permettant de commencer à reconstruire l'histoire évolutive du système olfactif de ces insectes. L'une de ces structures homologues semble être spécifiquement impliquée dans le traitement des hydrocarbures cuticulaires qui constituent des signaux de

reconnaissance cruciaux pour l'établissement des groupes sociaux. Nous pensons que le système olfactif des Hyménoptères (Aculéates) posséderait des préadaptations ayant favorisé les multiples émergences de l'eusocialité au sein de cet ordre.

L'étude du système olfactif de *V. velutina* nous a donc amenés à des questionnements évolutifs. Cependant le système olfactif et l'organisation du lobe antennaire n'ont été caractérisés que chez un nombre restreint d'espèces d'Hyménoptères. Ainsi, afin de mieux comprendre l'évolution du système olfactif des Hyménoptères et les potentielles adaptations à une niche écologique ou un mode de vie particulier, il conviendra maintenant d'étudier soigneusement et comparativement le lobe antennaire dans un grand nombre d'espèces voisines.

Finalement 130 ans après les travaux fondateurs d'Henri Viallanes (1887) sur le cerveau du frelon *V. crabro* et malgré les progrès impressionnants des techniques neuroanatomiques modernes, la démarche que ce chercheur proposait reste d'actualité : « *Ceci nous prouve qu'il ne faut espérer connaître bien le cerveau d'un Insecte que le jour où l'on aura étudié soigneusement et comparativement cet organe dans un certain nombre de types voisins. A un autre point de vue les recherches comparatives auront un intérêt considérable, car d'un ordre d'Insectes à l'autre, souvent même dans un même ordre, d'un genre à l'autre, nous voyons le cerveau présenter des différences vraiment inattendues. » […] « Un jour peut-être saurons-nous quels rapports existent entre le perfectionnement du cerveau et le perfectionnement des fonctions de l'Insecte? Mais aujourd'hui toute hypothèse serait téméraire; elle manquerait de base*. »

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ANNEXES

RESEARCH ARTICLE

Olfactory Pathway of the Hornet *Vespa velutina*: New Insights Into the Evolution of the Hymenopteran Antennal Lobe

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ABSTRACT

In the course of evolution, eusociality has appeared several times independently in Hymenoptera, within different families such as Apidae (bees), Formicidae (ants), and Vespidae (wasps and hornets), among others. The complex social organization of eusocial Hymenoptera relies on sophisticated olfactory communication systems. Whereas the olfactory systems of several bee and ant species have been well characterized, very little information is as yet available in Vespidae, although this family represents a highly successful insect group, displaying a wide range of life styles from solitary to eusocial. Using fluorescent labeling, confocal microscopy, and 3D reconstructions, we investigated the organization of the olfactory pathway in queens, workers, and males of the eusocial hornet *Vespa velutina*. First, we found that caste and sex dimorphism is weakly pronounced in hornets, with regard to both whole-brain morphology and antennal lobe organization, although several male-specific macroglomeruli are present. The *V. velutina* antennal lobe contains approximately 265 glomeruli (in females), grouped in nine conspicuous clusters formed by afferent tract subdivisions. As in bees and ants, hornets display a dual olfactory pathway, with two major efferent tracts, the medial and the lateral antennal lobe tracts (m- and l-ALT), separately arborizing two antennal lobe hemilobes and projecting to partially different regions of higher order olfactory centers. Finally, we found remarkable anatomical similarities in the glomerular cluster organizations among hornets, ants, and bees, suggesting the possible existence of homologies in the olfactory pathways of these eusocial Hymenoptera. We propose a common framework for describing AL compartmentalization across Hymenoptera and discuss possible evolutionary scenarios. J. Comp. Neurol. 524:2335–2359, 2016.

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Insects are influential models in the study of the evolution of olfactory processing and have revealed how selection pressures influence the organization and complexity of the olfactory system (Rospars, 1988; Kleineidam and Rössler, 2009; Ramdya and Benton, 2010; Hansson and Stensmyr, 2011). In insects, volatile molecules are detected by olfactory sensory neurons (OSNs) housed in cuticular sensilla on the antennae (Zacharuk, 1980). These neurons project to a primary olfactory center, the antennal lobe (AL), where they synapse onto local interneurons and projection neurons (PNs), within dense syn-

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aptic neuropils, the glomeruli (Hildebrand and Shepherd, 1997; Anton and Homberg, 1999; Vosshall et al., 2000). PNs convey the olfactory message processed within AL networks to higher order olfactory centers, the mushroom body (MB) and/or the lateral horn (LH).

Many previous works have revealed specific adaptations in AL organization, in relation to the particular biology and ecology of a wide range of insect species (Rospars, 1983; Martin et al., 2011; Clifford and Riffell, 2013). For instance, whereas the number of AL glomeruli is almost invariant among individuals of a given species, it varies greatly between species, which is thought to be related to the complexity of their olfactory environments (Anton and Homberg, 1999; Hansson and Anton, 2000; Kelber et al., 2009). Another striking example of such neuroanatomical and physiological adaptations is the existence of enlarged glomeruli, called *macroglomeruli*, dedicated to pheromone detection and processing (Burrows et al., 1982; Arnold et al., 1985; Hanson et al., 1992; Sandoz, 2006; Nishikawa et al., 2008; Nishino et al., 2012). In most cases, macroglomeruli are male dimorphic adaptations devoted to sex pheromone processing, but they have also been discovered in the females of some social Hymenoptera (Arnold et al., 1988; Kleineidam et al., 2005; Roselino et al., 2015). In leaf-cutting ants, for instance, a macroglomerulus found only in large workers is thought to process trail pheromone information (Kelber et al., 2009; Kuebler et al., 2010). Thus, the presence of hypertrophied glomeruli may inform about particular pheromonal communication channels in a species.

In some cases, neuronal adaptations of the olfactory pathway are found throughout a specific taxon. For instance, in Hymenoptera, uniglomerular PNs project to the MB and the LH through two parallel output tracts, the medial and the lateral antennal lobe tracts (m- and l-ALT). The AL is thus composed of two hemilobes which send their information separately to both higher order centers (Abel et al., 2001; Galizia and Rössler, 2010; Rössler and Zube, 2011; Rössler and Brill, 2013). Investigations in basal Hymenoptera showed that this neuronal organization is present in some sawfly (Symphyta) families but absent in others, leading to the hypothesis that it has evolved in basal Hymenoptera (Dacks and Nighorn, 2011; Rössler and Zube, 2011). The benefit of this parallel processing system and the selective pressures that favored its evolution remain unknown. Some authors hypothesized that it could represent a preadaptation to high demands on olfactory discrimination needed for particular life styles such as parasitism, central place foraging, or sociality (Galizia and Rössler, 2010; Rössler and Zube, 2011). These examples demonstrate that essential knowledge on the evolution of the insect olfactory system can be obtained from its comparative analysis in well-chosen taxa. Thus, comparing the organization of the AL and its specific adaptations in terms of glomerular number, size, and input–output connectivity may allow one to understand adaptations linked to changes in diet, host preference, or social life style in Hymenoptera (Rospars, 1983; Zube and Rössler, 2008; Kelber et al., 2009; Dacks et al., 2010; Ibba et al., 2010; Das and Fadamiro, 2013; Guidobaldi et al., 2014).

Social insects ensure colony defense and cohesion, division of labor, and behavioral regulation through a highly elaborate communication system (Vander Meer, 1998). Specific adaptations of the olfactory pathway can be observed among the different members of a eusocial insect colony, which are usually revealed through sex and caste polymorphism (Zube and Rössler, 2008; Groh and Rössler, 2008; Kuebler et al., 2010; Nakanishi et al., 2010; Stieb et al., 2011; Roselino et al., 2015). Eusociality has appeared several times in Hymenoptera, in at least three different families, Apidae (bees), Formicidae (ants), and Vespidae (wasps and hornets; Hines et al., 2007; Hughes et al., 2008; Cardinal and Danforth, 2011; Johnson et al.; 2013). Although possible adaptations of olfactory circuits related to the social life style have been investigated in several Apidae (honeybees, stingless bees; Arnold et al., 1985; Flanagan and Mercer, 1989; Galizia et al., 1999; Kelber et al., 2006; Nishino et al., 2009; Streinzer et al., 2013; Roselino et al., 2015) and Formicidae (several ant species; Nishikawa et al., 2008; Zube and Rössler, 2008; Mysore et al., 2009; Nakanishi et al., 2010; Stieb et al., 2011; Kuebler et al., 2010), Vespidae are poorly documented. This group, however, is a key group for studying the evolution of eusociality because it contains species with a wide range of social organizations, from solitary to eusocial (Hunt, 2007; Pickett and Carpenter, 2010).

In their highest level of sociality, Vespidae display a large repertoire of odor-guided behaviors, comparable to those of ants and bees, including kin recognition and cooperation for brood care, colony defense, and foraging (Spradbery, 1973; Edwards, 1980; Matsuura and Yamane, 1990; Oi et al., 2015). Hornets (*Vespa* sp.) are the largest eusocial wasps. They are generalist foragers that gather carbohydrates and proteins for their progeny, by exploiting flower nectar, tree sap, fruit pulp, and arthropod prey (Raver et Richter, 2000). For such task, they use odors emanating from the food sources but also intraspecific olfactory recruitment signals, which increase their foraging efficiency (Ono et al., 1995, 2003; Brodmann et al., 2009; Couto et al., 2014). Females can release scent marks that are used by both workers and males to locate the nest (Batra,

1980; Steinmetz et al., 2002; Tan, 2014). Within the nest, hornets live in organized colonies with a highly efficient task allocation system regulated through social pheromones acting on workers (Ikan et al., 1969; Ishay, 1973; Veith et al., 1984; Monceau et al., 2013). To prevent exploitation of such altruistic behavior by alien individuals, hornets are also able to discriminate nestmates and nonnestmates by means of cuticular hydrocarbons (Ruther et al., 1998; Ruther et al., 2002). In addition, queens produce a sex pheromone that triggers copulatory behavior in males (Batra, 1980; Ono and Sasaki, 1987; Spiewok et al., 2006). Therefore, both hornet males and females develop and live in a rich olfactory environment and use a wide range of inter- and intraspecific chemical cues (Bruchini et al., 2010).

To date very few works have described the central nervous system of Vespidae, and most studies date back more than a century (Flögel, 1878; Viallanes, 1887; Von Alten, 1910). Flögel (1878) proposed the first anatomical description of the wasp brain and observed striking differences in the morphology of its MBs compared with the brains of other eusocial Hymenoptera, such as ants and honeybees. This was confirmed by Viallanes (1887), who reported that despite the huge size of their MBs, the vertical (α) and medial (β) lobes appear atrophied. More recently, Ehmer and Hoy (2000) highlighted a strong anatomical divergence of MBs within this family, which could, however, not be related to the social organization level of the investigated species. Since then, neuroanatomical studies on the wasp or hornet brain have focused mainly on higher order centers (O'Donnell et al., 2004, 2011, 2015), but its olfactory pathway, and especially the AL, which may support important adaptations for foraging, pheromonal processing, and/or sociality, has been the subject of little consideration. According to Hanström (1928), the AL of social wasps would contain $\sim$1,000 small glomeruli arranged in multiglomerular layers, as in locusts (Hansson and Anton, 2000). In theory, such a high number of glomeruli would be consistent with the rich olfactory environment of these species. However, in light of recent data, serious doubts can be raised concerning Hanström's (1928) description (Kelber et al., 2009). First, none of the Hymenoptera studied so far displays such a high number of glomeruli, the maximum being 630 glomeruli in the ant *Apterostigma* cf. *mayri* (Kelber et al., 2009). Second, given current knowledge on the neural organization of the olfactory pathways of the different insect orders, it seems highly unlikely that the hornet AL presents a true locust-like organization, especially with regard to PN arborization. Whereas PNs are multiglomerular in locusts (Ignell, 2001; Ignell et al., 2001; Anton et al., 2002), Hymenoptera typically display two major tracts of uniglomerular PNs and three lesser tracts of multiglomerular PNs, which form a broad network in the protocerebrum (mediolateral ALT, ml-ALT; Abel et al., 2001; Kirschner et al., 2006; Galizia and Rössler, 2010; Rössler and Zube, 2011).

We performed a detailed neuroanatomical exploration of the olfactory pathway of a eusocial member of the Vespidae, the hornet *Vespa velutina*. This otherwise typical hornet species is particularly remarkable for its ecological success when introduced to new locations, such as in Europe, in which it has become an invasive species (Monceau et al., 2014; Arca et al., 2015). A nest founded by a single mated queen can produce up to 13,000 individuals, mostly sterile females (workers), which actively cooperate for the survival of the colony (Rome et al., 2015). They are vigorous predators of insects and very actively prey on honeybees, representing a threat for both local biodiversity and beekeeping activities (Monceau et al., 2014). Using fluorescent dye injection coupled with confocal microscopy, we compared AL features in queens, workers, and males. We asked whether the hornet olfactory system displays caste- or sex-specific adaptations, potentially related to the roles of the different members of a hornet colony. In particular, we looked for differences in the organization, size, and volume of AL glomeruli. Furthermore, from an evolutionary perspective, we investigated the input–output connectivity of the AL and compared it with the well-described olfactory pathways of ants and honeybees.

MATERIALS AND METHODS

Animals

Queens, workers, and males of the yellow-legged hornet, *Vespa velutina,* were used in the experiments. Queens and workers were obtained from field trapping in the area of INRA-Bordeaux Aquitaine (France) between April and November. Males were collected from wild nests in September in the same area. Males were identified by their specific morphological features such as long antennae (11 antennal segments instead of 10 in females) and the lack of a sting. Females were assigned to the reproductive or sterile caste by measuring their wing area according to Perrard et al. (2012). Wings were carefully flattened and clamped between two glass slides before capturing images with a film scanner (OpticFilm 7400, Plustek, Taipei, Taiwan). Wing size was evaluated in ImageJ (RRID:SCR_003070). A proxy measure of wing size was defined as the area between nine conspicuous vein intersections of the forewing (Fig. 1A, corresponding to landmarks 1–4, 9, 10, 13, 15, and 16 of Perrard et al., 2012). In hornets, queens are usually larger than workers

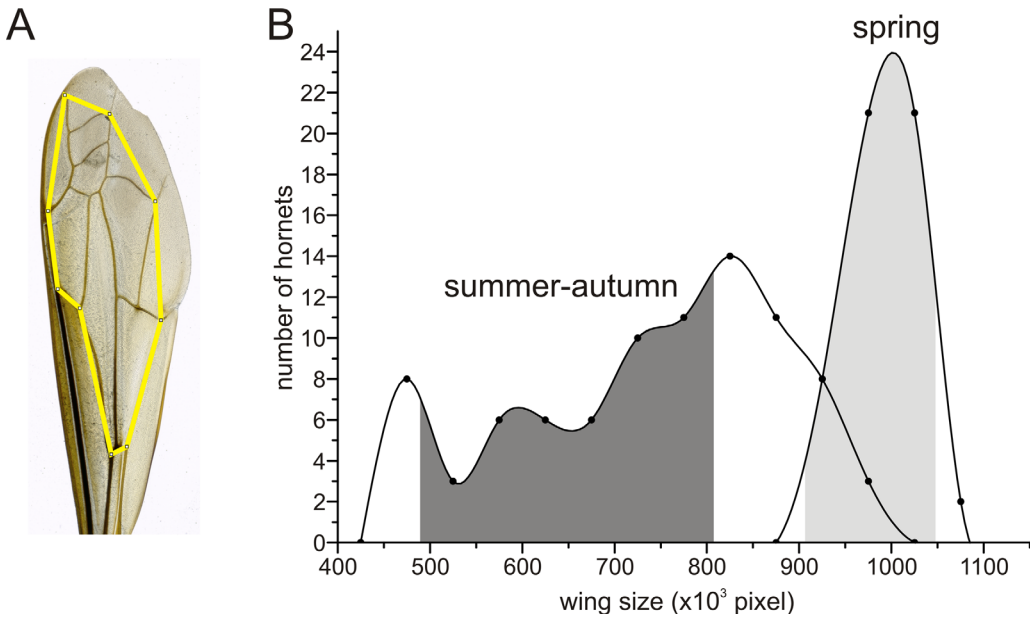

Figure 1. Wing size in *V. velutina* females. **A:** The area between nine vein intersections on the forewing (yellow polygon) was calculated as a proxy of wing size. **B:** Distribution of the wing sizes of all *V. velutina* females caught. Females caught in spring (April–May), at the start of the season, are necessarily founding queens. Females caught in summer–autumn (June–November) are mostly workers. Initially, workers are much smaller than queens. However, worker wing size increases throughout the colony cycle and may eventually (at the end of autumn) overlap with that of queens. Therefore, small individuals caught during summer and autumn (dark gray area) and large individuals trapped in spring (light gray area), which could be unambiguously assigned to workers and queens, respectively, were used in the experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Matsuura and Yamane, 1990). However, in *V. velutina*, this difference is not so clear-cut as in other species (Perrard et al., 2012). In particular, because of an increase in the size of emerging workers in the course of the colony cycle, wing areas of the two female castes may overlap in autumn, whereas they are clearly different in early summer. Therefore, females captured from April to June with a large wing area could be unambiguously assigned to queens, and small females captured from July to November could likewise be assigned to workers (Fig. 1B). Only such clearly identifiable individuals were used in this study (Fig. 1B; dark gray, workers; light gray, queens). For all experiments, hornets were anesthetized on crushed ice for 10 minutes before being placed in individual Plexiglas holders (see Sandoz et al., 2003, Fig. 1).

Neuroanatomical staining

Whole-brain neuropil staining

To visualize and reconstruct the hornet brain, Lucifer yellow was used as a neuropil background stain (Ai and Kanzaki, 2004; Rybak et al., 2010). The head of each hornet was fixed to the chamber with low-melting-point wax (Deiberit 502; Schöps and Dr. Böhme, Goslar, Germany), and the head capsule was opened. The brain was dissected out under phosphate-buffered saline (PBS) solution. It was then immediately transferred to 500 μl of fixative solution (4% paraformaldehyde [PFA] in PBS) containing 1 μl of 4% Lucifer yellow (Lucifer yellow CH, potassium salt, L-1177; Invitrogen, Eugene, OR) and kept for 4 days at 4°C.

Specific neuronal staining

To describe the glomerular organization of the antennal lobe, antennal sensory neurons were stained anterogradely. A window was cut into the scape of each antenna, and the antennal nerves were severed with a glass electrode loaded with crystals of fluoro-ruby (tetramethylrhodamine dextran, 10,000 MW, D-1817; Invitrogen; in 2% bovine serum albumin [BSA]). The preparation was then covered with saline solution (130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7) and kept for 24 hours in a dark room to let the dye diffuse. On the next day, the brain was dissected out and plunged into 4% PFA solution for 24 hours at 4°C.

For visualization of projections of antennal lobe tracts (ALTs) through the protocebrum toward higher order centers, a massive dye injection in the AL was performed. The hornet's head cuticle was removed by cutting a rectangle between the antennae and the compound eyes to expose the AL neuropil.

Subsequently, a glass electrode coated with fluoro-ruby was inserted into the AL and kept in this position until dissolution of the dye crystal, allowing uptake by injured AL neurons, including projection neurons (PNs). The electrode was then dislodged from the AL, and the brain was washed with saline solution to remove extra dye. As described above, preparations were kept for 24 hours in a dark room during dye migration before brains were dissected out and fixed in 4% PFA.

Double labeling

To assign AL glomeruli to one of the two main PN tracts (l-ALT, m-ALT or both), each tract was retrogradely labeled with different dyes in the same preparation. A piece of head cuticle was removed to expose the region between the ALs and the mushroom body calyces. The l-ALT was labeled by fluorescent tracer injection (dextran, Alexa Fluor 488, 10,000 MW, D-22910; Invitrogen; in 2% BSA) into the caudolateral area of the protocerebrum where the l-ALT runs between the AL and the lateral horn. The m-ALT was labeled by fluoro-ruby injection into the mediocaudal area of the protocerebrum, where the m-ALT runs between the AL and the MB. To avoid nonspecific staining, extra dye was removed after each injection by washing the brain with saline solution. After 24 hours of dye migration, the brains were dissected out and fixed in 4% PFA. To visualize olfactory and visual inputs to the MB calyces, AL projection neurons and visual output neurons were separately stained in the same preparation by mass tracer injections of Alexa Fluor into the AL and fluoro-ruby into the optic lobe (medulla and lobula).

Brain preparations

After being removed from the fixative solution, all brains were washed three times in PBS solution (10 minutes each), dehydrated in ethanol baths following an ascending series (50%, 70%, 90%, 95%, and 3 × 100% for 10 minutes each), and clarified in methylsalicylate (Sigma-Aldrich, Steinheim, Germany) for at least 3 days at 4°C. Brains were then mounted on aluminum slides with a central hole filled with methylsalicylate and covered by thin coverslips on both sides.

Confocal microscopy

Clarified brains were scanned from the ventral side with a laser scanning confocal microscope (LSM-700; Carl Zeis, Jena, Germany). Whole-brain optical sections were acquired at a resolution of 2.5 μm/pixels (x,y) with 3-μm intervals (z) using a water immersion objective (×10 achroplan 0.3 NA). AL optical sections were acquired at 0.52 μm/pixels (x,y) with 1-μm intervals (z) using a water immersion objective (×20 plan-apochromat 1.0 NA). Given the large size of the hornet brain, complete scans were obtained by stitching adjacent "tiles" (512 × 512 pixels) of optical sections with the tile function of the Zen software (Carl Zeiss; RRID:SCR_013672). Fluoro-ruby-labeled neurons were visualized with a 555-nm solid-state laser, whereas Lucifer yellow and Alexa Fluor were visualized with a 488-nm laser. Doubly stained neuropils were scanned sequentially to avoid emission overlap from distinct fluorescent dyes.

3D reconstructions and olfactory tract affiliation

Serial optical sections were saved as LSM files before adjusting brightness and contrast with ImageJ software and the Bio-formats plugin (RRID:SCR_000450). Adjusted pictures were then converted into TIFF files and imported into three-dimensional (3D) analysis software (Amira 5.4.3; FEI, Berlin, Germany; RRID:SCR_007353). Glomeruli were individually reconstructed by manual labeling in three planes (*xy*, *xz*, and *yz*) and using the Wrap function to obtain its 3D model. Larger structures, such as brain neuropils and macroglomeruli, were outlined in several stacks along one focal plane (*xy*), and the Interpolate function was used to create the 3D model.

Each reconstructed glomerulus was assigned to a glomerular cluster by closely following OSN bundles in the original scan. Because similar features such as input tract trajectories, glomerular morphologies, and output tract affiliations could be observed in different hymenopteran species, we attempted to unify olfactory tract nomenclature. Within each species, antennal sensory tracts had been previously named with numbers (T1–Tx), starting from the ventral and going to the dorsal surface of the AL (*Apis mellifera:* Suzuki, 1975; Flanagan and Mercer, 1989; Galizia et al., 1999; Kirschner et al., 2006; Nishino et al., 2009; *Camponotus* sp.: Zube et al., 2008, Nakanishi et al., 2010, Mysore et al., 2009; *Atta vollenweideri:* Kelber et al., 2010; *Cataglyphis* sp.: Stieb et al., 2011), resulting in a mismatch in tract designations between presumably homologous ones. The present study thus used a novel nomenclature, naming glomerular clusters with Roman letters (T_A–T_I), starting from the dorsal surface to the ventral surface because this arrangement better revealed similarities among different species. Among the species listed above, the hornet AL was especially compared with the honeybee and carpenter ant ALs because these species provide solid data on AL input–output connectivity. For honeybees, *A. mellifera*, we used the description of AL compartmentalization made by Kirschner et al. (2006) and Nishino et al. (2009) and our

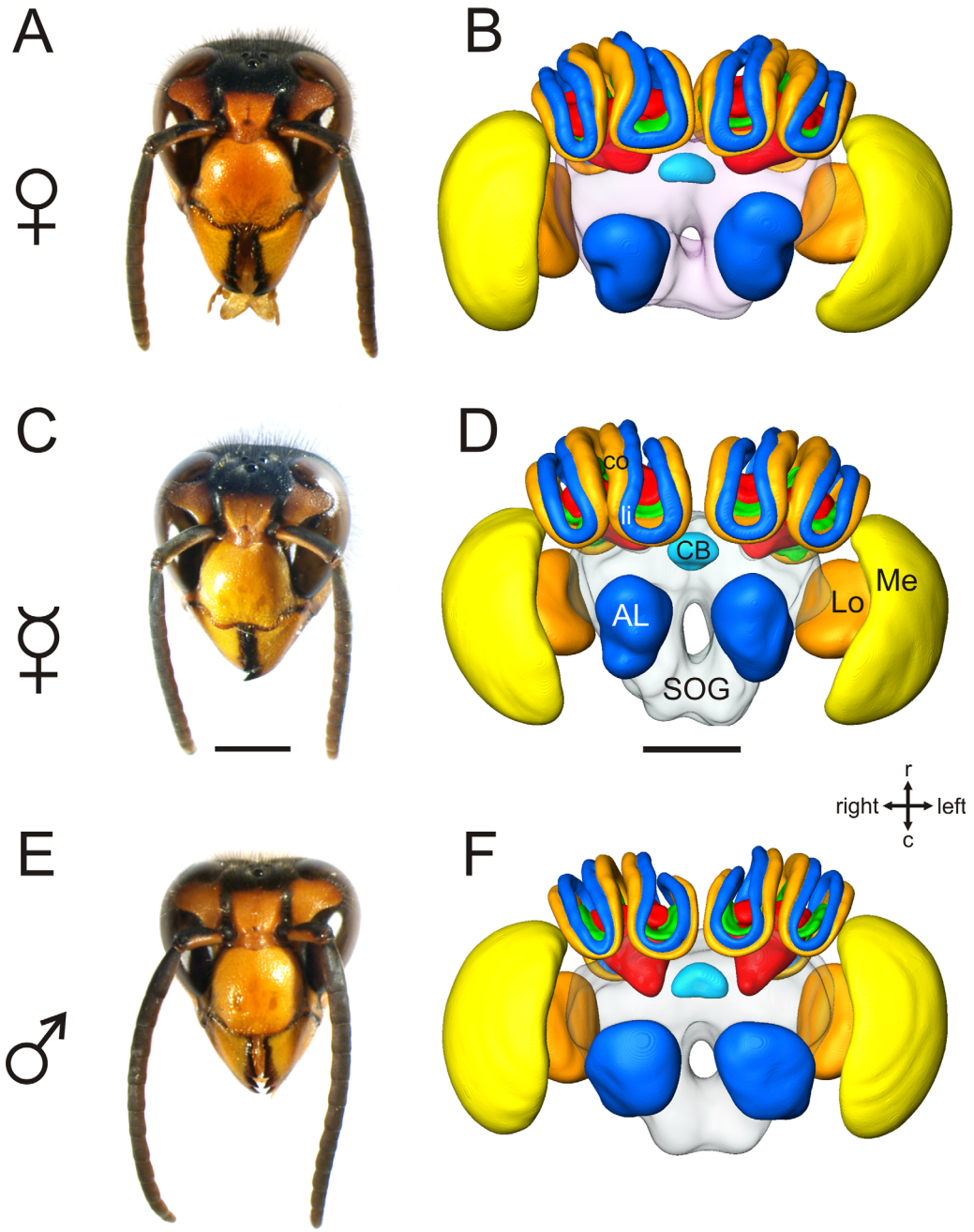

Figure 2. Head morphology and brain anatomy of the three main castes within a *V. velutina* colony. **A,C,E:** Photographs of *V. velutina* heads showing no noticeable morphological differences between queen (A) and worker heads (C) but a triangular head with longer antennae in males (E). **B,D,F:** 3D reconstructions of hornet brains. The brains of queen (B) and worker (D) do not show conspicuous differences in their organization, but slightly smaller calyces and thinner peduncles were observed in males (F). Heads and brains are shown from the ventral surfaces and are oriented following the same axis. r, Rostral; c, caudal; SOG, subesophageal ganglion; AL, antennal lobe; Me, medulla; Lo, lobula; CB, central body; li, lip; co, collar. Scale bars = 2 mm in C (applies to A,C,E); 500 μm in D (applies to B,D,F). Please click in the pdf file on D to activate the virtual content and then use the mouse to rotate the objects. Use the menu in the activated figure for further functions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

personal data. For carpenter ants, the descriptions by Zube et al. (2008) and Zube and Rössler (2008) for *Camponotus floridanus* and Nakanishi et al. (2010) for *C. japonicus* were used.

Spatial directions given in each figure are based on the neuraxis (Fig. 1H of Strausfeld, 2002). Consequently, rostral and caudal correspond, respectively, to anterior and posterior, according to the body axis. The

TABLE 1.
Volumes of the *V. velutina* Brain Neuropils[1]

	Protocerebrum	Medulla	Lobula	Antennal lobe	Collar	Lip	Basal ring	Peduncle	Central body	Total
Queen										
$\times 10^6$ μm^3	522.03	292.71	103.88	92.63	86.18	59.55	23.49	109.64	3.36	1.293.48
Percentage	40.36	22.63	8.03	7.16	6.66	4.60	1.82	8.48	0.26	100
Worker										
$\times 10^6$ μm^3	442.80	291.68	92.16	84.08	109.15	55.12	22.20	85.85	3.76	1.186.82
Percentage	37.31	24.58	7.77	7.08	9.20	4.64	1.87	7.23	0.32	100
Male										
$\times 10^6$ μm^3	428.01	273.23	94.85	97.46	58.73	30.83	15.98	58.17	3.43	1.060.70
Percentage	40.35	25.76	8.94	9.19	5.54	2.91	1.51	5.48	0.32	100

[1]The absolute volume (in cubic micrometers) and the relative volume (in percentage) of major brain structures are given for each caste. For multiple structures (such as the collar), the sum of all instances of this structure is given.

"frontal" surface of the brain corresponds to its most ventral region in the neuraxis.

Data and statistical analysis

To assess caste dimorphism in the number of glomeruli, a Mann-Whitney U test was used on six reconstructed ALs from each hornet caste. Potential type I errors induced by multiple comparisons were reduced by using a Bonferroni correction ($\alpha_{corr} = 0.05/2 = 0.025$).

Glomerular volumes were calculated from 3D reconstructions in Amira 5.4.3. To overcome the problem of AL size variability in different individuals, the volume of each glomerulus was normalized with respect to the size of the AL (calculated as the sum of all glomerular volumes; Fig. 4D). To identify macroglomeruli a quantitative threshold that defines outliers according to 80% of the distribution of glomerular volumes was used:

$$V_{outlier} > V_U + k\ (V_U - V_L),$$

where V_U is the upper percentile (90%) and V_L the lower percentile (10%) of glomerular volume distribution. We used $k = 3$ as a conservative value that successfully categorized macroglomeruli in several hymenopteran species (Kuebler et al., 2010; Streinzer et al., 2013; Roselino et al., 2015). Thus, glomeruli whose volume was above this threshold were considered macroglomeruli.

RESULTS

General brain morphology

V. velutina males and females can be distinguished based on head and abdomen morphology, but there are no evident morphological differences between the two female castes (worker and queen; Fig. 2). Queens (Fig. 2A) are usually larger than workers (Fig. 2C), but the sizes of these two castes sometimes overlap because of the increase in the body dimensions of workers produced throughout the colony cycle. Males' heads (Fig. 2E) show a typical triangular shape that is more elongated than females' heads, with slightly more bulbous compound eyes. In addition, males' antennae are longer than those of females, with shorter scapes but 11 flagellum segments instead of 10 in females. Confocal image stacks acquired from whole-brain staining in each caste allowed 3D reconstruction of selected neuropils (Fig. 2B,D,F). No clear differences in brain structure were observed between sexes or between female castes, the different neuropils occupying similar relative volumes in all these individuals (Table 1). In particular, both male and female hornets display voluminous visual sensory structures, with 30.6% of brain volume occupied by the medulla and the lobula in the queen, 32.3% in the worker and 34.7% in the male. Likewise, the primary olfactory centers, the ALs, display only slight sexual differences in their relative sizes, with 7.2% and 7.1% of brain volume in queen and worker, respectively, and 9.2% in the male.

Hornets' mushroom bodies have a characteristic shape, with two highly voluminous calyces on each brain hemisphere and comparatively small peduncle and lobes (Fig. 2B,D,F). As in other Hymenoptera (bees, ants, etc.), the calyces are composed of three concentric and morphologically distinct areas: the collar, the lip, and the basal ring. However, in each calyx, the lips are placed more centrally compared with the collar, which bulges around the lip, resembling a double-lip from an external viewpoint (Fig. 2B,D,F). Differential mass staining of optic lobe and AL revealed that the collars are targeted by visual output neurons, whereas the lips receive only olfactory input (Fig. 3A,B). Our staining procedure revealed strong olfactory input in the hornet basal ring, but we could observe only sparse visual input in this area. Although this differential staining was not aimed at detailing visual sensory

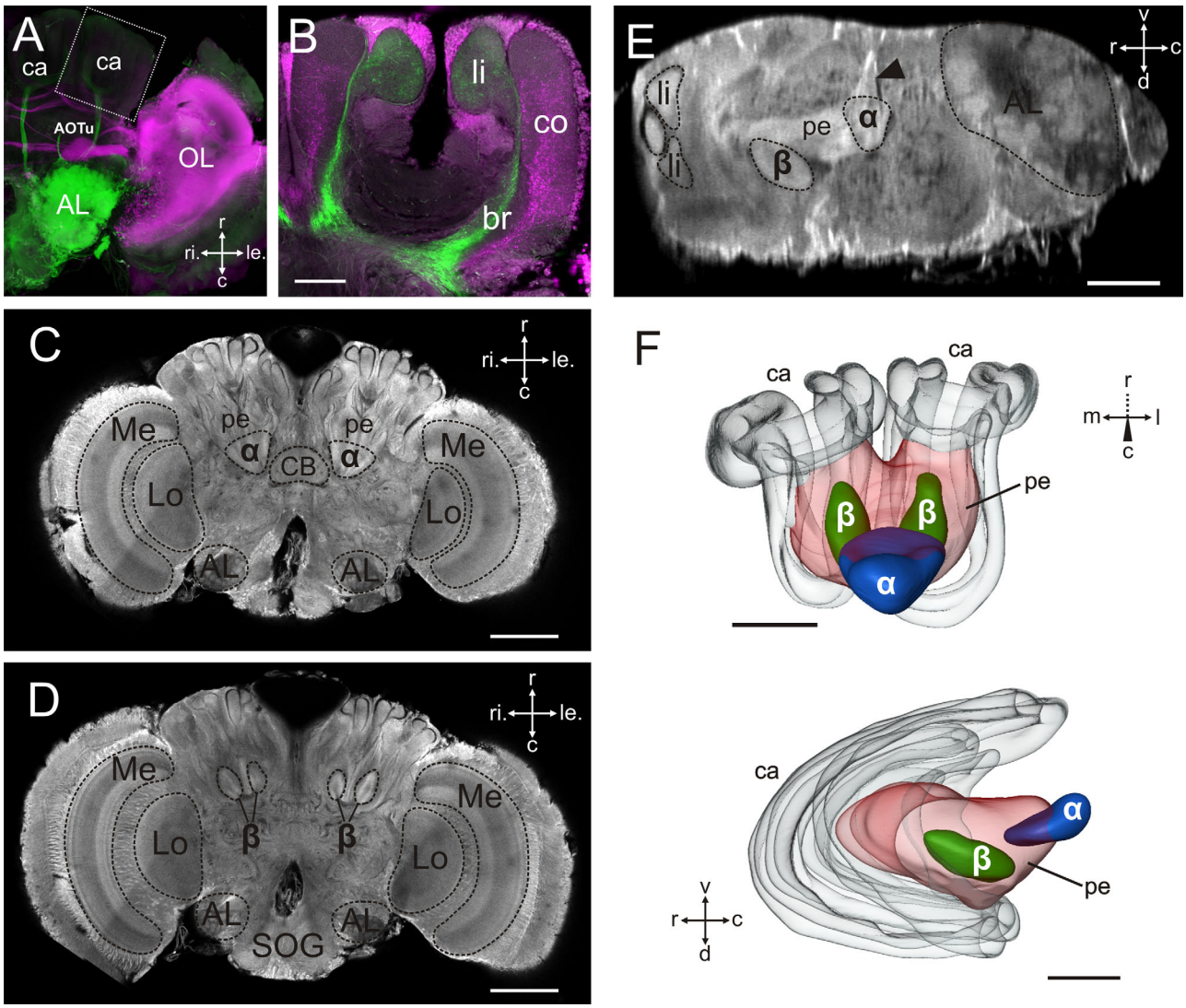

Figure 3. Mushoom body morphology in *V. velutina*. **A,B:** Frontal optical sections after mass staining in the optic lobe and in the AL with different fluorescent dyes. B: Section through the lateral mushroom body calyx (delimited in A). The collar receives visual input (magenta), whereas the lip receives olfactory input (green). **C,D:** Frontal sections through the brain at, respectively, 345 μm and 425 μm depths from the ventral surface. **E:** Sagital section of the brain. Each brain hemisphere contains a double β-lobe pointing dorsally and a single α-lobe projecting caudally, with only a fiber tract extending to the ventral surface (arrowhead). **F:** 3D model of a hornet mushroom body, showing the double β-lobe and the single α-lobe per hemisphere (left mushroom body). AL, antennal lobe; OL, optic lobe; Me, medulla; Lo, lobula; pe, peduncle; ca, calyces; li, lip; co, collar; CB, central body; r, rostral; c, caudal; v, ventral; d, dorsal; m, medial; l, lateral; ri, right; le, left. Scale bars = 80 μm in B; 400 μm in C,D; 200 μm in E,F. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

projections in the hornet brain, it suggested an arrangement of these projections similar to that in honeybees (Mobbs, 1984; Ehmer and Gronenberg, 2002; Mota et al., 2011). In brief, a prominent visual tract, the anterior optic tract (AOT), projects into the anterior optic tubercle, whereas in the rostral part of the brain a neuronal network composed of several subtracts connects the medulla and lobula with ipsi- and contralateral MB calyces. Direct interhemispheric connections between optic lobes were observed in more dorsal layers of the brain. At this level, diffuse ipsilateral projections into the dorsal protocerebrum are also found.

Hornets' MB lobes are clearly different from those described for honeybees and ants (Mobbs, 1982, 1984; Ehmer and Gronenberg, 2004; Gronenberg, 2008). In contrast to bees' or ants' MB in which the α-lobe extends vertically to the ventral surface of the brain, the *V. velutina* α-lobe points horizontally in a caudal direction, and only a fine fiber tract attributed to MB

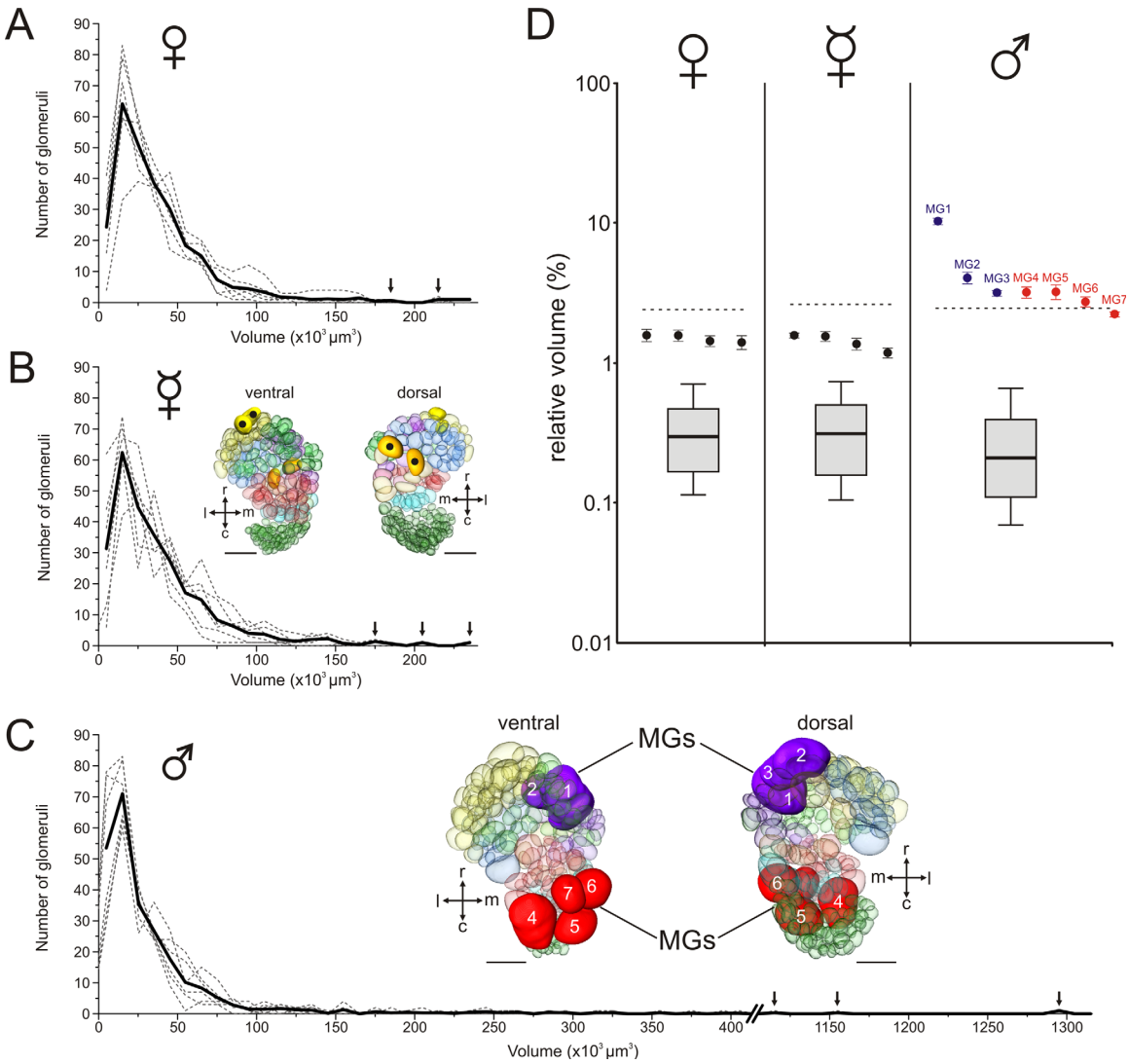

Figure 4. **A–C:** Glomerular volume distributions in the AL of six individuals (dotted lines) of each caste and their mean distributions (solid line). Queens (A) and workers (B) exhibit the same glomerular volume distribution. The largest glomeruli were identified at the same locations in the antennal lobes of workers and queens (black dots in B; see also Figs. (7 and 8D,E). In males (C), the upper part of the distribution extends to much greater volumes than in female castes. The AL of males contains seven hypertrophied glomeruli arranged in two clusters (MG1–7). Coloring of glomeruli relates to glomerular clusters as in Figure 7. **D:** Relative glomerular volume distribution in the three castes and positions of the largest glomeruli with regard to the statistical macroglomerulus threshold (dotted line). Dots indicate the average volume of the four (workers and queens) or seven (males) largest AL glomeruli of each caste. Only males possess glomeruli exceeding the statistical threshold. Bold lines indicate the median of glomerular volumes, boxes the first and the third quartiles (25–75%), and whiskers the 10th to 90th percentiles of the distribution. MGs, macroglomeruli; r, rostral; c, caudal; m, medial; l, lateral. Scale bars = 100 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

extrinsic neurons (see Ehmer and Hoy, 2000) extends to the ventral surface (Fig. 3C,E). Moreover, whereas honeybees' or ants' β-lobes extend horizontally toward the brain midline, the hornet β-lobe is split into two subparts, which point dorsally (Fig. 3D,E), in much the same way as previously described for the wasp *Vespula germanica* (Ehmer and Hoy, 2000).

Overall, we did not observe any clear polymorphism in the hornet brain organization except for MB calyces and peduncles, which are slightly thinner in males than in females (Fig. 2F; 21.6% of brain volume in the queen, 23% in the worker, and 15.4% in the male). The brains of the two female castes are not visually distinguishable.

AL organization

Confocal microscopic observations after mass staining of the antennal nerve allowed the segmentation of hornets' ALs into glomerular units (n = 6 individuals

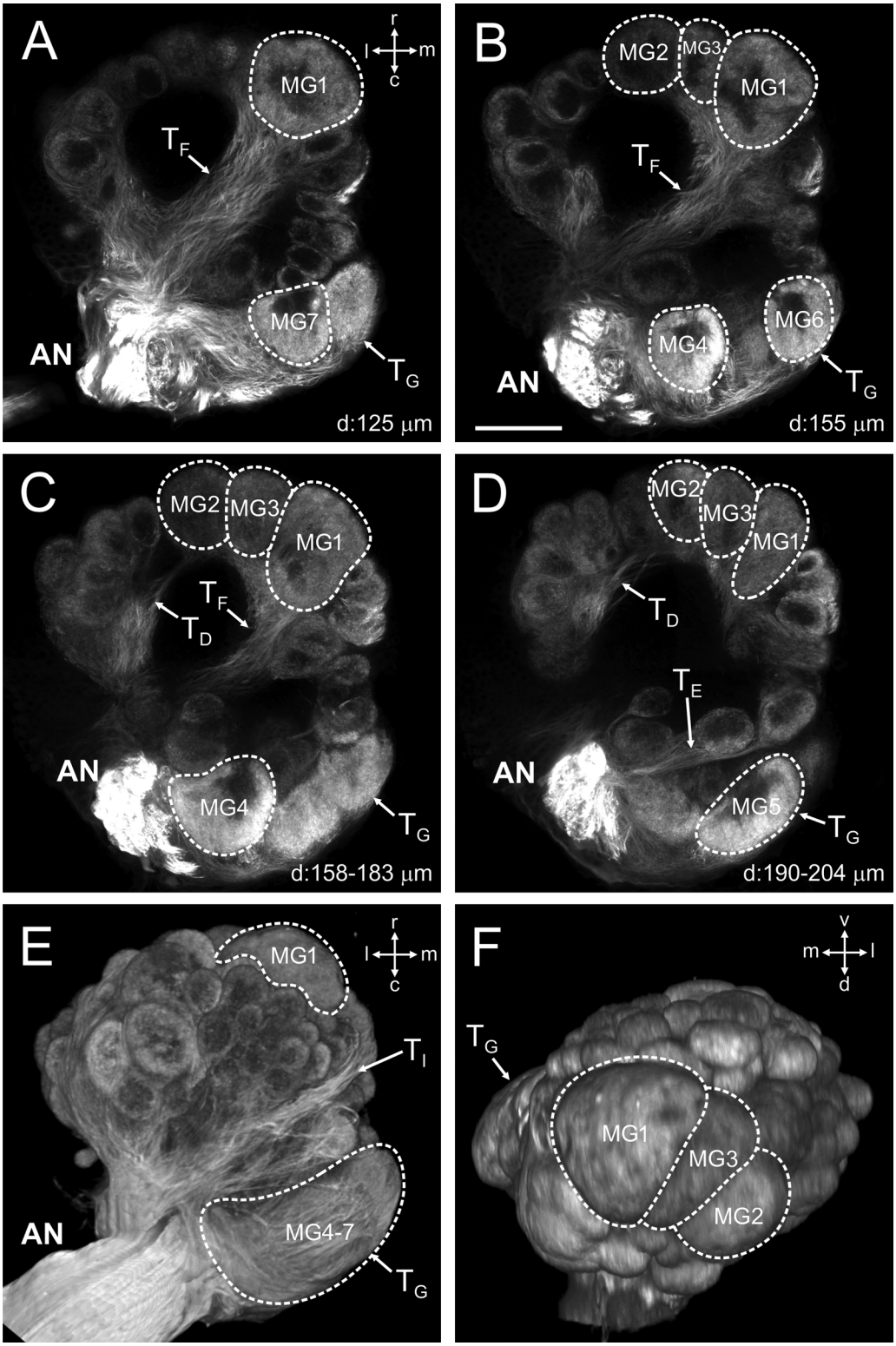

Figure 5. Macroglomeruli of the male antennal lobe. **A–D:** Confocal images (single section or z projection) at different depths from the ventral surface of a right AL in a *V. velutina* male. **E,F:** Ventral and rostral views, respectively, of a 3D volume rendering of the male AL. The macroglomeruli (dotted lines) are arranged in two clusters: the rostral cluster, receiving a thick olfactory tract through the AL, contains three macroglomeruli (MG1–3). The ventrocaudal cluster, located close to the antennal nerve entrance, contains four macroglomeruli (MG4–7). r, Rostral; c, caudal; v, ventral; d, dorsal; m, medial; l, lateral; AN, antennal nerve. Scale bar = 100 μm.

per caste). We found no difference between female castes in the number of AL glomeruli, with approximately 269 ± 9 glomeruli in queens (mean ± SD, n = 6) and 264 ± 7 glomeruli in workers (n = 6; Mann-Whitney test, U = 10, nonsignificant). We observed, however, a slight sexual dimorphism; the male AL contains 247 ± 6 glomeruli (n = 6), i.e., significantly fewer than in both female castes (Mann-Whitney test, male vs. queen, U = 1, $P < \alpha_{corr} = 0.025$; male vs. worker, U = 1, $P < \alpha_{corr} = 0.025$). From these 3D reconstructions, we measured the volumes of glomeruli in the ALs of each caste. Figure 4A–C shows individual and average distributions of glomerular volumes in queens, workers, and males (n = 6 in each group). In all castes, approximately 80% of glomerular volumes were distributed between 5×10^3 and 100×10^3 μm^3, most glomeruli displaying a volume of approximately 15×10^3 μm^3. In workers and queens, the largest glomeruli reached an absolute volume of approximately 225×10^3 μm^3. In males the distribution reached higher values, with the largest glomeruli approaching $1{,}300 \times 10^3$ μm^3. Thus, the three castes seem to share a common pattern of glomerular organization with the exception of a few substantially enlarged glomeruli in males. To determine whether these glomeruli are large enough to be termed *macroglomeruli*, we used a standard quantitative measure that defines outliers with regards to the distribution of glomerular volumes (Fig. 4D). In queens, as in workers, the four largest glomeruli are located in the most dorsal (two glomeruli) and in the ventrolateral regions (two glomeruli) of the AL (Fig. 4B, inset). They do not reach the statistical threshold and cannot be considered macroglomeruli. Similarly sized glomeruli are found at the same positions in males (see black dots, Fig. 7). By contrast, the male AL showed several enlarged glomeruli that reached the significance threshold (Figs. 4D, 5). They were grouped in two clusters, a rostral cluster containing the largest hornet macroglomerulus (MG1) and two smaller macroglomeruli (MG2, MG3), and a ventrocaudal cluster, positioned close to the antennal nerve, containing a group of four macroglomeruli. Three of them (MG4, MG5, and MG6) pass the threshold, but the fourth is marginally below the threshold. However, these four glomeruli show exactly the same OSN innervation pattern, which is much denser than that of all the other normal-sized glomeruli in this area. We thus consider this enlarged glomerulus as a possible macroglomerulus, termed *MG7*. We conclude from these observations that queens and workers have similar ALs without hypertrophied glomeruli, whereas males possess seven macroglomeruli distributed in two clusters.

AL afferent connection pattern

We next examined the precise projection pattern of OSNs in the AL of each caste. Because close inspection of projection patterns showed that queen and worker ALs are indistinguishable, they are often described together as "female" ALs hereafter. In hornets, the antennal nerve approaches the AL laterally from the ventral side. As in other Hymenoptera, part of the antennal nerve bypasses the AL and slopes down toward the antennal mechanosensory and motor center (AMMC). OSN axons separate from this main trunk at different depths with various orientations, forming nine distinct sensory tracts, which we termed T_A–T_I, from the most dorsal to the most ventral (Fig. 6). These tracts give rise to nine clusters of glomeruli, which display distinctive morphological features. Table 2 gives an overview of glomeruli numbers and volumes for each cluster.

1. In the most dorsal part of the AL, eight glomeruli are innervated by several loose bundle tracts (T_A) which were particularly brightly labeled in most preparations. These glomeruli are identifiable in the three castes by their typical shapes and positional arrangements (Fig. 6M–O). They are characterized by elongated or bent shapes and receive axon terminals penetrating the entire glomerulus. Their volumes are relatively large, especially for two of them that correspond to the largest female glomeruli (black dots in Fig. 7E,F).
2. The dorsocaudal region of the AL harbors a conspicuous cluster of numerous and comparatively smaller glomeruli than in the rest of the AL, rather lightly innervated in their cortex (Fig. 6J–O). This cluster is mostly responsible for the observed variability between sexes and among individuals in the total number of AL glomeruli. Because it was not brightly stained in some preparations, the variability observed in this cluster might be due in part to difficulties in discerning all glomeruli. We counted 93 ± 7 (mean ± SD) glomeruli there in queens, 89 ± 7 glomeruli in workers, and 71 ± 5 glomeruli in males. These glomeruli receive OSN axons from the T_B, which originates from the ventral side and points dorsomedially (Fig. 7E,F).
3. The T_C tract runs transversally through the AL, i.e., from the antennal trunk (on the lateral side) toward the dorsomedial side, determining a separation between the T_B cluster and the rest of the AL (Fig. 6G,H,J–L). It sparsely innervates approximately 15 glomeruli, mostly from their caudal periphery.
4. Facing this cluster, the T_D proceeds transversally through the inner dorsorostral part of the AL and

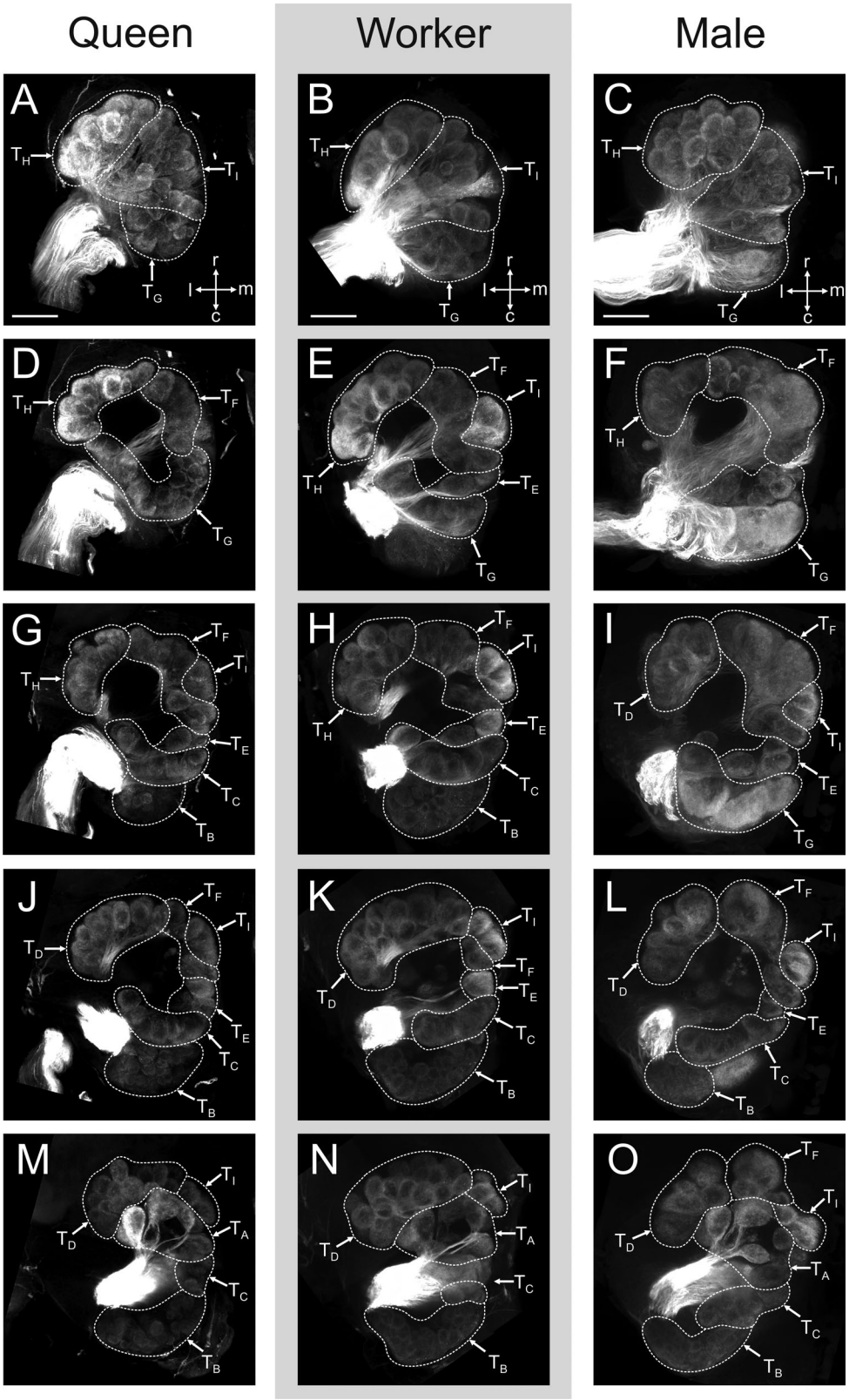

Figure 6. Sensory innervation of the AL of each caste. Projection views of approximately 50-μm-thick slices (~50 optical sections), starting from the ventral surface to the dorsal surface of a right AL after mass staining of the antennal nerve. The hornet AL contains nine sensory tracts (T_A–T_I) innervating distinct clusters of glomeruli. All antennal sensory tracts were observed in queens (**A,D,G,J,M**), workers (**B,E,H,K,N**), and males (**C,F,I,L,O**), but some tracts were thicker in males than in females (for instance, T_F in F compared with D and E). r, Rostral; c, caudal; m, medial; l, lateral. Scale bars = 100 μm in A (applies to A,D,G,J,M); 100 μm in B (applies to B,E,H,K,N); 100 μm in C (applies to C,F,I,L,O). The complete image stack of a worker AL is provided as Supporting Information online.

TABLE 2.
Cluster Characteristics in Hornets[1]

	T_A	T_B	T_C	T_D	T_E	T_F	T_G	T_H	T_I	Σ
Female										
Σ Glomeruli	8	90	15	29	3	22	33	29	37	266
Min volume ($\times 10^3$ μm^3)	51.3	3.3	11.3	10.5	58.9	4.6	8.1	2.5	10.3	
Max volume ($\times 10^3$ μm^3)	200.1	31.2	57.7	92.3	85.5	74.6	66.2	104.5	103.6	
Mean volume ($\times 10^3$ μm^3)	91.1	12.6	33.0	35.7	72.8	35.4	33.5	37.6	32.1	
Output tract	m-ALT (6 of 8)	m-ALT	m-ALT	l-ALT	m-ALT (2)/ both ALTs (1)	l-ALT	l-ALT (9)/ m-ALT (24)	l-ALT	l-ALT	m-ALT (138)/ l-ALT (127)
Male										
Σ Glomeruli	8	74	17	24	3	21	32	32	38	249
Min volume ($\times 10^3$ μm^3)	39.4	2.2	8.5	6.9	13.7	1.2	3.72	5.3	4.1	
Max volume ($\times 10^3$ μm^3)	136.1	25.2	47.4	250.7	38.4	763.1	285.6	87.3	62.9	
Mean volume ($\times 10^3$ μm^3)	67.1	8.9	21.8	44.1	25.8	90.9	51.3	30.8	22.7	

[1]Number, volume, and output tract affiliation of glomeruli belonging to the nine different clusters of the antennal lobe in female and male hornets.

densely innervates approximately 30 glomeruli in females and 25 glomeruli in males (Fig. 6I–O). We observed there a slight sexual dimorphism, with a thicker tract and relatively larger glomeruli in males, although none of them reached the macroglomerulus volume threshold (Table 2, Fig. 7E,F).

5. More ventrally, we found in both sexes a brightly labeled tract, T_E, that runs in the inner part of the AL, parallel to the T_C. This tract projects mediodorsally to only three glomeruli (Figs. 6E,G–I, 7C,D). The deepest T_E glomerulus shows a bent shape similar to that of T_A glomeruli and also receives axon terminals in its core region. It is, however, clearly innervated by the T_E tract.
6. The T_F is a thick tract that crosses the AL from the caudolateral side to the rostromedial side and innervates approximately 20 glomeruli in both males and females (Fig. 6D–F). Despite similar numbers of glomeruli between the sexes, the T_F is much thicker in males. It contains numerous axons that densely project to three macroglomeruli, MG1, MG2, and MG3, forming the rostral macroglomerular cluster (Fig. 7C,D).
7. Running at the ventral surface of the AL, the T_G separates from the antennal nerve and takes a mediocaudal direction. It innervates approximately 30 glomeruli mostly from the caudal periphery (Fig. 6A–F). In males, this tract is also thicker than in females and projects into four macroglomeruli close to the antennal nerve (MG4–7), forming the ventrocaudal macroglomerular cluster (Fig. 7A,B).
8. The T_H spreads from the antennal nerve to the rostral surface of the AL (Fig. 6A–H). This cluster consists of approximately 30 relatively large glomeruli and contains among the largest female glomeruli (black dots, Fig. 7A,B).
9. Finally, the T_I extends its axons within approximately 40 glomeruli from the lateral to the medial side of the AL, forming a cluster sided by T_G and T_H glomeruli (Fig. 6A–C). It innervates glomeruli on the ventral surface and then continues medially toward the dorsal side of the AL (see Fig. 6G–I).

AL efferent pathways

Mass staining of the AL with a fluorescent dye revealed that *V. velutina* has a typical hymenopteran olfactory pathway with five output PN tracts (Fig. 8A). They seem to correspond to the three multiglomerular (ml-ALT) and two uniglomerular (l- and m-ALT) PN tracts observed in other hymenopteran species (Abel et al., 2001; Kirschner et al., 2006; Zube et al., 2008). Among the ml-ALT tracts, two leave the AL medially and

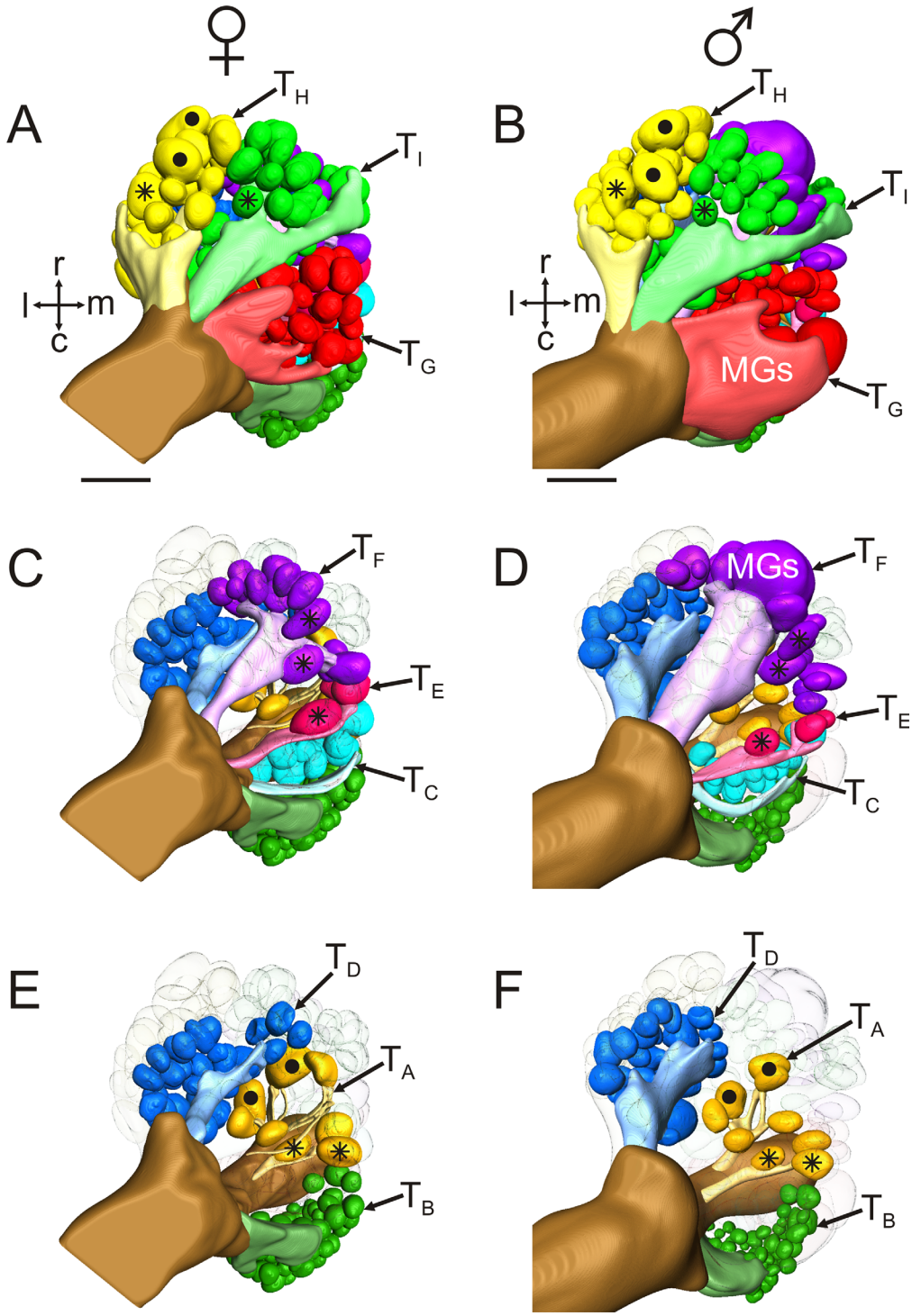

Figure 7. 3D reconstructions of the antennal lobes of *V. velutina* female (**A,C,E**) and male (**B,D,F**). The nine clusters of glomeruli and their afferent tracts (T_A–T_I) are color coded and are represented from the ventral to the dorsal surface (right AL). AL organization is highly similar in males and females, but sexually dimorphic clusters that contain macroglomeruli in males are innervated by thicker tracts (T_F and T_G). Dots indicate the four largest glomeruli in females and their homologs in males. Asterisks indicate landmark glomeruli. r, Rostral; c, caudal; m, medial; l, lateral. Scale bars = 100 μm in A (applies to A,C,E); 100 μm in B (applies to B,D,F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

project transversally to the lateral protocerebrum and the LH. They contribute to a lateral network, and their characteristics are highly similar to those of the honeybee ml-ACT1 and -2 (Abel et al., 2001; Kirschner et al., 2006). A third ml-ALT tract projects rostrally and bends around the fiber tract extending from the α-lobe,

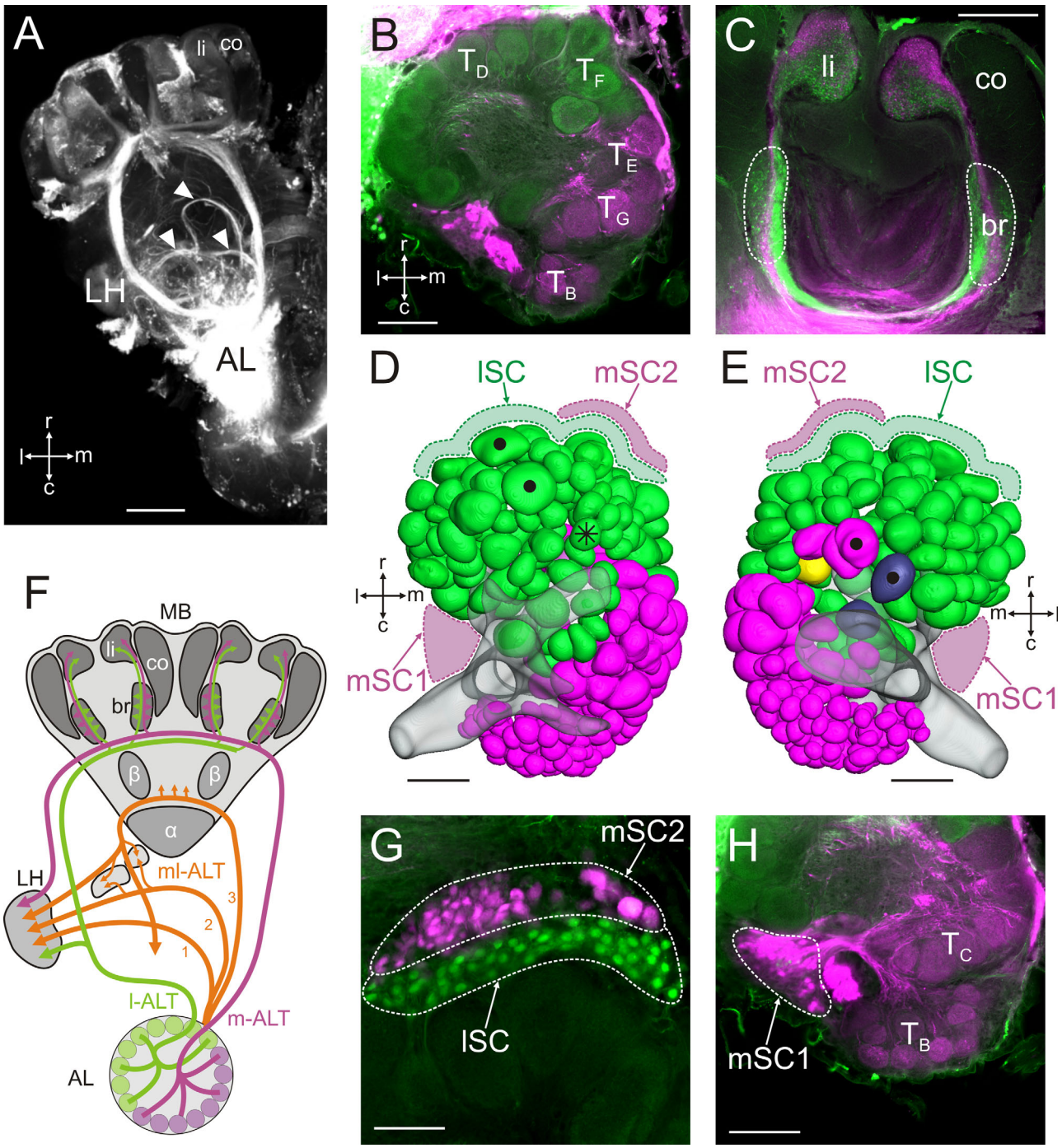

Figure 8. Efferent innervations of the AL of *V. velutina*. **A:** Projection view of an anterograde mass fill of all AL output tracts (300-µm-thick slice). Two prominent tracts (m- and l-ALT) project from the AL to the MB calyces and the LH, whereas three ml–ALTs (arrowheads) project broadly to the lateral protocerebrum and the LH. **B,C:** Optical section through the AL (B) and MB calyces (C) after differential labeling of the l-ALT (green) and m-ALT (magenta). The two output tracts arborize in segregated parts of the AL and project differentially to the lips and the basal ring of the MB calyces. **D,E:** 3D reconstruction of the *V. velutina* AL showing the affiliation of glomeruli with the two ALT output tracts (D, ventral view; E, dorsal view). Glomeruli innervated by the l-ALT (green) are in the rostroventral hemilobe of the AL, whereas m-ALT-innervated glomeruli (magenta) are located in the dorsocaudal hemilobe. Two glomeruli (dark blue) show no innervations from ALTs, whereas one glomerulus (yellow) receives processes from both m- and l-ALT. The somata of the l-ALT are spread along the rostral rim of the antennal lobe (ISC), whereas the somata of the m-ALT are distributed in a caudolateral cluster (mSC1) and a rostral cluster (mSC2). **F:** Schematic overview of the output network of the AL. **G:** Rostral somata clusters of l-ALT (ISC) and m-ALT (mSC2) neurons. **H:** Caudolateral somata cluster of the l-ALT (m-SC1). All views correspond to the right brain hemisphere. Scale bars = 200 µm in A; 100 µm in B–E,H; 50 µm in G. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 3.
Putative Homologies Between Glomerular Clusters in Three Well-Described Hymenopteran Species[1]

A. mellifera[2]			*V. velutina*			*C. floridanus*[3] and *C. japonicus*[4]		
Input tracts	Σ Glomeruli	Output tracts	Input tracts	Σ Glomeruli	Output tracts	Input tracts	Σ Glomeruli	Output tracts
T4	7	m-ALT/5 of 7	T_A	8	m-ALT/6 of 8	T7	6 or 10	m-ALT/5 of 6
T3b	12	m-ALT	T_B	90	m-ALT	T6	128 or 140	m-ALT
T3c	42	m-ALT	T_C	15	m-ALT	T5	36 or 35	m-ALT
			T_D	30	l-ALT	T4	78 or 95	l-ALT
T2	7	l-ALT/m-ALT/both	T_E	3	m-ALT/both			
T1	72	l-ALT	T_F	20	l-ALT			
T3a	24	l-ALT/m-ALT	T_G	30	l-ALT/m-ALT	T3	96 or 110	l-ALT/m-ALT
			T_H	30	l-ALT	T2	56 or 45	l-ALT
			T_I	40	l-ALT	T1	34 or 35	l-ALT

[1]Each line contains corresponding antennal sensory tracts in honeybees (*A. mellifera*), hornets (*V. velutina*), and carpenter ants (*C. floridanus* and *C. japonicus*), with reported numbers of glomeruli and their output tract affiliations. Empty cells indicate missing tracts in the given species. Putatively homologous tracts are aligned according to the new denomination implemented in hornets, from most dorsal to most ventral.
[2]After Kirschner et al. (2006) and Nishino et al. (2009).
[3]After Zube et al. (2008) and Zube and Rössler (2008).
[4]After Nakanishi et al. (2010).

sending collaterals toward the peduncle and the lateral network. This tract ramifies in several bundles, ending in the LH and in the caudal protocerebrum. Its trajectory and projections in the protocerebrum are therefore different from those of the honeybee's ml-ACT3 (Kirschner et al., 2006). Next to these ml-ALT PNs, two prominent tracts originate from the dorsal region of the AL and run through the protocerebrum along two widely differing routes, innervating both the LH and the MB calyces. Their course resembles greatly that observed in bees (Abel et al., 2001; Kirschner et al., 2006) and ants (Zube et al., 2008). One median tract (m-ALT) projects rostrally, then follows a mediolateral route to send first collaterals to both MB calyces and then continues onward to end in the LH. The lateral tract (l-ALT) bends laterally from the exit of the AL, first sends collaterals to the LH, and then continues rostrally, eventually forming multiple branches innervating the MBs calyces. We investigated the glomerular connectivity of these tracts in the AL by differentially labeling m-ALT (magenta) and l-ALT (green) PNs (n = 2 females; Fig. 8B–H). As in honeybees and ants, both tracts arborize in different hemilobes of the AL (Fig. 8B) and project to partly segregated areas in the lips (Fig. 8B,C) and the LH (not shown). The l-ALT arborizes in ~138 glomeruli in the ventral surface and the rostral region of the AL, whereas the m-ALT arborizes in ~127 glomeruli in the dorsal and the caudal regions (Fig. 8D,E). Thus, compared with the honeybee AL, the two hemispheres of the hornet AL seem to be rotated by approximately 45° along the rostrocaudal and mediolateral axes, similarly to what has been observed in the ant *C. floridanus* (Zube et al., 2008). Somata of l-ALT neurons encircle the rostral side of the AL (lSC; Fig. 8D,E,G). By contrast, m-ALT somata are distributed mainly in two clusters, a dorsolateral cluster close to the antennal nerve (mSC1; Fig. 8D,E,H) and a rostromedial cluster located next to l-ALT somata (mSC2; Fig. 8D,E,G).

AL input–output connectivity

To allocate glomerular clusters to either the m- or the l-ALT, we compared OSN and PN stainings of different individuals with the help of the remarkable tract architecture of the hornet AL and of landmark glomeruli (see asterisks and black dots in Figs. 7 and 8D,E). Table 2 presents the output tract affiliation of the different glomerular clusters. We observed that the T_A cluster is slightly innervated by m-ALT PNs, except for two large landmark glomeruli that were not stained by either of the uniglomerular tracts. Dorsocaudal clusters, T_B and T_C, clearly belong to the m-subsystem whereas ventrorostral clusters T_D, T_F, T_H, and T_I clearly belong to the l-subsystem. The T_E cluster is innervated by m-ALT PNs except for one glomerulus that receives processes from both m- and l-ALT neurons. Glomeruli of the T_G are divided into two groups; approximately 24 glomeruli belong to the m-subsystem and nine belong to the l-subsystem.

Common features of the hymenopteran AL

When studying the input–output connectivity of the hornet AL, we noticed conspicuous similarities to the ALs of two well-described hymenopteran species, the honeybee *A. mellifera* (Apidae) and the carpenter ants *Camponotus* sp. (Formicidae). Several glomerular clusters presented strikingly similar features in the different

TABLE 4.
Typical Features of Glomerular Clusters Across Species[1]

	Depth	Cluster position	Uniglomerular PN output	OSN input pattern	Relative glomerular size	Glomerular features/OSN innervation
T_A	Dorsal	Necklace arrangement	m-ALT/none	Several bundles	Large	Egg-shaped, dense innervation in both core and cortex
T_B	Dorsal	Caudal/medial	m-ALT	From caudoventral surface	Small	Clumped glomeruli, innervation in cortex
T_C	Relatively dorsal	Caudal/medial	m-ALT	From caudal to medial (running along TB)	Normal	Innervation in cortex, thin cortex
T_D	Relatively dorsal	Rostral/lateral	l-ALT	Thick, inner lateral part of the AL	Normal	Innervation in cortex
T_E	Medium	Medial	l-ALT/m-ALT/both	Through the inner medial part of the AL	Relatively large	Dense innervation in both core and cortex
T_F	Medium	Rostral	l-ALT	Transversally through AL midline	Normal (possible sex dimorphism)	Innervation in cortex
T_G	Relatively ventral	Medial	l-ALT/m-ALT	From caudal periphery to medial	Normal (possible sex dimorphism)	Innervation in cortex
T_H	Ventral	Lateral	l-ALT	From lateral periphery to rostral	Relatively large	Innervation in cortex
T_I	Ventral	Rostral	l-ALT	Caudal to rostral	Normal	Innervation in cortex

[1]Each line gives the relative depth, the localization, and the output tract affiliation of each cluster. The fifth column indicates the projection pattern (orientation) of the olfactory sensory tract. The sixth and the seventh columns specify typical glomerular features such as relative sizes, shapes and OSN innervation types (in the whole glomerulus or only in the glomerular cortex).

species, which led us to propose a common framework for describing these ALs. Table 3 presents the putative homologies among the ALs of the three model species, and Table 4 lists the typical features defining each cluster type. The most striking observation is that all glomerular clusters found in honeybees and in ants have an equivalent cluster in hornets, whereas some clusters found in ants are not found in bees and vice versa. For observing these homologies under the best conditions, the ALs should be oriented similarly in the three species, with the antennal nerve entrance on the same axis, and the separation plane between l-ALT and m-ALT hemilobes in the same orientation across species. We detail below the clues supporting the proposed homologies:

Glomerular cluster T_A is the most dorsal in hornets and presents very distinctive features, found in all three species. It contains a few large glomeruli with peculiar shapes and positional arrangements. It belongs mostly to the m-ALT subsystem, but, remarkably, two glomeruli are innervated neither by m-ALT nor by l-ALT PNs. In honeybees and carpenter ants, the most dorsal cluster (T4 in *A. mellifera,* T7 in *Camponotus*) shows the same features, which led previous authors to suggest their homology (Zube et al., 2008; Nakanishi et al., 2010). The "necklace" organization of glomeruli T_A-01, -03, -05, -06, and -08 in hornets greatly resembles that of glomeruli D01, D03, D05, D06, and D08 in honeybees (Fig. 9A; compare Fig. 5B of Kirschner et al., 2006). The homology is even more remarkable between the hornet T_A-02 glomerulus (Fig. 9A) and honeybee D02 glomerulus (see Fig. 3H,I of Kirschner et al., 2006); both are egg-shaped, positioned close to the AN, and lack any uniglomerular PN arborization.

The T_B is a dorsomedial cluster, partially segregated from the main part of the AL, which flanks the antennal nerve and generally contains smaller glomeruli than the main AL, innervated by m-ALT PNs (Fig. 9A). These features clearly correspond to those of the T6 cluster of *Camponotus* workers (compare Fig. 2C of Zube et al., 2008) and are also found in the T3b of *A. mellifera*, although it contains many fewer glomeruli than in the other species (see Fig. 4A–C of Kirschner et al., 2006).

Glomeruli belonging to the T_C also belong to the m-ALT subsystem. They occupy a caudomedial position within the AL of hornets and receive sensory innervations mostly from the caudal periphery of the AL. These features fit with those of the honeybee T3c and the ant T5 clusters, which have similar PNs arborization, locations, and afferent innervations (Fig. 9B; compare Fig. 3G of Kirschner et al., 2006; see also contralateral view in Fig. 2J–O of Nakanishi et al., 2010).

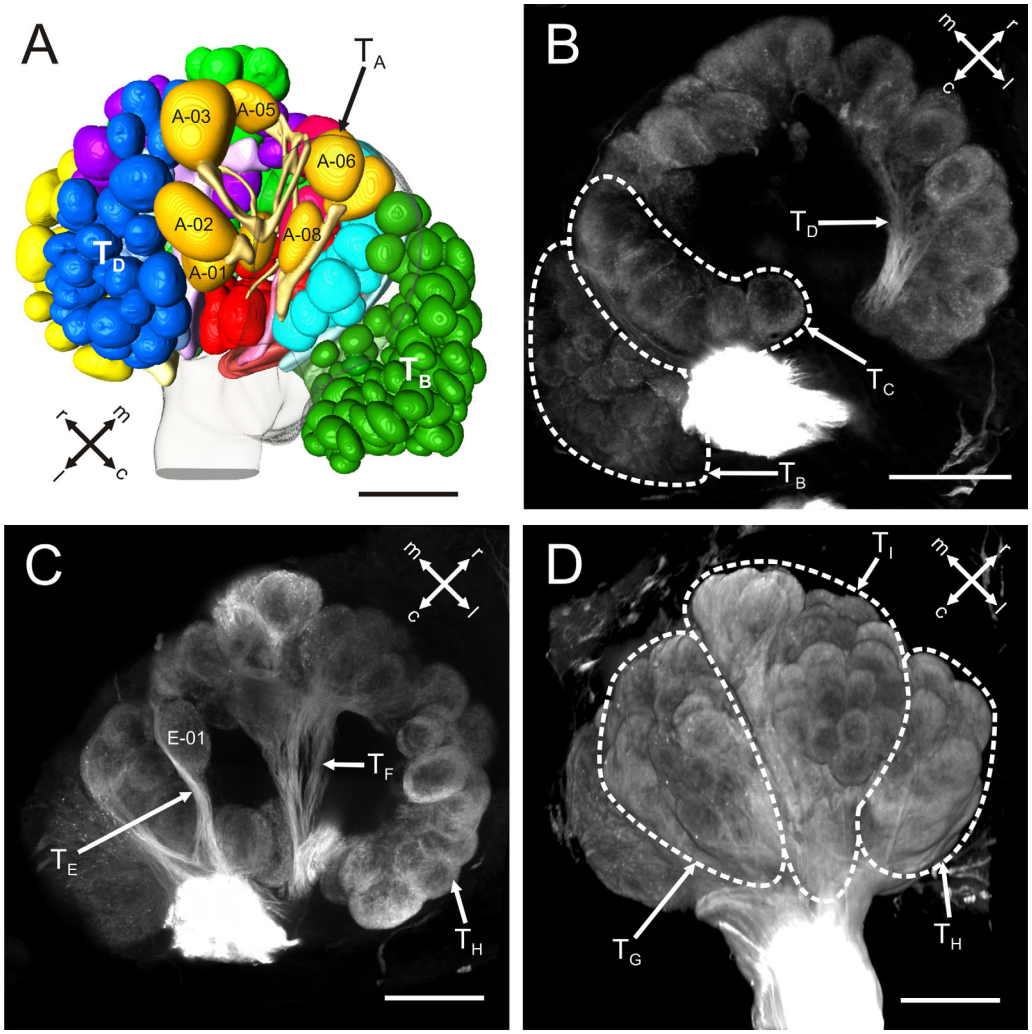

Figure 9. Morphological similarities between the AL of *V. velutina* and the ALs of honeybees and carpenter ants. **A:** Dorsal view of a 3D reconstruction of a *V. velutina* AL. Compare Figure 2B,C of Zube et al. (2008) and Figure 4A–C of Kirschner et al. (2006). **B:** Projection view of a 30-μm-thick slice of a left *V. velutina* AL after mass staining of the antennal nerve (depth 175–205 μm). Compare Figure 1C of Zube et al. (2008) and Figure 3G of Kirschner et al. (2006). **C:** As in B but at depth 120–150 μm. Compare Figure 3E of Kirschner et al. (2006). **D:** Ventral view of a 3D volume rendering of a left female AL. Compare Figure 3A of Zube and Rössler (2008) and Figure 2A–F of Nakanishi et al. (2010). For each picture, the antennal nerve orientation was aligned with that of bees and ants as presented in the cited studies. For comparison with previous studies, all images show a left AL. Remarkable glomerular clusters and landmark glomeruli are labeled with dotted lines and arrows. Scale bars = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The T_D glomeruli belong to the l-ALT subsystem and receive axon terminals from a highly characteristic thick tract that proceeds along the inner lateral part of the AL (Fig. 9B). Similar features were observed in *Camponotus* ants with the T4 tract (compare Fig. 1C of Zube et al., 2008), but we did not find any clear correspondence in *A. mellifera*.

Conversely, two other clusters, T_E and T_F, had clear correspondences in *A. mellifera* (with T2 and T1, respectively) but not in carpenter ants. The honeybee T2 was considered homologous to the hornet T_E because in both species this sensory tract runs through the inner medial part of the AL and projects to very few glomeruli with singular shapes (Fig. 9C; compare Fig. 3E of Kirschner et al., 2006). This homology is strongly supported by the features of a landmark glomerulus. The *A. mellifera* B02 glomerulus is innervated by both l- and m-ALT PNs and receives sensory axon terminals in both its cortex and its core (see Fig. 4F,H of Nishino et al., 2009). The hornet T_E-01 glomerulus shows exactly the same features (Fig. 9C).

On the other hand, the putative homology between the hornet T_F and the *A. mellifera* T1 is based on their similar affiliations to the l-ALT subsystem and to the conspicuous sensory tract projecting through the midline of the AL (Fig. 9C; compare Fig. 3E of Kirschner et al., 2006). In both species, it exhibits a clear sex dimorphism, being much thicker in males than in females and containing several macroglomeruli (Fig. 5B; see Fig. 5D–F of Nishino et al., 2009).

In *V. velutina* as well as in *A. mellifera* and carpenter ants, only one glomerular cluster is shared between the l- and the m-ALT subsystems, some of its glomeruli containing only l-ALT processes, others only m-ALT processes (they are termed T_G, *T3a*, and *T3*, respectively, in the three species). This cluster is located in the mediocaudal part of the AL in all species and contains glomeruli that are innervated mostly from their medial periphery (Fig. 9D; compare Fig. 1A of Zube et al., 2008; see also Fig. 3C–F of Kirschner et al., 2006).

Visually, the ventral surface of the hornet AL bears a strong resemblance to that of the carpenter ant, exhibiting similar clusters (Fig. 9D; compare Figs. 1A and 2B of Zube and Rössler, 2008). Glomeruli of the lateral surface receive sensory afferences from the T_H in *V. velutina* or the T2 in *Camponotus* sp. and belong to the l-ALT. Glomeruli from the hornet T_I or ant T1 cluster also belong to the l-ALT and are innervated by a sensory tract that first projects to the rostral part of the AL before sloping down dorsally (Fig. 9D; compare contralateral view Fig. 2A–F of Nakanishi et al., 2010). No equivalent of these clusters is, however, present in honeybees. All in all, these observations suggest that the hornet AL presents both honeybee and ant features.

DISCUSSION

We studied the neuronal olfactory pathway of the hornet brain and its sex- and caste-specific adaptations. Whole-brain staining revealed that the overall organization of the *V. velutina* brain is highly similar in the two sexes and between female castes. Whereas the morphology of the hornet MBs appears distinct from that of other Hymenoptera, we found homologous structures and the same subdivision of olfactory and visual inputs (lips and collars) in the MB calyces. Our data also show that the antennal lobe of *V. velutina* consists of ~265 glomeruli distributed in nine clusters innervated by distinct antennal sensory tracts. The number of glomeruli and their volume distribution are similar between the female castes. The AL of males contains fewer glomeruli but harbors seven macroglomeruli organized in two clusters. We observed five output (PN) tracts that arborize in the AL and project to higher order centers; two prominent tracts arborize in distinct AL hemilobes and project toward partially segregated areas of MB calyces and LH, in reverse order. Three relatively smaller output tracts project through the protocerebrum toward the LH. Finally, the input–output relationships of hornet ALs display striking similarities to those of honeybees and ants, allowing a common framework for describing the olfactory pathway in these species.

Low dimorphism in the hornet brain

Several observations that we made for hornets differ substantially from what is known in the other Hymenoptera described. First, a marked sex dimorphism within the central nervous system has been reported for many hymenopteran species and was assumed to be impelled by sexual selection (Ehmer and Gronenberg, 2004; Nishikawa, 2008; Streinzer et al., 2013; Roselino et al., 2015). The competition among males could act as a selective pressure on the sensory system for improving the localization of fertile females (Anderson and Iwasa, 1996). To assess a potential differential investment of the different sexes in particular sensory systems, the relative size of brain neuropils has been compared between males and females in several eusocial species. In these species, as in *A. mellifera* (honeybee), *Camponotus japonicus* (carpenter ant), *Atta vollenweideri* (leaf-cutting ant), or *Melipona scutellaris* (stingless bee), males present larger optic lobes (medulla and/or lobula) than females, suggesting sex differences in visual processing (Gronenberg and Hölldobler, 1999; Ehmer and Gronenberg, 2004; Nishikawa et al., 2008; Kuebler et al., 2010; Streinzer et al., 2013; Roselino et al., 2015). It was suggested that this sex-specific sensory adaptation is related to the importance of vision to detect and pursue fast movements of fertile females during mating flights (Ehmer and Gronenberg, 2004). Our data on the hornet *V. velutina* show that enlarged optic lobes in males are not a general feature of social Hymenoptera, as we did not find conspicuous size differences between males and females. In fact, behavioral observations in hornets in general, and *V. velutina* in particular, indicate that mating occurs on the nests or other solid substrate but not in flight (Batra 1980; Matsuura and Yamane, 1990; Monceau et al., 2014). These observations are in accordance with a less crucial role of vision in hornet males for finding their sexual partners than in other Hymenoptera. Alternatively, the hornet brain could be generally preadapted for highly demanding visual tasks (such as catching prey on the fly), so that the system is already sufficient for processing all the information relevant to mating.

Second, fewer AL glomeruli and smaller MBs in males are ubiquitous features in social Hymenoptera

and were assumed to be linked to a smaller behavioral repertoire in males (Arnold et al., 1985; Ehmer and Gronenberg, 2004; Kuebler et al., 2010; Smith et al., 2010; Streinzer et al., 2013). Typically, a lower number of glomeruli suggests a lower number of expressed olfactory receptors and can accordingly be linked to reduced olfactory discrimination abilities compared with females. In addition, smaller MBs, brain centers crucial for multimodal sensory integration, memory, and complex learning (Heisenberg, 1998; Farris, 2005; Strausfeld et al., 2009; Devaud et al., 2015), suggest lower cognitive capabilities in males. A recent comparative study underscored a less pronounced sexual dimorphism in number of glomeruli and MB size in solitary than in social bees (Streinzer et al., 2013). In contrast to males of eusocial species, which benefit from colonial altruism and leave the colony only for mating flights, males of solitary species require a higher level of behavioral plasticity, which might be responsible for such lower sex dimorphism (Stubblefield and Seger, 1994). Contrary to this general rule, we observed only ~20 (7%) fewer glomeruli in *V. velutina* males compared with females, which is clearly low compared with the ~30–40% reduction found in honeybees and carpenter ants (Arnold et al., 1985; Zube and Rössler, 2008; Nishino et al., 2009; Nakanishi et al., 2010). In the same way, the calyces and peduncle of hornet male MBs were only slightly thinner than those of females (see also Molina and O'Donnell, 2008). In hornets, males were observed to be rejected from their colony and then to perform foraging by their own means (Monceau et al., 2013, 2014). From these observations, a less pronounced sex dimorphism in the hornet brain could be attributed to a more elaborate male behavioral repertoire than in other species.

Hornet macroglomeruli

To estimate the presence of enlarged glomeruli in the AL of *V. velutina*, we measured glomerular volumes by confocal microscopy and 3D reconstruction. We did not find any macroglomeruli in hornet females, but the male AL presented two clusters with seven strongly enlarged glomeruli. These glomerular clusters were densely innervated by OSNs, resulting in thicker T_F and T_G tracts in males than in females. Whereas female macroglomeruli are adaptations related to the colony life style of eusocial insects, male macroglomeruli are found throughout insects because they are involved in mating (Arnold et al., 1985; Hansson et al., 1992; Nishikawa et al., 2008; Nishino et al., 2012). In many species, such as moths or cockroaches, male-specific macroglomeruli are located in the caudal region of the AL, close to the entrance of the antennal nerve (Rospars and Chambille, 1986; Skiri et al., 2005; Nishino et al., 2012; Das and Faddamiro, 2013). In the ant *Camponotus japonicus*, two macroglomeruli at similar locations were attributed to the T3 tract and were assumed to have a sex pheromone-receptive function (Nakanishi et al., 2010). By contrast, in honeybee drones, three macroglomeruli attributed to the T1 tract are located rostrally, one of which specifically responds to the sex pheromone compound 9-ODA (Arnold et al., 1985; Sandoz, 2006; Nishino et al., 2009). Compared with other species, hornet males present numerous macroglomeruli of both types, an ant-like cluster of four ventrocaudal macroglomeruli (T_G), located close to the entrance of the antennal nerve and a honeybee-like cluster of three rostral macroglomeruli (T_F). Such sex-specific investment in several hypertrophied glomerular structures should be connected to a crucial role played by several odorants in the mating behavior of *V. velutina* males. Observation of the mating behavior of a related species, *Vespa crabro*, highlighted the existence of at least two different pheromones (Ayasse et al., 2001). Hornet females produce a long range pheromone that attracts males from long distances (Ono and Sasaki, 1987; Spiewok et al., 2006). In addition, a contact pheromone on the queen's thorax elicits copulatory behavior in males (Batra, 1980). One possibility is that one of the macroglomerulus clusters of the male hornet AL is involved in long-range detection of the sex attractant pheromone, whereas the second would process contact recognition cues such as fertility signals. More extensive data on the mating behavior of *V. velutina* and on the implicated chemical signals are needed for substantiating these hypotheses.

A common reference for the hymenopteran AL

Despite the considerable variations observed in the number of glomeruli across hymenopteran species, previous authors have pointed out striking similarities, as well as important differences, in the organization of ant and honeybee ALs. The most remarkable similarity between these species resides in the subdivision of the AL into two hemilobes of almost equal size, separately innervated by two prominent output tracts (m- and l-ALT; Abel et al., 2001; Galizia and Rössler, 2010; Rössler and Zube, 2011). Such a dual olfactory pathway has been found in diverse taxa among Hymenoptera, except in some sawflies, suggesting its initial appearance in basal Hymenoptera (Dacks and Nighorn, 2011; Rössler and Zube 2011). In accordance with these observations, we show that Vespidae also possess such a dual pathway, which appears, as in the ant *C. floridanus,* rotated by 45° on the rostrocaudal and

mediolateral axis compared with that of the honeybee *A. mellifera*. However, we found other significant similarities among the ALs of these three species, which we attempted to integrate within a common framework (Table 3). Generally, the homologies we propose fit with and expand those proposed in an earlier work comparing bees and carpenter ants (Zube et al., 2008). We observed, based on OSN projection patterns, that the hornet AL contains nine clusters, each receiving differentially oriented branches of the antennal nerve. With similar classification rules, the AL of honeybees has been subdivided into six glomerular clusters (T1–T4, taking into account the T3a–c subdivision; Galizia et al., 1999; Kirschner et al., 2006; Nishino et al., 2009), whereas seven clusters have been identified in carpenter ants (Zube et al., 2008; Nakanishi et al., 2010). The possible homologies that we described are based mostly on neuroanatomical features related to glomerular output connectivity, OSN tract innervation patterns, cluster localizations, and average glomerular size. These features alone may not be sufficient to demonstrate these homologies unambiguously, and further comparative studies including functional data from these clusters would certainly be helpful for proving their validity. However, for some of them, available functional and neuroanatomical data already strongly support homologies across species. For instance, the T_A cluster contains a few (six to 10) large glomeruli located on the most dorsal region of the AL and harbors typical whole-glomerulus OSN projections, features that fit perfectly with those of the honeybee T4 cluster (seven glomeruli; Kirschner et al., 2006; Nishino et al., 2009) and of the ant T7 cluster (6 to 10 glomeruli; Zube et al., 2008; Nakanishi et al., 2010). These glomeruli are often described as homologous to the T10 cluster of the cockroach, containing hygro-/thermosensitive neurons (Nishino et al., 2009; Watanabe et al., 2010), so homologies for this cluster may reach beyond Hymenoptera. Another strongly supported homology concerns the T_B cluster. It is characterized in hornets by many small glomeruli forming a tight group slightly segregated from the main AL. This cluster has already been observed in the wasp *Polistes gallicus,* in the form of a glomerular compartment with distinct enzymatic activity compared with the main AL (Masson and Strambi, 1977). This compartment corresponds to the T6 cluster of carpenter ants (Zube et al., 2008; Nakanishi et al., 2010). This cluster is female specific in ants and bees and noticeably receives olfactory neurons housed in basiconic sensilla (Zube and Rössler, 2008; Kelber et al., 2010; Nakanishi et al., 2010; Nishikawa et al., 2012; Kropf et al., 2014). It is currently thought that these glomeruli might be involved in the processing of cuticular hydrocarbons, which are crucial for nestmate recognition in social insect colonies (Ozaki et al., 2005; Sharma et al., 2015). We recently obtained neuroanatomical and functional data suggesting a similar role in hornets, which supports the T_B homology (Couto et al., in preparation).

For other clusters with less conspicuous features, homologies could be more difficult to discern and demonstrate. The present data in hornets, however, allows resolving some previous uncertainties in the comparison between honeybees and ants. Thus, the *C. floridanus* T1, T2, and T4 clusters were all suggested as possible homologous clusters to the honeybee T1 cluster (Zube et al., 2008). The honeybee T1 projects rostrally, through the middle of the AL and, in males, contains three macroglomeruli. These features are strikingly similar to those of the hornet T_F (Kirschner et al., 2006; Nishino et al., 2009). In addition, we found other clusters more similar to the ants' T1, T2, and T4 tracts in the hornet AL (T_I, T_H and T_D, respectively). Our current view is thus that none of the carpenter ant clusters is homologous to the T1 cluster of honeybees. Future work should strive to provide additional data allowing corroboration of the putative homologies that we have proposed. A systematic investigation of sensillar equipment (morphological types of olfactory sensilla) on the antenna, and their AL afferences in the different species (see Kropf et al., 2014; Kelber et al., 2006), along with systematic immunohistochemistry for the main neurotransmitters and neuropeptides (Masson and Strambi, 1977; Zube and Rössler, 2008; Dacks et al., 2010; Siju et al., 2014), could provide pivotal clues for testing our model.

New insights into the evolution of the hymenopteran AL

Because the olfactory system is at the interface between the animal and its environment, it receives strong selection pressures, which induce the gain or loss of subcircuits depending on changes in the relative importance of particular chemical stimuli in the biology of the species (Ramdya and Benton, 2010). Glomerular clusters are conspicuous entities in the hymenopteran AL but it is still unclear whether different clusters are systematically involved in the detection of different odorant classes. As detailed above, for some remarkable clusters such as the T_A or T_B, current data points in this direction. For most other clusters, however, this may not be so clear-cut because even different AL hemilobes show strongly overlapping odor response spectra (Carcaud et al., 2012; Brill et al., 2013; but see Carcaud et al., 2015). It is nevertheless possible that, within each cluster, OSNs express groups of closely

related olfactory receptor (OR) genes, as suggested by data from fruit flies. In this insect, OR genes with the highest sequence similarity tend to be expressed in OSNs targeting neighbor glomeruli, suggesting such lineage constraints on OSN projection patterns (Couto et al., 2005; Vosshall et al., 2005). This may appear through the action of specific transcription factor genes controlling OSN axon targeting (Komiyama et al., 2004; Tichy et al., 2008). Thus, the antennal sensory tract ramifications observed in Hymenoptera might be the result of genetically encoded axonal-guidance factors, which could participate in a coarse odotopic representation of odors in the AL.

The evolution of the AL is thought to entail a continuous "birth-and-death" process of ORs, inducing the budding/pruning of glomeruli within each cluster, and, eventually, of whole clusters (Ramdya and Benton, 2010). The comparative analysis of AL neuroanatomy, and the homologies that we observed, could help in retracing the evolution of the AL in Hymenoptera. At this stage, the phylogeny of Hymenoptera is not totally resolved and remains controversial (Brothers, 1999; Pilgrim et al., 2008; Heraty et al., 2011; Sharkey et al., 2012). However, according to a recent phylogeny of stinging Hymenoptera, supported by extensive genomic and transcriptomic data sets (Johnson et al., 2013), Vespidae would be more basal among aculeates than Formicidae and Apoidea, which would be sister groups. If this phylogeny is confirmed, then one could speculate that the common ancestor of Vespidae, Formicidae, and Apoidea harbored a relatively complex AL with many sensory tract subdivisions and that different secondary losses took place in Formicidae (T_E and T_F) and Apoidea (T_D, T_H, and T_I). Clearly, these stimulating hypotheses can be substantiated only through the long-term study of AL neuroanatomical features and input–output relationships in a wider range of hymenopteran taxa. We note that the most strongly conserved clusters (T_A, T_B, and T_C) are related to the m-ALT PN tract, whereas the clusters that are related to the l-ALT output tract greatly vary among species (see Table 3). It seems remarkable that this output tract is the one that diverged more recently, probably in some basal Hymenoptera, whereas the m-ALT is the ancestral tract (Rössler and Galizia, 2010). If confirmed in other hymenopteran taxa, this could indicate that the invention of the l-ALT tract has provided Hymenoptera with a highly adaptable and plastic olfactory subsystem, potentially instrumental for their adaptation to a wide range of ecological niches and their ecological success. On the other hand, basic biological functions would be secured by the other, ancestral tract.

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CONFLICT OF INTEREST STATEMENT

All authors have confirmed that there is no identified conflict of interest, including any financial, personal, or other relationships with other people or organizations within 3 years of beginning this work that could inappropriately influence, or be perceived to influence, the work.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: AC, DT, JCS Acquisition of data: AC with help from BL. Analysis and interpretation of data: AC, JCS. Drafting of the manuscript: AC, JCS. Critical revision of the manuscript for important intellectual content: AC, DT, JCS. Obtained funding: JCS. Study supervision: JCS.

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RESEARCH ARTICLE

Olfactory Attraction of the Hornet *Vespa velutina* to Honeybee Colony Odors and Pheromones

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Abstract

Since the beginning of the last century, the number of biological invasions has continuously increased worldwide. Due to their environmental and economical consequences, invasive species are now a major concern. Social wasps are particularly efficient invaders because of their distinctive biology and behavior. Among them, the yellow-legged hornet, *Vespa velutina*, is a keen hunter of domestic honeybees. Its recent introduction to Europe may induce important beekeeping, pollination, and biodiversity problems. Hornets use olfactory cues for the long-range detection of food sources, in this case the location of honeybee colonies, but the exact nature of these cues remains unknown. Here, we studied the orientation behavior of *V. velutina* workers towards a range of hive products and protein sources, as well as towards prominent chemical substances emitted by these food sources. In a multiple choice test performed under controlled laboratory conditions, we found that hornets are strongly attracted to the odor of some hive products, especially pollen and honey. When testing specific compounds, the honeybee aggregation pheromone, geraniol, proved highly attractive. Pheromones produced by honeybee larvae or by the queen were also of interest to hornet workers, albeit to a lesser extent. Our results indicate that *V. velutina* workers are selectively attracted towards olfactory cues from hives (stored food, brood, and queen), which may signal a high prey density. This study opens new perspectives for understanding hornets' hunting behavior and paves the way for developing efficient trapping strategies against this invasive species.

Introduction

Since the beginning of the 20th century, as a result of globalization and the development of international exchanges, biological invasions have critically increased [1–4]. Invasive species frequently disturb biodiversity by competing with indigenous species for habitat and food resources and/or by exerting a strong predation pressure on local species. Due to the ecological and economical problems that ensue from biological invasions, they have become a major concern worldwide [5–6]. Introduced species do not however all become invasive and only ~1% actually lead to permanent invasions [7]. Social insects, like social wasps, are particularly efficient invaders. They live in populous colonies organized according to highly efficient task allocation systems, which confers them outstanding abilities for exploiting local food resources and outcompeting local species [8]. Social wasps gather food resources from the surroundings of the nest, mostly gathering carbohydrates and proteins [9]. These generalist hunters typically find protein resources by preying on numerous insect species including many pollinators. Because of the current decline of pollinator populations [10–11] and of the severe ecological consequences of the introduction of such generalist hunters in new ecosystems [12], acquiring a better knowledge of the predation behavior of social wasps has become a crucial endeavor.

Predation site selection by social wasps is strongly influenced by prey density [13–14], as a high number of preys at a particular location gives rise to conspicuous visual and olfactory cues. Social wasps are keen learners which are able to associate color or odor cues with food reward [15–16]. Thus, after several hunting bouts on a particular hunting site, these insects learn the cues that allow them to localize their prey [9, 17–21]. This high behavioral plasticity supported by excellent cognitive abilities has been proposed as another explanation for the invasive success of social wasps [22–24].

The yellow-legged hornet, *Vespa velutina*, is an attractive species for studying hornets' hunting behavior. Recently, this hornet originating from China has been accidently introduced to new locations in Western Europe and in Korea [25–27]. In Europe, this species was first observed in 2004 [27–28]. Like other wasps of the Vespinae subfamily, hornets prey on various arthropod species but also scavenge on dead vertebrate flesh (fish and meat) [9, 29–32]. Above all, however, *V. velutina* hunts domestic honeybees, including the Asian honeybee, *Apis cerana* (in their natural range) [33] and the European honeybee, *Apis mellifera* (both in their natural and introduction ranges) [34]. Because of the strong and immediate impact of this hornet's hunting pressure on honeybee colonies, several studies have been dedicated to honeybees' defense behavior against it. These studies essentially showed that most European honeybees are not able to display efficient anti-predator behaviors contrary to Asian honeybees which coevolved with several bee-hunting hornet species [35–42]. In invaded areas, hornets' feeding sites are thus primarily apiaries, because they present an abundant and defenseless prey source. However, little is known about the hunting behavior of *V. velutina* [43–44] and how they locate and choose these feeding sites.

At the peak of predation, as many as 20 hornets can be seen hovering simultaneously in front of a given hive, catching honeybee foragers on the fly. This hunting strategy strongly impairs the foraging efficiency of bees [43]. Two mechanisms may be involved in such high numbers of preying hornets. First, *V. velutina* workers could learn the characteristics and/or location of feeding sites, as the same foragers can be seen several times hunting on the same apiary [44]. Second, like other Vespine wasps, hornets may recruit nestmates to feeding sites [35, 45]. In any case, honeybee colonies are especially attractive to hornets, but the exact cues they may use to locate honeybee colonies in the first place remain unclear. Recently, a laboratory study showed that *V. velutina* workers can use both visual and olfactory cues to locate bees [46]. In the wild, visual cues are critical at short-range for catching flying honeybee foragers, while olfactory cues may be more relevant for long-range attraction to honeybees hives. To this day such odorants remain unknown despite their considerable interest for understanding *V. velutina* foraging tactic. In theory, predators can take advantage of different categories of olfactory cues [47]. The most immediate and accurate way to locate a prey is to use olfactory cues emanating directly from the prey (kairomones) [48–50]. However, if such odorants are not sufficient for guiding the predator, other strategies using indirect olfactory cues which emanate from the habitat or from the food of the prey have been reported [51–54]. Hence, hornets may be attracted to volatiles emitted by honeybees, to volatiles emitted by the bee hives as well as to odorants emitted by the flesh they sometimes scavenge on. In the present study, we tested the attraction behavior of *V. velutina* workers to olfactory stimuli in a multiple choice test in controlled conditions. We first asked which elements (hive products, honeybees or fish or meat samples) may attract hornets. Then, we tested hornets' relative attraction to a range of different molecules that are typical honeybee- or flesh-emitted volatiles. In particular, we tested a number of known honeybee pheromone that may act as kairomones for hornets.

Materials and Methods

Animal collection and caging procedure

Vespa velutina workers preying on honeybee hives were individually caught with a butterfly net between August and November 2013, at the experimental apiary of INRA Bordeaux-Aquitaine, France (GPS: N44°47′27.05″ W0°34′38.35″). Animals were then brought to a dark and temperature-controlled (22°C) experimental room in the laboratory containing a cubic cage (60 × 60 × 60 cm, described in [55]). Groups of 10 hornets were transferred to the cage and were allowed to move freely for 3 hours during a period of acclimatization prior to the experiments. The base of the cage contained a mobile drawer with a stainless steel plate allowing to securely deliver stimuli into the cage (Fig. 1A). A digital camcorder (HD pro webcam C920; Logitech, Lausanne, Switzerland) was placed inside the cage and attached to its roof to record *V. velutina* behavior during the tests. The cage was illuminated with red light by means of an incandescent light

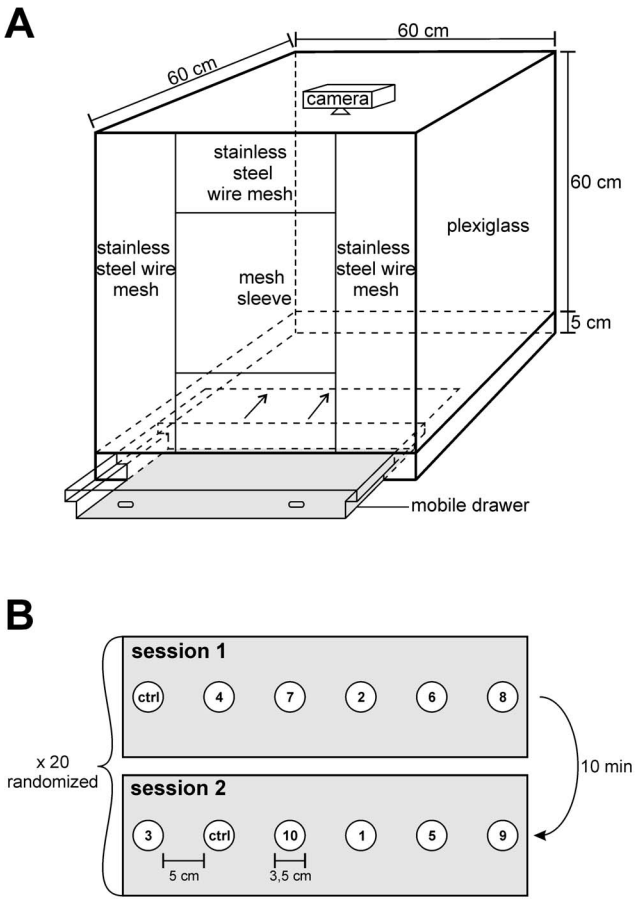

Fig. 1. Experimental setup. A. Schematic drawing of the cubic wooden cage (60 × 60 × 60 cm) used for the experiments. The front part was equipped with a mesh sleeve for introducing hornets into the cage. The base contained a 5 cm deep mobile drawer (in grey) and a stainless steel plate used to securely deliver tested stimuli. Inside the cage and attached to the roof, a digital camcorder allowed the recording of hornets' behavior. **B.** Experimental procedure used in the first experiment. Each of the 20 replications was divided in 2 consecutive sessions separated by a 10 minute interval. Within a session, 5 stimuli among the 10 tested (numbered circles) were placed 5 cm apart in the mobile drawer, and delivered together with a control (ctrl circle). The sequence of stimuli presentation and exact location in the drawer were fully randomized. The second experiment was similarly designed but contained 4 sessions of 5 stimuli among the 20 tested.

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bulb. Red light allowed filming the hornets while precluding their use of visual cues during the experiments, since, as shown for the closely related species *V. crabro,* hornets lack red-light sensitive photoreceptors [56]. The Virtualdub software (http://www.virtualdub.org) was used for recording high quality video at a frame rate of 22 fps.

Experiment 1

In order to screen attractive food sources or hive products, 10 different stimuli were used. For all stimuli, the same mass of substance was presented, i.e.

80 mg ± 2 mg, as it equates to the average weight of a honeybee worker. The stimuli were:

- *Honey*: organic raw mixed-flower honey (not warmed), obtained from a local beekeeper
- *Pollen*: organic, bee-collected, fresh pollen (not dried), obtained from a local beekeeper
- *Wax*: new honeybee comb foundation wax obtained from a local beekeeping shop
- *Propolis*: purified in ethanol (ethanol was evaporated after filtration in liquid phase)
- *Bee boost*: commercial synthetic honeybee queen mandibular pheromone (QMP) on a plastic strip (Contech, Victoria, BC, Canada). QMP is a blend of five constituents, 9-oxo-(E)-2-decenoic acid (9-ODA), two enantiomers of its biosynthetic precursor, (R)- and (S)-9-hydroxy-(E)-2-decenoic acid (9-HDA) and two other compounds, methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) [57].
- *Honeybee*: worker honeybee taken from a hornet-predated hive
- *Larva*: honeybee larva taken from a hornet-predated hive
- *Meat*: minced beef meat, obtained from a local supermarket
- *Fish*: salmon flesh, obtained from a local supermarket
- *Wet paper*: white cellulose wadding moisturized with distilled water

Stimuli were individually placed in Ø 35 mm Petri dishes (Petri dishes, EL46.1; Roth, Lauterbourg, France) and delivered into the cage using the mobile drawer. Six Petri dishes were placed at a time in the drawer, and were spaced 5 cm apart. Hornets were subjected to 2 consecutive sessions separated by a 10 minute interval (Fig. 1B). Each session presented 5 of the 10 stimuli plus a joint control (empty Petri dish). The order of presentation of the stimuli (session 1 or 2), as well as their location in the drawer, were fully randomized using Research Randomizer (http://www.randomizer.org). Before each session, the mobile drawer was thoroughly washed with ethanol to remove any stimulus residues. Following introduction of the Petri dishes, hornets' behavior in the drawer area was recorded for 15 minutes with the digital camcorder. This experiment was repeated 20 times (20 × 2 sessions) with different groups of 10 hornets. New stimuli were used for each test.

Experiment 2

Food odors are complex blends of odorant molecules present in variable ratios. To attempt to isolate attractive odorants for hornets, we selected 20 volatile compounds (Sigma-Aldrich, Steinheim, Germany) based on their occurrence at high concentration in the different food sources of *V. velutina*. Table 1 shows the list of tested compounds. For commodity, the tested stimuli were categorized in 3 main groups (honeybee, seafood and meat odors), although each stimulus can also be emitted by a variety of other sources. For each odorant, a filter paper piece (1 cm^2; Whatman, Maidstone, England) was soaked with 5 μL of the odor

Table 1. List of molecules tested in Experiment 2.

Chemical name	CAS	MW (g.mol^{-1})	Purity	Sources	References
isopentyl acetate	123-92-2	130.18	99%	**bee alarm pheromone**	[62, 76]
2-heptanone	110-43-0	114.18	99%	**bee alarm pheromone**	[62, 77–78]
geraniol	106-24-1	154.24	98%	**bee aggregation pheromone**	[61, 62–63]
citral	5392-40-5	152.23	95%	**bee aggregation pheromone**	[79–80]
homovanillyl alcohol	2380-78-1	168.18	99%	**bee queen pheromone**	[57–81]
10-hydroxydecanoic acid	1679-53-4	188.26	99%	**bee queen pheromone**	[82–83]
methyl 4-hydroxybenzoate	99-76-3	152.14	99%	**bee queen pheromone**	[84]
methyl palmitate	112-39-0	270.45	99%	**bee brood pheromone**	[85]
ethyl oleate	111-62-6	310.51	98%	**bee brood pheromone**	[86]
β-ocimene (E, Z mixture)	13877-91-3	136.23	90%	**bee brood pheromone**	[65]
heptanal	111-71-7	114.18	95%	**seafood**/plants/meat	[67, 87–89]
(E)-2-nonenal	18829-56-6	140.22	95%	**seafood/**aged beer	[67–68, 87, 90]
(E, E)-2,4-octadienal	5577-44-6	124.18	95%	**Seafood**	[67, 91]
(E, Z)-2,6-nonadienal	557-48-2	138.20	96%	**seafood/**meat/plants	[67–68, 87, 89, 92–93]
p-xylene	106-42-3	106.16	99%	**seafood/**meat/plants	[67–69, 89]
1-octen-3ol	3391-86-4	128.21	98%	**meat/**honey	[67–68, 87, 89, 94–95]
benzaldehyde	100-52-7	106.12	98%	**meat/**plant	[88–89, 94, 96–97]
octanoic acid	124-07-2	144.21	98%	**meat/**plant	[88, 97]
4-ethyloctanoic acid	16493-80-4	172.26	98%	**meat**	[98]
(E, E)-2,4-decadienal	25152-84-5	152.23	89%	**meat/**plant/seafood	[67, 87, 92, 94, 96, 99]

The table presents the chemical names of the 20 molecules tested in Experiment 2. CAS number, molecular weight and purity are indicated in the second, third and fourth column respectively. The fifth column shows a non exhaustive list of sources that emit these molecules, as mentioned in the literature cited in the last column.

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solution and placed in a Ø 35 mm Petri dish. Liquid substances were presented pure, while odorants in solid form at room temperature (homovanillyl alcohol, 10-hydroxydecanoic acid, methyl 4-hydroxybenzoate, methyl palmitate and ethyl oleate) were dissolved in 2-propanol and presented at a dose of 50 μg/μL. 2-propanol was allowed to evaporate for 1 min before presenting the stimuli. As in experiment 1, 5 stimuli and a control (1 cm^2 filter paper without a stimulus) were delivered at a time into the cage using the mobile drawer. The 20 stimuli were thus delivered in 4 consecutive sessions lasting 15 minutes each and separated by a 10 minute interval. To remove residual odors before each session, the drawer was thoroughly washed with ethanol. Sequence of stimulus presentation and location in the drawer were randomized as above. The experiment was repeated 20 times (20×4 sessions) on different groups of 10 hornets. New stimuli were used for each test.

Videos analysis

Preliminary observations of hornet's behavior showed that in Experiment 1, some food sources could be consumed within 10 minutes of the presentation of the

stimuli. Thus, in this experiment, hornet behavior was only analyzed during the first 10 minutes of each recording. In Experiment 2, none of the odorant sources were consumed, so hornet behavior was analyzed during the full 15 min of the experiment. Videos were visualized using Movie Maker V. 2011 software (Microsoft Corporation, Redmond, USA) allowing us to precisely record the number of visits and the amount of time hornets spent on each stimulus. At each separate occasion a hornet entered a Petri dish (at least with its entire head), times of entry and of exit were recorded. Hornet attraction to the different stimuli was measured according to two variables: 1) *Visit number*: the total number of hornet visits during each session; and 2) *Total visit duration*: the sum of all individual visit durations performed by hornets during this session.

Statistics

A Friedman test was used to compare visit frequencies and durations on the control stimuli during different sessions. To evaluate the attractiveness of the different stimuli, we compared the number of visits to each test stimulus with its joint control (i.e. with all control stimuli presented in the same sessions), using a Wilcoxon paired sample test. Likewise, the amount of time that hornets spent on each stimulus was compared to its joint control with a Wilcoxon paired sample test. As multiple comparisons were carried out with the same control (always 5 stimuli with 1 control), we used a Bonferroni correction to reduce type I errors ($\alpha_{corrected} = 0.05/5 = 0.01$). A p value below $\alpha_{corrected}$ was considered as statistically significant, while a value between $\alpha_{corrected}$ and the standard 0.05 threshold was only regarded as near-significant (but potentially biologically relevant). Significance levels are presented in the figures as follows: ***: $p<0.0001$; **: $p<0.001$; *: $p<0.01$; (*) $0.01<p<0.05$).

Results

The behavior of 400 hornets was observed in two experiments aiming to test their olfactory attraction towards food sources (Experiment 1) or individual odorants (Experiment 2).

Experiment 1

In the first experiment, *V. velutina* workers were presented with several food sources and different elements of a honeybee colony. The hornets could explore the drawer at the base of the cage, containing 6 Petri dishes presenting 5 stimuli and a control. Because hornets were free to move around the cage, visits to the stimuli may occasionally occur through random exploration. Visits to control stimuli therefore allows us to evaluate the intensity of hornets' exploration behavior. Stimulus exploration was similar between the first and second session, as quantified through the number of visits to the control (Wilcoxon test, $Z = 1.24$, $p = 0.21$, NS).

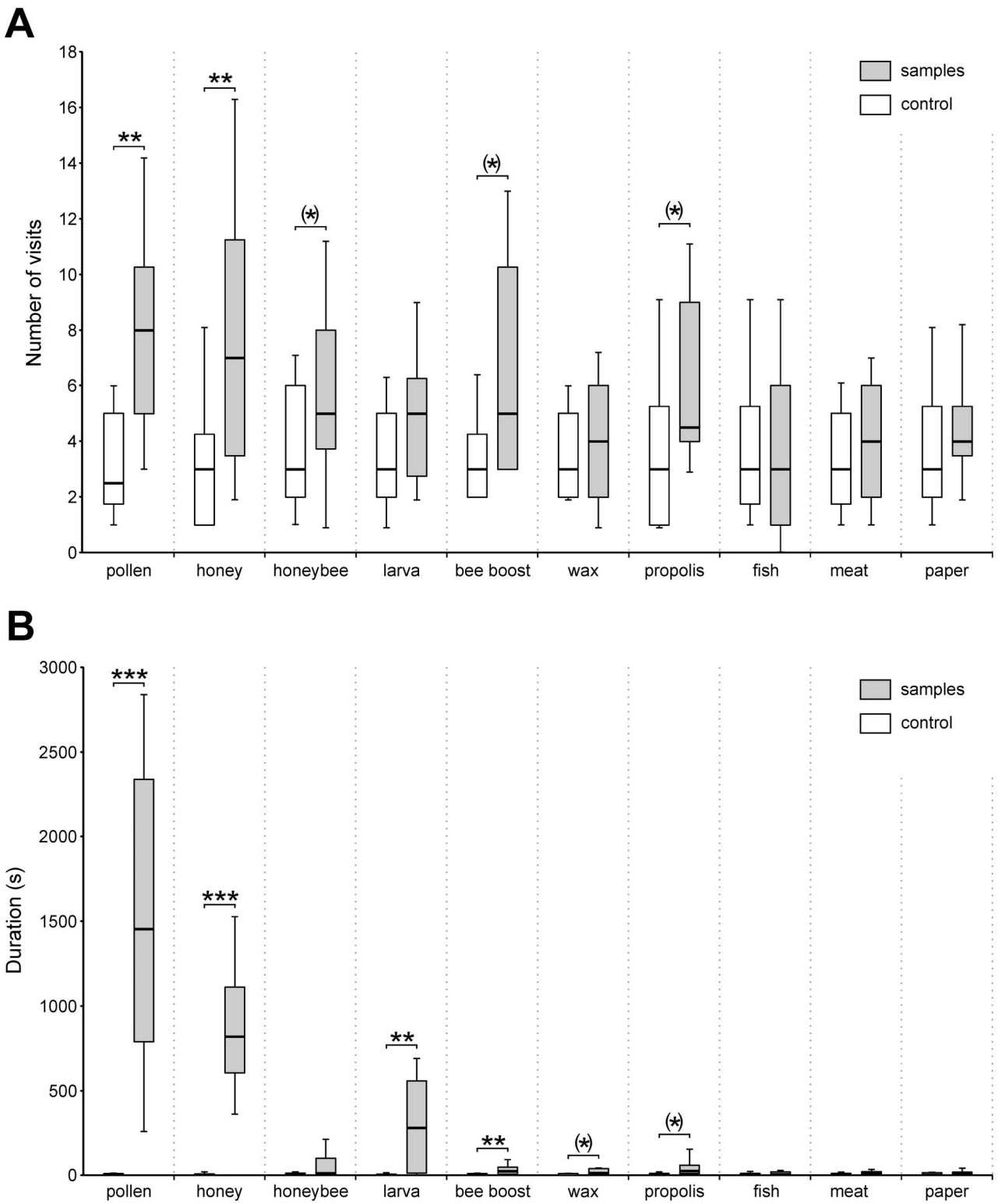

Fig. 2. Hornet attraction towards hive products and other food samples. A. Distribution of the number of visits on each sample for groups of 10 hornets. Bold lines show the median number of visits per session (20 replications, N =200 hornets). The boxes represent the first and third quartiles (25–75%), while whiskers represent the distribution from 10 to 90%. The thick vertical dashed lines separate the different sources emitting these molecules (from left to right:

honeybee, fish, meat). **B.** Distribution of the cumulative duration that groups of 10 hornets spent on each sample. Bold lines show the median duration by session (20 replications, N =200 hornets). Grey bars: samples; white bars: control. Comparison between samples and control were done with Wilcoxon tests (***: $p<0.0001$; **: $p<0.001$; *: $p<0.01$; (*) $0.01<p<0.05$).

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Visits to the control stimuli presented concomitantly with each stimulus were highly similar (Fig. 2A; median visit number between 2.5 and 3.0) and indicated that exploration behavior was homogenous in the whole experiment (Friedman test, $Chi^2=6.40$, $p=0.70$, NS, 9 df). When evaluating the attractiveness of the different stimuli, we observed that hornets visited *honey* and *pollen* significantly more often than the control stimulus (Wilcoxon test, $Z=3.33$, $p<0.001$ and $Z=3.57$, $p<0.001$). Although hornets showed no significant preference for other stimuli, some showed near-significant attraction ($\alpha_{corrected}=0.01<p<0.05$): *propolis* (Wilcoxon test, $Z=2.37$, $p=0.017$), *bee boost* (Wilcoxon test, $Z=2.48$, $p=0.013$) and *honeybee* (Wilcoxon test, $Z=2.07$, $p=0.038$).

When evaluating the duration that hornets spent visiting each stimulus (Fig. 2B), we observed that they consistently spent more time on *honey* and *pollen* than on the control (Wilcoxon test, $Z=3.92$, $p<0.0001$ and $Z=3.92$, $p<0.0001$, respectively). In addition, the duration spent on *larva* and *bee boost* was also significantly longer (Wilcoxon test, $Z=3.82$, $p<0.001$ and $Z=3.32$, $p<0.001$) than the control. The time hornets spent visiting *propolis* (Wilcoxon test, $Z=2.39$, $p=0.017$) and *wax* (Wilcoxon test, $Z=2.12$, $p=0.033$) was only near-significantly longer than on the control ($\alpha_{corrected}<p<0.05$).

Experiment 2

In the second experiment, we asked which food odorants or honeybee pheromones may attract *V. velutina* (Table 1). As in experiment 1, hornets explored the newly accessible drawer and visited the stimuli (Fig. 3A). When comparing visits to the control stimuli, we did not find any significant variation among the four sessions, although a trend for increased exploration between sessions 1 and 4 appeared (Friedman test, $Chi^2=7.77$, $p=0.051$, NS, 3 df). In any case, full randomization of stimulus sequence presentation allowed avoiding any influence of exploration changes on the results. Visits to control stimuli presented concomitantly with each stimulus were homogeneous (Fig. 3A, Median visit number between 3.0 and 5.0, Friedman test, $Chi^2=14.27$, $p=0.077$, NS, 19 df).

When evaluating the attractiveness of single odorant stimuli, we observed more visits on *geraniol* and *p-xylene* than on the control (Wilcoxon test, $Z=3.64$, $p<0.001$ and $Z=3.07$, $p<0.01$). Although hornets showed no significant preference for other stimuli, *homovanillyl alcohol* (Wilcoxon test, $Z=2.22$, $p=0.026$) and *β-ocimene* (Wilcoxon test, $Z=2.12$, $p=0.034$) showed near-significant attraction ($\alpha_{corrected}<p<0.05$).

Consistently with visit numbers, visit duration measurements showed that hornets spent more time on *geraniol* and *p-xylene* (Fig. 3B) than on the control (Wilcoxon test, $Z=3.81$, $p<0.001$ and $Z=3.92$, $p<0.0001$). Furthermore,

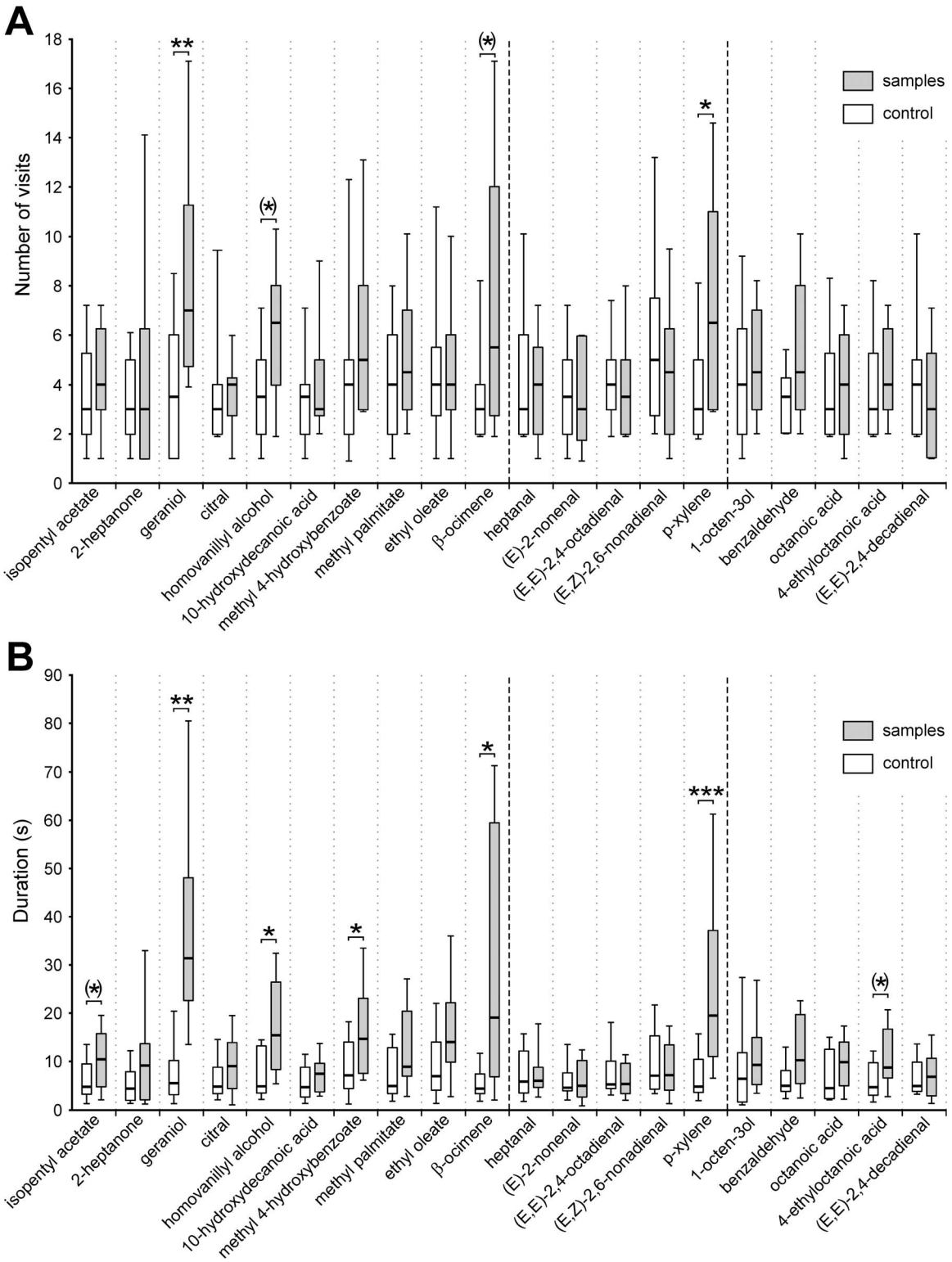

Fig. 3. Hornet attraction towards chemical substances. A. Distribution of the number of visits on each stimulus exhibited by groups of 10 hornets. Bold lines show the median number of visits per session (20 replications, N =200 hornets). The boxes represent the first and third quartiles, while whiskers represent the distribution from 10 to 90%. **B.** Distribution of the cumulative duration that groups of 10 hornets spent on each stimulus. Bold lines show the

median duration by session (20 replications, N =200 hornets). Grey bars: stimuli; white bars: control. Comparison between odor stimuli and control were done with Wilcoxon tests (***: $p<0.0001$; **: $p<0.001$; *: $p<0.01$; (*) $0.01<p<0.05$).

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hornets also spent longer periods of time on *homovanillyl alcohol* (Wilcoxon test, Z =2.69, $p<0.01$), *methyl 4-hydroxybenzoate* (Wilcoxon test, Z =2.87, $p<0.01$) and *β-ocimene* (Wilcoxon test, Z =2.95, $p<0.01$) than on the control. Lastly, the period of time hornets spent visiting *4-ethyloctanoic acid* (Wilcoxon test, Z =2.54, p =0.011) and *isopentyl acetate* (Wilcoxon test, Z =2.13, p =0.033) was near-significantly longer than on the control ($\alpha_{corrected} <p<0.05$).

Discussion

Using a controlled laboratory assay to measure hornets' olfactory attraction, we found that they are primarily attracted to (and spent more time on) two products from honeybee hives, *pollen* and *honey*. By contrast, the honeybees themselves were only near-significantly attractive. Among individual odorants, however, one honeybee aggregation pheromone, *geraniol*, induced significant attraction and longer visit durations than the control. Our experiments also allowed observing a few more subtle effects. Honeybee *larva* and *bee boost* induced longer visit durations than the control, and similar results were observed when presenting some of their compounds alone such as *β-ocimene* (larva pheromone), *homovanillyl alcohol* (HVA) or *methyl-4-hydroxybenzoate* (HOB) (queen pheromones present in the *bee boost*). Stimuli associated with protein food sources, such as fish and meat samples or typical compounds from these protein sources did not induce any particular attraction, with the exception of one compound, *p-xylene*. Thus some stimuli were attractive: *honey* and *pollen* in experiment 1 and *geraniol* and *p-xylene* in experiment 2 whereas some stimuli only induced longer visit durations: *larvae* and *bee boost* in experiment 1 and *homovanillyl alcohol* and *methyl 4-hydroxybenzoate* in experiment 2.

Removal of potential visual cues

Hornets are known to use both visual and olfactory cues to locate food sources [46]. In the present study, we aimed to compare the attractiveness of a range of olfactory cues without any influence from visual information provided by the samples or the experimental cage. Because of the need to film the hornets, our assays were conducted under low levels of red light. Intracellular recordings of photoreceptors have shown that the visible spectrum of hornets (in this case *V. crabro*) starts around 300 nm and quickly decreases around 600 nm [56] due to a lack of red photoreceptors as in many hymenopterans [58]. Thus, in this context, visual cues were, if not totally absent, at least very strongly reduced. In addition, to avoid any potential bias of each samples' location with respect to the red light, sample positions were placed randomly at each session. We are therefore

confident that hornets' orientation behavior in our experiments was influenced by olfactory cues and not by visual cues.

The prey itself or its products?

As many hornet species, and especially *V. velutina*, are keen honeybee hunters [33, 35, 37, 41] and the hornet workers we tested were caught while hunting on honeybee hives, we expected them to be primarily attracted to and to feed on their prey. This was not the case as the *honeybee* samples were only mildly attractive (Fig. 2A) and hornets did not remain on them when encountering them (Fig. 2B). By contrast, honeybee products, *pollen* and *honey*, were highly attractive (Fig. 2A). They strongly attracted hornets, sometimes even in groups and the hornets stopped for long periods of time on these samples, eventually feeding on them (Fig. 2B). Previous studies showed that the foraging behavior of some parasitic wasps [51, 53] and some social wasps [59–60], are influenced by cues associated with their prey but not emanating directly from the prey. For instance, the social wasp *Mischocyttarus flavitarsis* uses olfactory cues originating from a plant damaged by the feeding activity of its prey [60]. One important activity of honeybees during summer is to store high quantities of food within their colony and such stores may become a conspicuous signal for the predator. Taking advantage of cues emitted by hive stores could be more efficient for the hornet than searching for individual flying honeybees. We therefore hypothesize that *pollen* and *honey* odors emanating from the hives' food stores represent prominent distant cues helping *V. velutina* foragers to find their prey.

Hornets gather proteins to feed their larvae but also need carbohydrates (and possibly proteins) for their own needs. As *V. velutina* workers were sometimes seen feeding on *pollen* and *honey* in our experiment (Fig. 2B), we cannot exclude the possibility that the long time hornets spent on these stimuli may be related to their nutritional quality. However, the significant numbers of visits recorded to these stimuli demonstrate their olfactory attractiveness for hornets. In addition, during hunting, *V. velutina* workers usually hover at the hive entrance and catch flying honeybee foragers, some of which are loaded with pollen and/or nectar [43]. Hornets have never been observed feeding directly on pollen pellets from caught workers. Therefore, we rather favor the idea that hornets' attraction to pollen and honey odors was related to a potential role of these odors as long-distance signals for the presence of hives.

Could honeybee pheromones be used as kairomonal signals?

There is increasing literature reporting how prey pheromone can be counterused as a kairomonal signal by predators or parasitoids [47–50]. In addition to the observation that hive products strongly attract *V. velutina*, our experiments revealed that hornets can also be attracted to some honeybee pheromones. The only other honeybee-related stimulus that significantly attracted hornets was the honeybee worker aggregation pheromone component, *geraniol*. Hornets chose to

orient towards *geraniol* (Fig. 3A) and remained on this stimulus for long periods of time (Fig. 3B). Geraniol is a Nasanov gland secretion, which acts as an aggregation pheromone, attracting honeybee workers during swarming or at the colony entrance [61–63]. Because of its role in aggregating honeybees, geraniol may represent an honest signal for hornets, indicating high densities of honeybee workers. It may thus work as a kairomone, possibly together with hive products to help hornets finding honeybee colonies.

The other bee pheromone constituents tested in our work (Table 1) did not induce any significant increase in the number of visits, but some (*β-ocimene*, *homovanillyl alcohol* and *methyl-4-hydroxybenzoate)* were found to act as arrestants (Fig. 3B). Indeed, in this experiment, we noticed that visit duration increased when hornets exhibited typical arrestment responses [64] with klinotaxis behavior around the stimuli. *β-ocimene*, a volatile pheromone produced by honeybee larvae [65] showed only near-significant attraction but significant arrestment (Fig. 3. A, B). Consistently, *larvae*, when presented in the first experiment, did not induce any significant attraction but induced longer visit durations once hornets had found them (Fig. 2. A, B). These results suggest the existence of arrestment responses by hornets towards *β-ocimene,* which could also act as a kairomone, giving relevant cues for locating *larvae*.

A similar situation was observed for queen pheromones. While in the first experiment, the *bee boost*, an artificial blend of 5 components composing the honeybee Queen Mandibular Pheromones (QMP) showed near-significant attraction and significantly longer durations, two QMP components, *homovanillyl alcohol* (HVA) and *methyl-4-hydroxybenzoate* (HOB), showed the same pattern in experiment 2, with a significant arrestment to both stimuli. Here again, some honeybee queen pheromones may act as kairomones for hornets.

Such a result is consistent with previous studies on another hornet species, *V. bicolor*, which was shown to be attracted to (Z)-11-eicosen-1-ol, a major component in the alarm pheromone of both Asian and European honeybees [50]. Another prominent example is found in the wasp *Vespula germanica* which uses the pheromone produced by sexually active Mediterranean fruit fly males as olfactory cues to locate its prey [48]. Taken together, our results may indicate that hornets use a combination of honeybee-related odorants to locate hives from a distance (attraction to *honey*, *pollen* or *geraniol*). In addition, when a honeybee colony is weak, hornets sometimes enter the hive to plunder its resources. When in the hive, they may use particular honeybee pheromones as suggested by the significant arrestment observed to larvae, worker and queen pheromones.

Lack of attraction to generic animal protein sources

Vespine wasps are generalist foragers that hunt arthropod preys but also collect meat from vertebrates to feed their larvae [31–32]. They are notorious pests in butchers' and fishmongers' stalls where they cut small pieces of meat and fish and fly away [9, 29]. In this study, unlike hive product, neither *meat* nor *fish* attracted hornets significantly (Fig. 2.A). As also shown by the low time spent on these

stimuli (Fig. 2.B), we did not observe any hornets feeding on *meat* or *fish* samples. Thus, contrary to our expectations, hornets fed on only two stimuli considered as protein sources, mainly *pollen* and to a lesser extend *larva,* but neglected other animal proteins. Although carbonyl compounds resulting from lipid oxidation contribute strongly to the typical fishy odor [66–67], hornets exhibited significant attraction and arrestment responses only towards one fishy odor, *p-xylene* (Fig. 3. A, B). This compound can be a product of carotenoid degradation in fish flesh and has been reported as an active component in many seafood products [67, 68–69]. But, since *fish* flesh was not attractive in our experiment, the reason why *p-xylene* was of interest to *V. velutina* remains unclear. This chemical may also emanate from many different sources such as some fruit trees and some orchids [70–71].

Recruitement and learning as potential mechanisms

Hornets' attraction to particular stimuli in our experimental setup may be the product of both innate attraction and appetitive learning processes. Social insects are able to recruit nestmates to valuable food sources through active communication. For instance, honeybees can actively promote food sources to nestmates with a sophisticated dance [72–73]. Several foraging recruitment forms by means of scent marks have been reported in Vespoidea [35, 45, 74]. When the predation activity of *V. velutina* reaches its peak, up to 20 individuals can hunt on a single hive at the same time [43]. Therefore, the high number of hornets present at the hive entrance might be due to recruitment by conspecifics. Some of our hornets may have been caught during their first visits to the location and may have displayed innate attraction to the stimuli. However, taking into account the high learning abilities of Vespine wasps [15–16, 18, 20], tested individuals may also have learned hive cues in the course of preying visits to the hive. Indeed in a previous study the same individuals were observed several times hunting at the same site [44]. In the framework of associative learning, hive and honeybee odors may correspond to conditioned stimuli (CS) while the successful catch of a prey may act as reinforcement. If so, then our experiments reflect the attraction of honeybee-specialist hunters, which would explain the lack of attraction to meat/fish stimuli. Similar experiments comparing the behavior of *V. velutina* workers caught on different food sources should clarify how foraging specialization influences olfactory attraction in hornets.

Outlook

In our experiments, *V. velutina* hornets mainly exhibited interest to hive products and to honeybee emitted pheromones but not to honeybees themselves. All these attractive stimuli seem to share a common feature: they might signal not only the presence of honeybees, but their presence at a high density. Interestingly, *pollen* was strongly attractive and the prey spectrum of *V. velutina* includes a large variety of pollinators such as social Hymenoptera and hoverflies [75]. Preliminary

trapping experiments in the field gave promising results for using *pollen's* attractiveness for protecting hives from hornet aggression. Such field experiments will now be performed on a larger scale, testing the trapping properties of the stimuli identified in the present study, alone or in combination.

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Author Contributions

Conceived and designed the experiments: AC KM DT JCS. Performed the experiments: AC OB. Analyzed the data: AC KM JCS. Wrote the paper: AC KM DT JCS.

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« *L'étude du cerveau des Insectes présente, au point de vue de l'observation et de l'interprétation, des difficultés si considérables, que je ne me décide qu'avec un certain regret à publier mes recherches, car je suis convaincu que malgré tout le soin que j'ai apporté à mon travail, il contiendra beaucoup de lacunes, peut-être même quelques erreurs.*

Ceux qui se sont occupés d'un même genre de recherches comprendront aisément qu'il en soit nécessairement ainsi, rien n'est plus difficile que de débrouiller des trajets de fibres au sein d'un organe aussi compliqué qu'un cerveau d'Insecte.

Ainsi, qu'un faisceau nerveux ne se trouve, dans son parcours, exclusivement compris dans aucun des plans selon lesquels on oriente les coupes : il sera alors fort difficile sinon impossible d'en déterminer le trajet avec précision. Fort heureusement une telle circonstance, étant d'ordre purement topographique, peut se modifier d'un Insecte à l'autre, sans que pour autant la constitution du cerveau change en rien d'essentiel. Ainsi tel trajet de fibre qu'il est impossible de suivre chez un Insecte d'espèce donnée apparaîtra clairement chez le représentant d'une espèce voisine. Ceci nous prouve qu'il ne faut espérer connaître bien le cerveau d'un Insecte que le jour où l'on aura étudié soigneusement et comparativement cet organe dans un certain nombre de types voisins.

A un autre point de vue les recherches comparatives auront un intérêt considérable, car d'un ordre d'Insectes à l'autre, souvent même dans un même ordre, d'un genre à l'autre, nous voyons le cerveau présenter des différences vraiment inattendues. Ainsi on peut dire sans exagération que le cerveau d'un Coléoptère diffère du cerveau d'un Hyménoptère au moins autant que le cerveau d'un poisson diffère de celui d'un mammifère supérieur.

Un jour peut-être saurons-nous quels rapports existent entre le perfectionnement du cerveau et le perfectionnement des fonctions de l'Insecte? Mais aujourd'hui toute hypothèse serait téméraire; elle manquerait de base. »

Henri Viallanes 1887.

université PARIS-SACLAY
ÉCOLE DOCTORALE
Structure et dynamique des systèmes vivants (SDSV)

Titre : Etude neurophysiologique et comportementale de la perception olfactive chez une espèce invasive, le frelon *Vespa velutina*

Mots clés : insecte invasif, Hyménoptère, olfaction, communication sociale, lobe antennaire, comportement.

Résumé : Le frelon *Vespa velutina*, est une espèce invasive introduite en France il y a une dizaine d'années. Cet Hyménoptère social chasse de nombreux insectes, et plus particulièrement des abeilles, exerçant une forte pression de prédation sur l'entomofaune. Son établissement en Europe est donc susceptible d'avoir un impact néfaste sur les activités humaines et sur la biodiversité locale. L'olfaction joue un rôle central dans la biologie du frelon, que ce soit pour la communication intraspécifique (phéromones) ou la recherche de nourriture. Pour limiter l'impact de *V. velutina,* une stratégie prometteuse serait d'interférer avec son comportement olfactif. Nous avons donc cherché à acquérir une meilleure connaissance du système olfactif des frelons ainsi que des signaux chimiques impliqués dans leur comportement de prédation. Nous avons tout d'abord décrit et comparé l'organisation du système olfactif des différentes castes d'une colonie de frelons. Le premier centre olfactif du cerveau, le lobe antennaire, est constitué d'unités morphologiques et fonctionelles, les glomérules. Nous avons démontré l'existence de glomérules hypertrophiées (macroglomérules), potentiellement spécialisées dans la détection des phéromones sexuelles, chez les mâles. Nous avons également observé des similarités neuroanatomiques frappantes dans l'organisation du lobe antennaire chez des Hyménoptères de familles différentes (fourmis, abeilles, frelons), permettant de reconstruire une partie de l'histoire évolutive de cette structure, au sein de cet ordre. En particulier, nous avons démontré l'homologie d'un groupe de glomérules impliqué dans le traitement des hydrocarbures cuticulaires, signaux de reconnaissance intraspécifique, chez les frelons et les fourmis. Ce système a pu représenter une préadaptation pour l'apparition de comportements sociaux dans ces familles. De plus, nos résultats ont montré que le nombre de glomérules dans ce système varie fortement chez différentes espèces de frelons, probablement en relation avec les différents niveaux de sympatrie dans lesquels vivent ces espèces. Le lobe antennaire des frelons présente ainsi des adaptations concernant aussi bien le nombre de glomérules que leurs volumes respectifs. Enfin, nous avons effectué des essais comportementaux pour déterminer quels composés odorants sont impliqués dans l'attraction de *V. velutina* envers les ruches d'abeilles domestiques. Nos résultats suggèrent que les ouvrières de frelon sont sélectivement attirées par des odorants indiquant la présence de ruches, dont des phéromones d'abeille. Ces résultats permettent d'envisager le développement de stratégies de piégeage utilisant des appâts olfactifs spécifiques.

Title: Neurophysiological and behavioral study of olfactory perception in an invasive species, the hornet *Vespa velutina*

Keywords : invasive insect, Hymenoptera, olfaction, social communication, antennal lobe, behavior.

Abstract : The hornet *Vespa velutina* is an invasive species introduced from China to France about 10 years ago. This social hymenoptera preys on numerous insects species, but predominantly from honeybee colonies, hence exerting a stong predation pressure on insect fauna. Its etablishment to Europe can have strong detrimental effects on human activities and local biodiversity. Olfaction plays a central role in the biology and behavior of hornets, for both intraspecific communication or food search. To limit the impact of *V. Velutina*, a promising strategy would be to interfere with its olfactory behaviors. We thus aimed to acquire a better knowledge on the olfactory system of hornets and on the chemical signals involved in their predatory behavior. We first described and compared the organization of the olfactory system of the different castes of hornets. The first olfactory processing center, the antennal lobe, consists of morphological and functional units, the glomeruli. We have demonstrated the existence of enlarged glomeruli (macroglomeruli), potentially specialized in the detection of sex pheromones in males. We also observed striking neuroanatomical similarities in the antennal lobe organization of different Hymenoptera families (ants, bees, hornets) allowing to retrace part of the evolutionary history of this structure within that insect order. In particular, we demonstrated the homology of a glomerular cluster involved in the processing of cuticular hydrocarbons (intraspecific recognition signals) among hornets and ants. This systsem could be a preadaptation for the emergence of social behaviors in these families. In addition, our results showed that the number of glomeruli within this system varies across hornet species, probably due to the different sympatry levels these species experience. The hornet antennal lobe presents adaptations both in the number and the volume of glomeruli. Finally, we performed behavioral tests to identify odor compounds involved in the attraction of *V. Velutina* towards bee hives. Our results suggest that workers are selectively attracted to olfactory cues indicating the presence of hives, including some honeybee pheromones. These results allow considering the development of trapping strategies using specific baits.

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