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# Behavioral and neurobiological studies of visual learning in honey bees (*Apis mellifera*) in virtual reality

Alexis Buatois

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Université Fédérale



Toulouse Midi-Pyrénées

# THÈSE

En vue de l'obtention du

## DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

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Présentée et soutenue par

**Alexis BUATOIS**

Le 17 septembre 2018

### **Etudes comportementales et neurobiologiques de l'apprentissage visuel chez l'abeille (*Apis mellifera*) en réalité virtuelle**

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## Préface

Les différents paragraphes de cette thèse sont constitués des papiers prêts à être soumis, soumis, acceptés ou publiés dans des journaux scientifiques. Certaines répétitions étaient donc inévitables. Néanmoins, je considère ce format comme le plus adapté afin de mettre en avant le travail qui a été fourni au cours de cette thèse.

## Publications incluses dans ce manuscrit

- Schultheiss, P.\*, Buatois, A.\*, Avarguès-Weber, A., and Giurfa, M. (2017). Using virtual reality to study visual performances of honeybees. *Curr Opin Insect Sci* 24, 43–50. (\* First author is shared)
  - Constitue le chapitre 2
  
- Buatois, A., Pichot, M.C., Schultheiss, P., Sandoz, J.C., Lazzari, C., Chittka, L., Avarguès-Weber A. and Giurfa M. (2017). Associative visual learning by tethered bees in a controlled visual environment. *Sci Reports* 7(1), 127903.
  - Constitue le chapitre 3
  
- Buatois, A., Flumian C., Schultheiss, P., Avarguès-Weber A and Giurfa M. (in press). Transfer of visual learning between a virtual and a real environment in honey bees: the role of active vision. *Front Behav Neurosci*.
  - Constitue le chapitre 4

## Publications non incluses dans ce manuscrit

- Buatois, A. and Lihoreau, M. (2016). Evidence of trapline foraging in honeybees. *J Exp Biol*. 219, 2426-2429.

- M.Giurfa and A.Buatois: F1000Prime Recommendation of [Peckmezian T and Taylor PW, Anim Behav 2015, 107:87-95]. In F1000Prime, 28 Jan 2016; DOI: 10.3410/f.726099645.793513805. F1000Prime.com/726099645#eval793513805
- M.Giurfa and A.Buatois: F1000Prime Recommendation of [Terao K, Matsumoto Y, Mizunami M. Sci Rep. 2015; 5:8929]. In F1000Prime, 11 Mar 2016; DOI: 10.3410/f.725384030.793515400. F1000Prime.com/725384030
- M.Giurfa and A.Buatois: F1000Prime Recommendation of [Varga AG and Ritzmann RE, Curr Biol 2016, 26(14):1816-28]. In F1000Prime, 09 Nov 2016; DOI:10.3410/f.726494269.793525062.F1000Prime.com/726494269#eval7935 25062

### **Conférences et poster effectués pendant cette thèse**

- A.Buatois et al. Color learning by honey bees in virtual reality and the real world. Poster presentation at the international Society for neuroethology congress in Brisbane, Australia, 07/16-20/18
- A.Buatois et al. Associative visual learning by tethered honey bees in a controlled visual environment. CRCA PhD symposium, Toulouse, France 09/28/17
- A.Buatois et al. Associative visual learning by tethered honey bees in a controlled visual environment. Vision Camp, Castle Wildenstein, Leibertingen, Germany 06/30/17 to 07/01/17, invited speaker
- A.Buatois et al. How do honey bees discriminate virtual stimuli on a locomotion compensator? Poster presentation at the 18th meeting of invertebrate neurobiology club, Dijon, France 06/08-09/17

- A.Buatois et al. Associative visual learning by tethered and freely-moving bees. Learning and Memory: A synthesis of bees and flies, Aspet, France 06/02-04/17.
- A.Buatois et al. Behavioral assays on visual discrimination learning in tethered and freely-moving bees. 47th SFECA symposium, Gif-sur-Yvette, France 05/15-17/17
- A.Buatois et al. How do honey bees discriminate virtual stimuli on a locomotion compensator? Poster presentation at the Doctoral school BSB symposium, Toulouse, France 04/27-28/17
- A.Buatois. Cognition visuelle et spatiale chez l'abeille : du terrain au laboratoire. Présentation pour le club d'apiculture du comité d'entreprise de Thalès, Toulouse, 01/24/2017.
- A.Buatois et al. Behavioral assays on visual discrimination learning in tethered and freely-moving bees. Human frontiers program symposium, Aspet, 12/02-03/2016.
- A.Buatois et al. Neural bases of visual learning in honey bees. CBI symposium, Toulouse, France 08/01/16
- A.Buatois. Finding its way in the virtual world. Journée GREC, Toulouse 11/12/15.





# Introduction



L'environnement est composé de multiples stimulations sensorielles allant des odeurs à la lumière en passant par les sons. Ces stimulations sont en constante évolution et apportent des informations concernant le milieu qui nous entoure. Afin d'agir de manière optimale, il est alors primordial de pouvoir associer ces informations à des événements particuliers. Par exemple, chez les mammifères des cris d'alarme sont largement utilisés pour prévenir d'un danger imminent. Ces cris ne sont alors utiles que s'ils sont reconnus et mémorisés comme prédictifs du danger. C'est le cas chez le rat qui est par exemple prédisposé à apprendre un appel d'une fréquence précise de 22 kHz à cet effet (Endres et al., 2007). Ce n'est pas sans rappeler notre capacité par exemple à associer une alarme à un incendie. Ces facultés à agir en fonction de l'environnement ont une grande importance pour se nourrir, se reproduire, trouver son chemin ou encore éviter les prédateurs.

Parmi ces signaux, les stimulations visuelles ont une très grande place. En effet, le système visuel va permettre à un individu d'identifier avec précision des éléments de son environnement et ce même s'il est privé de ses autres sens. Il pourra alors mémoriser cet élément et l'associer à un événement particulier afin de lui donner une valence positive, négative ou neutre. La cognition visuelle regroupe les capacités des individus à identifier, associer et mémoriser toutes ces informations visuelles pour les utiliser à bon escient le moment venu. Ce domaine a été largement étudié que ce soit chez l'humain (Kozbelt, 2001; Summerfield and Egner, 2009; Tallon-Baudry, 2009), les Vertébrés (Bussey et al., 2008; Colwill et al., 2005; Murray et al., 2007; Zoccolan et al., 2009) ou les Invertébrés (Avarguès-Weber et al., 2011; Giurfa and Menzel, 1997; Zeil, 2012; Zylinski, 2014) .

Au cours de cette thèse je me suis focalisé sur les capacités d'apprentissage visuel chez l'abeille mellifère (*Apis mellifera*) dont la biologie est étudiée depuis des siècles en passant par Aristote ou Charles Darwin. L'étude de la cognition visuelle chez l'abeille a vraiment pris son envol après la publication des travaux de Karl von Frisch (von Frisch, 1914), et de sa disciple Mathilde Hertz (E.g. Hertz, 1935) dans lesquels ils ont montré que les abeilles étaient capables d'associer une couleur, une forme ou une combinaison des deux avec une récompense sucrée. En effet, on a depuis observé que les abeilles utilisaient une stratégie de butinage particulière appelée « la constance florale » qui consiste à ne butiner qu'une seule espèce de fleurs jusqu'à ce que celle-ci arrête de fournir des ressources, après quoi elles partent butiner une autre espèce plus profitable (Chittka and Raine, 2006). Cette stratégie est permise par ces même capacités mises en évidence



par Karl von Frisch il y a de cela déjà un siècle. Une centaine d'années et des dizaines de publications plus tard, nous sommes maintenant en mesure de dire que les abeilles sont capables d'apprentissage visuel simple, mais aussi d'apprentissage plus complexe dans lesquelles des informations visuelles simples sont extrapolées afin d'apprendre des règles, des concepts ou la reconnaissance d'objet plus complexe (Avarguès-Weber et al., 2012).

L'abeille possède un cerveau miniature composé d'un million de neurones soit cent mille fois moins qu'un cerveau humain. L'objectif aujourd'hui est de comprendre comment il est possible avec un cerveau dont la structure est très simple d'apprendre des associations visuelles simples et complexes. Malgré des avancées considérables, nous ne sommes malheureusement pas à ce jour capable de caractériser les aires cérébrales impliquées dans ce type d'apprentissage. Ceci pour une simple raison, combiner des méthodes invasives telles que la neuropharmacologie ou l'électrophysiologie est à ce jour impossible avec les protocoles d'apprentissage visuel habituellement utilisés pour étudier la cognition visuelle chez l'abeille.

L'enjeu au cours de ma thèse a donc été d'étudier la possibilité de tester des abeilles attachées et en conditions contrôlées. Les parties suivantes ont pour vocation de présenter tous les points essentiels à la compréhension des différents chapitres de mon manuscrit.

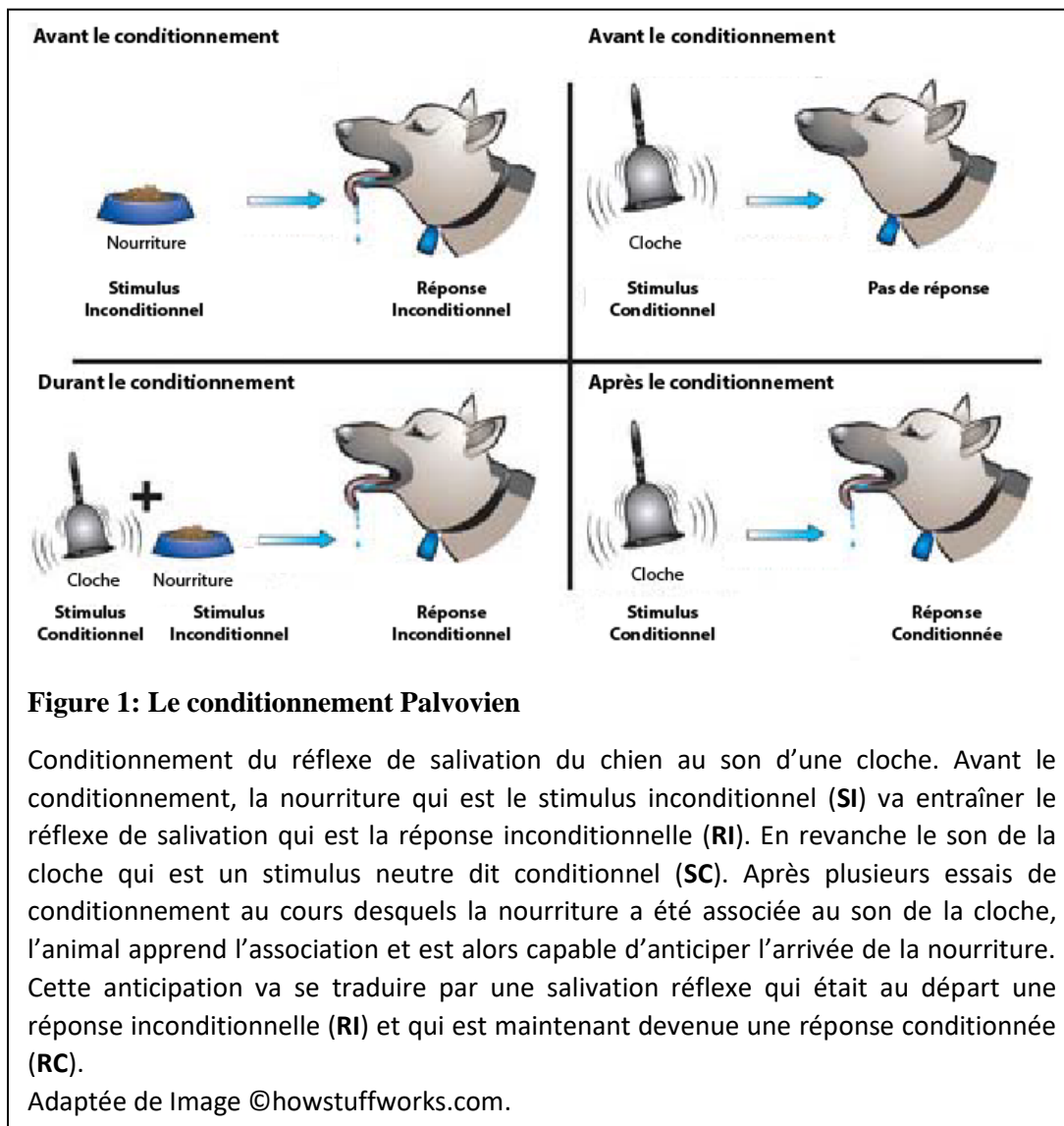
## I. Les bases de l'apprentissage

L'apprentissage est un des domaines de recherche les plus important de la psychologie contemporaine. A ce jour, la définition la plus commune est la compréhension et les connaissances acquises par l'expérience. Cette définition est quelque peu vulgarisée et reste assez floue. Une définition populaire qui tend à mieux expliquer l'apprentissage est un changement permanent relatif dans le comportement qui découle comme un résultat d'une pratique renforcée (Kimble, 1961). L'apprentissage est une notion vaste qui englobe différents types du social au visuel basés sur des théories bien précises, mais aussi différents degrés de complexité qui vont être définis dans les sections suivantes.

### a. Apprentissage associatif élémentaire

L'apprentissage associatif élémentaire est un apprentissage qui va être permis par la mise en place d'un lien entre 2 événements (ou plus). Par exemple, pour revenir au cas de l'incendie, un individu pourra apprendre un lien élémentaire entre le son de l'alarme et le départ de l'incendie. En parallèle, il pourra aussi apprendre que le panneau sortie de secours indique un lieu de sûreté. Ces deux associations sont non ambiguës et vont être spécifiques, entraînant ainsi des comportements propres à chacune des stimulations.

L'exemple le plus courant et le plus connu lorsque l'on parle d'apprentissage associatif est le **conditionnement classique** ou **conditionnement pavlovien** mis en évidence par Ivan Pavlov

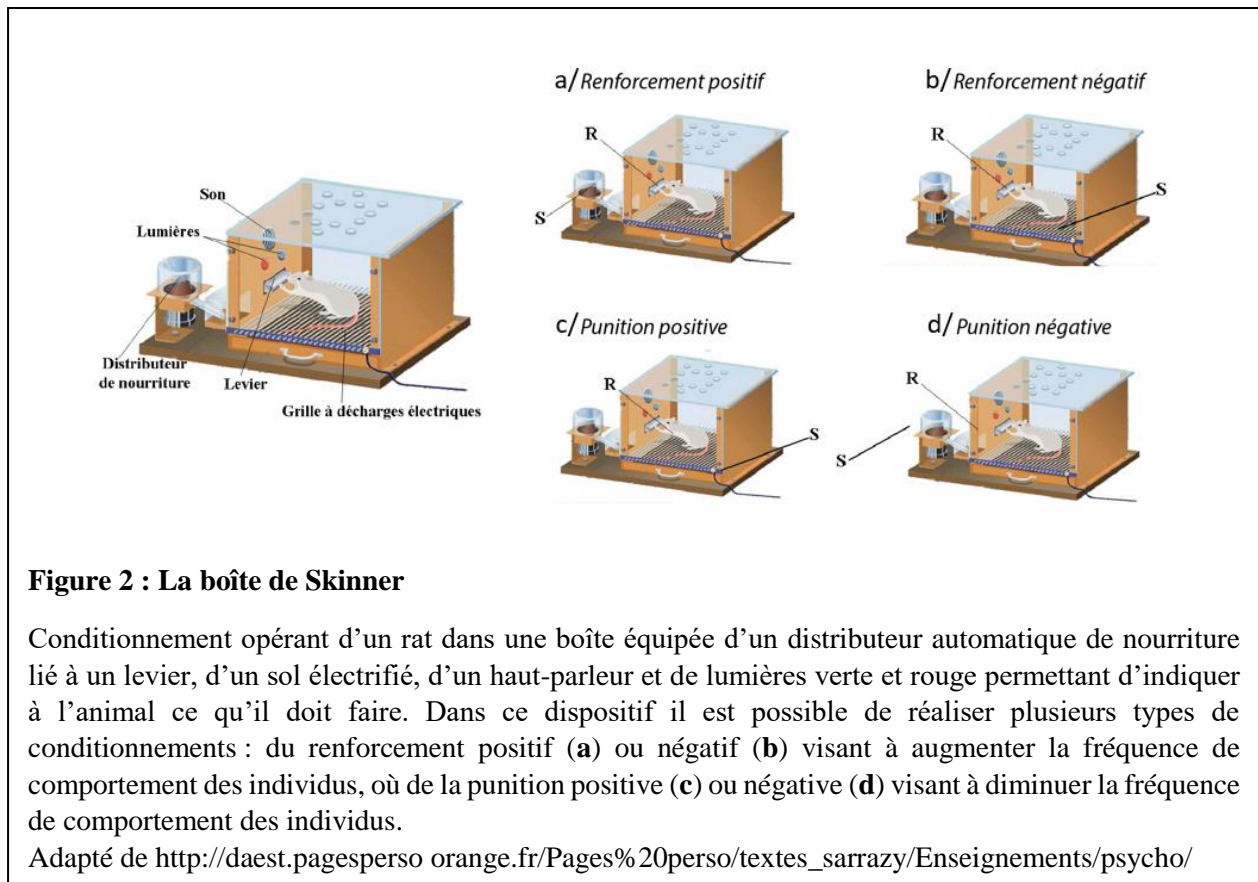


au début du 20<sup>ème</sup> siècle. Ce scientifique russe qui nourrissait son chien chaque jour à la même heure, s'est vite rendu compte que le chien produisait plus de salive qu'a l'accoutumé avant l'heure de son repas. Il décida alors de mettre en place une expérience afin de tester sa théorie de l'apprentissage d'une association entre des stimuli de l'environnement et des réactions automatiques de l'organisme (**Figure 1**). Cet apprentissage est basé sur le principe simple qu'un stimulus inconditionnel **SI**, par exemple de la nourriture, va entraîner une réponse réflexe dite réponse inconditionnelle **RI** qui est ici la salivation pour le chien. En revanche, le son d'une cloche qui est normalement neutre pour le chien et qui est considéré comme un stimulus conditionnel **SC**, ne va pas entraîner de salivation chez le chien. Si maintenant le chien reçoit sa nourriture à plusieurs reprises précédée du son de la cloche, celui-ci va apprendre que le son de la cloche prédit l'arrivée de la nourriture. Une fois cette association apprise, la réponse inconditionnelle va devenir une réponse conditionnée **RC** qui se traduit par la salivation du chien dès qu'il entend le son de la cloche.

Très rapidement un second type d'apprentissage est apparu soulevé par Burrhus Frederic Skinner et appelé **conditionnement opérant (ou instrumental)**. Lors de cet apprentissage, c'est le comportement propre de l'animal qui est pris en compte, ainsi que les conséquences de ce comportement. Un exemple courant est celui de la boîte de skinner qui permet facilement de définir les différents types de conditionnement opération (**Figure 2**). Cette boîte, dans laquelle un rat est disposé, est constituée d'un distributeur de nourriture relié à un levier, ainsi que d'une grille électrique au sol. Un microphone, ainsi que des lumières colorées constituent les différentes stimulations sensorielles permettant d'indiquer à l'individu la règle à suivre. Par exemple, lorsque la lumière verte est allumée le rat peut appuyer sur le levier, à l'inverse si la lumière est rouge il ne peut pas. Plusieurs types de conditionnement sont ainsi possibles :

- **Le renforcement positif** : L'augmentation de la fréquence du comportement de l'individu entraîne une récompense positive. Le rat reçoit de la nourriture s'il appuie sur le levier (**Figure 2a**).
- **Le renforcement négatif** : L'augmentation de la fréquence du comportement de l'individu entraîne la diminution du renforcement négatif. Le rat arrête de recevoir des chocs électriques lorsqu'il appuie sur le levier (**Figure 2b**).

- **La punition positive** : L'augmentation de la fréquence du comportement de l'individu entraîne un renforcement négatif : Le rat reçoit un choc électrique lorsqu'il appuie sur le levier (**Figure 2c**).
- **La punition négative** : L'augmentation de la fréquence du comportement de l'individu entraîne une disparition du renforcement positif. Le rat se voit enlever sa nourriture lorsqu'il appuie sur le levier (**Figure 2d**).



Les apprentissages associatifs sont largement étudiés chez de nombreuses espèces que ce soit chez les Vertébrés (e.g., Homme: Daum et al., 1993; Lapin: Lockhart and Moore, 1975; Rat: Mcallister et al., 1972; Chat: Norman et al., 1977; Souris: Watkins et al., 1998; Singes: Wolpaw and Dowman, 1988; Poisson: Zala and Määttänen, 2013) ou les Invertébrés (e.g., Abeille : Bitterman et al., 1983; Aplysie: Brembs et al., 2002; Seiche: Cole and Adamo, 2005; Fourmi: Dupuy et al., 2006; Guerrieri and d'Ettoire, 2010; Grillon: Matsumoto and Mizunami, 2000; Mouche: Quinn et al., 1974; Blatte: Watanabe et al., 2003).

Parmi ces apprentissages on retrouve plusieurs types de paradigme. On distingue ainsi le **conditionnement absolu**, dans lequel un seul stimulus conditionnel (**SC**) est présenté et associé à un stimulus inconditionnel (**SI**) qui peut être un renforcement positif ou négatif. Le **conditionnement différentiel** quant à lui va mettre l'individu face à deux stimuli conditionnels. L'un sera renforcé positivement (**SC+**) alors que l'autre ne sera pas renforcé ou renforcé négativement (**SC-**). Ces deux types de conditionnement diffèrent par les associations nécessaires. En effet, lors d'un conditionnement absolu l'individu apprend un seul stimulus récompensé et ne génère donc qu'un profil excitateur qui l'entraîne à avoir une réponse uniquement pour ce stimulus. En revanche, lors d'un conditionnement différentiel, l'individu va devoir apprendre un stimulus générant un profil excitateur, ainsi qu'un stimulus générant un profil inhibiteur. Il va donc y avoir une opposition et l'individu en plus d'apprendre la valence de chaque stimulus va aussi apprendre cette opposition.

#### b. Apprentissage non élémentaire

Créer un lien élémentaire entre chaque stimulus et événement semble être une stratégie payante sur le court terme, mais peu optimale à grande échelle. En effet, dans la nature, un animal peut être amené à rencontrer des milliers de stimulus différents, il deviendrait donc rapidement compliqué de mémoriser un tel nombre de lien élémentaire. De plus, chez de nombreuses espèces, des zones cérébrales bien particulières sont allouées à la mémoire (Heisenberg, 2003; Squire et al., 1992) et de ce fait un nombre limité de connexion peuvent être construites dans le cerveau. Un trop grand nombre de lien élémentaire forcerait le réarrangement des connexions dans ces zones, et engendrerait ainsi des coûts énergétiques élevés. Il devient alors nécessaire d'extrapoler des règles et des concepts des apprentissages passés, ce qui permettra de faciliter la mise en place du comportement adéquat dans chaque situation en se reposant sur des connexions cérébrales déjà existantes.

L'apprentissage non élémentaire est donc un apprentissage au cours duquel il n'est pas possible d'utiliser simplement les liens élémentaires formés entre une stimulation et un renforcement. Voici une liste non exhaustive de plusieurs types d'apprentissage qui rentre dans cette catégorie.

### i. L'apprentissage de contexte

L'animal va devoir apprendre que dans un contexte 1, une stimulation A est récompensée et qu'une stimulation B ne l'est pas, alors que dans un contexte 2 c'est la situation inverse. L'individu ne peut alors pas s'appuyer uniquement sur les informations apportées par les stimulations A et B qui apparaissent comme étant autant récompensées l'une que l'autre. Il est aussi impossible de se baser uniquement sur les contextes qui sont eux aussi autant récompensés l'un que l'autre. Un animal démontrera une capacité d'apprentissage contextuel à la condition d'apprendre la configuration des stimulations et du contexte.

Un des paradigmes les plus utilisés chez les vertébrés est celui du conditionnement au contexte de la peur (Humain: Glotzbach et al., 2012; Poisson zèbre: Kenney et al., 2017; Rongeur: Saxe et al., 2006). L'expérience consiste ici à apprendre qu'un des contextes est puni par un choc électrique et donc d'éviter celui-ci.

### ii. L'apprentissage inversé successif

Ce type d'apprentissage est certainement le plus pertinent écologiquement du fait de l'obligation dans la nature pour n'importe quelle espèce animale de modifier les valences données à une stimulation du fait de la variabilité de l'environnement au cours du temps. Comme son nom l'indique il s'agit ici pour l'individu d'apprendre dans un premier temps à associer une stimulation **A** avec un renforcement positif et une stimulation **B** avec rien. Ensuite, au cours d'une seconde phase, l'animal devra associer **B** avec une récompense et **C** avec rien. Lors de la 3<sup>ème</sup> phase, il devra cette fois-ci associer **C** à une récompense et **D** à rien. Pour finir, **D** sera pendant la dernière phase associer à la récompense alors que la stimulation **A** de départ n'est cette fois-ci pas renforcée. L'individu va ainsi être confronté à une inversion des valences des stimulations au cours du temps, afin d'être optimal et de réagir en conséquence, l'animal doit apprendre chaque paire de stimulation comme appartenant à une configuration particulière.

Ce paradigme a lui aussi été largement adopté chez de nombreuses espèces vertébrées et invertébrées, fournissant ainsi un nouvel outil pour une meilleure compréhension des mécanismes

comportementaux et neuronaux sous-jacents à la mémoire (Bond et al., 2007; Gossette, 1968; Kamil et al., 1977; Komischke et al., 2002; Schusterman, 1964).

### iii. Les apprentissages de concepts

Contrairement au précédent apprentissage décrit, il est ici question d'être capable d'associer une récompense à la relation en plusieurs stimulations. Il existe deux paradigmes très connus permettant par exemple de tester la capacité d'un animal à apprendre le concept de similarité et le concept de différence entre deux stimulations.

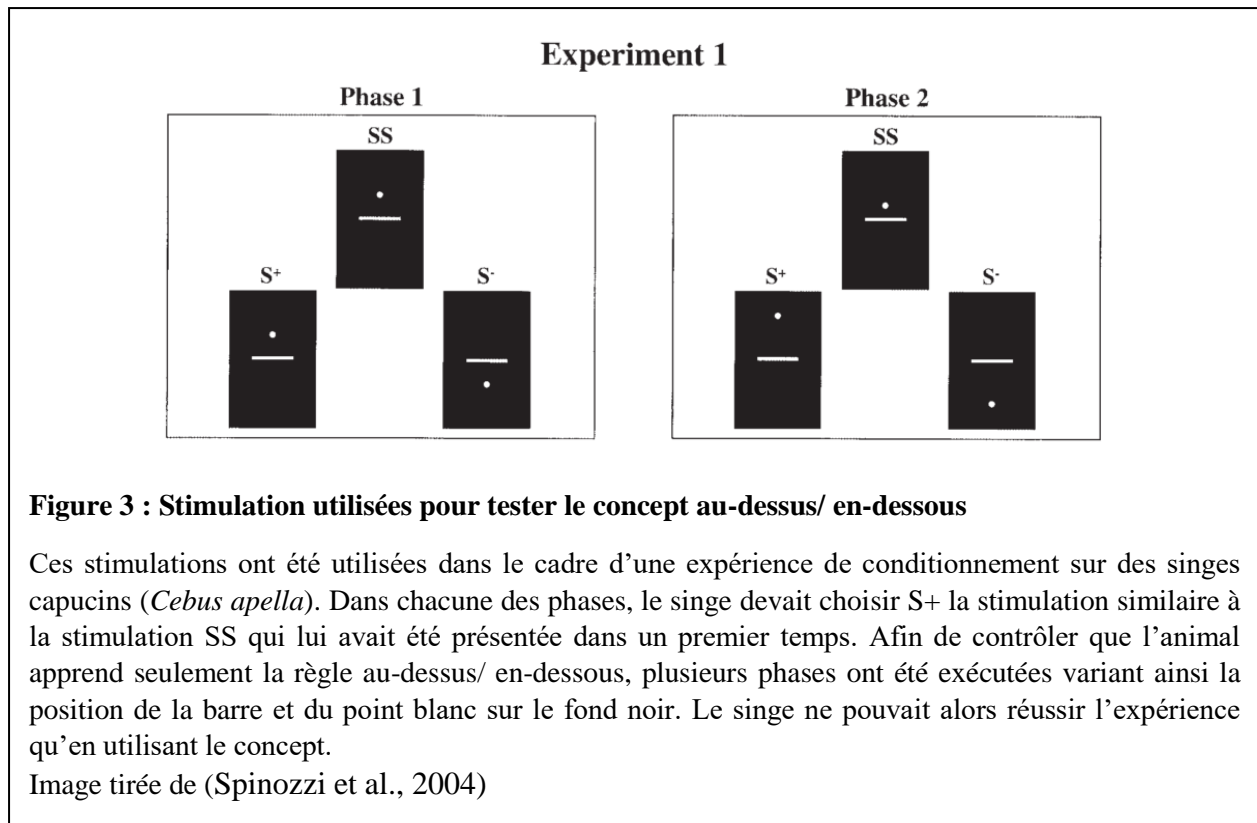
#### 1. Le concept de similarité

Le DMTS où « Delayed Matching-To-Sample » est un paradigme qui permet d'étudier la capacité d'un individu à apprendre le concept de similarité. Le principe de ce procédé est qu'une stimulation va être présentée à l'animal dans un premier temps, puis dans un second temps celui-ci va être confronté à deux stimulations dont l'une est la même que présentée précédemment. L'individu doit alors apprendre que la stimulation renforcée positivement est celle qui lui avait été présentée auparavant. Afin de s'assurer de l'apprentissage de la règle et non des caractéristiques physico-chimiques des stimulations, la nature des stimuli utilisés est modifiée à chaque essai de conditionnement.

À l'inverse, le DNMTS où « Delayed Non Matching-To-Sample » va permettre d'étudier la capacité d'un individu à apprendre le concept de différence. Le principe reste le même, à la différence que cette fois-ci l'individu doit toujours choisir la stimulation différente de celle présentée plus tôt s'il veut recevoir un renforcement positif.

Ce type de paradigme a été largement utilisé depuis des dizaines d'années pour étudier si les animaux possédaient des capacités d'apprentissage de concepts ou de symboles (Finch, 1942; Giurfa et al., 2001; Herman et al., 1989; Kangas et al., 2010; Kuśmierk and Kowalska, 1998; Nakagawa et al., 2004; Pack et al., 1991; Weinstein, 1941; Yerkes and Nissen, 1939), ou pour étudier les effets de drogues ou de lésion du cerveau sur la mémoire (Dunnett et al., 1990; Horel et al., 1984; Sloan et al., 2006; Stanhope et al., 1995).

## 2. Le concept de relation spatiale



Ici les caractéristiques physico-chimiques de la stimulation n'ont aucune importance, seule la position est importante. Il est d'ailleurs possible d'utiliser le paradigme de DMTS afin de tester la capacité des individus à apprendre ce genre de concept. En effet, il suffit d'utiliser des stimulations où la seule différence est la position d'un des éléments (**Figure 3**). L'individu sera alors obligé d'extraire l'information de position pour réussir à obtenir sa récompense. Ce type d'expérience a été réalisée chez plusieurs espèces allant des Vertébrés aux Invertébrés, ici encore pour mettre en évidence leur capacité à apprendre des concepts (Avargues-Weber et al., 2011a; Avarguès-Weber et al., 2012; Dépy et al., 1999; Quinn, 1994; Spinozzi et al., 2004)

## 3. Le concept de numérosité

La capacité de compter des objets est une mesure très populaire pour évaluer les capacités cognitives d'un animal. Depuis des années, des études sur ce type de capacité ont d'ailleurs été



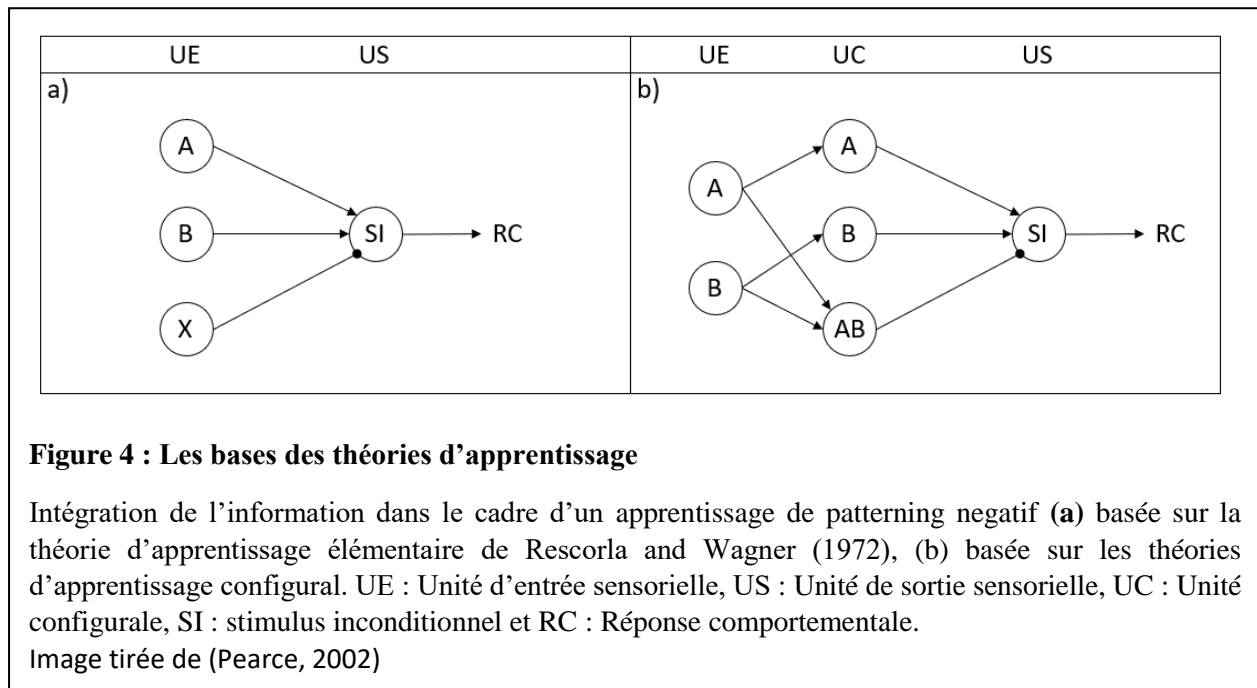
largement réalisées que ce soit chez des mammifères (Abramson et al., 2013; West and Young, 2002), des oiseaux (Pepperberg, 2006; Roberts et al., 2002; Rugani et al., 2007), des poissons (Agrillo et al., 2007), des salamandres (Uller et al., 2003) ou encore des insectes (Chittka and Geiger, 1995). Il ne s'agit encore une fois plus d'apprendre les caractéristiques d'un objet, mais cette fois-ci d'être capable de compter le nombre d'objets présentés et ainsi être capable de discriminer une situation dans laquelle cinq objets sont présentés et une autre où 10 objets sont présentés.

#### iv. Les apprentissages configuraux

Contrairement aux apprentissages élémentaires pour lesquels on observe la création d'un lien stimulus-réponse pour des stimuli individuels (Rescorla and Wagner, 1972), lors d'un apprentissage configuratif un individu va être capable de construire un lien spécifique et unique avec chaque stimulus, ou chaque configuration incluant ces stimuli (Gulliksen and Wolfle, 1938). Le meilleur moyen d'introduire la théorie d'apprentissage configuratif (Pearce, 2002) est de la comparer à celle de l'apprentissage élémentaire de Rescorla and Wagner (1972) pendant un apprentissage de type « Patterning négatif ». Ce type d'apprentissage nécessite comme l'apprentissage inversé successif la capacité de considérer une paire de stimulations comme une configuration particulière. Dans ce type de discrimination, deux stimulations vont être renforcées (A+ et B+), alors que le motif incluant A et B lui ne sera pas renforcé. L'individu va alors devoir apprendre à ne répondre qu'à A et B mais pas au motif. Cette discrimination n'est possible que si le motif AB n'est pas considéré comme une simple somme de A et B. En effet, cette discrimination est impossible si on s'appuie sur la théorie élémentaire (**Figure 4a**). Si AB est associé au SI, X représente l'unique indice lié au composé AB. Cette théorie prédit que X va acquérir une valence négative et aura une influence négative sur la réponse comportementale. Cette valence négative pourrait entièrement couvrir la valence positive acquise pour A et B seul et entraîner une inhibition complète de toute réponse comportementale pour AB. En revanche, pour A et B présentés seuls, X ne sera pas activé et la réponse comportementale ne sera liée qu'à l'association du stimulus conditionnel et du stimulus inconditionnel. En s'appuyant sur cette théorie, le résultat attendu serait une généralisation pour A, B et AB. Force est de constater que ce n'est pas le comportement observé. La théorie d'apprentissage configuratif (**Figure 4b**), met en avant l'existence d'une unité configurale entre les

unités d'entrée et les unités de sortie. Contrairement à la théorie élémentaire, ici lorsque AB n'est pas renforcé, une connexion inhibitrice va se former entre l'unité configurale AB et l'unité US. Ainsi, lorsque A est présenté, il va activer fortement sa propre unité configurale et faiblement l'unité configurale AB. Il en va de même pour B. Lorsque AB est présenté, la forte activation de l'unité configurale AB va s'opposer à la faible activation des unités configurales A et B. De ce fait, la réponse comportementale pour AB va être très faible. Cette théorie explique pleinement les comportements observés lors de ce type de protocole d'apprentissage.

La capacité à résoudre ce type d'apprentissage a d'ailleurs été observée chez de nombreuses espèces que ce soit pour des modalités olfactives ou visuelles (Bellingham et al., 1985; Davidson et al., 1993; Deisig et al., 2001; Harris et al., 2008; Kehoe and Graham, 1988; Livesey et al., 2011).



#### v. La catégorisation

Ce type d'apprentissage nécessite la capacité de traiter différentes stimulations comme équivalentes et de ce fait d'avoir une réponse comportementale similaire quel que soit celui présenté. C'est un processus qui comme précisé précédemment va permettre de réelles économies quant au processus cognitif à appliquer et donc une économie d'énergie.

Dans ce type d'apprentissage, l'individu va devoir intégrer que le renforcement positif n'est cette fois-ci pas lié à une seule stimulation mais à un panel de stimulations liées entre elles par une caractéristique physico-chimique commune. Un animal capable de catégorisation va alors montrer, après avoir reçu un renforcement positif pour plusieurs objets sphériques, le même type de réponse comportementale pour un nouvel objet inconnu mais sphérique lui aussi. De nombreuses publications ont montré des capacités de catégorisation chez les Vertébrés tels que l'homme (De Brigard et al., 2017), les singes (Antzoulatos and Miller, 2014), les oiseaux (Wahlheim and DeSoto, 2017) ou encore les Invertébrés tels que l'abeille (Benard et al., 2006).

Cette première partie a permis de présenter un panel des nombreuses formes d'apprentissage. Force est de constater que ces modalités ont été étudiées chez de nombreuses espèces en passant par l'homme, les singes, certains oiseaux tels que le pigeon, le dauphin et enfin les rongeurs. Bien que les performances de toutes ces espèces soient remarquables dans chaque type d'apprentissage présenté, elles présentent malheureusement une limite. En effet, leur cerveau est particulièrement complexe et structuré autour d'un nombre incalculable de neurones et de connexions rendant ainsi l'étude de cette structure compliquée. L'insecte quant à lui ne présente pas cet inconvénient du fait de la petite taille du cerveau et du faible nombre de neurones. Les insectes constituent ainsi un modèle de choix pour étudier les bases neurales de l'apprentissage visuel. **Mais pourquoi l'abeille ?** En effet, de nombreuses publications tendent à montrer que des insectes tels que la drosophile ou le cricket sont des modèles fournissant des outils puissants pour étudier le cerveau chez l'insecte (Grima et al., 2004; Joesch et al., 2010; Matsumoto et al., 2016). **La section suivante répondra à cette question par la mise en avant des nombreuses avancées sur nos connaissances à propos de l'apprentissage visuel chez les abeilles tant en terme comportementale que neuronale.**

## II. L'abeille : un modèle pour l'étude de la cognition visuelle

L'abeille est un hyménoptère social dont le nid occupe une place centrale. La colonie est en effet divisée en plusieurs castes afin de séparer les différentes tâches et ainsi permettre le maintien de la survie de la colonie qui compte pas moins de plusieurs dizaines de milliers d'individus (Winston, 1991). On retrouve ainsi dans un premier temps des individus reproducteurs, la reine et les mâles

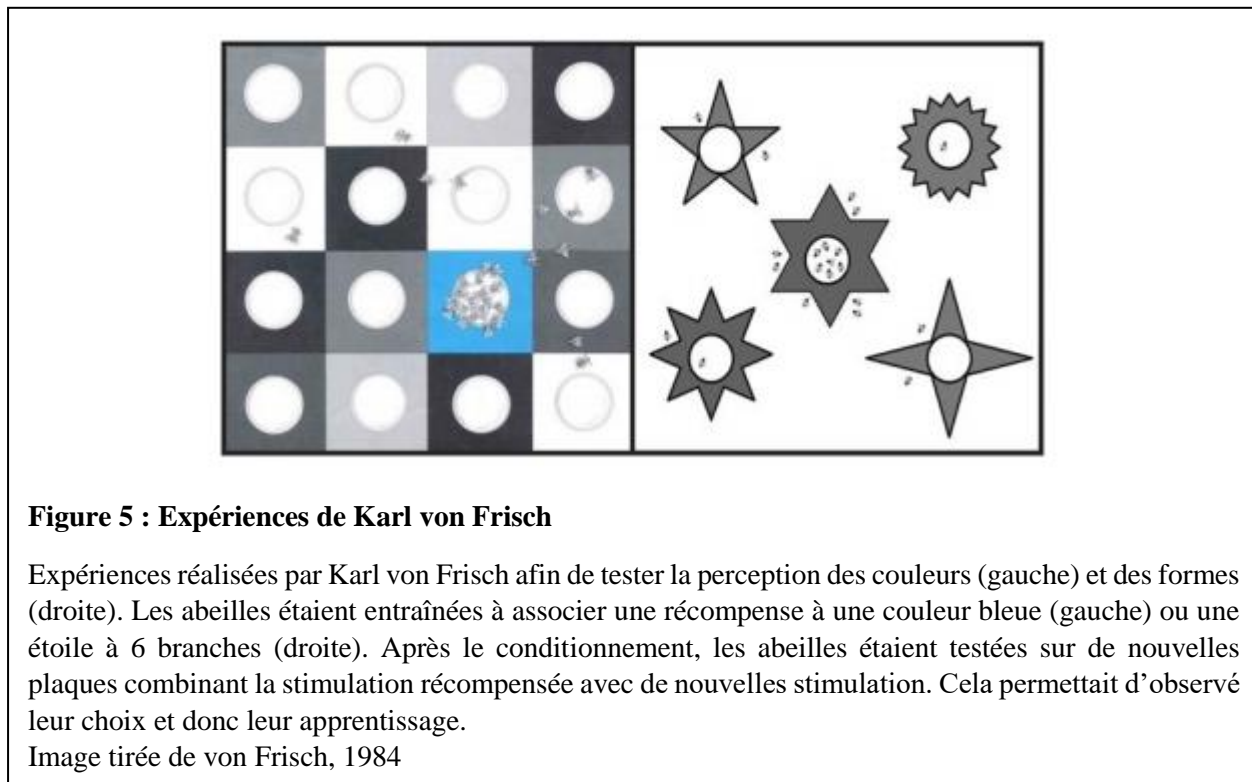
(drones ou faux-bourçons). La reine unique et exclusive à la colonie va pondre plus de 2000 œufs par jour à l'intérieur de cellules hexagonales qui vont être recouvertes et dont le regroupement sera appelé « couvain ». Les faux-bourçons quant à eux n'apparaîtront qu'au printemps et ne représenteront qu'une faible partie des effectifs de la ruche. Leur rôle est uniquement d'aller se reproduire avec d'autres reines. Passé cette période de reproduction, les mâles vont être expulsés des ruches. La majorité de la population de la colonie est en fait composée d'ouvrières dont le rôle va évoluer au cours du temps. À leur naissance, les abeilles nettoient les alvéoles du couvain pendant les deux premiers jours. Durant les 10 jours suivants leur fonction va évoluer vers un nourrissage des jeunes larves. Va s'ensuivre une courte période au cours de laquelle cette abeille va se consacrer à plusieurs activités telles que l'élaboration du miel, la construction des rayons. Pour finir, celle-ci deviendra une gardienne et défendra l'entrée de la ruche. Finalement, l'abeille va endosser son dernier rôle, celui de butineuse. C'est un des rôles les plus importants car de son activité va découler les ressources de la colonie pour le nourrissage du couvain et pour pouvoir passer l'hiver. Au cours de ma thèse, c'est cette caste qui a été privilégiée. En effet, lors de leur butinage, ces abeilles sont amenées à effectuer de nombreux allers-retours chaque jour entre la ruche et des sources de nourriture. Par conséquent, ces abeilles ont développé d'incroyables capacités de navigation basées sur l'utilisation de la lumière polarisée (Evangelista et al., 2014; Menzel et al., 1996) , mais aussi de divers repères visuels et du flux optique pour déterminer les distances de vol (Menzel et al., 1996; Srinivasan et al., 1996). De plus, comme cela était expliqué au début de l'introduction, les abeilles utilisent la « constance florale » comme stratégie de butinage (Chittka and Raine, 2006). Ces stratégies de navigation et de butinage en font un modèle invertébré privilégié pour l'étude des mécanismes comportementaux et neuronaux de la cognition visuelle (Avarguès-Weber et al., 2011; Giurfa, 2013, 2015). De plus, la facilité d'entraînement de cet insecte et la simplicité de la structure de son cerveau permettent aisément de mettre en place différents types de protocoles.

#### a. Apprentissage et mémoire visuel chez l'abeille : du terrain au laboratoire

Depuis plus d'un siècle et les découvertes de Karl von Frisch (von Frisch, 1914), nombre d'études ont été réalisées pour déterminer les limites de la cognition visuelle chez l'abeille. Ces expériences ont été en majorité réalisées en libre vol. Mais les ambitions visant à étudier les corrélats neuronaux

impliqués ont poussé les chercheurs à développer des protocoles d'apprentissage visuel en laboratoire.

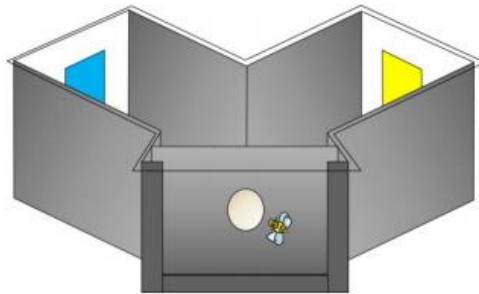
i. Les expériences en libre vol



Il est possible de marquer au préalable une abeille afin de l'identifier et de la conditionner à associer une stimulation à une récompense sucrée en libre vol. C'est sur cette base protocolaire que Karl von Frisch s'est reposé afin de mettre en évidence la capacité des abeilles à associer une couleur, une forme ou un motif à une récompense (von Frisch, 1914). Il présentait horizontalement divers stimuli visuels dont seul un était associé à la solution sucrée. De cette manière il a pu montrer que les abeilles possédaient une vision chromatique en les confrontant à une stimulation colorée et des stimulations achromatiques de même luminosité (**Figure 5**). De la même manière, il a aussi montré leur capacité à percevoir les formes (**Figure 5**).

Depuis, bien que les principes aient été conservés, les méthodes, elles, ont évoluées. Ainsi, d'une présentation horizontale, nous sommes passés à une présentation verticale permettant une constance de l'orientation de la stimulation quel que soit l'angle d'arrivée de l'abeille (Wehner,

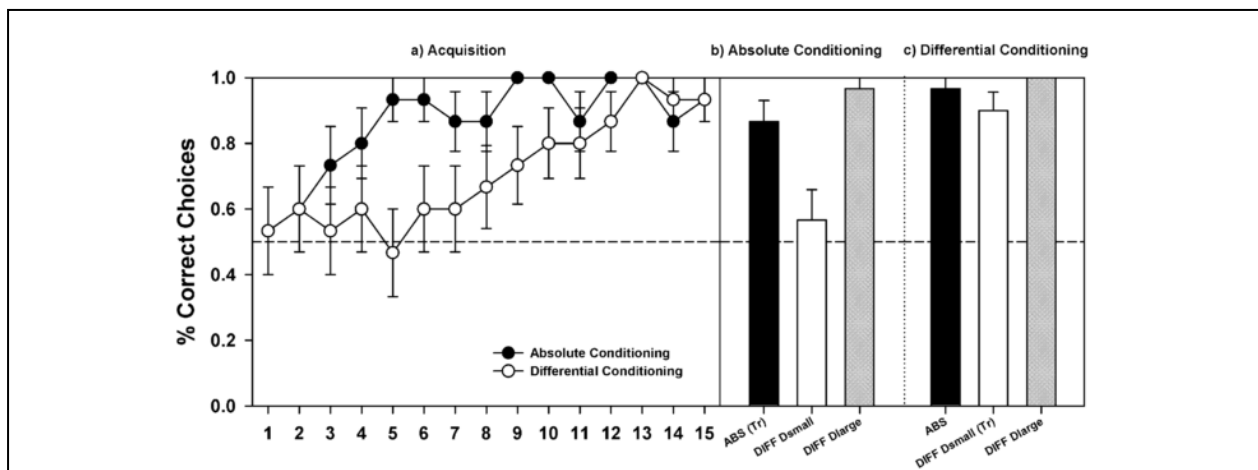
1967). De plus, l'utilisation de labyrinthe en Y s'est généralisée (**Figure 6**). Cela a permis d'isoler une abeille de ses congénères pendant le conditionnement, et aussi de réaliser des protocoles de discrimination plus contrôlés. Une majorité des expériences d'apprentissage visuel ont ainsi été réalisées dans ce type de dispositif.



**Figure 6 : Labyrinthe en Y**

Labyrinthe en Y composé de 3 branches, une de départ, et deux de choix. L'abeille entre par la branche de départ, après quoi une trappe se ferme afin de l'isoler. Elle se retrouve alors confrontée à deux stimuli visibles seulement une fois la partie centrale atteinte. L'abeille va alors faire un choix entre une des deux branches où elle trouvera un renforcement positif ou négatif au niveau du stimulus. Le labyrinthe est recouvert d'un plexiglas transparent au rayons ultraviolets afin de ne pas perturber la vision de l'abeille.

Des études sur l'acuité visuelle des abeilles ont pu être mises en place grâce au contrôle précis des angles entre les stimuli fournis par le labyrinthe en Y (Giurfa et al., 1996; Srinivasan and Lehrer, 1988). Ces expériences étaient essentielles car elles permettent à présent de déterminer si une abeille perçoit correctement une stimulation visuelle. Ce nouveau dispositif a aussi permis de mettre en évidence qu'en augmentant le niveau d'attention de l'abeille (isolation de l'environnement), la finesse de perception était augmentée. Cela a par exemple permis de montrer que les abeilles sont capables de discriminer des motifs plus complexes qu'une simple couleur ou forme (Giurfa et al., 1999). Les différents paradigmes d'apprentissage associatif élémentaire présentés plus haut ont ainsi été testés dans les labyrinthes en Y, en passant de l'apprentissage absolu à l'apprentissage discriminatif (Giurfa, 2004, **Figure 7**). Dans cette étude, des abeilles ont appris à discriminer deux ronds colorés au cours d'un conditionnement absolu ou différentiel. Les abeilles étaient ensuite testées sur leur apprentissage au cours d'un test en absolu et en différentiel. Les abeilles conditionnées en absolu généralisaient beaucoup plus lorsqu'elles étaient testées en différentiel. En revanche, conditionnées en différentiel, elles restaient tout aussi efficaces pendant les tests quelques soit leurs natures. **De plus, cette publication a été une référence au cours de ma thèse concernant le nombre d'essai à utiliser afin d'obtenir les meilleures performances (12 essais, Figure 7).**

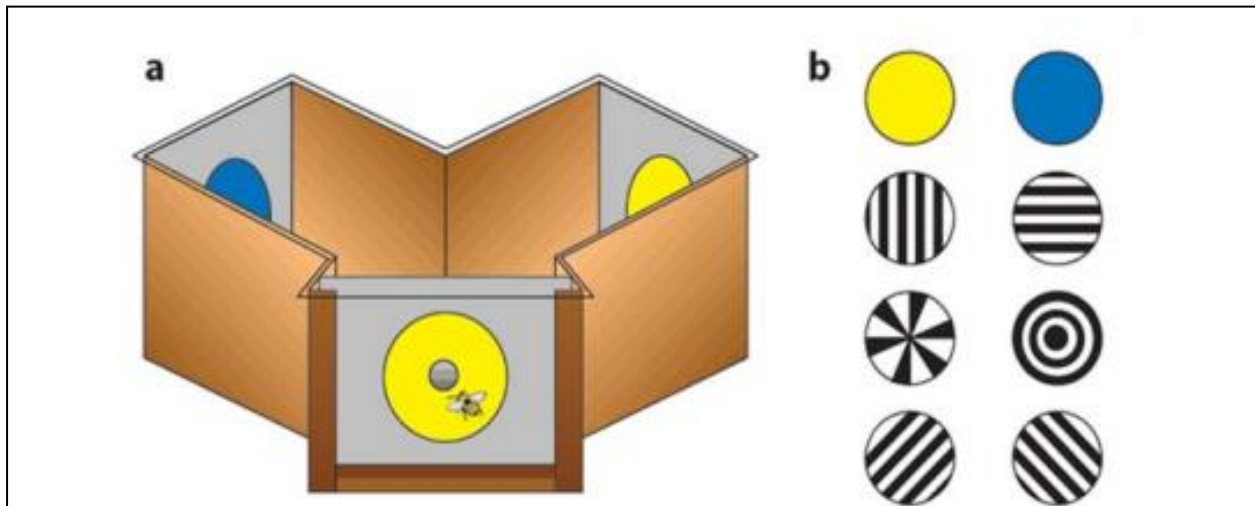


**Figure 7 : Performances d'abeilles lors d'un apprentissage visuel en libre vol**

Performances lors du conditionnement (a) pendant un conditionnement absolu (noir) ou discriminatif (blanc). Le pourcentage de choix correct augmente avec les essais. Performances lors du test pour les abeilles après un conditionnement absolu (b) et un conditionnement discriminatif (c). Après chaque conditionnement les abeilles étaient testées en conditions absolue (noir), discriminatif avec la même taille pour les deux stimuli (blanc) ou une taille différente (gris).

Figure tirée du papier (Giurfa, 2004).

L'apprentissage non élémentaire a lui aussi été exploré grâce au labyrinthe en Y. Ainsi, la capacité des abeilles à apprendre de nombreux concepts lors de protocoles de « Delayed Matching-To-Sample » nous a été démontrée (Avarguès-Weber and Giurfa, 2013; Zhang, 2006). En effet, les abeilles semblent disposées à pouvoir apprendre le concept de « similarité/différence » (**Figure 8**, Giurfa et al., 2001), mais aussi de « au-dessus/ en-dessous » (Avarguès-Weber et al., 2011b; Avarguès-Weber et al., 2012). Force est de constater que les abeilles sont aussi capables d'extraire des informations visuelles pour apprendre à catégoriser des objets en fonction de leurs caractéristiques visuelles (Benard et al., 2006), ou encore de les différencier en utilisant la conformation des différents éléments pour discriminer des visages par exemples (Avarguès-Weber et al., 2010).



**Figure 8 : Concept de similarité chez l'abeille**

Dispositif (a) et stimulation visuelle (b) utilisés pour étudier les capacités des abeilles à apprendre le concept de similarité/différence. L'abeille devait apprendre que le stimulus récompensé à l'intérieur du labyrinthe en Y était le même que celui présenté à l'entrée. Dans cet exemple, le rond jaune était donc récompensé. Afin d'éviter que l'abeille apprenne des caractéristiques de la stimulation ou le côté du labyrinthe récompensé, la nature des stimuli utilisés variait au cours de l'expérience, ainsi que la branche récompensée. Pour finir et pour être certain que le concept était appris, un test de transfert était réalisé en utilisant des stimuli très différents (par exemple des grilles noire et blanche, b).

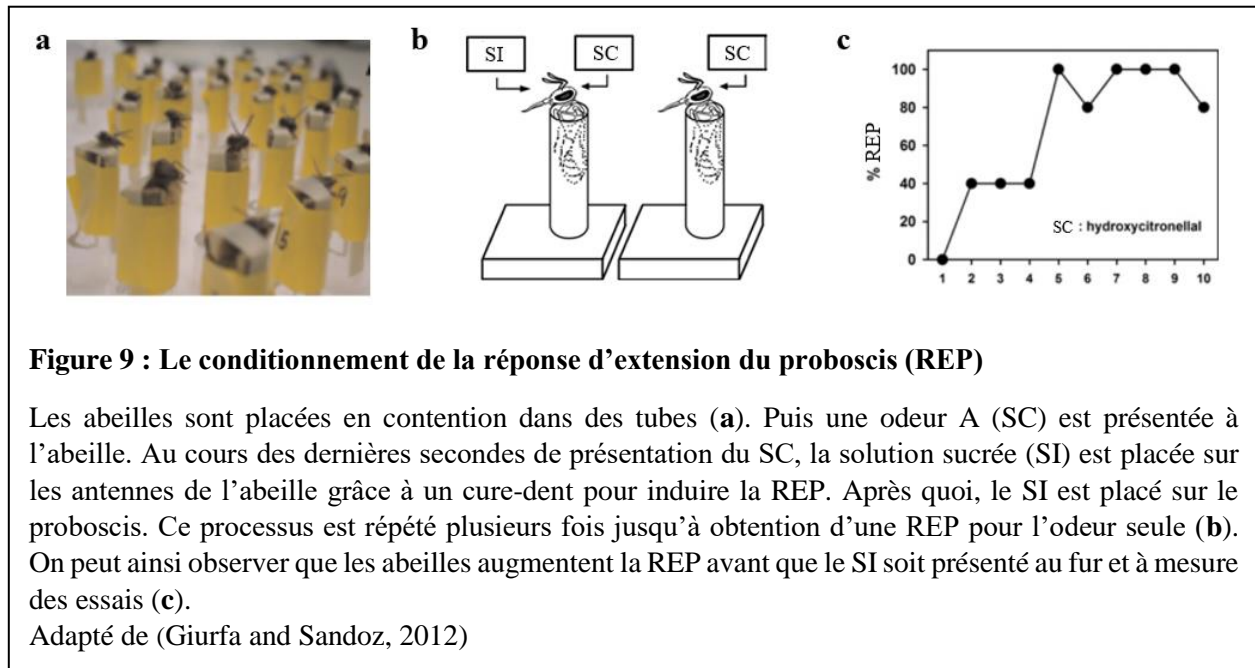
Figure tirée de (Avarguès-Weber et al., 2011a) basée sur l'expérience de (Giurfa et al., 2001)

De plus, les abeilles ont aussi démontré des capacités d'apprentissage visuel configurale tel que le patterning négatif (Schubert et al., 2002). Dans cette étude, des abeilles en libre vol ont réussi à discriminer un damier jaune et un damier violet récompensés et un damier jaune et violet non récompensé, prouvant ainsi leur capacité à réaliser un apprentissage configurale en modalité

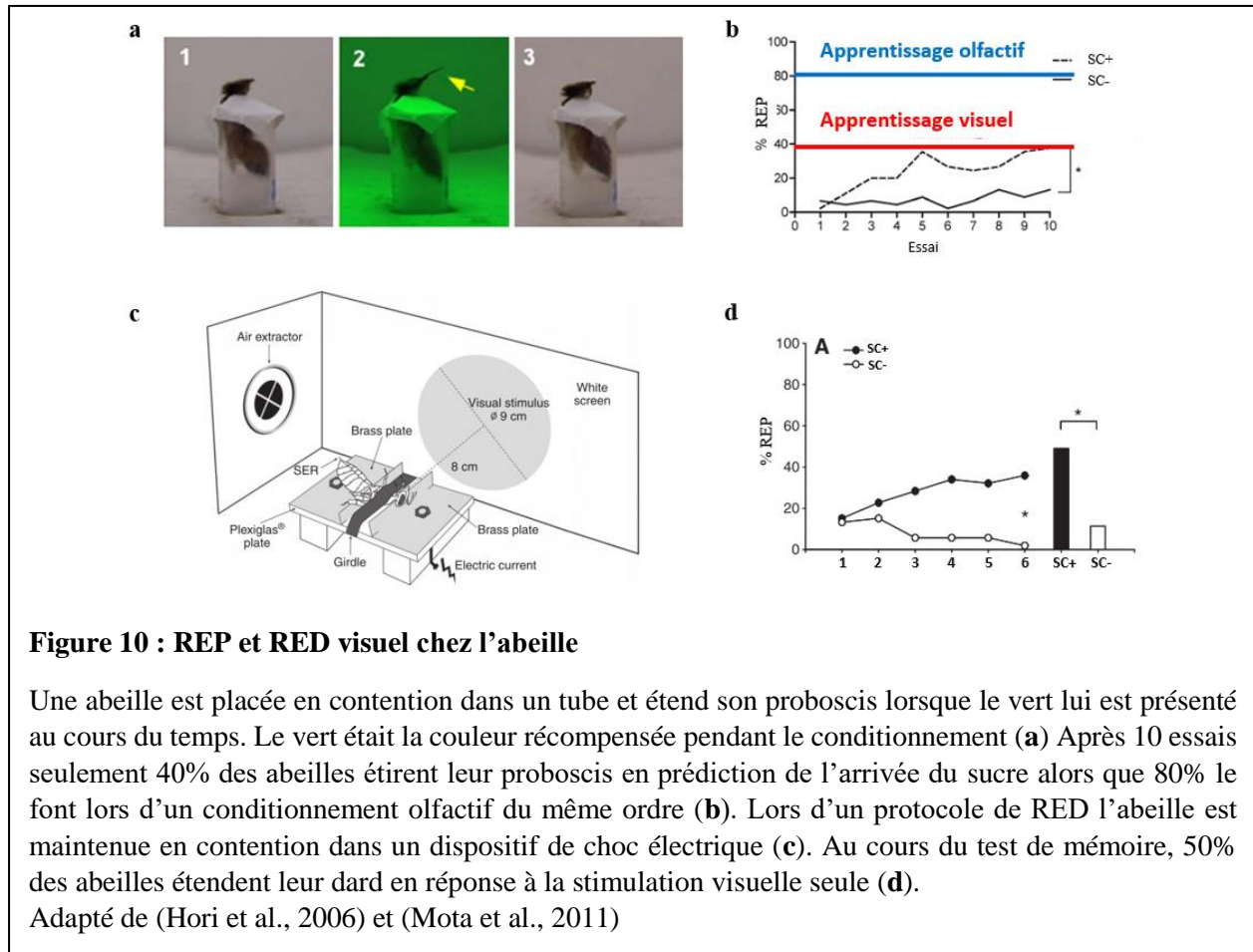


visuelle. Cette étude a constitué une référence solide afin de mettre en place un paradigme d'apprentissage visuel non élémentaire avec des abeilles fixées au cours de ma thèse.

## ii. Les expériences contrôlées en laboratoire

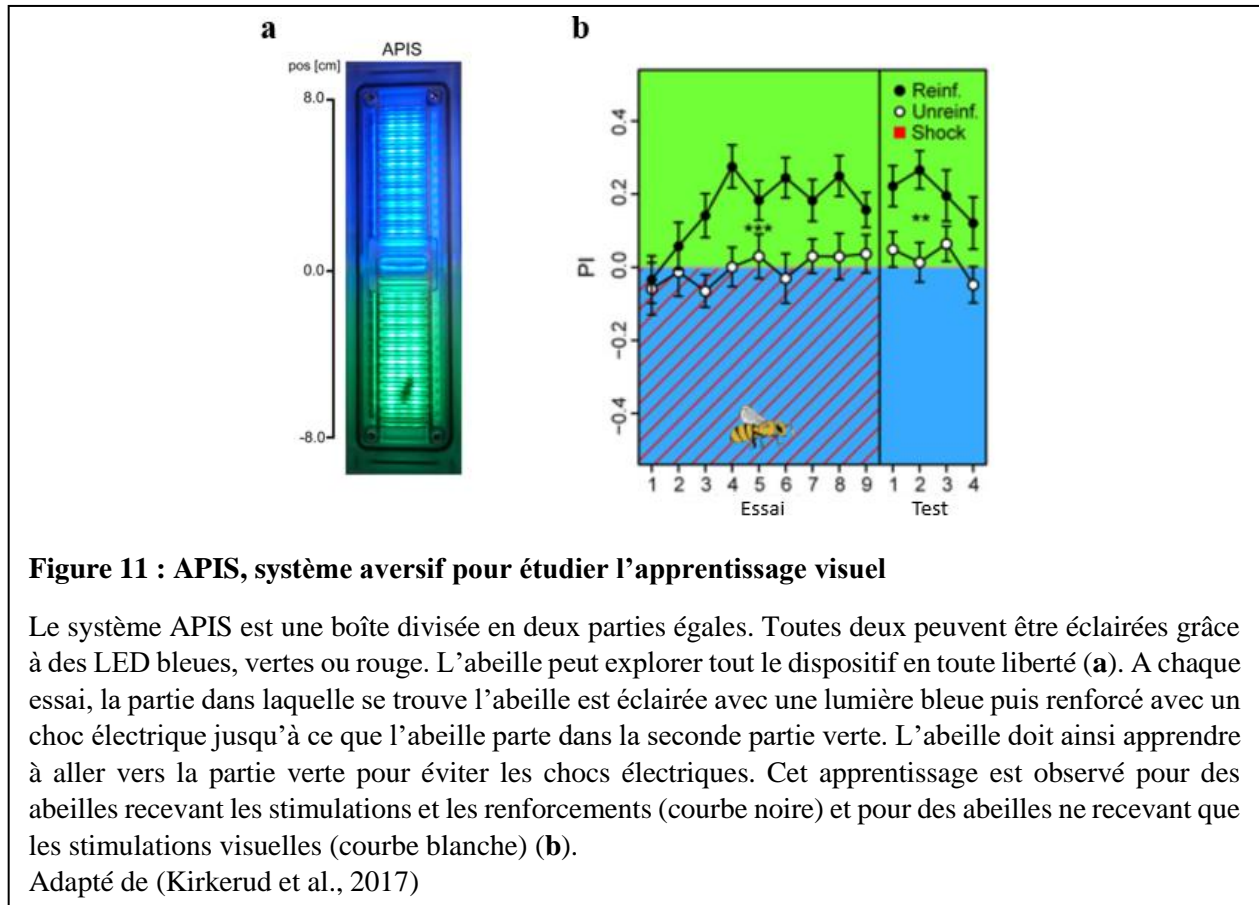


La compréhension des mécanismes neuronaux sous-jacents à ces apprentissages est un enjeu majeur et a poussé les chercheurs à adapter les paradigmes utilisés en libre-vol afin de les utiliser en laboratoire. Pour ce faire, l'idée a été de transformer ces conditionnements opérants en conditionnements classiques. C'est alors un des conditionnements classiques les plus réputés pour étudier l'apprentissage olfactif chez l'abeille qui a été choisi : le conditionnement de la Réponse d'Extension du Proboscis (**REP**) (Giurfa and Sandoz, 2012). Ce conditionnement est réalisé sur des abeilles placées en contention dans des tubes (**Figure 9a**). Une odeur A va leur être présentée grâce à un canon à odeur. Pendant l'odeur A, grâce à un cure-dent une récompense sucrée va être déposée dans un premier temps sur les antennes, entraînant ainsi de la réponse d'extension du proboscis (langue). Le cure-dent est alors mis sur le proboscis afin de permettre à l'abeille de se nourrir quelques secondes. Cette association entre l'odeur A et la solution sucrée va être répétée plusieurs fois, puis au cours d'un test l'odeur seule va être présentée. Si l'individu a appris il va étendre son proboscis pour l'odeur seule car il aura appris que cette odeur était prédictive de



l'arrivée de la solution sucrée (Figure 9b). On peut ainsi observer qu'au fur et à mesure des essais de conditionnement les abeilles vont de plus en plus étendre leur proboscis avant même que la solution sucrée arrive (Figure 9c).

Le protocole pour l'étude de l'apprentissage associatif visuel repose sur le même principe. Des abeilles sont placées en contention dans des tubes et font l'expérience d'un conditionnement absolu ou différentiel au cours duquel elles doivent apprendre qu'un stimulus visuel est prédictif d'une récompense (Figure 10a). L'adaptation de ce protocole pour étudier l'apprentissage visuel n'a malheureusement pas fourni de résultats concluants. En effet, pour un protocole d'apprentissage discriminatif simple entre deux couleurs, les abeilles présentent des performances relativement faibles (Figure 10b, 40% de REP contre 80 à 100% en olfactif). Afin de remédier à cela de nombreuses études ont été menées en modifiant les différents paramètres du REP tel que l'inclinaison des abeilles, la liberté de la tête, la manière dont les stimulations visuelles étaient produites (Avarguès-weber and Mota, 2016). Ces expériences reposaient sur des récompenses



positives. Certains ont donc tenté d'utiliser un autre protocole de conditionnement absolu réputé chez l'abeille, le conditionnement de Réponse d'Extension du Dard (RED). Ce paradigme repose sur les mêmes principes expérimentaux que le REP mais avec un renforcement aversif. En effet, l'abeille est cette fois-ci placée en contention sur le dos dans un dispositif de choc électrique (Figure 10c). L'abeille apprend alors que la stimulation visuelle est prédicteur d'un choc électrique et va donc étendre son dard pour la réponse seule au cours du test de mémoire. Cette expérience n'a pas non plus rivalisé avec l'apprentissage obtenu en REP (Figure 10d).

Ces expériences sont basées sur des principes de conditionnement pavlovien, restreignant en tout point la mobilité de l'abeille. Une des hypothèses majeure des faibles performances lors de ces conditionnements est d'ailleurs l'impossibilité pour les abeilles d'utiliser la vision active qui est un élément essentiel au cours de la navigation et du butinage (Srinivasan and Zhang, 2004). Ces dernières années, un premier pas vers une augmentation de la liberté de mouvement de l'abeille en laboratoire a été réalisé. En effet, on a vu un nouveau dispositif apparaître nommé APIS (pour « Automatic Performance Index System ») (Kirkerud et al., 2013). Ce dispositif est un système

aversif basé sur l'utilisation de choc électrique (**Figure 11a**). Au cours de ces expériences de conditionnement opérant les abeilles apprennent à éviter la zone colorée qui est renforcée avec un choc électrique (Kirkerud et al., 2017, **Figure 11b**).

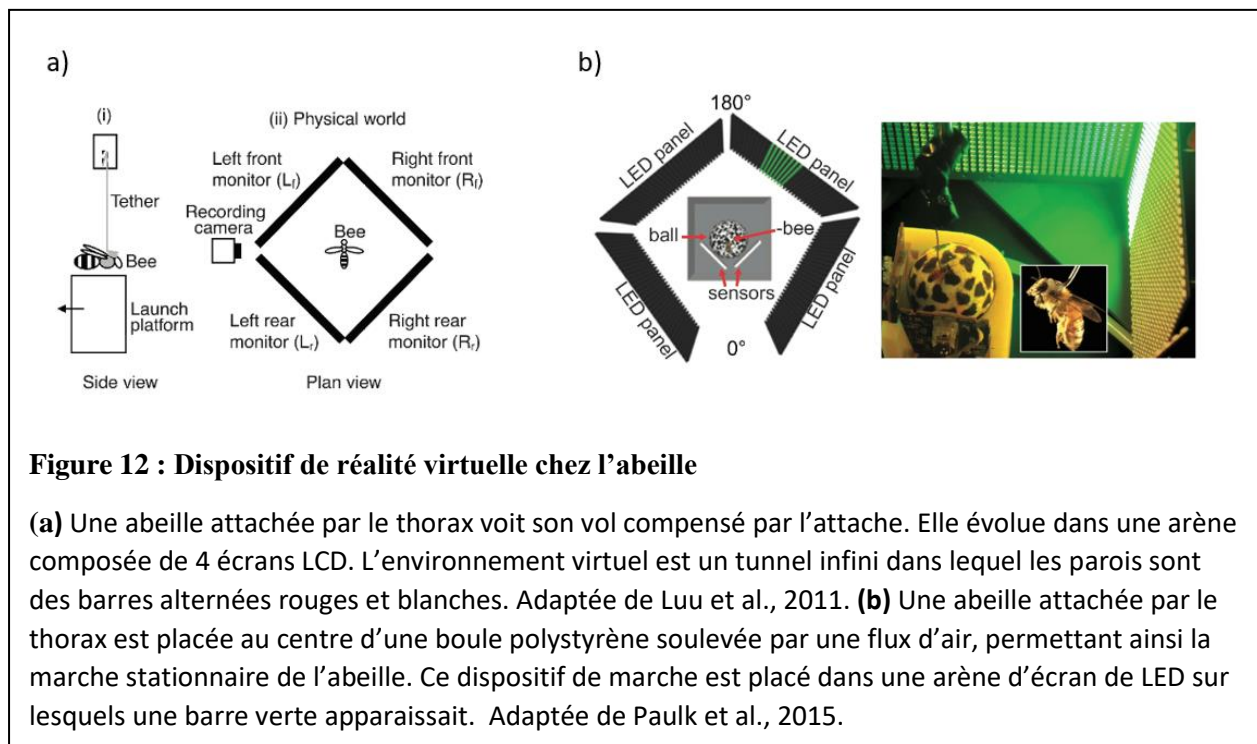
Ce procédé a aussi été utilisé afin de tester la capacité inverse des abeilles à ne pas aller vers une zone colorée, de la même manière que dans un protocole de conditionnement de peur (Dinges et al., 2017). Ce nouveau dispositif a déjà permis la mise en évidence de certaines zones du cerveau impliquées dans les apprentissages visuels aversif grâce à des injections ciblées (Plath et al., 2017). Mais il ne permet pas d'aller plus loin dans l'exploration du cerveau et ne fournit ici qu'un support pour l'apprentissage aversif. La porte reste donc grande ouverte quant au développement d'un nouveau dispositif permettant de l'apprentissage appétitif combiné à des méthodes invasives.

### iii. La réalité virtuelle : un compromis entre terrain et laboratoire

Les sciences ont toujours évolué grâce à la modernisation toujours plus rapide des technologies. Par exemple, les microscopes deviennent de plus en plus précis, permettant ainsi la mise en évidence de structures cellulaires jusqu'alors non observables. Force est de constater que depuis quelques années, la nouvelle tendance est à la réalité virtuelle. La réalité virtuelle pourrait être définie comme un environnement simulé, perçu par l'individu placé en son sein, et qui va évoluer en fonction des mouvements de cet individu (Dombeck and Reiser, 2012). Particulièrement mise en avant ces dernières années par l'industrie du jeu vidéo, la réalité virtuelle a néanmoins très rapidement attiré l'attention des scientifiques, notamment pour faciliter l'étude des comportements et du cerveau humain (Bennett et al., 2018 ; Reggente et al., 2018). L'utilisation à l'échelle humaine était simple et déjà adaptée, mais de nombreux chercheurs face à cette nouveauté ont décidé d'aller plus loin et d'adapter ce type de technologies à des modèles autres que l'humain. Nous avons ainsi pu voir émerger de nombreux systèmes pour les souris, les poissons et les insectes (Peckmezian and Taylor, 2015 ; Van De Poll et al., 2015 ; Zhang et al., 2014). L'abeille n'a pas dérogé à l'utilisation de la réalité virtuelle et de nombreux dispositifs ont ainsi été mis en place pour étudier la vision en simulateur de vol (**Figure 12a**, Luu et al., 2011) ou de marche (**Figure 12b**, Paulk et al., 2015). Ces dispositifs ont à ce jour permis d'étudier la navigation et les processus attentionnels chez l'abeille, mais jamais de l'apprentissage visuel. Au cours d'une revue bibliographique (Annexe 1), j'ai recensé toutes les technologies existantes chez l'abeille ainsi que les questions qui

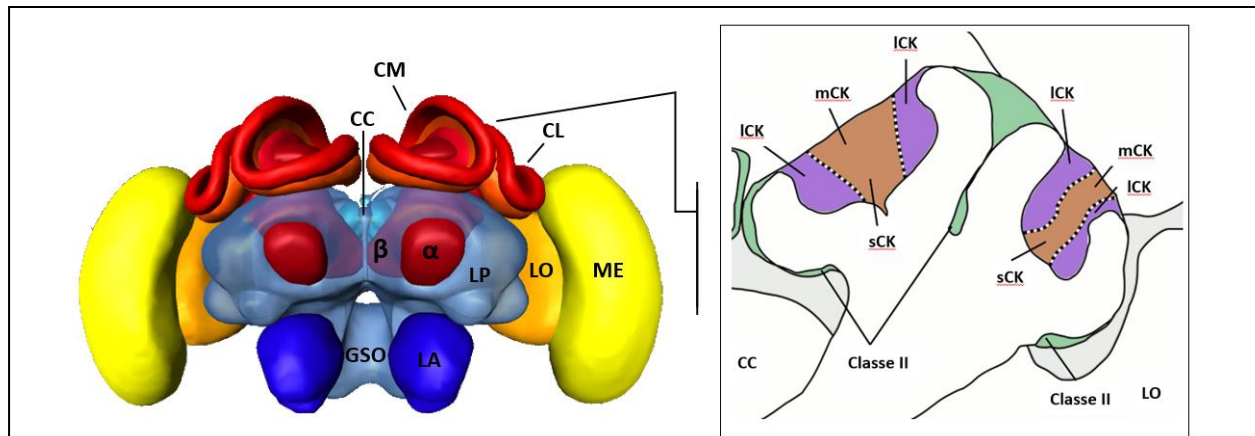
ont été abordées grâce à elles. La conclusion de cette revue est que la réalité virtuelle, qui jusqu'à maintenant n'avait jamais été utilisée pour étudier l'apprentissage visuel chez l'abeille, représentait une alternative de choix pour réaliser nos études sur des abeilles attachées mais avec une possibilité de mouvement. En effet, grâce à ce type de dispositif, effectuer des expériences d'apprentissage visuel contrôlées pour chaque individu, tout en accédant au cerveau devient entièrement possible.

**Cette partie nous a permis de mettre en évidence la richesse cognitive dont peut faire preuve l'abeille testée en condition visuelle. De plus, la réalité virtuelle semble être une bonne alternative à toutes les méthodes déjà existantes et n'a jusque-là pas été utilisée pour étudier la cognition visuelle chez l'abeille. Au cours de ma thèse, j'ai donc exploré les possibilités offertes par la réalité virtuelle. Trouver le bon dispositif et les bons paradigmes est essentiel mais ne permet pas de comprendre comment l'abeille avec un cerveau si simple, peut montrer de telles capacités cognitives. Nous sommes loin de pouvoir répondre à cette question, et comprendre comment son cerveau est organisé constitue la première étape.**



## b. Le cerveau de l'abeille

Le cerveau de l'abeille est composé de 3 parties : le protocerebrum, le deutocerebrum et le tritocerebrum (**Figure 13**).



**Figure 13 : Architecture 3D d'un cerveau d'abeille**

Reconstruction 3D en vue frontale à partir de donnée d'imagerie confocale. Les lobes optiques sont en jaune composé de la lamina (non visible), la medulla (ME) et la lobula (LO). Les corps pédonculés, considérés comme le centre de la mémoire sont en rouge et sont composés de deux sous unités qui constituent la voie d'entrée de l'information : les calices latéraux et médian (CL et CM). Les lobes  $\alpha$  et  $\beta$  sont les régions de sortie de l'information. Le complexe centrale (CC) régit le contrôle moteur. En transparence apparaissent le lobe protocérébrale (LP) et le ganglion suboesophagien (GSO). On observe une symétrie de la majorité des structures. Chaque calice est constitué de cellules de Kenyon extérieure (Classe II) ou intérieure (Classe I). Cette dernière classe est divisé en sous-population : les cellules larges (ICKs), les cellules moyennes (mCKs) et les cellules petites (pCKs). Ces sous-populations ont été déterminé en fonction de la taille et de la position des corps cellulaires.

Adapté de (Brandt et al., 2005) et (Suenami et al., 2016)

Le protocerebrum est constitué de trois structures : le lobe optique, le corps pédonculé et le complexe central. Le lobe optique et le corps pédonculé sont au nombre de deux dans le cerveau et sont fusionnés dans chaque hémisphère. Les lobes optiques (lamina, medulla et lobula), qui reçoivent et traite l'information visuelle et constituent donc le centre de traitement primaire de ces informations (Kien and Menzel, 1977). Les corps pédonculés sont composés d'un calice latéral et d'un calice médian connectés aux lobes  $\alpha$  et  $\beta$  grâce au pédoncule. Cette structure comprend un grand nombre de neurones (environ 170000) appelés les cellules de Kenyon. Les extensions dendritiques forment la périphérie des calices. On distingue différentes sous populations de cellule de Kenyon en fonction de leur profil morphologique et de leur position : les classes I localisées à l'intérieur du calice et les classes II localisées à l'extérieur du calice. Les classes I sont classifiées

en trois sous-catégories en fonctions de leur position et de leur taille : les cellules larges (lCKs) sur les bords intérieurs du calice, les cellules petites (pCKs) dans le corps central du calice.

Entre ces deux types de cellules on trouve les cellules moyennes (mCKs). Les cellules petites et larges ont des projections dendritiques dans les calices et agissent dans le traitement des informations sensorielles (**Figure 13**, Fahrbach, 2006; Kaneko et al., 2013; Mobbs, 1982; Strausfeld, 2002; Suenami et al., 2016). Le complexe central occupe comme son nom l'indique une place centrale au sein du cerveau. Composé du pont protocérébrale et du corps central, il est divisé en une partie haute et basse et possède une paire de nodules. Il est, de par son emplacement et ses connexions, le lien direct entre les deux hémisphères cérébraux. On lui connaît un rôle dans le contrôle moteur, ainsi que dans la perception d'informations visuelles telles que la lumière polarisée (Pfeiffer and Homberg, 2014). Il a été mis en évidence que cette structure avait aussi un rôle dans l'apprentissage visuel aversif chez l'abeille (Plath et al., 2017) et dans la mémoire visuelle chez la drosophile (Pan et al., 2009).

Le deutocerebrum est composé des lobes antennaires et des lobes dorsaux. Les lobes antennaires sont les centres primaires de l'intégration de l'information olfactive et sont composés d'environ 160 glomérules. Chaque partie du deutocerebrum reçoit les fibres sensorielles primaires venants des récepteurs sensoriels des antennes. La majorité des axones des récepteurs olfactifs du 3<sup>ème</sup> segment antennaire (le flagelle) termine dans le lobe antennaire. En revanche, le lobe dorsal va lui recevoir les axones des deux autres segments antennaires (Scape et Pédicelle) et être innervé par les dendrites des motoneurones des muscles antennaires (Homberg et al., 1989). Un nombre précis de gènes récepteurs olfactifs ont été identifiés dans le génome de l'abeille (Robertson and Wanner, 2006). Ce nombre correspond au nombre de glomérules ce qui a confirmé l'hypothèse que l'information envoyée par un récepteur moléculaire était bien traitée dans un glomérule du lobe antennaire.

Le tritocerebrum quant à lui est composé de deux lobes bilatéraux adjacents au ganglion sub-oesophagien (GSO). Le ganglion sub-oesophagien renferme plusieurs motoneurones qui innerve les muscles du cou et permet donc le contrôle de la tête (Goodman et al., 1987). Il contient aussi les projections sensorielles des récepteurs localisés au niveau des pièces buccales et les motoneurones liés aux muscles de la bouche (Rehder, 1989). C'est donc le centre sensoriel et moteur de l'alimentation. De plus, on retrouve au sein de ce ganglion le soma d'un neurone bien

spécifique, le neurone VUMmx1 qui a un rôle de médiateur dans le traitement des renforcements (Hammer, 1993)

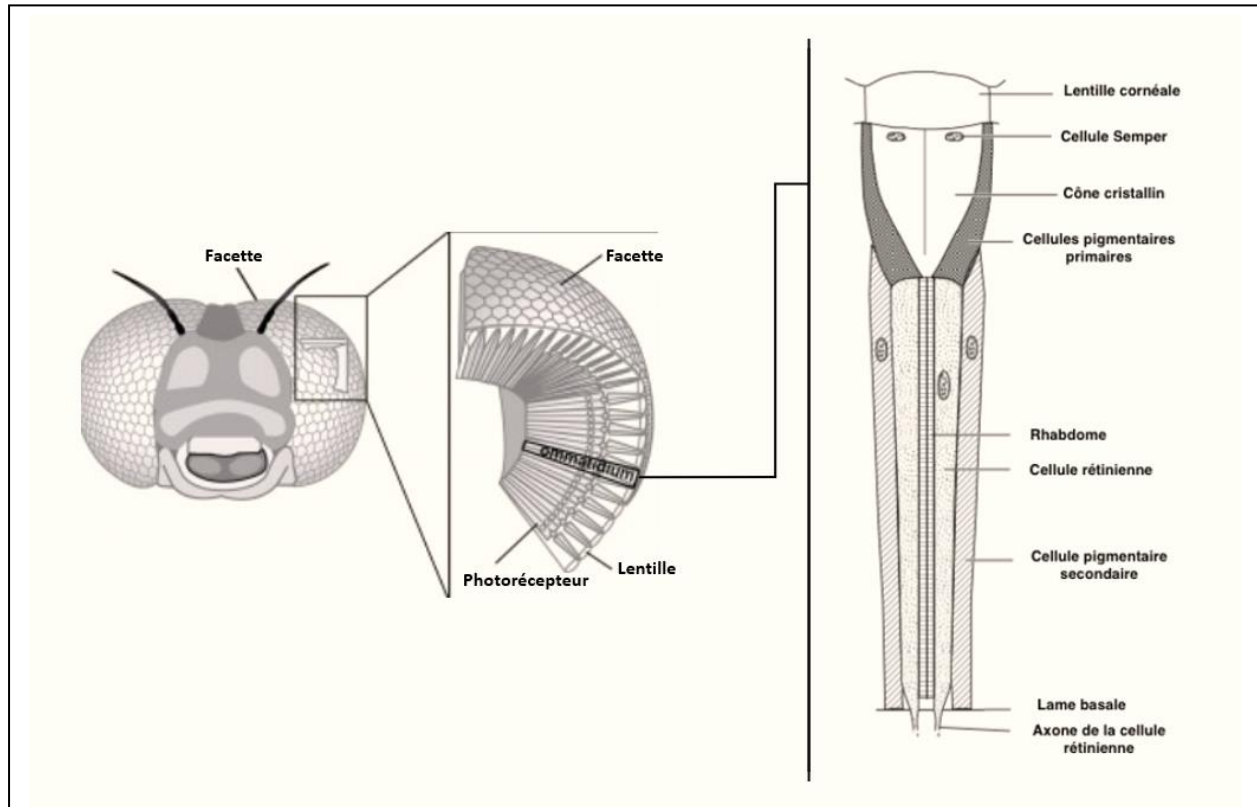
**Au cours de ma thèse, je me suis focalisé sur l'apprentissage visuel chez l'abeille. Lors de ces apprentissages les abeilles devaient associer une stimulation visuelle à une récompense sucrée. Afin de mieux comprendre les événements au cours de ces apprentissages, le système visuel ainsi que le codage de l'information visuel seront décrits, puis la voie du renforcement sera à son tour détaillée.**

### c. Système visuel

Le système visuel de l'abeille est composé de deux yeux composés qui lui permette d'avoir une vision à presque 360 degrés. Ces yeux composés vont permettre de réceptionner l'énergie lumineuse au niveau des photorécepteurs qui convertissent cette énergie lumineuse en énergie électrique afin de transmettre au cerveau par l'intermédiaire de neurone spécifique. Chaque œil composé est constitué d'ommatidies (**Figure 14**, environ 5500) au sein desquelles on trouve 9 cellules photoréceptrices (R1-9, Strausfeld, 1976). Le rhabdomère renferme des microvillosités du photorécepteur dans lesquelles sont localisées les molécules de pigments telles que la rhodopsine (Strausfeld, 1976). L'association de tous les rhabdomères forme le rhabdome qui va s'étendre sur toute la longueur de la cellule photoréceptrice. Les principaux photorécepteurs (R1-8) vont



constituer les microvillosités de la totalité du rhabdom alors que le R9 ne va fournir des microvillosités qu'à la base de l'ommatidie (Menzel and Blakers, 1976).



**Figure 14 : Structure de l'œil composé**

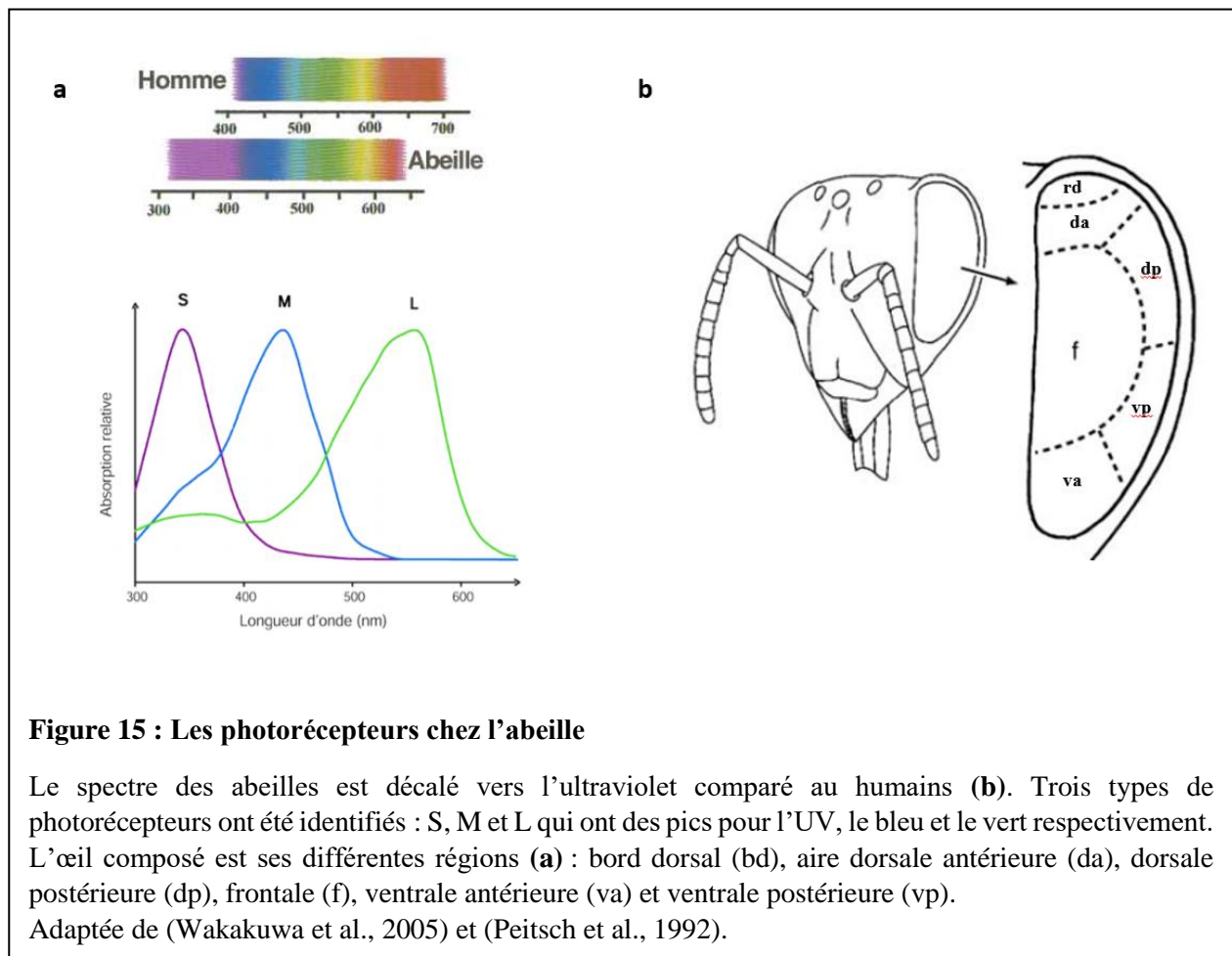
L'œil composé est constitué d'une multitude d'ommatidie (environ 5500) au sein desquelles on retrouve 9 cellules photoréceptrices (photorécepteurs). Les microvillosités des photorécepteurs vont se rejoindre pour former le rhabdome qui va parcourir toute l'ommatidie. Les cellules primaires et secondaires vont permettre de soutenir la structure.

Adaptée à partir de (Chapman, R, 2013)

Trois types de photorécepteurs ont été identifiés dans la rétine des abeilles (**Figure 15a**) : les récepteurs « UV » dits S (longueurs d'ondes courtes, pic à 344nm), « bleus » dits M (longueurs d'ondes moyennes, pic à 436nm) et « verts » dits L (longueurs d'ondes longues, pic à 544nm) (Peitsch et al., 1992). Des expériences ont permis de mettre en évidence le rôle des récepteurs L dans le traitement des informations achromatiques et de tous les types de récepteurs pour les informations chromatiques (Giurfa et al., 1996, 1997; Menzel and Backhaus, 1991). Les ommatidies sont divisées en 3 types (Wakakuwa et al., 2005). Tous les types contiennent six

récepteurs L. En revanche, les types I (44% des ommatidies) contiennent un récepteur S et un récepteur M. Les types II (46%) contiennent deux récepteurs S et les types III (10%) contiennent deux récepteurs M. A la base de chaque ommatidie on peut aussi trouver un 9<sup>ème</sup> récepteur dont la fonction est à ce jour inconnue. La distribution de ces différents types d'ommatidies dans l'œil composé semble complètement aléatoire (Wakakuwa et al., 2005). Cependant, cette règle n'est pas vraie dans deux zones particulière de l'œil (**Figure 15b**) : le bord dorsal (bd) dans lequel on trouve énormément de récepteur S (UV) tous disposés perpendiculairement les un par rapport aux autres permettant ainsi le traitement de la lumière polarisée (Labhart and Meyer, 2002; Spaethe, 2005); et la région ventrale antérieure (va) dans laquelle on retrouve plus d'ommatidie de type III et donc une population de récepteurs M plus dense que dans les autres régions.

À ces yeux composés viennent s'ajouter trois ocelles qui sont cette fois des yeux simples placés au sommet de la tête. Elles vont permettre de détecter les changements d'intensité lumineuse (Mizunami, 1995).



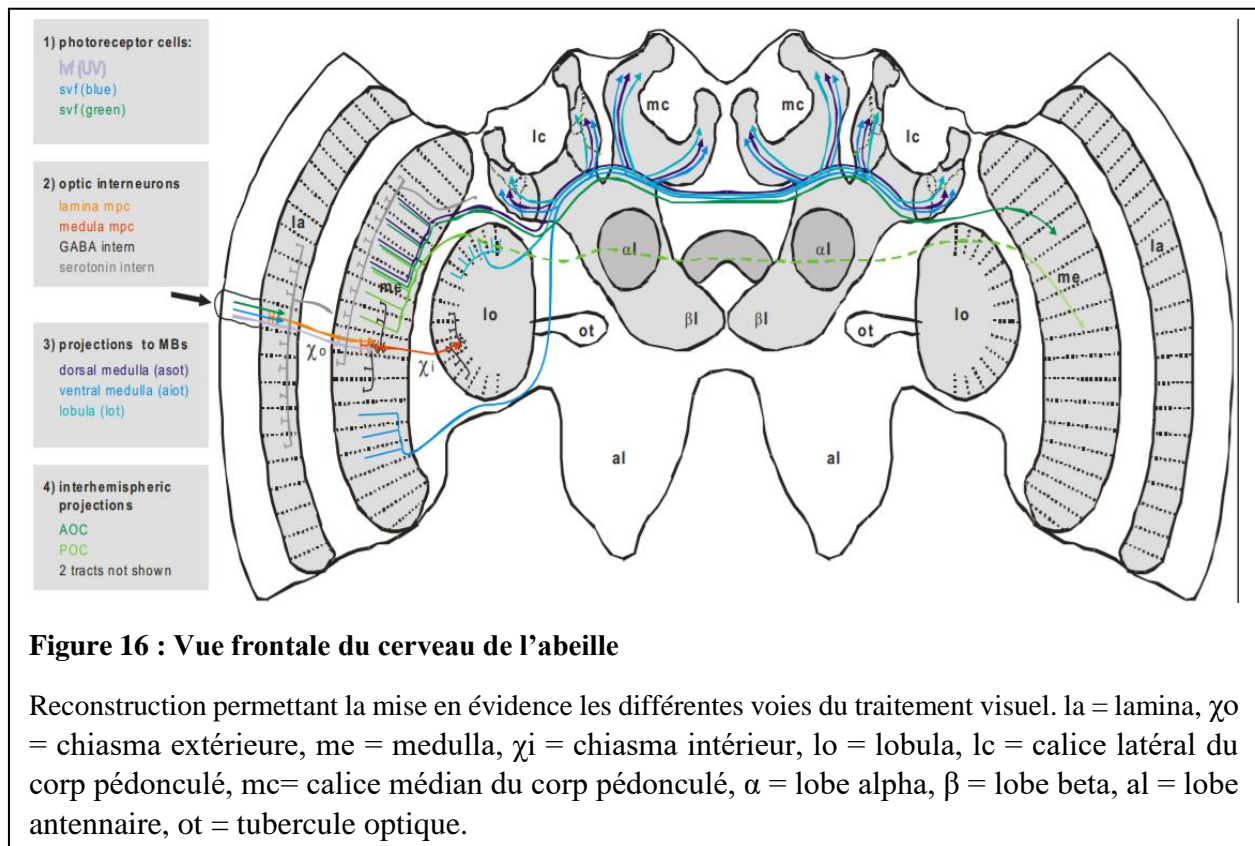
Cette organisation de l'œil permet donc à l'abeille d'avoir une vision trichromatique balayant de 300nm à 650nm. Cette vision diffère donc de la nôtre tant en termes du spectre d'absorption (400-700nm pour l'homme) qu'en terme de résolution temporelle. En effet, les abeilles ont une fréquence de balayage de 200Hz (Srinivasan and Lehrer, 1984), alors que celle des humaines est de 23Hz (Anderson and Burr, 1985). Leur résolution spatiale est en revanche 100 fois inférieure à la nôtre (Srinivasan and Lehrer, 1984). Cette vision va permettre aux abeilles de réaliser les nombreux types d'apprentissage décrits plus haut mais pour cela il est dans un premier temps nécessaire de traiter l'information.

#### d. Comment est codée l'information visuelle ?

Afin d'être traitée, l'information visuelle va être prise en charge par différents niveaux successivement : la lamina, la medulla, la lobula. L'information va ensuite être relayée au niveau du corp pédonculé et des zones centrales du cerveau.

##### i. La lamina

La lamina est la première couche du lobe optique et elle reçoit essentiellement des axones des récepteurs L qui vont participer à la voie permettant de traiter les mouvements et les informations achromatiques (Menzel, 1974). Cette lamina va contenir de nombreux neurones dont la variation de réponse est très faible pour une large variété de longueurs d'onde (Menzel, 1974). Les axones des récepteurs L de la lamina vont se connecter aux cellules monopolaires de la lamina (CML, Menzel, 1974). Ainsi, la lamina est composée de « cartouches optiques » regroupant l'information provenant des neuf photorécepteurs de chaque ommatidie (**Figure 16**). Au sein de chaque « cartouche optique » on retrouve le même arrangement spatial des axones des photorécepteurs et des CMLs et ce dans toute la lamina. Cet arrangement spatial implique une organisation rétinotopique.

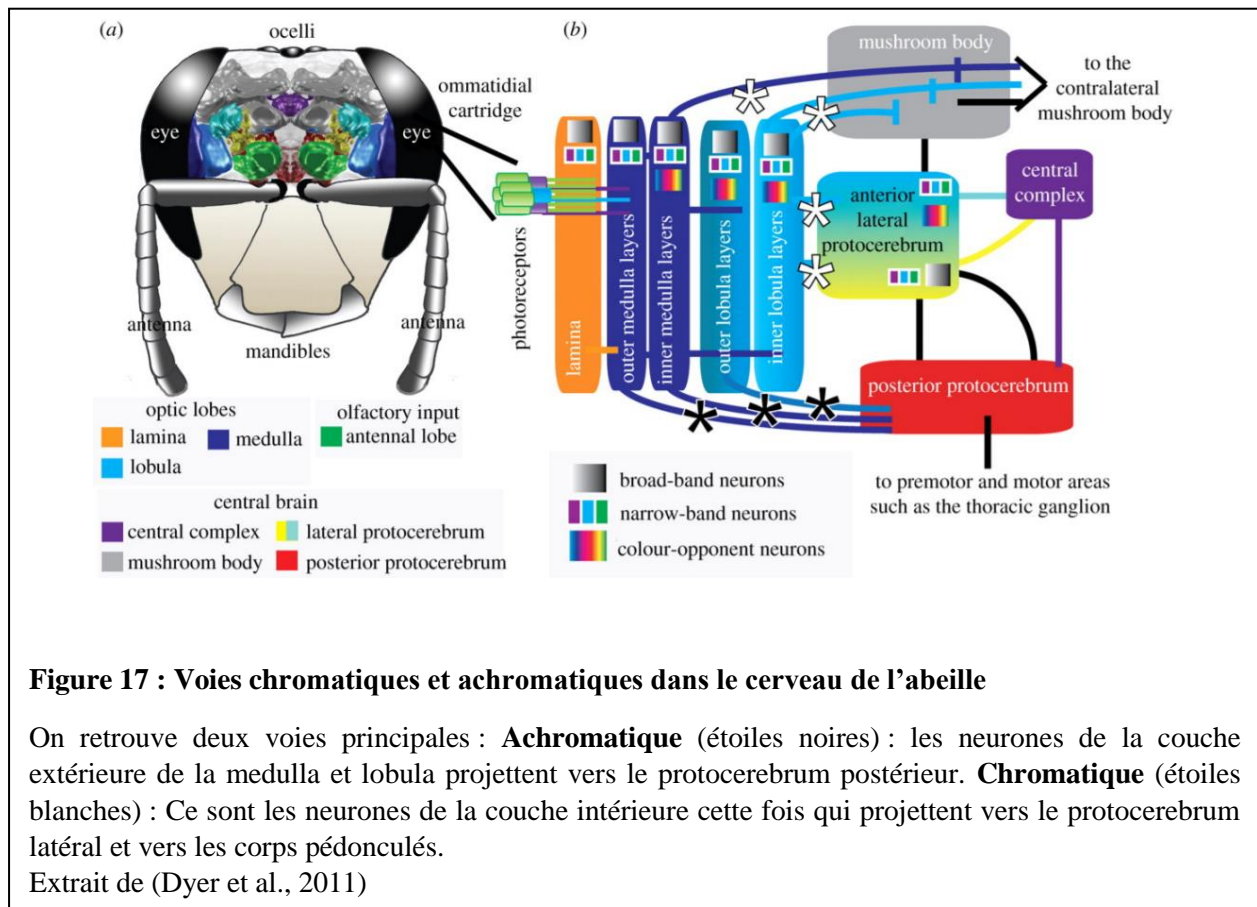


## ii. La medulla

Les axones des CMLs et les photorécepteurs S et M vont continuer vers la medulla (seconde couche du lobe optique). Cette structure contient la majorité des neurones impliqués dans le système visuel de l'abeille (Ribi and Scheel, 1981). La connexion entre la lamina et la medulla est assurée par le chiasma extérieur ( $\chi_0$ , **Figure 16**). Les fibres des CMLs qui partent de la partie antérieure de la lamina projettent vers la partie postérieure de la medulla. À l'inverse, les fibres provenant de la partie postérieure de la lamina vont projeter vers la partie antérieure de la medulla. Ainsi l'organisation rétinotopique est préservée mais inversée. Les neurones de la medulla répondent différemment (excitation ou inhibition) en fonction du type d'information entrante dans le photorécepteur (Paulk et al., 2009). Ces neurones dit « antagonistes de la couleur » sont à la base de la vision des couleurs chez l'abeille (**Figure 17**). Au moins 10 de ces neurones ont été décrits. Il semble qu'ils soit basés sur l'interaction entre les différentes voies des photorécepteurs S, M et L (Yang et al., 2004).

### iii. La lobula

La lobula est la dernière couche du lobe optique. La medulla est la lobula sont connectées grâce au chiasma intérieur ( $\chi_i$ , **Figure 16**). L'organisation rétinotopique est maintenue et de nouveau inversée antéropostérieurement. Les caractéristiques chromatiques des neurones de la medulla sont conservées et amplifiées dans la lobula qui contient aussi beaucoup de « neurones antagonistes de



la couleur» (Paulk et al., 2008). Il a aussi été décrit dans la lobula différents types de neurones « antagonistes des régions de l'œil » qui présentent une activation ou une inhibition opposées en fonction de la région de l'œil qui reçoit l'information visuelle (Yang et al., 2004).

### iv. Le corps pédonculé

Le corps pédonculé va être lié au lobe optique par différentes voies (**Figure 16, 17**). La majorité des afférences visuelles reçues au niveau du corps pédonculé sont dites sensibles à la couleur (Paulk and Gronenberg, 2008). Les neurones de la medulla et de la lobula vont se projeter par différentes voies jusqu'aux corps pédonculés des deux hémisphères cérébraux (Mobbs, 1984). De plus, les lobes optiques des deux yeux vont aussi être connectés (Ehmer and Gronenberg, 2002; Mobbs, 1984), notamment par la « commissure optique postérieure » (COP) et la « commissure optique antérieure (COA) dont l'importance a été démontré pour le codage spectral (Mobbs, 1984). Les différentes afférences visuelles semblent se diviser entre la zone du collier (spécifiquement visuelle) et dans l'anneau basal au sein duquel on retrouve des afférences visuelles et olfactives (Gronenberg, 2001).

#### v. Zones centrales du cerveau

Des études récentes ont montré chez le bourdon que les voies chromatiques et achromatiques étaient localisées dans le protocérébrum antérieur et postérieur respectivement (**Figure 17**, Paulk et al., 2009). Les couches internes des neurones de la medulla et de la lobula (qui sont plus susceptibles d'avoir une réponse antagoniste à la couleur) vont projeter vers le corps pédonculé et le protocérébrum latéral antérieur ((Paulk and Gronenberg, 2008). En revanche, la couche externe des neurones de la lobula et les couches internes et externes des neurones de la medulla projettent vers le protocerebrum postérieur (Paulk et al., 2008).

#### e. Comment sont codés les renforcements ?

Cette partie est basée sur des résultats obtenus lors de conditionnements olfactifs notamment utilisant le conditionnement de la réponse d'extension du proboscis (REP). Il est donc important de garder à l'esprit que ce qui est vrai pour l'olfactif ne l'est peut-être pas pour le visuel.

Le concept de récompense et de punition a commencé avec Loeb (Loeb, 1918) qui définissait les récompenses comme des stimuli induisant un comportement d'approche et une punition comme un stimulus induisant un comportement d'évitement. Cette définition est surtout

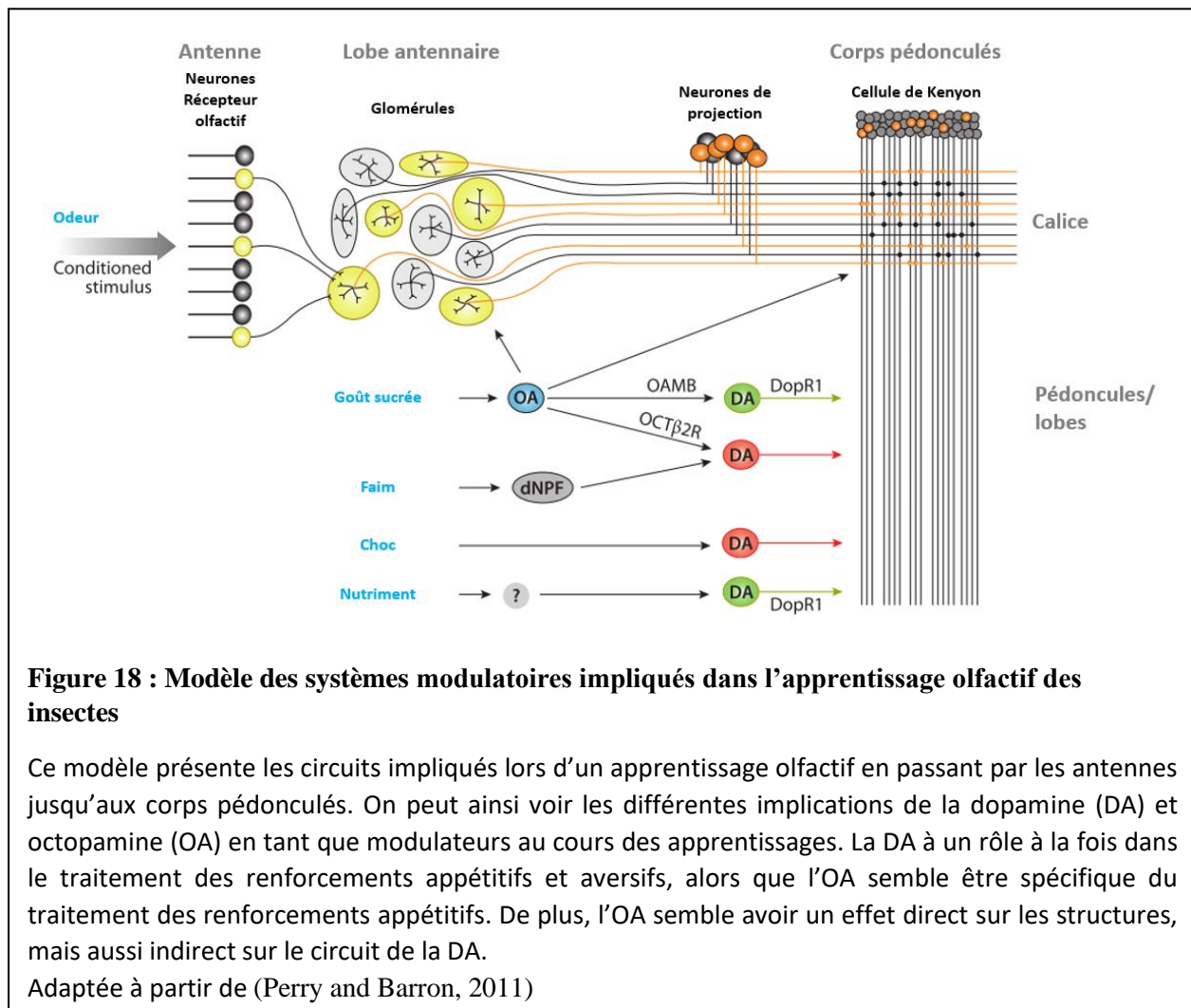
vraie dans un contexte opérant dans lequel l'individu peut contrôler l'arrivée du renforcement. La neuroanatomie du système de récompense chez les mammifères est déjà largement décrite (Leknes and Tracey, 2008). Il semble que chez le mammifère le traitement de la récompense se fait essentiellement par le biais des voies dopaminergiques. Les analyses neuroanatomiques des insectes tendent à montrer que le traitement des renforcements est un peu différent, en tout cas concernant les récompenses (Perry and Barron, 2011). En effet, deux voies seraient impliquées. Une première basée sur le signal prédictif envoyé par un interneurone unique appelé VUMmx1, et une seconde basée sur des neuromodulateurs.

#### i. Le rôle de VUMmx1

Le neurone médian non apparié ventral 1 du neuromère maxillaire (VUMmx1) est connu pour son rôle dans le traitement de l'information liée à la récompense. Le corps cellulaire de ce neurone est localisé dans les profondeurs du ganglion suboesophagien (GSO) et projette vers la partie dorsale du GSO, les glomerules des lobes antennaires et vers les calices des corps pédonculés (Hammer, 1993). Ce neurone répond fortement lors de l'utilisation de récompense sucrée. Après plusieurs essais de conditionnement dans lequel une odeur A (SC+) est récompensée avec une solution sucrée (SU), VUMmx1 déchargeait au moment de la présentation du SC+ avant même que la solution sucrée soit présentée. Cette décharge a alors été caractérisée comme un signal prédictif de la récompense (Hammer, 1993). L'identification de cet interneurone a permis la mise en évidence du circuit lié à la récompense, mais a ouvert de nouvelles questions quant à la modulation de ce signal et à l'existence d'un système permettant de traiter les informations de punition. Il s'avère que c'est le rôle des neuromodulateurs.

#### ii. Le rôle des neuromodulateurs

Les neuromodulateurs sont dans le cas du traitement des renforcements des amines biogènes : la dopamine et l'octopamine. Chez les insectes, l'octopamine est considéré comme la voie de réponse lorsqu'une récompense est utilisée (Roeder, 1999). De plus, il a été proposé que VUMmx1 soit octopaminergique (Hammer and Menzel, 1998). En effet, des injections d'octopamine suffisent à substituer l'utilisation d'une solution sucrée au cours d'un conditionnement classique (Hammer



and Menzel, 1998). Durant plusieurs années, les différentes études neuropharmacologiques tendaient à fournir une version plutôt manichéenne du rôle des neuromodulateurs, avec la dopamine qui influence les apprentissages par punition et l'octopamine les apprentissages par récompense. Avec le temps et l'apparition de nouveaux outils génétiques, le rôle de la dopamine a pu être éclairci chez la drosophile. En effet, il semblerait que des signaux dopaminergiques sont observés lors de



punitions ou de récompense et que les voies dopaminergiques et octopaminergiques interagissent (Burke et al., 2012).

Les connexions entre ces différentes voies et les autres structures cérébrales sont bien connues dans le cadre de l'apprentissage olfactif chez l'insecte (**Figure 18**). Ce sont ces connexions qui permettent d'expliquer en grande partie les mécanismes de l'apprentissage, et à ce jour nous ne sommes pas capables de caractériser les connexions impliquées dans l'apprentissage visuel. Pour autant, de nombreuses techniques sont à notre disposition pour mettre en évidence les bases neurales de l'apprentissage.

#### f. Comment identifier les bases neurales de l'apprentissage visuel chez l'abeille ?

Identifier une méthode afin de tester des abeilles fixées pendant un apprentissage visuel est une étape primordiale afin de faire un pas vers la compréhension des bases neurales. Mais il est aussi important de choisir les bonnes méthodes afin d'identifier les activations des différentes zones du cerveau sans interagir négativement avec le comportement. Cette dernière partie a donc pour vocation de mettre en évidence les différentes méthodes utilisables, ainsi que leurs avantages et inconvénients.

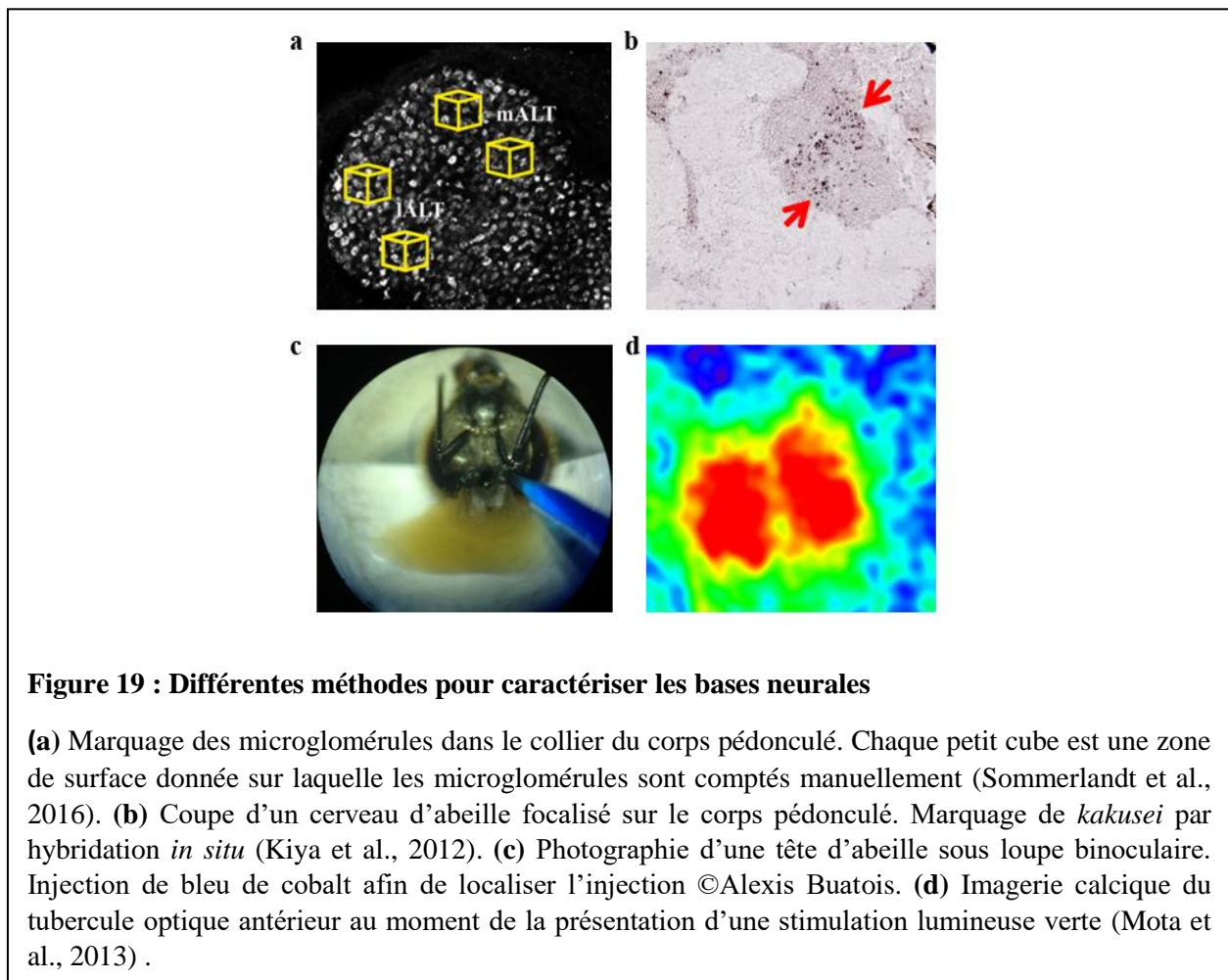
#### i. Les méthodes a posteriori

##### 1. Neuroanatomie

La neuroanatomie est essentiellement basée sur l'utilisation de colorant ou de molécules fluorochromes. Il est ainsi possible d'observer en microscopie des structures particulières grâce à la spécificité de ces produits. Par exemple, il est possible de marquer spécifiquement les microglomérules situés au niveau des corps pédonculés. Les microglomérules sont des complexes synaptiques formés par les terminaisons axonales d'un neurone de projection qui vient d'une zone sensorielle du cerveau et qui forme des connections avec les dendrites des neurones des corps pédonculés. Relevé le nombre et la densité de ces microglomérules a posteriori peut permettre de mettre en évidence la modification des connexions synaptiques. Cette méthode a déjà permis de

montrer que les corps pédonculés sont impliqués dans la rétention de mémoire olfactive à long terme. En effet, après un conditionnement olfactif, des abeilles testées 3 jours après le conditionnement présentaient une augmentation du nombre de microglomérules au niveau de la lèvre du corp pédonculé et donc un réarrangement synaptique (Hourcade et al., 2010).

En modalité visuelle, cette méthode n'a, à ce jour, été utilisée que pour caractériser les réorganisations synaptiques pour la mémoire à long termes après un protocole de discrimination de couleur. Il semblerait d'ailleurs qu'aucune réorganisation synaptique n'ai été engendré au cours de ce protocole (**Figure 19a**, Sommerlandt et al., 2016). Les résultats de cette étude sont néanmoins discutables du fait de la difficulté à contrôler l'expérience des abeilles en libre vol. En effet, cette méthode est particulièrement sensible et une simple exposition à la lumière peut déjà



faire varier le nombre de microglomérules (Scholl et al., 2014) ce qui rend très difficile son utilisation dans le domaine du visuel.

## 2. Quantification génétique

Les gènes sont le support de l'ADN et vont permettre la synthèse protéique. La quantification de l'expression de ces gènes est donc un moyen indirect de mettre en évidence des modifications au niveau du cerveau. Les gènes précoces immédiats (GPIs) sont des gènes bien particuliers. En effet, ces gènes sont activés très rapidement par une large variété de stimuli cellulaires. Ils constituent en général les premiers facteurs de transcription après une stimulation. Depuis quelques années, certains GPIs ont été mis en évidence comme marqueur de l'activité neurale chez l'abeille (Kiya et al., 2007; Ugajin et al., 2013, 2017). Cela permet donc de mettre en évidence l'activité neurale dans des zones particulières soit par quantification en qPCR, soit par marquage en hybridation *in situ* (**Figure 19b**).

Bien que cette méthode soit efficace pour mettre en évidence une activation neurale dans une zone spécifique, elle ne permet en revanche pas d'avoir d'informations sur la nature des neurones activés. Les résultats produits vont permettre d'identifier des zones du cerveau impliquées dans l'apprentissage pour ensuite pousser les investigations avec d'autres techniques dans ces mêmes zones.

### ii. Les méthodes invasives

Ce sont ces dernières méthodes qui vont permettre de mettre en évidence de manière plus fine les bases neurales. En effet, à la différence des précédentes expériences, ces manipulations sont réalisées *in vivo*. Ceci permet une acquisition des données en direct et de cibler plus précisément certaines structures.

#### 1. Injection neuropharmacologique

La majorité du temps, le meilleur moyen de savoir si une structure est impliquée dans un comportement c'est de la supprimer. Pour ce faire, pendant de nombreuses années, les chercheurs créaient des lésions ou même réalisaient des ablations de certaines structures par chirurgie ou modifications au cours du développement (Carcaud et al., 2016; Komischke et al., 2005; Malun et

al., 2002). Ces méthodes ont malheureusement l'inconvénient de ne pas permettre de revenir en arrière. Les injections (**Figure 19c**), à l'inverse, permettent d'agir sur une zone particulière et ce à cours termes et par plusieurs voies (Søvik et al., 2016). Il est en effet possible d'utiliser toutes sortes de produits pharmacologiques allant de l'anesthésique (Devaud et al., 2015) au bloqueur de transcription (Eisenhardt, 2014).

C'est par cette voie que la majorité des avancées concernant les bases neurales de l'apprentissage chez l'abeille ont été réalisées. Ces résultats ont permis une meilleure compréhension des structures impliquées, ainsi que des bases moléculaires sous-tendant les différentes formes de mémoires (Devaud et al., 2015; Eisenhardt, 2014; Hammer and Menzel, 1998; Lefer et al., 2012). Récemment, les injections ont d'ailleurs permis de mettre en évidence le rôle des corps pédonculés et du complexe central lors d'un apprentissage visuel aversif (Plath et al., 2017), ainsi que le rôle des amines biogènes lors d'un apprentissage discriminatif des couleurs (Rodrigues Vieira et al., 2018). Néanmoins, malgré les nombreux résultats qui en découlent, cette méthode reste invasive et nécessite la mise en place de protocole impliquant des individus dont la tête a été ouverte et donc une forte mortalité. De ce fait, tout protocoles impliquant des tests de mémoire après plus de 24h deviennent compliqués à effectuer

## 2. Imagerie calcique

L'imagerie calcique est un des outils qui a permis d'incroyables avancées sur la compréhension des bases neurales de l'apprentissage olfactif chez l'abeille (Faber et al., 1999; Rath et al., 2011). Cette méthode repose sur l'enregistrement de l'activité des ions calciums au sein des neurones. En effet, ces ions jouent un rôle constitutif dans la fonction du neurone et permettent une identification directe de leur activation grâce à la fluorescence. Il est ainsi possible d'observer comment un apprentissage associatif peut modifier l'activation des glomérules au sein du lobe antennaire (Faber et al., 1999).

Cette méthode a aussi été utilisé pour caractériser les voies chromatiques chez l'abeille (**Figure 19d**, Mota et al., 2013). Cette étude bien que prometteuse a souligné les inconvénients itinérants à l'utilisation de l'imagerie calcique pour caractériser les voies neurales impliquées dans l'apprentissage visuel. En effet, les abeilles doivent être fixées et leurs têtes ouvertes afin de

permettre l'observation de fluorescence. De plus, afin d'exciter le fluorochrome il est nécessaire de stimuler le cerveau avec une lumière monochromatique. De ce fait, il est primordial d'établir une séparation physique entre les yeux et le cerveau afin de ne pas biaiser l'apprentissage visuel avec le laser, ou l'excitation du fluorochrome avec la lumière des stimulations visuelles utilisées au cours de l'apprentissage, ce qui rend la possibilité que l'abeille apprenne dans de telles conditions improbable.

### 3. Electrophysiologie

Cette dernière technique est certainement la plus précise car elle permet d'enregistrer l'activité d'un neurone unique ou d'un réseau de neurones. Son caractère invasif repose sur le fait qu'on implante des électrodes au sein du cerveau de l'individu afin d'enregistrer l'activité électrique entre une électrode A et une électrode B. Cela a par exemple permis de mettre en évidence les cellules de lieu chez les mammifères, récemment mis en lumière par un prix Nobel (Hartley et al., 2013). Force est de constater qu'il est nettement plus compliqué d'utiliser ce type de technique sur un insecte tel que l'abeille. Néanmoins, de nombreuses études ont déjà exploré les mécanismes sous-jacents à la vision grâce à l'électrophysiologie (Erlangung and Doktor, 2014; Paulk et al., 2014, 2015; Paulk and Gronenberg, 2008). Il n'y a pour autant pas à ce jour d'étude électrophysiologique explorant l'activation de neurones dans le cadre d'apprentissages visuels. Comme la méthode précédente, elle nécessite de réaliser l'expérience sur un individu dont la tête est ouverte, et de garder des électrodes dans le cerveau. La mortalité liée à ses expériences est donc particulièrement élevée.

### III. Objectifs

L'enjeu de cette thèse est de progresser quant à nos connaissances sur l'apprentissage visuel chez l'abeille et plus particulièrement sur les bases neurales sous-jacentes. Cette introduction a mis en lumière la difficulté à étudier ces mécanismes malgré les nombreux outils à notre disposition. En effet, à ce jour, les protocoles expérimentaux en libre vol ne permettent que très difficilement

l'utilisation de ces outils. Mon but a donc été de mettre en place un nouveau dispositif permettant l'utilisation de paradigmes d'apprentissages visuels élémentaires et non élémentaires avec des abeilles attachées. Les différentes étapes sont décrites dans les chapitres suivants :

## Chapitre I : Apprentissage visuel discriminatif dans un milieu visuel contrôlé

- Les abeilles sont-elles capables de discriminer deux stimuli visuels générés par un vidéoprojecteur en libre marche ?
- Les abeilles sont-elles capables de discriminer deux stimuli visuels générés par un vidéoprojecteur dans un système en circuit ouvert ?
- La nature du renforcement négatif a-t'il un impact sur les performances des abeilles testées dans le système en circuit ouvert ?

Ces questions ont été abordées dans une première publication :

**Buatois A, Pichot C, Schultheiss P, Sandoz JC, Lazzari C R, Chittka L, Avarguès-Weber A, Giurfa M (2017)**

**Associative visual learning by tethered bees in a controlled visual environment.**

Scientific Report 7:12903

## Chapitre II : Apprentissage visuel discriminatif en réalité virtuelle

- Les abeilles sont-elles capables d'apprendre à discriminer des stimuli visuels simples dans un système en réalité virtuelle ?
- La vision active est-elle importante au cours de l'apprentissage visuel des abeilles ? Les stratégies mise en place sont-elles les mêmes en fonction de la liberté de vision active ?

- Les abeilles sont-elles capables de transférer un apprentissage de la réalité virtuelle au monde réelle ? et vice versa ?

Ces questions nous ont mené à la rédaction d'une troisième publication (soumise) :

**Buatois A, Flumian C, Schultheiss P, Avarguès-Weber A, Giurfa M (in press)**

**Transfer of visual learning between a virtual and a real environment in honey bees: the role of active vision?**

Frontiers in Behavioral Neurosciences

### Chapitre III : Apprentissage non-élémentaire en réalité virtuelle

- Les abeilles sont-elles capables de réaliser un apprentissage non-élémentaire de type « patterning négatif » en réalité virtuelle ?

Cette question est à l'origine de ce manuscrit :

**Buatois A, Laroche L, Avarguès-Weber A, Giurfa M**

**Non-elemental learning of honeybees in virtual reality.**

### Chapitre IV : Bases neurales de la mémoire des couleurs chez l'abeille

- Quelles aires cérébrales sont activées au cours d'un test de mémoire de couleur ?
- Quelles aires cérébrales sont potentiellement des centres de stockage de l'information de couleur ?

Ces questions ont été à l'origine de ce dernier manuscrit en préparation :

**Buatois A, Avarguès-Weber A, Massou I, Giurfa M.**

**Immediate early genes as a tool to identify the neural bases of color memory in honey bees**

#### IV. Références

- Abramson, J. Z., Hernández-Lloreda, V., Call, J., and Colmenares, F. (2013). Relative quantity judgments in the beluga whale (*Delphinapterus leucas*) and the bottlenose dolphin (*Tursiops truncatus*). *Behav. Processes* 96, 11–19. doi:10.1016/j.beproc.2013.02.006.
- Agrillo, C., Dadda, M., and Bisazza, A. (2007). Quantity discrimination in female mosquitofish. *Anim. Cogn.* 10, 63–70. doi:10.1007/s10071-006-0036-5.
- Anderson, S. J., and Burr, D. C. (1985). Spatial and temporal selectivity of the human motion detection system. *Vision Res.* 25, 1147–54. doi:10.1016/0042-6989(85)90104-X.
- Antzoulatos, E. G., and Miller, E. K. (2014). Increases in functional connectivity between prefrontal cortex and striatum during category learning. *Neuron* 83, 216–225. doi:10.1016/j.neuron.2014.05.005.
- Avarguès-Weber, A., Deisig, N., and Giurfa, M. (2011). Visual cognition in social insects. *Annu. Rev. Entomol.* 56, 423–443. doi:10.1146/annurev-ento-120709-144855.
- Avarguès-Weber, A., Dyer, A. G., Combe, M., and Giurfa, M. (2012). Simultaneous mastering of two abstract concepts by the miniature brain of bees. *Proc. Natl. Acad. Sci. U. S. A.* 109, 7481–7486. doi:10.1073/pnas.1202576109.
- Avarguès-Weber, A., Dyer, A. G., and Giurfa, M. (2011a). Conceptualization of above and below relationships by an insect. *Proc. R. Soc. B Biol. Sci.* 278, 898–905. doi:10.1098/rspb.2010.1891.
- Avarguès-Weber, A., Dyer, A. G., and Giurfa, M. (2011b). Conceptualization of above and below relationships by an insect. *Proc. R. Soc. B Biol. Sci.* 278, 898–905. doi:10.1098/rspb.2010.1891.
- Avarguès-Weber, A., and Giurfa, M. (2013). Conceptual learning by miniature brains. *Proc. R. Soc. B Biol. Sci.* 280, 20131907. doi:10.1098/rspb.2013.1907.
- Avarguès-weber, A., and Mota, T. (2016). Advances and limitations of visual conditioning



- protocols in harnessed bees. *J. Physiol. - Paris*. doi:10.1016/j.jphysparis.2016.12.006.
- Avargues-Weber, A., Portelli, G., Benard, J., Dyer, A., and Giurfa, M. (2010). Configural processing enables discrimination and categorization of face-like stimuli in honeybees. *J. Exp. Biol.* 213, 593–601. doi:10.1242/jeb.039263.
- Avarguès Weber, A., Mota, T., and Giurfa, M. (2012). New vistas on honey bee vision. *Apidologie* 43, 244–268. doi:10.1007/s13592-012-0124-2.
- Bellingham, W. P., Gillette-Bellingham, K., and Kehoe, E. J. (1985). Summation and configuration in patterning schedules with the rat and rabbit. *Anim. Learn. Behav.* 13, 152–164. doi:10.3758/BF03199268.
- Benard, J., Stach, S., and Giurfa, M. (2006). Categorization of visual stimuli in the honeybee *Apis mellifera*. *Anim. Cogn.* 9, 257–270. doi:10.1007/s10071-006-0032-9.
- Bennett, C. R., Bailin, E. S., Gottlieb, T. K., Bauer, C. M., Bex, P. J., and Merabet, L. B. (2018). Assessing Visual Search Performance in Ocular Compared to Cerebral Visual Impairment Using a Virtual Reality Simulation of Human Dynamic Movement. *Proc. Technol. Mind, Soc. ZZZ - TechMindSociety '18*, 1–6. doi:10.1145/3183654.3183674.
- Bitterman, M. E., Menzel, R., Fietz, A., and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* 97, 107–119. doi:10.1037//0735-7036.97.2.107.
- Bond, A. B., Kamil, A. C., and Balda, R. P. (2007). Serial Reversal Learning and the Evolution of Behavioral Flexibility in Three Species of North American Corvids (*Gymnorhinus cyanocephalus*, *Nucifraga columbiana*, *Aphelocoma californica*). *J. Comp. Psychol.* 121, 372–379. doi:10.1037/0735-7036.121.4.372.
- Brandt, R., Rohlfing, T., Rybak, J., Krofczik, S., Maye, A., Westerhoff, M., et al. (2005). Three-dimensional average-shape atlas of the honeybee brain and its applications. *J. Comp. Neurol.* 492, 1–19. doi:10.1002/cne.20644.
- Brembs, B., Lorenzetti, F. D., Reyes, F. D., Baxter, D. A., and Byrne, J. H. (2002). Operant reward learning in *plysia*: neuronal correlates and mechanisms. *Science (80- )*. 296, 1706–1709. doi:10.1126/science.1069434.

- Burke, C. J., Huetteroth, W., Oswald, D., Perisse, E., Krashes, M. J., Das, G., et al. (2012). Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* 492, 433–437. doi:10.1038/nature11614.
- Bussey, T. J., Padain, T. L., Skillings, E. A., Winters, B. D., Morton, A. J., and Saksida, L. M. (2008). The touchscreen cognitive testing method for rodents: How to get the best out of your rat. *Learn. Mem.* 15, 516–523. doi:10.1101/lm.987808.
- Carcaud, J., Giurfa, M., and Sandoz, J. C. (2016). Parallel Olfactory Processing in the Honey Bee Brain: Odor Learning and Generalization under Selective Lesion of a Projection Neuron Tract. *Front. Integr. Neurosci.* 9, 1–13. doi:10.3389/fnint.2015.00075.
- Chapman, R. F. (2013). *The insects: Structure and Function.*, eds. S. J. Simpson and A. E. Douglas Fifth Edition.
- Chittka, L., and Geiger, K. (1995). Can honey bees count landmarks? *Anim. Behav.* 49, 159–164. doi:10.1016/0003-3472(95)80163-4.
- Chittka, L., and Raine, N. E. (2006). Recognition of flowers by pollinators. *Curr. Opin. Plant Biol.* 9, 428–435. doi:10.1016/j.pbi.2006.05.002.
- Cole, P. D., and Adamo, S. A. (2005). Cuttlefish (*Sepia officinalis*: Cephalopoda) hunting behavior and associative learning. *Anim. Cogn.* 8, 27–30. doi:10.1007/s10071-004-0228-9.
- Colwill, R. M., Raymond, M. P., Ferreira, L., and Escudero, H. (2005). Visual discrimination learning in zebrafish (*Danio rerio*). *Behav. Processes* 70, 19–31. doi:10.1016/j.beproc.2005.03.001.
- Daum, I., Schugens, M. M., Ackermann, H., Lutzenberger, W., Dichgans, J., and Birbaumer, N. (1993). Classical Conditioning After Cerebellar Lesions in Humans. *Behav. Neurosci.* 107, 748–756. doi:10.1037/0735-7044.107.5.748.
- Davidson, T. L., McKernan, M. G., and Jarrard, L. E. (1993). Hippocampal Lesions Do Not Impair Negative Patterning: A Challenge to Configural Association Theory. *Behav. Neurosci.* 107, 227–234. doi:10.1037/0735-7044.107.2.227.
- De Brigard, F., Brady, T. F., Ruzic, L., and Schacter, D. L. (2017). Tracking the emergence of

- memories: A category-learning paradigm to explore schema-driven recognition. *Mem. Cogn.* 45, 105–120. doi:10.3758/s13421-016-0643-6.
- Deisig, N., Lachnit, H., Giurfa, M., and Hellstern, F. (2001). Configural olfactory learning in honeybees: negative and positive patterning discrimination. *Learn. Mem.* 8, 70–78. doi:10.1101/lm.38301.
- Dépy, D., Fagot, J., and Vauclair, J. (1999). Processing of above/below categorical spatial relations by baboons (*Papio papio*). *Behav. Processes* 48, 1–9. doi:10.1016/S0376-6357(99)00055-8.
- Devaud, J.-M., Papouin, T., Carcaud, J., Sandoz, J.-C., Grünewald, B., and Giurfa, M. (2015). Neural substrate for higher-order learning in an insect: Mushroom bodies are necessary for configural discriminations. *Proc. Natl. Acad. Sci.* 112, E5854–E5862. doi:10.1073/pnas.1508422112.
- Dinges, C. W., Varnon, C. A., Cota, L. D., Slykerman, S., and Abramson, C. I. (2017). Studies of Learned Helplessness in Honey Bees (*Apis mellifera ligustica*). *J. Exp. Psychol. Learn. Cogn.* 43, 147–158. doi:10.1037/xan0000133.
- Dombeck, D. A., and Reiser, M. B. (2012). Real neuroscience in virtual worlds. *Curr. Opin. Neurobiol.* 22, 3–10. doi:10.1016/j.conb.2011.10.015.
- Dunnett, S. B., Martel, F. L., and Iversen, S. D. (1990). Proactive interference effects on short-term memory in rats: II. Effects in young and aged rats. *Behav. Neurosci.* 104, 666–670.
- Dupuy, F., Sandoz, J. C., Giurfa, M., and Josens, R. (2006). Individual olfactory learning in *Camponotus* ants. *Anim. Behav.* 72, 1081–1091. doi:10.1016/j.anbehav.2006.03.011.
- Dyer, A. G., Paulk, A. C., and Reser, D. H. (2011). Colour processing in complex environments: insights from the visual system of bees. *Proc. R. Soc. B Biol. Sci.* 278, 952–959. doi:10.1098/rspb.2010.2412.
- Ehmer, B., and Gronenberg, W. (2002). Segregation of visual input to the mushroom bodies in the honeybee (*Apis mellifera*). *J. Comp. Neurol.* 451, 362–373. doi:10.1002/cne.10355.
- Eisenhardt, D. (2014). Molecular mechanisms underlying formation of long-term reward memories and extinction memories in the honeybee (*Apis mellifera*). *Learn. Mem.* 21, 534–42.

doi:10.1101/lm.033118.113.

- Endres, T., Widmann, K., and Fendt, M. (2007). Are rats predisposed to learn 22 kHz calls as danger-predicting signals? *Behav. Brain Res.* 185, 69–75. doi:10.1016/j.bbr.2007.07.012.
- Erlangung, Z., and Doktor, G. (2014). Dissertation Neural circuits underlying colour vision and visual memory in *Drosophila melanogaster*.
- Evangelista, C., Kraft, P., Dacke, M., Labhart, T., and Srinivasan, M. V. (2014). Honeybee navigation: critically examining the role of the polarization compass. *Philos. Trans. R. Soc. B Biol. Sci.* 369, 20130037–20130037. doi:10.1098/rstb.2013.0037.
- Faber, T., Joerges, J., and Menzel, R. (1999). Associative learning modifies neural representation of odors in the insect brain. *Nat. Neurosci.* 2, 74–78.
- Fahrbach, S. E. (2006). Structure of the Mushroom Bodies of the Insect Brain. *Annu. Rev. Entomol.* 51, 209–232. doi:10.1146/annurev.ento.51.110104.150954.
- Finch, G. (1942). Delayed matching-from-sample and non-spatial delayed response in chimpanzees. *J. Comp. Psychol.* 34, 315–319. doi:10.1037/h0062821.
- Giurfa, M. (2004). Conditioning procedure and color discrimination in the honeybee *Apis mellifera*. *Naturwissenschaften* 91, 228–231. doi:10.1007/s00114-004-0530-z.
- Giurfa, M. (2013). Cognition with few neurons: higher order learning in insects. *Trends Neurosci.* 35, 285–294.
- Giurfa, M. (2015). Learning and cognition in insects. *Wiley Interdiscip. Rev. Cogn. Sci.* 6, 383–395. doi:10.1002/wcs.1348.
- Giurfa, M., Hammer, M., Stach, S., Stollhoff, N., Müller-Deisig, N., and Mizyrycki, C. (1999). Pattern learning by honeybees: Conditioning procedure and recognition strategy. *Anim. Behav.* 57, 315–324. doi:10.1006/anbe.1998.0957.
- Giurfa, M., and Menzel, R. (1997). Insect Visual perception: Complex abilities of simple nervous systems. *Curr. Opin. Neurobiol.* 7, 505–513. doi:10.1016/S0959-4388(97)80030-X.
- Giurfa, M., and Sandoz, J.-C. (2012). Invertebrate learning and memory: Fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* 19, 54–66.

doi:10.1101/lm.024711.111.

- Giurfa, M., Vorobyev, M., Brandt, R., Posner, B., and Menzel, R. (1997). Discrimination of coloured stimuli by honeybees: Alternative use of achromatic and chromatic signals. *J. Comp. Physiol. - A Sensory, Neural, Behav. Physiol.* 180, 235–243. doi:10.1007/s003590050044.
- Giurfa, M., Vorobyev, M., Kevan, P., and Menzel, R. (1996). Detection of coloured stimuli by honeybees: minimum visual angles and receptor specific contrasts. *J. Comp. Physiol. A* 178. doi:10.1007/BF00227381.
- Giurfa, M., Zhang, S., Jenett, A., Menzel, R., and Srinivasan, M. (2001). The concepts of “sameness” and ‘difference’ in an insect. *Nature* 410, 2–5. Available at: <http://www.nature.com/nature/journal/v410/n6831/abs/410930a0.html>.
- Glotzbach, E., Ewald, H., Andreatta, M., Pauli, P., and Mühlberger, A. (2012). Contextual fear conditioning predicts subsequent avoidance behaviour in a virtual reality environment. *Cogn. Emot.* 26, 1256–1272. doi:10.1080/02699931.2012.656581.
- Goodman, L. J., Fletcher, W. A., Guy, R. G., Mobbs, P. G., and Pomfrett, C. D. J. (1987). Motion Sensitive Descending Interneurons, Ocellar LD Neurons and Neck Motoneurons in the Bee: A Neural Substrate for Visual Course Control in *Apis mellifera*. in *Neurobiology and Behavior of Honeybees*, eds. R. Menzel and A. Mercer (Berlin, Heidelberg: Springer Berlin Heidelberg), 158–171.
- Gossette, R. L. (1968). Examination of Retention Decrement Explanation of Comparative Successive Discrimination Reversal Learning by Birds and Mammals. *Percept. Mot. Skills*, 1147–1152.
- Grima, B., Chélot, E., Xia, R., and Rouyer, F. (2004). Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. *Nature* 431, 869–873. doi:10.1038/nature02935.
- Gronenberg, W. (2001). Subdivisions of hymenopteran mushroom body calyces by their afferent supply. *J. Comp. Neurol.* 435, 474–489. doi:10.1002/cne.1045.
- Guerrieri, F. J., and d’Ettorre, P. (2010). Associative learning in ants: Conditioning of the maxilla-labium extension response in *Camponotus aethiops*. *J. Insect Physiol.* 56, 88–92.

doi:10.1016/j.jinsphys.2009.09.007.

Gulliksen, H., and Wolfle, D. L. (1938). A theory of learning and transfer. *Psychometrika* 3, 127–149. doi:10.1007/BF02288482.

Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* 366, 59–63.

Hammer, M., and Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn. Mem.* 5, 146–56. doi:10.1101/lm.5.1.146.

Harris, J. A., Livesey, E. J., Gharaei, S., and Westbrook, R. F. (2008). Negative Patterning Is Easier Than a Biconditional Discrimination. *J. Exp. Psychol. Anim. Behav. Process.* 34, 494–500. doi:10.1037/0097-7403.34.4.494.

Hartley, T., Lever, C., Burgess, N., and O’Keefe, J. (2013). Space in the brain: how the hippocampal formation supports spatial cognition. *Philos. Trans. R. Soc. B Biol. Sci.* 369, 20120510–20120510. doi:10.1098/rstb.2012.0510.

Heisenberg, M. (2003). Mushroom body memoir: from maps to models. *Nat. Rev. Neurosci.* 4, 266–275. doi:10.1038/nrn1074.

Herman, L. M., Hovancik, J. R., Gory, J. D., and Bradshaw, G. L. (1989). Generalization of Visual Matching by a Bottlenosed Dolphin (*Tursiops truncatus*): Evidence for Invariance of Cognitive Performance with Visual and Auditory Materials. *J. Exp. Psychol. Anim. Behav. Process.* 15, 124–136. doi:10.1037/0097-7403.15.2.124.

Hertz, M. (1935). Die untersuchungen ueber den formensinn der honigbiene. *Naturwissenschaften*, 628–624.

Homberg, U., Christensen, T. A., and Hildebrand, J. G. (1989). Structure and Function of the Deutocerebrum in Insects. *Annu. Rev. Entomol.* 34, 477–501. doi:10.1146/annurev.en.34.010189.002401.

Horel, J. A., Voytko, M. L., and Salsbury, K. G. (1984). Visual learning suppressed by cooling the temporal pole. *Behav. Neurosci.* 98, 310–324. doi:10.1037//0735-7044.98.2.310.

- Hori, S., Takeuchi, H., Arikawa, K., Kinoshita, M., Ichikawa, N., Sasaki, M., et al. (2006). Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera* L. *J. Comp. Physiol. A* 192, 691–700. doi:10.1007/s00359-005-0091-4.
- Hourcade, B., Muenz, T. S., Sandoz, J.-C., Rössler, W., and Devaud, J.-M. (2010). Long-term memory leads to synaptic reorganization in the mushroom bodies: a memory trace in the insect brain? *J. Neurosci.* 30, 6461–6465. doi:10.1523/JNEUROSCI.0841-10.2010.
- Joesch, M., Schnell, B., Raghu, S. V., Reiff, D. F., and Borst, A. (2010). ON and off pathways in *Drosophila* motion vision. *Nature* 468, 300–304. doi:10.1038/nature09545.
- Kamil, A. C., Jones, T. B., Pietrewicz, A., and Mauldin, J. E. (1977). Positive transfer from successive reversal training to learning set in blue jays (*Cyanocitta cristata*). *J. Comp. Physiol. Psychol.* 91, 79–86. doi:10.1037/h0077295.
- Kaneko, K., Ikeda, T., Nagai, M., Hori, S., Umatani, C., Tadano, H., et al. (2013). Novel Middle-Type Kenyon Cells in the Honeybee Brain Revealed by Area-Preferential Gene Expression Analysis. *PLoS One* 8. doi:10.1371/journal.pone.0071732.
- Kangas, B. D., Vaidya, M., and Branch, M. N. (2010). Titrating-delay matching-to-sample in the pigeon. *J. Exp. Anal. Behav.* 94, 69–81. doi:10.1901/jeab.2010.94-69.
- Kehoe, E. J., and Graham, P. (1988). Summation and Configuration: Stimulus Compounding and Negative Patterning in the Rabbit. *J. Exp. Psychol. Anim. Behav. Process.* 14, 320–333. doi:10.1037/0097-7403.14.3.320.
- Kenney, J. W., Scott, I. C., Josselyn, S. A., and Frankland, P. W. (2017). Contextual fear conditioning in zebrafish. *Learn. Mem.*, 516–523. doi:10.1101/lm.045690.117.24.
- Kien, J., and Menzel, R. (1977). Chromatic properties of interneurons in the optic lobes of the bee - I. Broad band neurons. *J. Comp. Physiol. - A* 113, 17–34. doi:10.1007/BF00610451.
- Kimble, G. A. ed. (1961). *Hilgard and Marquis' "Conditioning and Learning."* East Norwalk, CT, US: Appleton-Century-Crofts.
- Kirkerud, N. H., Schlegel, U., and Giovanni Galizia, C. (2017). Aversive Learning of Colored

- Lights in Walking Honeybees. *Front. Behav. Neurosci.* 11, 1–17. doi:10.3389/fnbeh.2017.00094.
- Kirkerud, N. H., Wehmann, H.-N., Galizia, C. G., and Gustav, D. (2013). APIS—a novel approach for conditioning honey bees. *Front. Behav. Neurosci.* 7, 1–19. doi:10.3389/fnbeh.2013.00029.
- Kiya, T., Kunieda, T., and Kubo, T. (2007). Increased neural activity of a mushroom body neuron subtype in the brains of forager honeybees. *PLoS One* 2. doi:10.1371/journal.pone.0000371.
- Kiya, T., Ugajin, A., Kunieda, T., and Kubo, T. (2012). Identification of kakusei, a nuclear non-coding RNA, as an immediate early gene from the honeybee, and its application for neuroethological study. *Int. J. Mol. Sci.* 13, 15496–15509. doi:10.3390/ijms131215496.
- Komischke, B., Giurfa, M., Lachnit, H., and Dagmar, M. (2002). Successive Olfactory Reversal Learning in Honeybees. *Learn. Mem.*, 122–129. doi:10.1101/lm.44602.
- Komischke, B., Sandoz, J. C., Malun, D., and Giurfa, M. (2005). Partial unilateral lesions of the mushroom bodies affect olfactory learning in honeybees *Apis mellifera* L. *Eur. J. Neurosci.* 21, 477–485. doi:10.1111/j.1460-9568.2005.03879.x.
- Kozbelt, A. (2001). Artists as experts in visual cognition. *Vis. cogn.* 8, 705–723. doi:10.1080/13506280042000090.
- Kuśmierk, P., and Kowalska, D. M. (1998). Effect of experimental setting on learning and performance of Auditory Delayed Matching- to-Sample task in dogs. *Acta Neurobiol. Exp. (Wars)*. 58, 291–307.
- Labhart, T., and Meyer, E. P. (2002). Neural mechanisms in insect navigation: Polarization compass and odometer. *Curr. Opin. Neurobiol.* 12, 707–714. doi:10.1016/S0959-4388(02)00384-7.
- Lefer, D., Perisse, E., Hourcade, B., Sandoz, J., and Devaud, J.-M. (2012). Two waves of transcription are required for long-term memory in the honeybee. *Learn. Mem.* 20, 29–33. doi:10.1101/lm.026906.112.
- Leknes, S., and Tracey, I. (2008). A common neurobiology for pain and pleasure. *Nat. Rev. Neurosci.* 9, 314–320. doi:10.1038/nrn2333.



- Livesey, E. J., Thorwart, A., and Harris, J. A. (2011). Comparing positive and negative patterning in human learning. *Q. J. Exp. Psychol.* 64, 2316–2333. doi:10.1080/17470218.2011.605153.
- Lockhart, M., and Moore, J. W. (1975). Classical differential and operant conditioning in rabbits (*Oryctolagus cuniculus*) with septal lesions. *J. Comp. Physiol. Psychol.* 88, 147–54. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/1120791>.
- Loeb, J. (1918). *Forced movements, tropisms, and animal conduct*. Philadelphia, PA, US: J B Lippincott Company doi:10.1037/13037-000.
- Luu, T., Cheung, A., Ball, D., and Srinivasan, M. V. (2011). Honeybee flight: a novel “streamlining” response. *J. Exp. Biol.* 214, 2215–2225. doi:10.1242/jeb.050310.
- Malun, D., Plath, N., Giurfa, M., Moseleit, A. D., and Müller, U. (2002). Hydroxyurea-induced partial mushroom body ablation in the honeybee *Apis mellifera*: Volumetric analysis and quantitative protein determination. *J. Neurobiol.* 50, 31–44. doi:10.1002/neu.10015.
- Matsumoto, Y., Matsumoto, C. S., Takahashi, T., and Mizunami, M. (2016). Activation of NO-cGMP Signaling Rescues Age-Related Memory Impairment in Crickets. *Front. Behav. Neurosci.* 10, 1–11. doi:10.3389/fnbeh.2016.00166.
- Matsumoto, Y., and Mizunami, M. (2000). Olfactory learning in the cricket *Gryllus bimaculatus*. *J. Exp. Biol.* 203, 2581–2588.
- Mcallister, D. E., Mcallister, W. R., and Brooks, C. I. (1972). MAGNITUDE AND SHIFT OF REWARD IN INSTRUMENTAL AVERSIVE LEARNING IN RATS. *J. Comp. Physiol. Psychol.* 80, 490–501.
- Menzel, R. (1974). Spectral sensitivity of monopolar cells in the bee lamina. *J. Comp. Physiol.* ■ A 93, 337–346. doi:10.1007/BF00606801.
- Menzel, R., and Backhaus, W. (1991). Colour vision in insects. *Vis. Vis. Dysfunct.* 6, 262–293.
- Menzel, R., and Blakers, M. (1976). Colour receptors in the bee eye - Morphology and spectral sensitivity. *J. Comp. Physiol.* ■ A 108, 11–13. doi:10.1007/BF00625437.
- Menzel, R., Geiger, K., Chittka, L., Joerges, J., Kunze, J., and Müller, U. (1996). The knowledge base of bee navigation. *J. Exp. Biol.* 199, 141–146. doi:Article.

- Mizunami, M. (1995). Functional diversity of neural organization in insect ocellar systems. *Vision Res.* 35, 443–452. doi:10.1016/0042-6989(94)00192-O.
- Mobbs, P. G. (1982). The Brain of the Honeybee *Apis Mellifera* . I . The Connections and Spatial Organization of the Mushroom Bodies. *Society*, 309–354.
- Mobbs, P. G. (1984). Neural networks in the mushroom bodies of the honeybee. *J. Insect Physiol.* 30, 43–58. doi:10.1016/0022-1910(84)90107-0.
- Mota, T., Gronenberg, W., Giurfa, M., and Sandoz, J.-C. (2013). Chromatic Processing in the Anterior Optic Tubercle of the Honey Bee Brain. *J. Neurosci.* 33, 4–16. doi:10.1523/JNEUROSCI.1412-12.2013.
- Mota, T., Roussel, E., Sandoz, J.-C., and Giurfa, M. (2011). Visual conditioning of the sting extension reflex in harnessed honeybees. *J. Exp. Biol.* 214, 3577–3587. doi:10.1242/jeb.062026.
- Murray, E. A., Bussey, T. J., and Saksida, L. M. (2007). Visual Perception and Memory: A New View of Medial Temporal Lobe Function in Primates and Rodents. *Annu. Rev. Neurosci.* 30, 99–122. doi:10.1146/annurev.neuro.29.051605.113046.
- Nakagawa, S., Etheredge, R. J. M., Foster, T. M., Sumpter, C. E., and Temple, W. (2004). The effects of changes in consequences on hens' performance in delayed-matching-to-sample tasks. *Behav. Processes* 67, 441–451. doi:10.1016/j.beproc.2004.07.005.
- Norman, R., Buchwald, J., and Villablanca (1977). Classical conditioning with auditory discrimination of the eye blink in decerebrate cats. *Science* (80-. ). 196, 551–553. doi:10.1126/science.850800.
- Pack, A. A., Herman, L. M., and Roitblat, H. L. (1991). Generalization of visual matching and delayed matching by a California sea lion (*Zalophus californianus*). *Anim. Learn. Behav.* 19, 37–48. doi:10.3758/BF03197858.
- Pan, Y., Zhou, Y., Guo, C., Gong, H., Gong, Z., and Liu, L. (2009). Differential roles of the fan-shaped body and the ellipsoid body in *Drosophila* visual pattern memory. *Learn. Mem.* 16, 289–295. doi:10.1101/lm.1331809.

- Paulk, A. C., Dacks, A. M., and Gronenberg, W. (2009). Color processing in the medulla of the bumblebee (Apidae: *Bombus impatiens*). *J. Comp. Neurol.* 513, 441–456. doi:10.1002/cne.21993.
- Paulk, A. C., and Gronenberg, W. (2008). Higher order visual input to the mushroom bodies in the bee, *Bombus impatiens*. *Arthropod Struct. Dev.* 37, 443–458. doi:10.1016/j.asd.2008.03.002.
- Paulk, A. C., Kirszenblat, L., Zhou, Y., and van Swinderen, B. (2015). Closed-Loop Behavioral Control Increases Coherence in the Fly Brain. *J. Neurosci.* 35, 10304–10315. doi:10.1523/JNEUROSCI.0691-15.2015.
- Paulk, A. C., Phillips-Portillo, J., Dacks, A. M., Fellous, J.-M., and Gronenberg, W. (2008). The processing of color, motion, and stimulus timing are anatomically segregated in the bumblebee brain. *J. Neurosci.* 28, 6319–6332. doi:10.1523/JNEUROSCI.1196-08.2008.
- Paulk, A. C., Stacey, J. A., Pearson, T. W. J., Taylor, G. J., Moore, R. J. D., Srinivasan, M. V., et al. (2014). Selective attention in the honeybee optic lobes precedes behavioral choices. *Proc. Natl. Acad. Sci.* 111, 5006–5011. doi:10.1073/pnas.1323297111.
- Pearce, J. M. (2002). Evaluation and development of a connectionist theory of configural learning. *Anim. Learn. Behav.* 30, 73–95. doi:10.3758/BF03192911.
- Peckmezian, T., and Taylor, P. W. (2015). A virtual reality paradigm for the study of visually mediated behaviour and cognition in spiders. *Anim. Behav.* 107, 87–95. doi:10.1016/j.anbehav.2015.06.018.
- Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi:10.1007/BF00190398.
- Pepperberg, I. M. (2006). Grey parrot numerical competence: A review. *Anim. Cogn.* 9, 377–391. doi:10.1007/s10071-006-0034-7.
- Perry, C. J., and Barron, A. B. (2011). Neural Mechanisms of Reward in Insects. *Annu. Rev. Entomol.* 58, 120928130709004. doi:10.1146/annurev-ento-120811-153631.
- Pfeiffer, K., and Homberg, U. (2014). Organization and Functional Roles of the Central Complex

- in the Insect Brain. *Annu. Rev. Entomol.* 59, 165–184. doi:10.1146/annurev-ento-011613-162031.
- Plath, J. A., Entler, B. V., Kirkerud, N. H., Schlegel, U., Galizia, C. G., and Barron, A. B. (2017). Different Roles for Honey Bee Mushroom Bodies and Central Complex in Visual Learning of Colored Lights in an Aversive Conditioning Assay. *Front. Behav. Neurosci.* 11. doi:10.3389/fnbeh.2017.00098.
- Quinn, P. C. (1994). The Categorization of above and below Spatial Relations by Young. *Child Dev.* 65, 58–69.
- Quinn, W. G., Harris, W. A., and Benzer, S. (1974). Conditioned behavior in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 71, 708–12. doi:VL - 71.
- Rath, L., Galizia, C. G., and Szyszka, P. (2011). Multiple memory traces after associative learning in the honey bee antennal lobe. *Eur. J. Neurosci.* 34, 352–360.
- Reggente, N., Essoe, J. K.-Y., Aghajani, Z. M., Tavakoli, A. V., McGuire, J. F., Suthana, N. A., et al. (2018). Enhancing the Ecological Validity of fMRI Memory Research Using Virtual Reality. *Front. Neurosci.* 12, 1–9. doi:10.3389/fnins.2018.00408.
- Rehder, V. (1989). Sensory pathways and motoneurons of the proboscis reflex in the suboesophageal ganglion of the honey bee. *J. Comp. Neurol.* 279, 499–513. doi:10.1002/cne.902790313.
- Rescorla, R. A., and Wagner, A. R. (1972). A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. *Class. Cond. II Curr. Res. Theory* 21, 64–99. doi:10.1101/gr.110528.110.
- Ribi, W. A., and Scheel, M. (1981). The second and third optic ganglia of the worker bee - Golgi studies of the neuronal elements in the medulla and lobula. *Cell Tissue Res.* 221, 17–43. doi:10.1007/BF00216567.
- Roberts, W. A., Roberts, S., and Kit, K. A. (2002). Pigeons presented with sequences of light flashes use behavior to count but not to time. *J. Exp. Psychol. Anim. Behav. Process.* 28, 137–150. doi:10.1037/0097-7403.28.2.137.

- Robertson, H. M., and Wanner, K. W. (2006). The chemoreceptor superfamily in the honey bee., *Genome Res.*, 1395–1403. doi:10.1101/gr.5057506.
- Rodrigues Vieira, A., Salles, N., Borges, M., and Mota, T. (2018). Visual discrimination transfer and modulation by biogenic amines in honeybees. *J. Exp. Biol.*
- Roeder, T. (1999). Octopamine in invertebrates. *Prog. Neurobiol.* 59, 533–561. doi:10.1016/S0301-0082(99)00016-7.
- Rugani, R., Regolin, L., and Vallortigara, G. (2007). Rudimental numerical competence in 5-day-old domestic chicks (*Gallus gallus*): Identification of ordinal position. *J. Exp. Psychol. Anim. Behav. Process.* 33, 21–31. doi:10.1037/0097-7403.33.1.21.
- Saxe, M. D., Battaglia, F., Wang, J.-W., Malleret, G., David, D. J., Monckton, J. E., et al. (2006). Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc. Natl. Acad. Sci.* 103, 17501–17506. doi:10.1073/pnas.0607207103.
- Scholl, C., Wang, Y., Krischke, M., Mueller, M. J., Amdam, G. V., and Rössler, W. (2014). Light exposure leads to reorganization of microglomeruli in the mushroom bodies and influences juvenile hormone levels in the honeybee. *Dev. Neurobiol.* 74, 1141–1153. doi:10.1002/dneu.22195.
- Schubert, M., Lachnit, H., Francucci, S., and Giurfa, M. (2002). Nonelemental visual learning in honeybees. *Anim. Behav.* 64, 175–184. doi:10.1006/anbe.2002.3055.
- Schusterman, R. J. (1964). Successive discrimination-reversal training and multiple discrimination training in one-trial learning by chimpanzees. *J. Comp. Physiol. Psychol.* 58, 153–156. doi:10.1037/h0044309.
- Sloan, H. L., Good, M., and Dunnett, S. B. (2006). Double dissociation between hippocampal and prefrontal lesions on an operant delayed matching task and a water maze reference memory task. *Behav. Brain Res.* 171, 116–126. doi:10.1016/j.bbr.2006.03.030.
- Sommerlandt, F. M. J., Spaethe, J., Rössler, W., and Dyer, A. G. (2016). Does fine color discrimination learning in free-flying honeybees change mushroom-body calyx neuroarchitecture? *PLoS One* 11, 1–17. doi:10.1371/journal.pone.0164386.

- Søvik, E., Plath, J. A., Devaud, J.-M., and Barron, A. B. (2016). Neuropharmacological Manipulation of Restrained and Free-flying Honey Bees, *Apis mellifera*. *J. Vis. Exp.*, 1–11. doi:10.3791/54695.
- Spaethe, J. (2005). Molecular characterization and expression of the UV opsin in bumblebees: three ommatidial subtypes in the retina and a new photoreceptor organ in the lamina. *J. Exp. Biol.* 208, 2347–2361. doi:10.1242/jeb.01634.
- Spinozzi, G., Lubrano, G., and Truppa, V. (2004). Categorization of above and below spatial relations by tufted capuchin monkeys (*Cebus apella*). *J. Comp. Psychol.* 118, 403–412. doi:10.1037/0735-7036.118.4.403.
- Squire, L. R., Zola-Morgan, S., Haist, F., and Musen, G. (1992). Memory and the Hippocampus: A Synthesis From Findings With Rats, Monkeys, and Humans. *Psychol. Rev.* 99, 195–231. doi:10.1037/0033-295X.99.3.582.
- Srinivasan, M., Zhang, S., Lehrer, M., and Collett, T. (1996). Honeybee navigation en route to the goal: visual flight control and odometry. *J. Exp. Biol.* 199, 237–44. doi:10.1006/anbe.1998.0897.
- Srinivasan, M. V., and Lehrer, M. (1984). Temporal acuity of honeybee vision: behavioural studies using flickering stimuli. *Physiol. Entomol.* 9, 447–457. doi:10.1111/j.1365-3032.1984.tb00787.x.
- Srinivasan, M. V., and Lehrer, M. (1988). Spatial acuity of honeybee vision and its spectral properties. *J. Comp. Physiol. A* 162, 159–172. doi:10.1007/BF00606081.
- Srinivasan, M. V., and Zhang, S. (2004). Visual Motor Computations in Insects. *Annu. Rev. Neurosci.* 27, 679–696. doi:10.1146/annurev.neuro.27.070203.144343.
- Stanhope, K. J., McLenachan, A. P., and Dourish, C. T. (1995). Dissociation between cognitive and motor/motivational deficits in the delayed matching to position test: effects of scopolamine, 8-OH-DPAT and EAA antagonists. *Psychopharmacology (Berl)*. 122, 268–280. doi:10.1007/BF02246548.
- Strausfeld, N. J. (1976). *Atlas of an Insect Brain*. Springer-Verlag.

- Strausfeld, N. J. (2002). Organization of the honey bee mushroom body: Representation of the calyx within the vertical and gamma lobes. *J. Comp. Neurol.* 450, 4–33. doi:10.1002/cne.10285.
- Suenami, S., Paul, R. K., Takeuchi, H., Okude, G., Fujiyuki, T., Shirai, K., et al. (2016). Analysis of the differentiation of Kenyon cell subtypes using three mushroom body-preferential genes during metamorphosis in the honeybee (*Apis mellifera* L.). *PLoS One* 11, 1–29. doi:10.1371/journal.pone.0157841.
- Summerfield, C., and Egner, T. (2009). Expectation (and attention) in visual cognition. *Trends Cogn. Sci.* 13, 403–409. doi:10.1016/j.tics.2009.06.003.
- Tallon-Baudry, C. (2009). The roles of gamma-band oscillatory synchrony in human visual cognition. *Front. Biosci.* Volume, 321. doi:10.2741/3246.
- Ugajin, A., Kunieda, T., and Kubo, T. (2013). Identification and characterization of an Egr ortholog as a neural immediate early gene in the European honeybee (*Apis mellifera* L.). *FEBS Lett.* 587, 3224–3230. doi:10.1016/j.febslet.2013.08.014.
- Ugajin, A., Uchiyama, H., Miyata, T., Sasaki, T., Yajima, S., and Ono, M. (2017). Identification and initial characterization of novel neural immediate early genes possibly differentially contributing to foraging-related learning and memory processes in the honeybee. *Insect Mol. Biol.* 27, 154–165. doi:10.1111/imb.12355.
- Uller, C., Jaeger, R., Guidry, G., and Martin, C. (2003). Salamanders (*Plethodon cinereus*) go for more: Rudiments of number in an amphibian. *Anim. Cogn.* 6, 105–112. doi:10.1007/s10071-003-0167-x.
- Van De Poll, M. N., Zajackowski, E. L., Taylor, G. J., Srinivasan, M. V., and van Swinderen, B. (2015). Using an abstract geometry in virtual reality to explore choice behaviour: visual flicker preferences in honeybees. *J. Exp. Biol.* 218, 3448–3460. doi:10.1242/jeb.125138.
- von Frisch, K. (1914). Der farbesinn und Formensinn der Bienen. *Zool. Jahrbucher Physiol.* 35, 1–188.
- Wahlheim, C. N., and DeSoto, K. A. (2017). Study preferences for exemplar variability in self-regulated category learning. *Memory* 25, 231–243. doi:10.1080/09658211.2016.1152378.

- Wakakuwa, M., Kurasawa, M., Giurfa, M., and Arikawa, K. (2005). Spectral heterogeneity of honeybee ommatidia. *Naturwissenschaften* 92, 464–467. doi:10.1007/s00114-005-0018-5.
- Watanabe, H., Kobayashi, Y., Sakura, M., and Matsumoto, Y. (2003). Classical Olfactory Conditioning in the Cockroach *Periplaneta americana*. *Zoolog. Sci.* 20, 1447–1454.
- Watkins, R. W., Gurney, E., and Cowan, D. P. (1998). Taste-aversion conditioning of house mice (*Mus domesticus*) using the non-lethal repellent, cinnamamide. *Appl. Anim. Behav. Sci.* 57, 171–177. doi:10.1016/S0168-1591(96)01182-3.
- Wehner, R. (1967). Pattern Recognition in Bees. *Nature* 215.
- Weinstein, B. (1941). Matching-fkrom-sample by rhesus monkeys and by children. *J. Comp. Psychol.* 31, 195–213.
- West, R. E., and Young, R. J. (2002). Do domestic dogs show any evidence of being able to count? *Anim. Cogn.* 5, 183–186. doi:10.1007/s10071-002-0140-0.
- Winston, M. (1991). *The Biology of the Honey Bee*. Harvard Un. Cambridge, Massachusetts.
- Wolpaw, J. R., and Dowman, R. (1988). Operant conditioning of primate spinal reflexes: effect on cortical SEPs. *Electroencephalogr. Clin. Neurophysiol.* 69, 398–401. doi:10.1016/0013-4694(88)90012-0.
- Yang, E. C., Lin, H. C., and Hung, Y. S. (2004). Patterns of chromatic information processing in the lobula of the honeybee, *Apis mellifera* L. *J. Insect Physiol.* 50, 913–925. doi:10.1016/j.jinsphys.2004.06.010.
- Yerkes, R. M., and Nissen, H. W. (1939). Prelinguistic sign behavior in chimpanzee. *Science* (80-), 585–587.
- Zala, S. M., and Määttänen, I. (2013). Social learning of an associative foraging task in zebrafish. *Naturwissenschaften* 100, 469–472. doi:10.1007/s00114-013-1017-6.
- Zeil, J. (2012). Visual homing: An insect perspective. *Curr. Opin. Neurobiol.* 22, 285–293. doi:10.1016/j.conb.2011.12.008.
- Zhang, S. (2006). Learning of abstract concepts and rules by the honeybee. *Int. J. Comp. Psychol.*

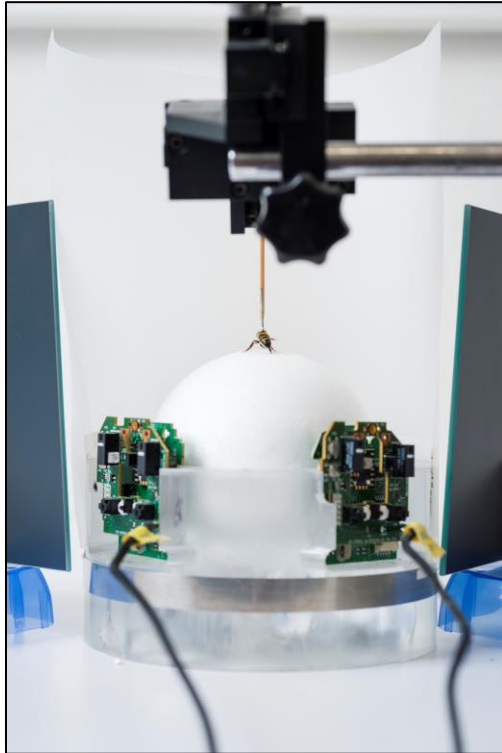


19. Available at:  
<http://escholarship.org/uc/item/3nr4d3r6.pdf%5Chttp://escholarship.org/uc/item/3nr4d3r6>.

Zhang, S., Yang, H., and Singh, L. (2014). Predatory Fish Select for Coordinated Collective Motion in Virtual Prey. *CEUR Workshop Proc.* 1225, 41–42. doi:10.1023/A.

Zoccolan, D., Oertelt, N., DiCarlo, J. J., and Cox, D. D. (2009). A rodent model for the study of invariant visual object recognition. *Proc. Natl. Acad. Sci.* 106, 8748–8753. doi:10.1073/pnas.0811583106.

Zylinski, S. (2014). “Visual cognition in deep-sea cephalopods : what we don’t know and why we don’t know it,” in *Cephalopod cognition* (Cambridge University Press), 224–243.



# Chapitre 1



# SCIENTIFIC REPORTS

## OPEN Associative visual learning by tethered bees in a controlled visual environment

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Les abeilles en libre vol montrent des capacités cognitives remarquables mais les bases neurales de ces capacités ne peuvent pas être étudiées chez des insectes en vol. À l'inverse, le cerveau des abeilles immobilisées est accessible mais ne permet pas d'explorer l'apprentissage visuel. Pour dépasser cette limite, notre objectif a été d'établir un dispositif avec un environnement visuel contrôlé dans lequel une abeille attachée marchant sur un compensateur de locomotion apprend à discriminer des stimuli visuels vidéo projetés devant elle. Des abeilles en libre vol ont été entraînées à marcher dans un labyrinthe en Y miniature dans lequel les stimuli ont été projetés dans un environnement obscur. Ces abeilles apprennent à discriminer efficacement les deux stimuli visuels lorsque l'un est récompensé avec du sucrose (CS+) et l'autre puni avec de la quinine (CS-). En adaptant cette discrimination au compensateur de locomotion, une abeille attachée était capable de discriminer les stimuli et préférait le CS+ au CS- après entraînement. L'apprentissage était meilleur dans le labyrinthe, ce qui suggère l'importance de la liberté de mouvement, de la vision active et du contexte comportemental pour l'apprentissage visuel. La nature de la punition associée au CS- peut aussi affecter l'apprentissage. En effet, la quinine et l'eau distillée améliorent la proportion d'abeilles qui apprennent. Ainsi, l'apprentissage visuel est répliquable dans un milieu visuel contrôlé dans lequel une abeille attachée doit apprendre des stimuli visuels, un résultat plus qu'encourageant pour les futures études neurobiologiques en réalité virtuelle.



# Associative visual learning by tethered bees in a controlled visual environment

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## Abstract

Free-flying honeybees exhibit remarkable cognitive capacities but the neural underpinnings of these capacities cannot be studied in flying insects. Conversely, immobilized bees are accessible to neurobiological investigation but display poor visual learning. To overcome this limitation, we aimed at establishing a controlled visual environment in which tethered bees walking on a spherical treadmill learn to discriminate visual stimuli video projected in front of them. Freely flying bees trained to walk into a miniature Y-maze displaying these stimuli in a dark environment learned the visual discrimination efficiently when one of them (CS+) was paired with sucrose and the other with quinine solution (CS-). Adapting this discrimination to the treadmill paradigm with a tethered, walking bee was successful as bees exhibited robust discrimination and preferred the CS+ to the CS- after training. As learning was better in the maze, movement freedom, active vision and behavioral context might be important for visual learning. The nature of the punishment associated with the CS- also affects learning as quinine and distilled water enhanced the proportion of learners. Thus, visual learning is amenable to a controlled environment in which tethered bees learn visual stimuli, a result that is important for future neurobiological studies in virtual reality.

**Keywords:** Vision, Visual Discrimination, Visual Learning, Active Vision, Honey Bees, Treadmill, Reinforcement

## Introduction

Decades of research on associative learning and memory have established a few insect species as standard models for the study of these capacities. Fruit flies, bees, crickets and ants, among others, can learn simple associations and their neural underpinnings can be understood at the cellular and molecular levels<sup>1-7</sup>. Among these, the honeybee *Apis mellifera* is a powerful model for the study of various forms of visual and olfactory learning<sup>2,8-10</sup>. In the olfactory domain, a Pavlovian conditioning protocol<sup>2,11,12</sup> is useful to dissect associative learning in bees at the circuit and molecular levels<sup>2,8,9,13-15</sup>. In this protocol, individually harnessed hungry bees, which extend their proboscis (proboscis extension response - PER) if sugar water touches their antennae, learn to associate an odor presentation with sugar and thus respond with PER to the odor alone. The sugar water acts as an unconditioned stimulus (US) and the odor as the conditioned stimulus (CS). The fact that bees learn and memorize odors despite being immobilized constitutes the key to success of this protocol as it allows combining behavioral recordings with a variety of invasive techniques to characterize neural activity during olfactory acquisition and retention<sup>8,9</sup>.

In the visual domain, freely flying honey bees can be trained to solve visual problems of high complexity in experimental setups where they are rewarded with sucrose solution for the correct choice of visual stimuli (colors, shapes and patterns, depth and motion cues, among others<sup>8,16-18</sup>). The protocols used reveal that when bees are confronted with complex visual tasks, they show learning capacities on a par with those of some vertebrates<sup>10,19-26</sup>. Indeed, freely flying bees can categorize objects based on common visual features<sup>21,23,27</sup>, and master non-elemental discriminations based on relational concepts such as identity (“sameness” and “difference” relationships)<sup>22</sup>, numerosity<sup>28-30</sup> or on spatial concepts such as “above” or “below” with respect to a constant reference<sup>19,26,31</sup>. Yet, the exploration of the neural bases of visual cognitive processing is not possible in freely flying insects. Attempts to establish a visual variant of PER conditioning have been disappointing until now, as the resulting learning performances are usually poor, even in simple color discrimination tasks (see review in<sup>32</sup>). It seems, therefore, that full immobilization, as imposed by PER conditioning, restricts the possibility of studying more sophisticated forms of visual learning in a way that would permit neurophysiological recordings. Thus, developing alternative protocols that overcome the historical limitations that have impeded understanding and characterizing the neural architecture underlying visual learning in bees is crucial.



Air-cushioned spheres used as treadmills and virtual environments have become popular to study a series of stereotyped responses to olfactory and visual stimuli in a variety of arthropods<sup>33-43</sup>. Yet, attempts to reproduce associative learning in this context have been so far unsuccessful or limited to the observation of learning that occurred beforehand, in unrestrained conditions<sup>38,40</sup>. Here we introduce an experimental protocol in which a tethered bee walking stationary on a treadmill learns to discriminate an appetitive from an aversive visual stimulus in a controlled visual surrounding. We asked 1) if freely flying bees trained to walk into a miniature Y-maze displaying video projected stimuli in a dark environment learn efficiently the visual discrimination when one of them (CS+) is paired with sucrose and the other with quinine solution (CS-); 2) if the miniature-maze situation is adaptable to the controlled environment provided by the treadmill, i.e. if tethered, walking bees learn associations between visual-stimuli and reinforcement/punishment in this paradigm; and 3) if the nature of the punishment associated with the aversive visual stimulus influences learning performances in the treadmill.

We report a systematic analysis of the influence of several experimental factors such as phototactic responses, reinforcement quality and tethered condition vs. free walking. We discuss the necessity of active vision for visual discrimination learning in tethered bees. In active vision, an observer varies his viewpoint in order to investigate the environment and extract better information from it. This strategy is used by flying bees<sup>24,44-47</sup> to extract the borders of objects for better recognition<sup>48,49</sup> via a series of flight maneuvers. We thus consider our results from this perspective and discuss the extent to which tethering preparations allow for active vision compared to free-flight conditions. More generally, we identify the appropriate conditions for the bees to successfully learn a visual discrimination under these novel experimental conditions, and highlight the importance of aversive reinforcement and active vision for visual learning in honey bees.

## Results

### Experiment 1: visual discrimination under free walking conditions

Efficient and sophisticated learning performances have been observed in freely flying bees in contrast to their poor performance in visual PER conditioning, which imposes total immobility, except for the antennae and mouthparts (see Introduction). The establishment of a controlled treadmill paradigm with a tethered, walking bee discriminating video projected stimuli while being

in the darkness requires demonstrating that freely moving bees set in comparable conditions learn to discriminate visual stimuli based on differential reinforcement. We thus investigated to what extent these conditions affect the behavioral performance of freely walking bees trained to collect sucrose solution in a miniature maze (Fig. 1a).

The bees ( $n = 21$ ) were marked with a color spot in the thorax to facilitate individual recognition upon video recording of behavior. They flew freely between the hive and the laboratory and once they entered the maze set in a dark surrounding, they could only walk due to the maze's reduced size. The visual stimuli, a blue square (RGB:-1,-1, 1) and a green disc (RGB:-1, 0.2,-1), were projected onto tracing-paper screens placed vertically at the ends of the two arms using a video projector (Acer K135i, Roissy, France). Stimulus intensity was  $14000 \mu\text{W}/\text{cm}^2$  for the blue square and  $1800 \mu\text{W}/\text{cm}^2$  for the green disc; these values were adjusted to suppress a homogeneous spontaneous preference for the green disc uncovered by preliminary assays. The blue square and the green disc displayed the same total area ( $5.5 \text{ cm}^2$ ) and subtended a similar visual angle to the bee eyes (square, from edge to edge:  $26.3^\circ$ ; disc:  $29.6^\circ$ ). This angular range ensures that bees engaged their chromatic vision to achieve the visual discrimination<sup>50,51</sup>. Small Eppendorf tube covers containing 1 M sucrose solution or 60 mM quinine solution were placed at the end of the arm presenting the training visual stimulus. The setup was placed under a red ceiling to ensure a dark environment for the bees. Thus, bees were trained to choose video projected stimuli while walking, similarly to the situation imposed by a treadmill to a tethered bee. Maze bees were, nevertheless, free and returned to the hive between foraging bouts.

We first evaluated spontaneous preferences between the blue square and the green disc in a pre-test in which both stimuli were presented simultaneously in the absence of reinforcement (Fig. 2a). Afterwards, we conditioned bees to discriminate a rewarding stimulus paired with sucrose solution (CS+) from a non-rewarding stimulus paired with quinine solution (CS-). Bees experienced 12 conditioning trials (6 CS+ and 6 CS- presentations in a pseudorandom sequence) in which only one stimulus was visible at a time, on either the right or left arm of the maze. In the opposite arm, a black screen was projected (Fig. 2a). At each trial, the visual target was presented during 30 s and paired with its corresponding reinforcement. Right after the choice of the CS+, the bees were allowed to feed from the sucrose solution *ad libitum* to promote their regular return to the setup from the hive. Thus, the inter-trial interval was set by the bee itself. Training was balanced so that for a group of bees the blue square and the green disc were the CS+ and the CS-,

respectively, while for another group of bees the contingencies were reversed. Finally, we evaluated learning-induced preferences in a post-test presenting simultaneously the CS+ and the CS- in the absence of reinforcement (Fig. 2a).

***Test performance: proportion of learners.*** Spontaneous preferences between the blue square and the green disc were recorded during the pre-test, which lasted for the 30 s of stimulus presentation. The bees' preference was recorded for all bees ( $n = 21$ ) in terms of the first choice performed upon entrance into the mini maze. We calculated the percentages of bees choosing first the maze-arm corresponding to the blue square or the green disc, or not choosing any stimulus. In the pre-test, these percentages were 48%, 48% and 4%, respectively (Fig. 3a). There were no differences between the values corresponding to the choice of the blue square and of the green disc (GLMM binomial family with Tukey method for multiple comparison; blue square vs. green disc:  $z_{124} = 0$ ,  $p = 1$ ). The proportion of bees choosing both stimuli was significantly different from that of bees not choosing (blue square vs. no choice:  $z_{124} = 2.604$ ,  $p = 0.025$ , green disc vs. no choice:  $z_{124} = 2.604$ ,  $p = 0.025$ ). After conditioning, all bees (100%;  $n = 21$ ) exhibited perfect learning in the post-test (Fig. 3b). The totality of bees chose the CS+ and none of the bees chose the CS- or did not choose any stimulus in the maze. These results thus indicate that when bees had total freedom to choose video projected stimuli differentially reinforced, while walking in a dark environment, discrimination learning was fully successful.

***Test performance: time spent in each maze arm during the pre-test and the post-test.*** In addition to the proportion of bees choosing either stimulus, we computed the time spent searching within arms displaying the CS+ or the CS- during the 30 s of stimulus presentation both during the pre-test and the post-test. During the pre-test, bees tended to spend a reduced proportion of the 30s in the arms of the maze, probably due to their lack of familiarity with the setup (Figs. 3c,d; white box plots). At this stage, the time spent in the arms displaying the CS+ and the CS- did not differ (Wilcoxon test;  $U=81$ ,  $p=0.51$ ). After conditioning (Figs. 3c,d; grey box plots), bees increased the time spent in the CS+ arm and simultaneously decreased the time spent in the CS- arm (CS+ arm:  $U = 0$ ,  $p < 0.0001$ ; CS- arm:  $U = 183$ ,  $p < 0.001$ ). As a consequence, bees spent significantly more time in the CS+ arm than in the CS- arm ( $U = 0$ ,  $p < 0.0001$ ), thus showing a clear learning effect.

***Acquisition performance.*** Bees experienced 6 CS+ and 6 CS- presentations in a pseudorandom sequence in which only one stimulus was visible at a time, on either the right or left arm of the

maze. The bees' performance was recorded in terms of the percentage of individuals choosing either the CS+ or the CS- when confronted against the black screen present in the alternative arm of the maze.

Bees showed a strong tendency to choose the single visual stimulus presented during the first trial, be it CS+ or CS- (Fig. 3e: CS +, 75%; CS -, 83%). Yet, a progressive differentiation in responses occurred during conditioning trials: while responses to the CS+ remained high (Fig. 3e, red curve), responses to the CS- decreased (Fig. 3e, black curve). This resulted in a non-significant CS effect (GLMM binomial family; df: 1,  $\chi^2 = 0.99$ ,  $p = 0.32$ ), a significant trial effect (df: 5,  $\chi^2 = 11.26$ ,  $p = 0.046$ ) and a significant interaction (CS\*trial effect; df: 5,  $\chi^2 = 13.04$ ,  $p = 0.023$ ). These results reveal that bees learned the visual discrimination during training.

Finally, the proportion of time spent in each arm of the maze during the 30 s of CS display was also recorded during conditioning trials (Supplementary Fig. S1). During CS+ trials, bees spent almost the entire 30 s of CS+ display in the CS+ arm. In contrast, during CS- trials, bees tended to spend only one third of this time (ca. 10 s) in the CS- arm when the CS- was shown. These results show that during CS- trials, bees avoided choosing between two options, which did not yield the reward outcome they were searching.

Taken together, the results of this experiment show that freely flying bees trained to walk into a miniature Y-maze displaying video projected stimuli in a dark environment learn efficiently the visual discrimination when one of them is paired with sucrose and the other with quinine solution. This result sets, therefore, the basis for treadmill experiments in which a tethered, walking bee learns equivalent visual discriminations in a better-controlled visual environment.

## **Experiment 2: visual learning at the treadmill**

We determined if tethered honeybee foragers learn to discriminate the same two visual stimuli used in the previous experiment. Tethered bees (Fig. 1b) were placed on a treadmill in a dark room (Fig. 1c), which provided the same dark environment as in the mini-maze experiment. The setup was positioned in front of a semi-cylindrical screen of white tracing paper onto which visual stimuli were video projected (Fig. 1d). The projection background was the same as the one used in the Y-maze experiments. A set of mirrors was used to project the stimuli on the lateral parts of the semicircular screen (Fig. 1d) without deforming them. The visual stimuli appeared for 30 s on the

screen and were 7 cm distant from the bee's head. As in the mini-maze experiment, only one stimulus was shown at a time during conditioning, at  $50^\circ$  to either the left or right of the bee's body axis (same angular position as in the maze experiment). The stimulus side was pseudorandomized from trial to trial (see Methods). Stimuli were equal in their total area ( $9 \text{ cm}^2$ ). They subtended a similar visual angle to the bee eyes (square, from edge to edge:  $24.2^\circ$ ; disc:  $27.3^\circ$ ), which was consistent with that of the mini-maze experiment to ensure that chromatic vision was engaged for visual discrimination<sup>50,51</sup>. As in the maze experiment, stimulus intensity was adjusted to suppress a homogeneous spontaneous preference for the green disc uncovered by preliminary assays. The position of the visual stimuli was independent of the bees' movements (open-loop condition) and did not vary during conditioning or testing. As stimuli were displayed at  $50^\circ$  on either side of the bee, a choice was counted if the cumulated rotation angle of the sphere exceeded  $50^\circ$  to the right or to the left during the 30 s of stimulus presentation. As cumulative rotation could exceed  $360^\circ$  during this measurement period, cumulative-heading values ( $^\circ$ ) were represented as multiples of  $360^\circ$ .

Three groups of bees were studied in parallel, interspersing bees from all three groups every day: a *paired group* ( $n = 38$ ), a *CS (conditioned stimuli) group* ( $n = 32$ ) and a *US (unconditioned stimuli) group* ( $n = 32$ ). As in the mini-maze experiment, a pre-test assessed potential naïve preference for the green disc or the blue square presented simultaneously during 30 s in the absence of reinforcement (Fig. 2b). The percentage of bees choosing these stimuli or not choosing any stimulus (i.e. with a cumulative heading not exceeding  $50^\circ$  in either direction) and the cumulative heading of these bees were quantified during this period.

Subsequently, the *paired group* experienced a differential conditioning protocol (Fig. 2c) using positive and negative reinforcements as in the mini-maze experiment<sup>52</sup>. Bees in this group were trained with 12 conditioning trials spaced by one minute (Fig. 2b) in which one stimulus (CS+) was rewarded with 1M sucrose solution while the other stimulus (CS-) was associated with a 60 mM quinine solution. The sucrose and quinine solutions were delivered to the proboscis by means of toothpick if it was extended after stimulating the antenna on the side of the stimulus presentation. During training, only one stimulus (CS+ or CS-) was displayed on the screen in order to follow as closely as possible the procedure of the previous experiment with the maze (Fig. 2a,b). The sequence of CS+ and CS- was also pseudorandom (see Methods). Two subgroups were trained in order to balance reinforcement experience with the green disc and the blue square (i.e., one

subgroup experienced the green disc rewarded and the blue square punished, while the other subgroup experienced the reversed contingency). The *CS group* experienced only visual stimulation (CS1, CS2) matched to that of the *paired group* (Fig. 2c), i.e. six green-disc and six blue-square presentations, but without any US. The *US group* experienced a sequence of 12 US presentations (6 sucrose and 6 quinine presentations) matching that of the *paired group* (Fig. 2c).

After the end of training, all three groups experienced a post-test in which bees were presented with the green disc and the blue square simultaneously, without any reinforcement (Fig. 2b). The percentage of bees choosing these stimuli or not choosing any stimulus and the corresponding cumulative headings were again quantified. In this way, performance in the post-test could be compared to that in the pre-test prior to training.

**Test performance: proportion of learners.** We first focused on spontaneous choices in the pre-test. We quantified the percentage of bees that chose the green disc, the blue square or did not choose any stimulus (Fig. 4a,b,c). A significantly higher proportion of bees of the *paired group* (69%) preferred spontaneously the blue square (Fig. 4a). The proportion of bees choosing the green disc or not choosing any stimulus were 0% and 31%, respectively (GLMM binomial family with Tukey method for multiple comparison; blue square vs. green disc:  $z_{915} = 4.415$ ,  $p = 0.0003$ , blue square vs. no choice:  $z_{915} = 4.52$ ,  $p = 0.002$ , green disc vs. no choice:  $z_{915} = 2.10$ ,  $p = 0.47$ ). In the *CS group* (Fig. 4b), the proportion of bees spontaneously choosing the blue square was 56%, whereas 19% chose the green disc and 25% did not choose any stimulus. These proportions did not differ significantly from each other despite the tendency of bees to prefer the blue square (blue square vs. green disc:  $z_{915} = 2.98$ ,  $p = 0.08$ , blue square vs. no choice:  $z_{915} = 2.49$ ,  $p = 0.24$ , green disc vs. no choice:  $z_{915} = 0.60$ ,  $p = 1.00$ ). Finally, in the *US group* (Fig. 4c), the proportions of bees spontaneously choosing the blue square, the green disc or not choosing any stimulus were 50%, 25% and 25%, respectively. As for the *CS group*, these proportions did not differ significantly from each other despite the higher proportion of bees choosing the blue square (blue square vs. green disc:  $z_{915} = 2.03$ ,  $p = 0.52$ , blue square vs. no choice:  $z_{915} = 2.03$ ,  $p = 0.52$ , green disc vs. no choice:  $z_{915} = 0$ ,  $p = 1.00$ ). Consequently, a comparison between the three groups showed that they had different patterns of spontaneous responses (GLMM binomial family; group\*choice effect: df: 4,  $\chi^2 = 10.58$ ,  $p = 0.03$ ). As the three groups were tested in parallel and experienced identical conditions in the pre-test, the significant spontaneous preference for the blue square in one of the three groups was likely due to a random sampling of individuals.

After the 12 trials experienced by each group after the pre-test, bees were again presented with the green disc and the blue square simultaneously in a post-test in the absence of reinforcement (Figs. 4d,e,f). The three groups differed in their stimulus choice during this post-test. In the *paired group* (Fig. 4d), a significant proportion of bees preferred the CS+ irrespective of the nature of the positively reinforced stimulus (blue square or green disc; see inset of Fig. 4d; reinforced-stimulus effect; df: 1,  $\chi^2 = 2.32$ ,  $p = 0.12$ ; reinforced-stimulus\*choice effect: df: 2,  $\chi^2 = 3.37$ ,  $p = 0.18$ ). The absence of a significant reinforced-stimulus effect allowed pooling data from both subgroups (Fig. 4d). Globally, 60% of the bees preferred the CS+, while 20% preferred the CS- and another 20% exhibited no choice during the post-test (choice effect; df: 2,  $\chi^2 = 10.61$ ,  $p = 0.005$ ). These results thus show that learning occurred within the *paired group*.

For bees in the *CS group* (Fig. 4e), there was no reinforcement associated with the visual stimulation (i.e. there was neither a CS+ nor a CS-). Thus, the post-test performance was represented in terms of the proportion of bees choosing the green disc, the blue square or not choosing any stimulus (Fig. 4e). The same representation was adopted for the *US group* (Fig. 4f), which never experienced a CS stimulus but only positive and negative reinforcements on the left or right antenna corresponding to a theoretical presentation of a CS on the left or right of the screen, respectively, as performed in the paired group. The *CS group* did not show significant differences in the proportions of bees preferring either stimulus in the post-test (Fig. 4e). The proportion of bees spontaneously choosing the blue square was 34%, whereas 16% chose the green disc and 50% did not choose any stimulus. These values did not differ statistically from each other (blue square vs. green disc:  $z_{1536} = 1.69$ ,  $p = 0.87$ , blue square vs. no choice:  $z_{1536} = -1.26$ ,  $p = 0.98$ , green disc vs. no choice:  $z_{1536} = 2.80$ ,  $p = 0.18$ ). Similarly, in the *US group* (Fig. 4f), no significant differences were found between the bees preferring the blue square (38%), the green disc (28%) or not choosing (34%). These proportions did not differ from each other statistically (blue square vs. green disc:  $z_{1536} = 0.79$ ,  $p = 1.00$ , blue square vs. no choice:  $z_{1536} = -0.26$ ,  $p = 1.00$ , green disc vs. no choice:  $z_{1536} = 0.54$ ,  $p = 1.00$ ). Thus, neither the *CS* nor the *US group* varied their performance between the pre-test and the post-test (test effect; df: 1,  $\chi^2 = 3.01$ ,  $p = 0.08$ ; group effect; df: 1,  $\chi^2 = 0.07$ ,  $p = 0.79$ ; test\*group effect; df: 1,  $\chi^2 = 0.28$ ,  $p = 0.59$ ). Only the training experienced by the *paired group* induced a significant increase in the proportion of bees preferring the CS+, which was particularly visible for bees rewarded on the green disc (compare Fig. 4a and inset in Fig. 4d).

**Test performance: cumulative heading towards conditioned stimuli.** These conclusions were confirmed by the quantitative analysis of the cumulative heading of the bees during the pre-test (Fig. 5, white box plots) and the post-test (grey box plots). Consistent with the absence of learning, bees of the *CS* (Fig. 5a) and the *US* groups (Fig. 5b) did not show any stimulus orientation during the post-test (*CS* group:  $U = 126$ ,  $p = 0.08$ ; *US* group:  $U = 325$ ,  $p = 0.26$ ). While bees of the *US* group did not change their heading direction between the pre-test and the post-test (Fig. 4b;  $U = 272$ ,  $p = 0.89$ ), bees of the *CS* group changed slightly their orientation and prioritized the left side (Fig. 4a;  $U = 375$ ,  $p = 0.037$ ), even if this did not induce any significant stimulus preference. In the case of the *paired* group (Fig. 5c), bees exhibited no stimulus preference during the pre-test ( $U = 461$ ,  $p = 0.19$ ), but oriented significantly towards the CS + during the post-test after training ( $U = 567$ ,  $p = 0.004$ ). This change in orientation was significant ( $U = 223$ ,  $p = 0.03$ ) and confirmed that learning occurred within the *paired* group.

To determine to what extent this learning effect was masked by performance of non-learners in this group, we focused our analysis on bees that chose the CS+ during the post-test (Fig. 5d; “learners”;  $n = 22$ ). During the pre-test, these bees did not show any stimulus preference ( $U = 169$ ,  $p = 0.17$ ) but headed significantly more towards the CS+ after training ( $U = 253$ ,  $p < 0.001$ ). This change in orientation was even more significant ( $U = 220$ ,  $p = 0.002$ ) than that of the entire *paired* group. Taken together, both the cumulative heading and the percentage of bees choosing the two stimulus alternatives during the post-test revealed a significant visual learning in the *paired* group trained on the treadmill. This effect was neither apparent in the *CS* nor in the *US* group.

**Acquisition performance.** We next focused on the responses of the four groups of bees during their respective training schedules. For the *paired* group, the proportion of bees responding to the visual stimuli before US delivery was quantified. We evaluated both the entire group, as well as just the learners within the group. For the *CS* group, the same parameter was quantified, with the difference that these bees never experienced reinforcement associated with visual stimuli. Responses were thus evaluated during the CS period preceding a virtual US. For the *US* group, quantification of responses to CS stimuli was not possible as only reinforcement was delivered; nevertheless, we quantified responses occurring in the interval of time prior to US delivery on the left or right antenna, which corresponds to a theoretical CS stimulation on the left or right of the screen, respectively.



Figure 6 shows the response curves obtained for the four groups during the 12 training trials. As expected from their test performances, neither the *CS group* nor the *US group* exhibited any discrimination (Fig. 6a, b). The *CS group* (Fig. 6a), which experienced a succession of green disc / blue square stimulations matching that of the *paired group* (CS1 / CS2), did not respond preferentially to any stimulus during the 12 trials (CS effect; df: 1,  $\chi^2 = 0.15$ ,  $p = 0.69$ ). Yet responses decreased similarly along trials for both stimuli (trial effect; df: 5,  $\chi^2 = 14.63$ ,  $p = 0.01$ ; CS\*trial effect; df: 5,  $\chi^2 = 6.90$ ,  $p = 0.23$ ), which indicates that in the absence of reinforcement, bees responded progressively less to the green disc and the blue square, a phenomenon akin to habituation. The *US group* (Fig. 6b) did not respond preferentially during the interval preceding the sucrose solution ('theoretical CS+') or the quinine solution ('theoretical CS-'), simply because they had no predictive cue enabling this discrimination. In this case, their response along trials remained invariable (CS effect; df: 1,  $\chi^2 = 1.90$ ,  $p = 0.16$ ; trial effect; df: 5,  $\chi^2 = 6.68$ ,  $p = 0.24$ ; CS\*trial effect; df: 5,  $\chi^2 = 5.77$ ,  $p = 0.33$ ). Interestingly, the performance of the entire *paired group* (Fig. 6c) was similar to those of the *CS* and *US groups* as no discrimination was visible along trials (CS effect; df: 1,  $\chi^2 = 3.08$ ,  $p = 0.08$ ; trial effect; df: 5,  $\chi^2 = 4.06$ ,  $p = 0.54$ ; CS\*trial effect; df: 5,  $\chi^2 = 6.26$ ,  $p = 0.28$ ). Such an absence of discrimination could have been due to a potential masking of the learners' performance by that of non-learners in the entire *paired group*. To examine this possibility, we restricted the analysis to learners. The resulting acquisition curves (Fig. 6d) were surprisingly similar to those of the *CS group* and of the entire *paired group*, thus revealing an apparent absence of discrimination (CS effect; df: 1,  $\chi^2 = 3.37$ ,  $p = 0.07$ ; trial effect; df: 5,  $\chi^2 = 5.19$ ,  $p = 0.39$ ; CS\*trial effect; df: 5,  $\chi^2 = 3.46$ ,  $p = 0.63$ ). Yet, as shown by post-test performances, bees of the *paired group* (entire group and learners) had learned the visual discrimination.

A strong tendency to choose the single visual stimulus presented during the first trial, be it CS1 or CS2, or CS+ or CS-, was clearly visible both in the *CS* and in the *paired groups* (Fig. 6a: *CS group*; CS1: 56%, CS2: 59%; Fig. 6c: entire *paired group*; CS+: 76 %, CS-: 61%; Fig. 6d: learners of the *paired group*; CS+: 68 %, CS-: 45%). This tendency was similar in the first trial of all three groups (all between-group comparisons NS) and occurred before any reinforcement delivery in the *paired groups*. This shows that bees were responding to the mere presence of the first visual stimulus displayed on the screen and simply kept responding alternately to the CS+ and the CS- during successive trials. This non-specific CS attraction suggests that positive phototaxis and/or object fixation drove the bees towards the visual stimulus displayed on the screen during a

training trial and were the cause for the similar performance of the *CS* and the *paired groups*. The *US group* (Fig. 6b) showed a level of responses which was significantly lower than that of the other three groups during the 12 trials (group effect;  $\chi^2 = 9.30$ , df: 3,  $p = 0.02$ ; *paired (entire) vs. US group*:  $z_{5948} = 11.51$ ,  $p < 0.0001$ ; *paired (learners) vs. US group*:  $z_{5948} = 8.5$ ,  $p < 0.0001$ ; *CS vs. US group*:  $z_{5948} = 7.27$ ;  $p < 0.0001$ ). This confirms that responses during training of the *CS* and the *paired groups* were driven by object fixation and/or positive phototaxis, two components that were absent in the *US group*, which did not experience any visual stimulus.

An analysis of the mean cumulative heading angle during the conditioning phase performed for all groups (Supplementary Fig. S2 a, b, c, d) confirmed the presence of a strong positive phototaxis or object fixation in bees of the *CS group* (Supplementary Fig. S2a) and of the *paired groups* (entire group: Supplementary Fig. S2c; learners: Supplementary Fig. S2d). These bees headed towards the visual stimulus presented (Supplementary Fig. S3), independently of its contingency (in the case of the *paired group*; i.e. they headed towards the CS+ or the CS- when these stimuli were presented alternately during training), its nature (in the case of the *CS group*, i.e. they headed towards the green disc or the blue square) or its position (right or left). By contrast, the bees from the *US group* (Supplementary Fig. S2b) which were not attracted by visual stimulation, walked in a rather straightforward way but in the absence of visual stimulation, they tended to walk less.

We thus conclude that presentation of a single-color stimulus during training induces strong phototaxis or object fixation at the treadmill. This confounding factor impedes measuring acquisition of a visual discrimination in our controlled conditions, even if a significant percentage of bees in the *paired group* learned the associations between visual stimuli and reinforcement. Thus, in addition to the clear evidence for learning found in the *paired group* during the tests with simultaneous presentation of trained stimuli, phototaxis and object fixation are also factors that need to be considered under certain training regimes.

### **Experiment 3: the influence of negative unconditioned stimuli on visual learning at the treadmill**

An important topic in associative learning studies relates learning strength to the relative strength of the US in terms of its ability to promote conditioning to the CS<sup>53</sup>. In this experiment, we aimed

at determining which aversive US had a higher relative strength to promote visual conditioning in a joint manner with the appetitive sucrose US. In differential olfactory PER conditioning, concentrated saline solution is more efficient than quinine solution as a negative US<sup>54</sup>. Here we asked if changing the nature of the US associated with the CS- also modulates learning success at the treadmill.

We studied the performance of four groups of bees that experienced the conditioning schedule of the *paired group* of the previous experiment. All groups experienced 1 M sucrose solution as the US paired with the CS+, but differed in the nature of the US associated with the CS-, which could be 60 mM quinine (n = 32), 3 M NaCl (n = 32), distilled water (n = 33) or contact with a dry toothpick (n = 32). The *quinine group* therefore reproduces the treatment experienced by the *paired group* of the previous experiment (Experiment 2).

**Test performance: proportion of learners.** In the pre-test (Figs. 7a,c,e,g), the *distilled water group* showed a spontaneous preference for the blue square (Fig. 7a). The proportions of bees spontaneously choosing the blue square, the green disc or not choosing any stimulus in this group were 49%, 15% and 36%, respectively (blue square vs. green disc:  $z_{392} = 2.78$ ,  $p = 0.014$ , blue square vs. no choice:  $z_{392} = 1.00$ ,  $p = 0.58$ , green disc vs. no choice:  $z_{392} = 1.92$ ,  $p = 0.13$ ). In the *dry-toothpick group* (Fig. 7c), these proportions were 38%, 19% and 44%, respectively (blue square vs. green disc:  $z_{380} = 1.64$ ,  $p = 0.23$ , blue square vs. no choice:  $z_{380} = -0.51$ ,  $p = 0.87$ , green disc vs. no choice:  $z_{380} = 2.11$ ,  $p = 0.08$ ). In the *quinine group* (Fig. 7e), the proportions of bees choosing the blue square, the green disc or not choosing any stimulus were 41%, 6% and 53%, respectively (blue square vs. green disc:  $z_{380} = 2.86$ ,  $p = 0.01$ , blue square vs. no choice:  $z_{380} = -0.99$ ,  $p = 0.58$ , green disc vs. no choice:  $z_{380} = 3.49$ ,  $p = 0.001$ ). Finally, in the *NaCl group* (Fig. 7g), these proportions were 31%, 31% and 38% respectively (blue square vs. green disc:  $z_{380} = 0$ ,  $p = 1.00$ , blue square vs. no choice:  $z_{380} = -0.53$ ,  $p = 0.86$ , green disc vs. no choice:  $z_{380} = 0.53$ ,  $p = 0.86$ ). A comparison between all four groups did not reveal significant differences (group effect:  $\chi^2 = 0.70$ , df: 3,  $p = 0.87$ ; choice effect, including both CSs and no-choice:  $\chi^2 = 4.73$ , df: 2,  $p = 0.09$ ; CS effect, including both CSs only:  $\chi^2 = 0.57$ , df: 1,  $p = 0.44$ ; group\*choice effect:  $\chi^2 = 3.96$ , df: 6,  $p = 0.68$ ; group\*choice\*CS effect:  $\chi^2 = 7.84$ , df: 11,  $p = 0.72$ ). The fact that the choice effect was close to significance was probably due to the *distilled water group* and the *quinine group*, in which more bees preferred significantly the blue square in the pre-test.

After conditioning (Figs. 7b,d,f,h), the performance in the post-test was independent of the stimulus (blue square or green disc) chosen by the experimenter as CS+ or CS- (CS effect:  $\chi^2 = 0.57$ , df: 1,  $p = 0.44$ ; group\*choice\*CS effect:  $\chi^2 = 7.68$ , df: 11,  $p = 0.74$ , see insets in Figs. 6 e, f, g, h). Thus, for each group, performance was represented in terms of the proportion of bees choosing the CS+, the CS- or not choosing any option. In the *distilled water group* (Fig. 7b), a significant learning effect was found: 58% of the bees preferred the CS+, while 15% preferred the CS- and 27% exhibited no choice during the post-test (CS+ vs. CS-:  $z_{392} = -3.38$ ,  $p = 0.002$ , CS+ vs. no choice:  $z_{392} = 2.44$ ,  $p = 0.03$ , CS- vs. no choice:  $z_{392} = -1.19$ ,  $p = 0.46$ ). In the *dry-toothpick group* (Fig. 7d), no variation was found in the proportion of bees choosing the CS+, the CS- or not choosing any option. Percentages for these three categories were 41%, 31% and 28%, respectively, and did not differ from each other (CS+ vs. CS-:  $z_{380} = -0.78$ ,  $p = 0.71$ , CS+ vs. no choice:  $z_{380} = 1.05$ ,  $p = 0.55$ , CS- vs. no choice:  $z_{380} = 0.27$ ,  $p = 0.96$ ). In the *quinine group* (Fig. 7f), a significant learning effect was found as 50% of the bees preferred the CS+, while 9% preferred the CS- and another 41% exhibited no choice during the post-test (CS+ vs. CS-:  $z_{380} = -3.23$ ,  $p = 0.004$ , CS+ vs. no choice:  $z_{380} = 0.75$ ,  $p = 0.73$ , CS- vs. no choice:  $z_{380} = -2.68$ ,  $p = 0.02$ ). Finally, in the *NaCl group* (Fig. 7h), no significant learning was found as the proportions of bees choosing the CS+, the CS- or not choosing any option were 34%, 34% and 32%, respectively (CS+ vs. CS-:  $z_{380} = 0$ ,  $p = 1.00$ , CS+ vs. no choice:  $z_{380} = 0.27$ ,  $p = 0.96$ , CS- vs. no choice:  $z_{380} = 0.27$ ,  $p = 0.96$ ). A comparison between all four groups showed no differences (group effect:  $\chi^2 = 2.94$ , df: 3,  $p = 0.40$ ; choice effect:  $\chi^2 = 4.73$ , df: 2,  $p = 0.09$ ; group\*choice effect:  $\chi^2 = 3.96$ , df: 6,  $p = 0.68$ ). This was confirmed by a Tukey test for multiple comparisons between proportions, which allowed comparing the proportion of learners between all four groups (*distilled-water group*: 19/33 learners; *quinine group*: 16/32 learners; *dry-toothpick group*: 11/32 learners; *NaCl group*: 13/32 learners;  $q_{\infty,4} < 3,633$  for all comparisons, NS). However, significant differences in favor of the CS+ were only found in the post-test of the *quinine* and the *distilled-water groups* while no differences between choice categories were found in the post-test of the *toothpick* and the *NaCl groups*. This difference indicates that learning occurred only in the *quinine* and *distilled groups*, in which more CS+ choosers were found as a consequence of conditioning, but not in the *toothpick* and *distilled groups* where bees distributed equally between CS+ choosers and CS- choosers in the post-test. Thus, quinine solution and distilled water were more efficient as negative US and

supported discrimination learning while contact with the dry toothpick or the 3 M NaCl solution did not.

**Acquisition performance.** The analysis of the acquisition curves revealed again a phototactic/object fixation effect similar to that found in Experiment 2. Bees in all groups exhibited a strong tendency to choose the single visual stimulus presented, be it CS+ or CS-, from the very first trial and kept doing so during the 12 trials. Focusing on learners in all four groups did not change this conclusion (Supplementary Figs. S4 a,b,c,d), thus showing that either phototaxis or object fixation overshadowed learning, as in the previous experiment. An analysis of the mean cumulative heading angle of learners during conditioning confirmed this conclusion: despite exhibiting a significant cumulative heading towards the CS+ during the tests, learners in all four groups did not show any evidence of learning during the 12 conditioning trials when the same behavioral parameter (cumulative heading) was considered (Supplementary Fig. S5 a,b,c,d).

We thus concluded that presenting a single training stimulus at a time overshadows learning in the treadmill. Learning occurred in all four paired groups but not all US associated with the CS- yielded the same learning success. Indeed, quinine and distilled water were more effective as negative US as in these cases significantly more learners were found.

## Discussion

We aimed at establishing the bases for the study of visual learning in honeybees in a controlled laboratory environment allowing future explorations of its neural underpinnings and the use of virtual reality to this end. We tethered individual bees and allowed them to walk on a treadmill while displaying visual stimuli in front of them by means of a video projector. The ultimate goal was to reproduce visual learning performances by free-flying bees<sup>8,16</sup>, while partially constraining the freedom of movement. Such a constraint is necessary to allow the simultaneous study of the neural architectures underlying different forms of visual learning, a goal that has proven elusive in bees until now. Our results show that bees walking into a miniature maze set in a dark environment successfully learn to discriminate visual stimuli projected onto the back walls of the maze (Experiment 1). Adapting this experimental situation to a treadmill setup (Experiment 2) showed

that learning performances were better in the maze, i.e. that full movement freedom and behavioral context are important for visual learning. Yet, robust visual learning occurred at the treadmill, thus showing that it is possible to reproduce elemental forms of visual learning in this setup. Despite the experimental constraints imposed by tethering and by setting the bees in a dark surrounding, a significant percentage of animals exhibited discrimination learning performances and preferred, after training, the visual stimulus associated with sucrose reward (CS+) over a negative stimulus (CS-). We also show that the nature of the US associated with the CS- affects the learning performance (Experiment 3). Indeed, quinine and distilled water were more effective as in these cases significantly more bees learned the visual discrimination. These results set the bases for further explorations and refinements of the protocols described here.

The learning success (proportion of learners) at the treadmill varied between 50% and 60% (Experiments 2 & 3). These values are non-negligible compared to the lower percentage of learners (usually, between 30% and 40%) that are reported in experiments on visual PER conditioning<sup>55-58</sup>. Full immobilization is imposed by PER conditioning, contrary to our experimental situation in which bees walk stationary on the treadmill. Yet, although the results obtained in our work are promising for further explorations of visual learning, they seem to be distant from the 100% found in the mini maze (Experiment 1) and usually reported in visual learning experiments with free-flying bees<sup>8,10,16</sup>. Note, however, that studies on visual learning in freely flying bees usually discard individuals that for some reasons do not learn the task (e.g. bees that do not return to the experimental site and that do not complete, therefore, the training, or bees that develop a tendency to turn always to the same side in a maze). Had these works included a thorough analysis of non-learners, as in this work, the proportion of success under the two situations - treadmill and free flight - could be perhaps more similar.

It seems, nevertheless, that the critical factor for successful visual learning is that bees dispose of a certain freedom of movement, as shown by Experiment 1. It could be argued that the lack of freedom affects the animal's appetitive motivation, thus decreasing performance success in protocols that do not grant such freedom. Yet, this argument can be discarded by considering that olfactory PER conditioning, in which bees are fully immobilized, yields a learning success that typically reaches 90-100%<sup>2,11,12</sup>. Thus, if a decrease in motivation is not a likely explanation for the decrease in learning success observed when movement is restricted, what could be the cause for such a learning impairment? We suggest that the key factor is the impaired possibility for

exerting active scanning while learning a visual task. In active vision, animals vary the viewpoint in order to investigate the environment and extract better information from it. Honeybees scan visual scenes sequentially, rather than being able to taking them in “at a glance”<sup>59,60</sup>. This need for serial scanning means that shape differences can only be identified with difficulty if stimuli are presented for short intervals, or when bees cannot move their eyes, as happens when they are fully restrained. Performing flight maneuvers allows extracting and acquire information about the nature and structure of visual targets<sup>44,61,62</sup>. Bees, flies and other flying insects actively shape the dynamics of the image flow on their eyes, a strategy that facilitates the solving of spatial vision tasks in an efficient way. In this context, active vision is crucial to segregate rotational from translational optic flow components. Stereotyped circling or scanning flights are used to this end<sup>24,44-47</sup>, which help, in the case of 2D stimuli like the ones used in our work, to extract the borders of objects for better recognition<sup>48,49,63</sup>. An alternative explanation may put the accent on the difference in behavioral contexts between the treadmill and the mini maze. In the mini-maze, the bees flew back to the hive between trials and returned to the setup on their own accord, conditions which might facilitate associative learning performances. In the treadmill, on the contrary, bees were not free to choose when to enter the experimental trials, and were not able to return to the hive between trials. Note that these limitations also apply to PER experiments, which do not affect efficient olfactory learning and memory formation. Yet, it could be that olfactory learning is less sensitive to these aspects than visual learning.

Both the stimuli displayed by the video projector and the illumination conditions were the same in the miniature maze and in the treadmill setup. The mini-maze used in Experiment 1 certainly imposed a differently structured environment (e.g., maze arms vs open visual field at the treadmill) but the visual stimuli were in the same angular range as in the treadmill. In addition, the associative framework occurring in both scenarios had comparable components: learning in the Y-maze experiment was mainly operant, since reinforcement outcome depended on the bees’ choice<sup>64</sup>. Yet, as in most operant protocols using discriminative stimuli, learning also included Pavlovian components as the bees learned the association between a given visual stimulus (conditioned stimulus, CS) and reinforcement/punishment (unconditioned stimulus, US). In the treadmill experiments, there were also obvious Pavlovian components as the CS+ and the CS- (and their respective US at the end of each CS) were delivered in a pseudorandom yet fixed sequence for every bee. Yet operant associations cannot be excluded due to the strong tendency of bees to

walk towards the single illuminated stimulus displayed during training, which resulted in the delivery of an appetitive or an aversive US. In other words, associations between walking towards a CS+ and the appetitive US, or between walking towards a CS- and an aversive US, could also occur, despite the fixed sequence of presentation of the CS+ and CS- and their respective US, over which bees had no control.

A main difference between the maze and the treadmill scenarios lies in the possibility of moving and actively scanning the visual targets, and of seeing subsequent changes in stimulus properties following this active sensing. These features may account for differences between these experimental contexts. In the maze, scanning and visual stimulus control were possible while they were reduced and/or inexistent in the treadmill, in particular because of the open loop conditions imposed on the bees. Consequently, no change in stimulus perceptual properties occurred following the bee's translation on the treadmill. Thus, performing these or other experiments under closed-loop conditions in which the projected stimuli update in real time in response to the bee's movements could be crucial to improve learning success.

Our original discrimination protocol used 1M sucrose solution as positive US paired with the CS+, and 60 mM quinine solution as a negative reinforcement paired with the CS-. In doing this, we aimed at reproducing conditions that have been used repeatedly in visual learning experiments with free-flying bees<sup>52,65</sup> and that were suggested to enhance attentional mechanisms and improve discrimination performances<sup>66</sup>. Consistent with our findings, prior studies showed that not all aversive US have the same consequences for discrimination learning. Free-flying bumble bees trained to discriminate between two perceptually similar colors, one associated with 1.75 M sucrose solution, and the other with water or quinine solution 120 mM, perform better if they experience quinine on the CS- targets rather than water<sup>65</sup>.

In differential conditioning, the higher the contrast between the subjective value of the CS+ and the CS-, the better the learning. In the case of the toothpick (Experiment 3), the antennal stimulation was purely mechanical and did not generate an appetitive expectation. This stimulus did not offer, therefore, a marked contrast with the appetitive sucrose solution experienced upon CS+ presentation. It is thus understandable that the toothpick stimulation did not induce a higher proportion of learners. In the case of quinine solution and distilled water, the animals experienced an aqueous solution on the antennae which induces in the first trial PER, followed by contact of



the proboscis with these solutions. Here the contrast between the CS+ and the CS- existed in terms of expectation and real outcome as bees extended the proboscis expecting an appetitive stimulus and experienced water or quinine, which do not correspond to their expectancy. The fact that the results were similar for quinine solution and water may be explained by prior works indicating that bees have a reduced sensitivity to bitter substances at the level of their antennae and may thus detect an aqueous solution rather than a bitter solution<sup>67</sup>. The fact that until now, no bitter-taste receptor genes have been identified in the honey bee genome<sup>68</sup>, contrary to other insects, strengthens this conclusion. It has, therefore, been suggested that the aversive effect of concentrated quinine solution could have two main explanations: 1) in the long delay, a malaise effect induced by the consumption of this substance<sup>69,70</sup>; 2) in the short delay, the successive negative contrast experienced by a bee searching for sucrose solution and receiving instead a watery solution that does not fulfill its appetitive expectation<sup>71</sup>. The fact that we found the same improvement with distilled water and 60 mM quinine solution supports the second explanation. It is, nevertheless, intriguing that a concentrated NaCl solution paired with the CS- did not support learning. Indeed, in olfactory discriminations with harnessed bees, this solution induced better learning performances (i.e. had more aversive strength) than the same quinine solution used in our experiments<sup>54</sup>. Further studies should explore why NaCl did not have the expected aversive strength in our experimental conditions.

In Experiments 2 and 3 performed at the treadmill, the acquisition curves for tethered bees did not reveal successful discrimination learning (see Fig. 6 and Supplementary Fig. S4) even if such learning occurred and was afterwards visible in the post-test performances (see Figs. 4 & 5 and 7). This was due to a confounding effect of either phototaxis or object fixation, which led bees to choose the single illuminated visual target that was displayed at a time on the projection screen, irrespectively of its nature and reinforcement. During successive trials, bees kept choosing the single stimulus that was shown in a given trial, thus masking discrimination learning. Setting the setup in darkness was necessary to display salient stimuli using the video projector. This could have enhanced phototactic tendencies but Experiment 1 offered the same illumination conditions in the mini maze, and although a phototactic/object fixation effect was seen in the first trial (see Fig. 3e), it did not overshadow the bees' discrimination learning. Thus, darkness was not the primary cause for phototactic/object-fixation overshadowing of learning during training at the treadmill. The reason for this effect is to be found in our training schedule. By offering a single

illuminated stimulus in any conditioning trial, bees were compelled to orient towards it. In Experiment 1, when an equivalent situation was offered in CS- trials in the mini maze, bees decided not to enter the maze arm in almost half of the time of CS- display (Supplementary Fig. S1), yet in this case they had the freedom to do so. Thus, reconfiguring the training in order to display both the CS+ and the CS- in any conditioning trial should decrease both spontaneous phototaxis and object fixation, and force the animals to choose between alternatives with different reinforcement outcomes. The simultaneous presentation of both the CS+ and the CS- during training requires closed-loop conditions, in which responses are tracked and used to update the next ‘view’ of the virtual environment in real time. These conditions allow the bee to ‘move’ the stimulus chosen towards her and thus to improve the nature of the associations established upon reward or punishment delivery during training.

We conclude that visual learning in honey bees is amenable to a laboratory preparation in which tethered animals learn visual stimuli in a controlled visual environment. This result is important for further analyses of the neural bases of such learning, which have remained poorly explored until now. We have identified potential caveats and suggested further improvements for future studies under these experimental conditions: simultaneous presentation of the CS+ and the CS- during training, stimulus display under closed-loop conditions, careful choice of the reinforcement associated with the CS- and enhancement of active vision, for instance through 3D stimulus display, are possible ways to improve learning performances in our setup. Such experiments will allow us to learn more about the mechanisms of visual learning and perception in the honey bee, thus reinforcing the model status of this insect for this research field.

## Methods

Honey bees (*Apis mellifera*) were obtained from the apiary located at the campus of the University Paul Sabatier. Only foragers caught upon landing on a gravity feeder filled with a 0.9 M sucrose solution were used in our experiments to ensure high appetitive motivation.

### Experiment in the mini-maze

Bees were fully free to fly from the hive to the laboratory where they approached and entered the mini-maze to collect sucrose solution. Bees were previously trained to fly to a gravity feeder filled with 0.9 M sugar water where they were marked with acrylic paints on the thorax and/or abdomen for individual identification. A single marked bee was randomly chosen from the feeder and moved progressively to the mini-maze by means of an Eppendorf tube cover offering sucrose solution. Similar small mazes have been used to successfully train walking bees to collect sucrose solution<sup>54,72</sup>. The maze was placed in a dark room, with illumination conditions that were similar to those set for the treadmill. Each bee was trained systematically to enter the Y-maze by itself in order to find sucrose solution at the small feeder. No visual stimulus was offered during this pre-training phase. The reduced size of the maze prevented the bee from flying. It could nevertheless freely walk inside the maze arms to search and collect the sucrose solution. Only one bee at a time was present inside the maze.

**The mini Y-maze.** The maze (Fig. 1a) consisted of three arms shaped in a Y. Each arm had a length of 5 cm and a cross-section of 3x3 cm. The visual stimuli were projected by a video projector (Acer K135i, Roissy, France; Fig. 1c) onto tracing-paper screens placed vertically at the ends of the two arms. The stimuli were, therefore, at 5cm from the decision point of the maze (arm intersection). Small Eppendorf tube covers (Eppendorf tube 3810x, Hamburg, Germany) containing 60µl of 1 M sucrose solution or 60 mM quinine solution were placed at the end of the arm presenting the respective visual stimulus. The setup was placed under a red ceiling to ensure a dark environment for the bees.

**Visual stimuli.** The visual stimuli to be discriminated were a blue square (RGB:-1,-1, 1) and a green disc (RGB:-1, 0.2,-1). The blue square was 2.34x2.34 cm and the green disc was 2.64 cm in diameter. Their intensity was 14000 µW/cm<sup>2</sup> for the blue square and 1800 µW/cm<sup>2</sup> for the green disc. Both stimuli displayed the same total area (5.5 cm<sup>2</sup>). They subtended a similar visual angle to the bee eyes (square, from edge to edge: 26.3°; disc: 29.6°), which was in the same range as those presented at the treadmill. This angular range ensures that bees engaged their chromatic vision to achieve the visual discrimination<sup>50,51</sup>.

**Conditioning and testing procedure.** The conditioning and testing procedures (Fig. 2a) mirrored as closely as possible those used at the treadmill (Experiment 2, paired group; Experiment

3, quinine group; see below). The bees were first trained to forage on an external feeder, which was progressively moved close to the mini maze inside the dark room. A single marked bee was trained gradually to enter the maze to collect sucrose solution. No visual stimulus was presented therein. Once the bee entered the maze by itself four consecutive times (i.e. after four foraging bouts), the experiment started. A pre-test was conducted in which bees were presented during 30 s with both visual stimuli simultaneously to check for spontaneous preferences (Fig. 2a). The stimulus position (left or right arm) was randomized between bees (see above). One minute of darkness followed this pre-test and the differential conditioning started following the same procedure as at the treadmill.

Bees were subjected to 12 conditioning trials (6 CS+ and 6 CS- trials in a pseudorandom succession, which was the same for all trained bees and reproduced the one used at the treadmill, Fig. 2a). One stimulus (CS+, paired with 1M sucrose, or CS-, paired with 60 mM quinine) was visible at a time in one arm of the maze while the other arm presented a black screen associated with an empty feeder. The side of stimulus presentation was varied between trials in the same way as in the treadmill. Bees were allowed to drink the sucrose solution *ab libitum* before leaving for the hive. Because of this, the time spent in the maze during CS+ trials was longer than for CS- trials. Yet, for the sake of comparative analyses, responses were quantified during the first 30 s in both types of trials. The bees were in full control of the interstimulus interval and the intertrial interval. The latter was defined by the return time of the bee to the maze and lasted usually 3 to 5 min.

After the last conditioning trial and upon the next return to the maze, bees were subjected to a post-test in which both stimuli, CS+ and CS-, were presented simultaneously during 30 s in the absence of reinforcement (Fig. 2a).

**Data analysis and statistics.** During the tests and conditioning trials, we quantified the bees' first choice and calculated on this basis the percentage of bees belonging to the three categories defined for the treadmill experiments (individuals choosing the CS+, the CS- or not making a choice; see above). The first choice was defined as the first arm (CS+, CS-) entered by a walking bee upon arrival to the maze, which was followed by a touch of the screen displaying the stimulus at the end of the arm. The bees' performance was represented with its corresponding 95% confidence interval. The time spent within each arm during the 30 s of stimulus presentation was also quantified and

represented in terms of medians with corresponding quartiles, as it did not follow a normal distribution.

For the pre-test and the post-test, the proportions of individuals choosing the CS+, the CS- or not making a choice were compared within groups by means of a generalized linear mixed model (GLMM) for binomial family in which the individual identity (Bee) was considered as a random factor (individual effect). The Tukey method was used for multiple comparisons; z values with corresponding degrees of freedom are reported throughout for this kind of analysis. Comparisons between groups were done using a GLMM for binomial family with Group, Choice and interaction Group x Choice as main effects;  $\chi^2$  values with corresponding degrees of freedom are reported throughout for these analyses. The time spent in the arms displaying the CS+ and the CS- and its variation between the pre-test and the post-test was analyzed by means of a Wilcoxon U test.

For the acquisition, the proportion of bees, which chose the CS+ or the CS-, was analyzed by means of a generalized linear mixed model (GLMM) for binomial family in which Trial was considered as a continuous factor (Trial effect) and the individual identity (Bee) as a random factor (individual effect). For within-group analyses,  $\chi^2$  values are reported for CS effect, Trial effect and CS x Trial effect. Multiple comparisons were performed using Tukey's method (z values reported).

All statistical analyses were done with R 3.2.3 (R Core Team 2016). Packages lme4 and lsmeans were used for GLMMs and Tukey's method for multiple comparisons, respectively.

## **Experiments on the treadmill**

**Preparation of bees for experiments on the treadmill.** Captured bees were brought to the laboratory where they were placed on ice for five minutes in order to reduce their activity. This facilitated the fixation of a vertical tethering attachment to the thorax of each bee. The attachment was composed of a toothpick and a small piece of metal glued to the thorax with UV-curing opaque dentine (3M Symphony D01-D05, DT&SHOP, Bad Bocklet, Germany) (Fig.1b). The toothpick was used to clip the bee to a micromanipulator (M3301, WPI, Sarasota, USA) and adjust its position on the treadmill. Each bee was placed in a dark, humid box, and attached to the box's lid by its tether for at least one hour before the start of the experiment, in order to habituate it to the tethering. These steps were done in a dark room under red light.

**Treadmill.** The apparatus (Fig.1 c,d) was composed of a polystyrene ball (diameter: 10 cm, weight: 8 g) held by a 3D-printed support and floating on a constant air flow produced by an air pump (air flow: 555ml/s; Aqua Oxy CWS 2000, Oase, Wasquehal, France). To accurately position the bee on top of the ball, the tether was mounted on the micromanipulator. The treadmill was placed in front of a semi-cylindrical screen of tracing paper (height: 25 cm; distance to the bee: 7 cm), onto which visual stimuli were projected using the same video projector used for projecting visual stimuli in the mini maze. A set of mirrors was used to present stimuli on the lateral parts of the screen without deformation. The stimuli appeared on the screen at 50° to the left and right of the central axis of the bee body. To have full control of temporal parameters in our experiments (CS time, US time, trial duration and intertrial interval), we used an open-loop condition for both spatial variables, direction and distance, i.e., the bees' movement modified neither the relative position, nor the apparent size of the stimuli.

The treadmill translates the movements of the walking bee into rotations of the ball. These were recorded by two infrared optic-mouse sensors (Logitech M500, 5700 dpi, Logitech, Lausanne, Switzerland), which were placed on the ball support, at 90° from each other. The rotational speed of the ball around the vertical axis was calculated to account for the directional walking movements of the bee ("instant heading"; one data point every 250 ms) using the following equation:

$$\text{Instant heading} = - \left( \frac{\frac{X1 + X2}{2}}{5700} \right) * \left( \frac{25.4 * 180}{R\pi} \right)$$

X1 and X2 are, respectively, the translational movement in dots recorded in the horizontal axis of each sensor and 5700 is the sampling rate of the sensors in dots/inch. Multiplying the obtained value by 25.4 allows conversion into millimeters while multiplying it by  $2\pi R$  (with R being the radius of the ball) converts the measured distance from millimeters into radians. Finally, multiplying by  $180/\pi$  converts radians to degrees.

A *cumulative heading* was calculated for the whole duration of each trial or test session by summing up the instant headings. This measure was used to categorize the walking behavior of bees as 'right choice' if the cumulative heading was larger than 50°, 'left choice' if it was smaller than -50°, or 'no choice' if it was between -50° and 50°. The cutoff value of 50° corresponds to the position of the stimuli on the screen with respect to the bee's central axis (see above) when the

insect is placed on the tracking ball. This value corresponds consequently to the rotation that a bee should make to orient its body axis towards the stimulus. Cumulative headings could exceed  $360^\circ$  during a test/trial and were thus represented as multiples of a  $360^\circ$  rotation.

**Visual stimuli.** The visual stimuli to be discriminated were a blue square (3x3 cm; RGB:-1,-1, 1, with dominant wavelength at 450 nm) and a green disc (diameter: 3.4 cm; RGB:-1, 0.2,-1, with dominant wavelength at 530 nm) (Fig. 2b). Stimulus intensity was measured at the level of the bee eye, inside the arena. It was the same as in the mini maze experiments, i.e.  $14000 \mu\text{W}/\text{cm}^2$  for the blue square and  $1800 \mu\text{W}/\text{cm}^2$  for the green disc. These values were chosen to suppress a homogeneous original attraction towards the green light. This was possible in all experimental groups, but it induced in 3 out of 8 cases a higher proportion of bees preferring the blue square to the green disc (*paired group* in Fig. 4 and *distilled-water* and *quinine group* in Fig. 7). In the other 5 cases (*freely-walking bees* in Fig. 3, *CS* and *US groups* in Fig. 4, and *distilled-water* and *dry-toothpick groups* in Fig. 7) the proportion of bees preferring the blue square and the green disc did not differ. Preliminary experiments showed that higher-intensity values for the green disc reestablished a homogeneous preference for this stimulus, and were, therefore, discarded.

Stimuli were equal in their total area ( $9 \text{ cm}^2$ ) and subtended a similar visual angle to the bee eyes (square, from edge to edge:  $24.2^\circ$ ; disc:  $27.3^\circ$ ). As in the mini maze experiment, this angular range ensures that bees engaged their chromatic vision to achieve the visual discrimination<sup>50,51</sup>. Both during the training and during the pre- and the post-tests, the visual stimuli were presented during 30 s.

**Conditioning and testing at the treadmill.** Experiments (Fig. 2b) started with a ‘pre-test’ in which the stimuli to be discriminated were presented simultaneously during 30 seconds (at  $50^\circ$  on each side of the bee’s body axis) to check for spontaneous preferences. The stimulus position (left or right of the bees’ body axis) was randomized between bees. After this pre-test, the bees were offered one minute without stimulation (black screen). Thereafter, the conditioning protocol started.

Bees were trained to discriminate the two visual stimuli based on their different reinforcement outcomes (differential conditioning). They experienced a succession of 12 trials in which they were presented either with one stimulus (CS+) paired with 1 M sucrose solution or with the other stimulus (CS-) paired with 60 mM quinine solution. In each trial, the CS was displayed

during 30 s either at  $-50^\circ$  or at  $+50^\circ$  from the bee's body axis. The stimulus side was pseudorandomized from trial to trial and the side sequence was the same from bee to bee (L, L, R, R, L, L, L, R, R, L, R, R). The US (sucrose or quinine) was provided during the last 5 s of CS presentation by means of a wooden toothpick that contacted the antenna on the side of the visual stimulus. The US was then delivered to the proboscis when it was extended. If no extension occurred upon delivery of quinine (or other negative reinforcement, see below), the solution was nevertheless approached to the proboscis in the first two trials; afterwards, negative reinforcements remained at the level of the antenna to avoid excessive disturbance. Delivery of reinforcements via a toothpick could provide uncontrolled visual cues. However, this delivery procedure was used for both appetitive and aversive reinforcements and did not offer discriminative cues. Moreover, acquisition data reported correspond to conditioned responses to the visual stimuli occurring *before* reinforcement delivery, i.e. before toothpick use. In addition, during the tests, no toothpick was presented. These facts exclude the potential confounding factor of toothpick movement in the performances reported.

The bees experienced 6 CS+ and 6 CS- trials in a pseudorandom sequence (CS+, CS-, CS+, CS-, CS-, CS+, CS-, CS-, CS+, CS+, CS-, CS+), which was the same from bee to bee. Trials were separated by an intertrial interval of one minute. Such a short interval was chosen to diminish the impact of a possible decrease in the bee's motivation to walk. If a bee did not respond with a proboscis extension to any sucrose-rewarding trial, or if it did not walk during the experiment, it was discarded from the analyses (<10 % in each group).

One minute after the end of the last conditioning trial, bees were subjected to a 'post-test' in which they saw simultaneously the CS+ and the CS- during 30 seconds in the absence of reinforcement. Performance in the post-test revealed if bees learned the discrimination or not, as it could be compared with performance in the pre-test before conditioning.

**Experiment 2: visual learning at the treadmill.** Bees were randomly allocated to three different groups (Fig. 2c): 1) the *paired group* experienced a differential conditioning protocol, which followed the steps described above; two subgroups were trained in order to balance reinforcement experience with the green disc and the blue square (i.e., one subgroup experienced the green disc rewarded and the blue square punished, while the other subgroup experienced the reversed contingency); 2) the *CS group* experienced only visual stimulation matching that of the



*paired group*, i.e. 12 CS presentations (6 green discs and 6 blue squares) but without any US; 3) the *US group* experienced a sequence of 12 US presentations (6 sucrose and 6 quinine presentations) matching that of the *paired group*, i.e. a given US was delivered on the right or left antenna, corresponding to a theoretical CS stimulation on the right or on the left of the screen, respectively.

**Experiment 3: the effect of negative US on visual learning at the treadmill.** Bees were randomly allocated to four groups that experienced the conditioning schedule of the *paired group* of Experiment 1. All groups received 1 M sucrose as positive US but differed in the nature of the negative US associated with the CS-, which could be a solution of 60 mM quinine, 3 M NaCl, distilled water or contact with a dry toothpick. The *quinine group* therefore reproduces the treatment experienced by the *paired group* of Experiment 1. All groups were balanced in terms of the association stimulus (green disc, blue square) - reinforcement (sucrose, negative reinforcement).

**Data analysis and statistics.** During the tests and conditioning trials, the response of the bee was categorized as ‘choice of the right side’, ‘choice of the left side’ or ‘absence of choice’, depending on the value of the cumulative heading (see above). In the *paired groups*, the bee’s choice was then recorded as correct (CS+ side), incorrect (CS- side) or as a non-choice. In the *CS group*, it was recorded as CS1 choice, CS2 choice or non-choice. In the *US group*, it was recorded as ‘theoretical CS+’ choice, ‘theoretical CS-’ choice or non-choice. Within each category, individual data were converted into a binomial format (0 or 1 if the bee’s choice belonged to this category or not, respectively) to calculate the proportion of individuals which chose the CS+, the CS-, or which didn’t make a choice. Data were bootstrapped (Decrouez and Hall, 2014) to plot these proportions  $\pm$  their corresponding 95% confidence interval. Analyses were also performed on the cumulative heading itself, quantified during the 30 s of stimulus presentation. In this case, data did not follow a normal distribution. They are thus presented as medians with corresponding quartiles.

For the pre-test and the post-test, the proportions of individuals choosing first the CS+, the CS- or not making a choice during the 30 s of stimulus presentation were computed. This duration was chosen after determining that shorter durations did not induce significant differences between these categories. Indeed, although some bees chose stimuli during the first seconds of stimulus

presentations, their number was not sufficient to generate significant differences. This is well illustrated by a reanalysis of the *quinine* and the *NaCl* groups of Fig. 7 using only the first 10 s of stimulus presentation (Supplementary Fig. S7). These two groups are interesting as the former exhibited a learning-induced increase in the proportion of CS+ choosers with respect to CS- choosers (Fig. S7b), while the latter did not show differences in the proportions of CS+ choosers, CS- choosers and non-choosers after conditioning (Fig. S7h). After reducing the period of analysis to the first 10 s, significance was lost in the *quinine* group (Fig. S7d) while the results of the *NaCl* group remained non-significant (Fig. S7j). Restricting the analysis to the subsequent period of 10 – 30 s did not change these results. The *quinine* group did not reach the clear significant difference visible when considering the entire 30 s period (Fig. S7f), and the *NaCl* group maintained its non-significant distribution (Fig. S7l). These results illustrate why the 30 s period was appropriate for computing preferences based on a cumulative proportion of bees choosing either stimulus, or not making a choice.

Test proportions were compared within groups by means of a generalized linear mixed model (GLMM) for binomial family in which the individual identity (Bee) was considered as a random factor (individual effect). The Tukey method was used for multiple comparisons;  $z$  values with corresponding degrees of freedom are reported throughout for this kind of analysis. Comparisons between groups were done using a GLMM for binomial family with Group, Choice and interaction Group x Choice as main effects;  $\chi^2$  values with corresponding degrees of freedom are reported throughout for these analyses. The cumulative heading was compared with a theoretical orientation of  $0^\circ$  (no choice) by means of a one-sample Mann Whitney test. Furthermore, the within-group variation in cumulative heading between the pre-test and the post-test was analyzed by means of a Wilcoxon U rank test. Cumulative-heading performances were compared between groups using a Kruskal-Wallis test.

For the acquisition, the proportion of bees which chose the CS+ or the CS- was analyzed by means of a generalized linear mixed model (GLMM) for binomial family in which Trial was considered as a continuous factor (Trial effect) and the individual identity (Bee) as a random factor (individual effect). For within-group analyses,  $\chi^2$  values are reported for CS effect, Trial effect and CS x Trial effect. Multiple comparisons were performed using Tukey's method ( $z$  values reported).

All statistical analyses were done with R 3.2.3 (R Core Team 2016). Packages lme4 and lsmeans were used for GLMMs and Tukey's method for multiple comparisons, respectively.

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## **Author Contributions**

A.B., P.S., A.A.-W. and M.G. designed the experiments. C.L. and J.C.S. provided facilities for pilot experiments. The experiments were conducted by A.B. and C.P. A.B. analyzed the results and prepared figures and tables. A.B., A.A.-W., L.C., C.L., J.C.S. and M.G. wrote the manuscript. All authors reviewed and approved the final manuscript.

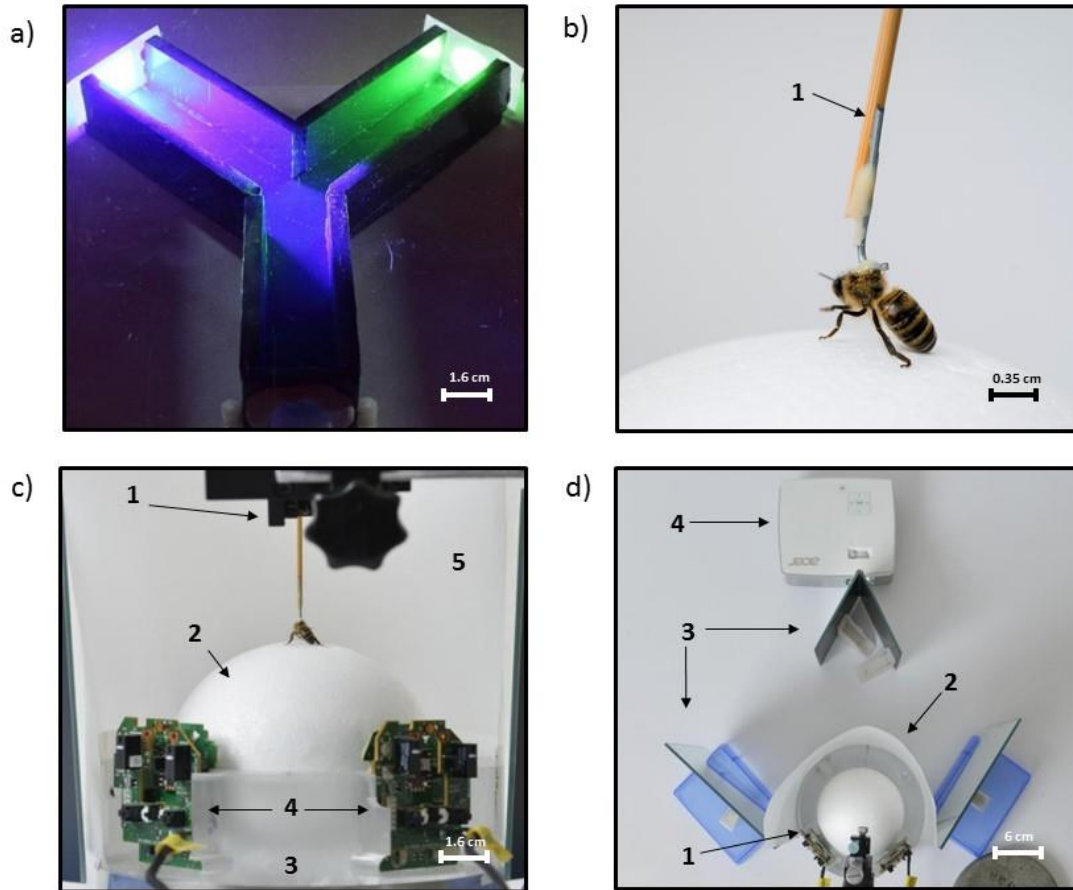
## **Additional Information**

**Supplementary information:** Four supplementary figures accompany this paper.

**Competing Interests:** The authors declare no competing financial interests.

## Figures and figure captions

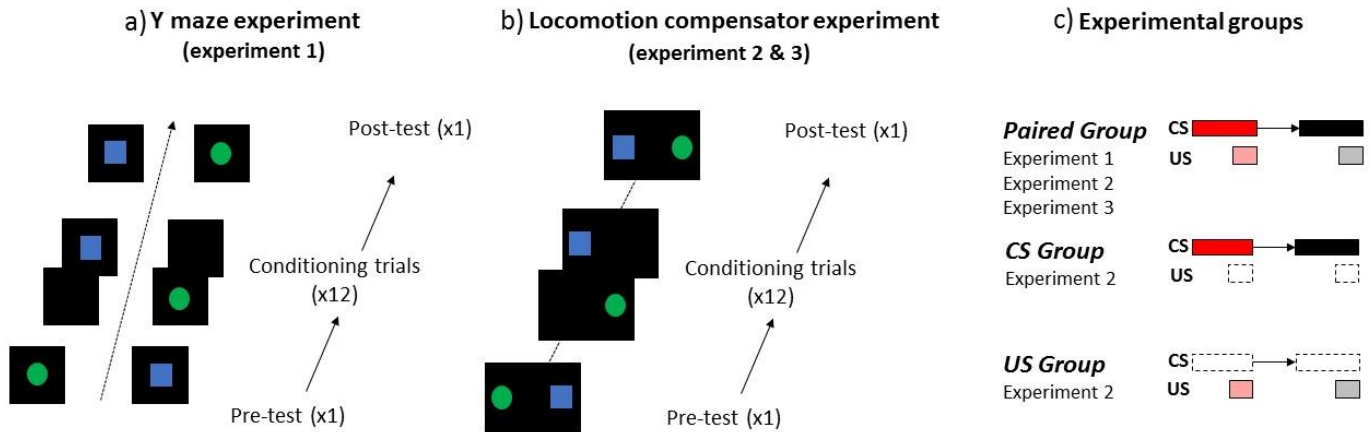
### Figure 1



**Figure 1. Experimental setups: the mini Y-maze (a) and the spherical treadmill (b-d).** (a) Top view of the mini Y-maze. Arms were 5 cm in length and 3x3 cm in section. Bees walked inside the maze to collect sucrose solution. Stimuli were projected by the video projector onto paper screens placed at the ends of the maze arms. (b) A bee tethered by the thorax by means of a vertical attachment (1) made of a wooden toothpick and an L-shaped metal piece glued to the thorax. The toothpick was held by a micromanipulator, which allowed adjusting the position of the bee on the treadmill (© Cyril Frésillon/CNRS). (c) The bee held by the micromanipulator (1) walked stationary on the treadmill. The setup was composed of a polystyrene ball (2) floating on a constant airflow (3). Two optic-mouse sensors (4) were placed on the ball support, at 90° of each other to record the ball movements. The setup translates the movements of the walking bee into rotations of the ball (© Cyril Frésillon/CNRS). (d) Top view of the treadmill (1) placed behind a semi

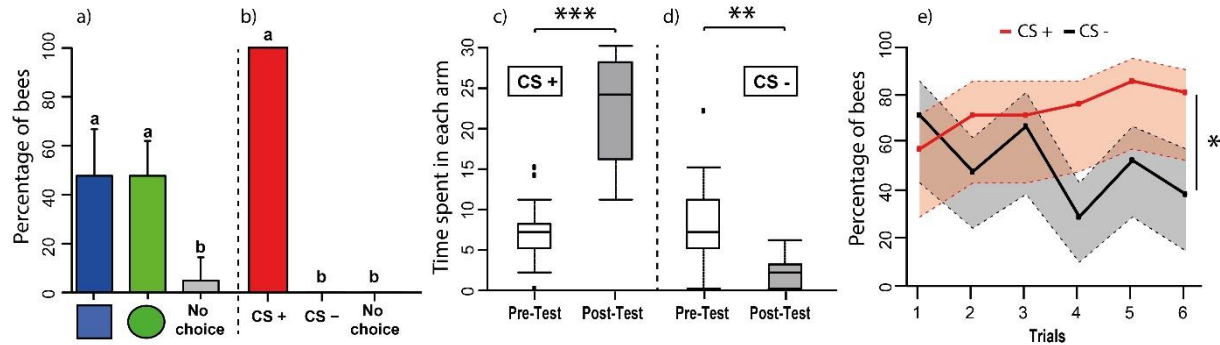
cylindrical paper screen (2) onto which visual stimuli were projected. A set of mirrors (3) was placed between the video projector (4) and the screen to allow projecting the stimuli on the lateral parts of the semicircular screen without deforming their shapes (© Cyril Frésillon/CNRS).

Figure 2



**Figure 2. Training, testing and experimental groups at the miniature Y-maze and at the spherical treadmill.** (a) Experimental sequence at the Y-maze (Experiment 1). Experiments started with a pre-test in which both stimuli were shown in the absence of reinforcement to check for spontaneous preferences. The pre-test was followed by 12 conditioning trials in which a single reinforced stimulus was shown in one arm of the maze. A black screen was visible in the alternative arm of the maze. After conditioning, a post-test in which both stimuli were shown simultaneously in the absence of reinforcement allowed to determine whether bees learned the visual discrimination. (b) Experimental sequence at the treadmill (Experiments 2 and 3). Experiments started with a pre-test in which both stimuli were shown simultaneously in the absence of reinforcement to check for spontaneous preferences. The pre-test was followed by 12 conditioning trials in which a single reinforced stimulus was shown at a time. After conditioning, a post-test in which both stimuli were shown simultaneously in the absence of reinforcement allowed to determine whether bees learned the visual discrimination. (c) Experimental groups. In Experiments 1, 2 and 3, the performance of a *paired group* was studied. Bees in this group experienced one visual stimulus (CS+, red rectangle) paired with sucrose solution (S, pink square) and another visual stimulus (CS-, black rectangle) paired with quinine solution (Q, grey square). In Experiment 2, the performances of a *CS group* and of a *US group* were also studied. Bees in the *CS group* experienced only visual stimulation (CS1, CS2) matching that of the *paired group* but without any

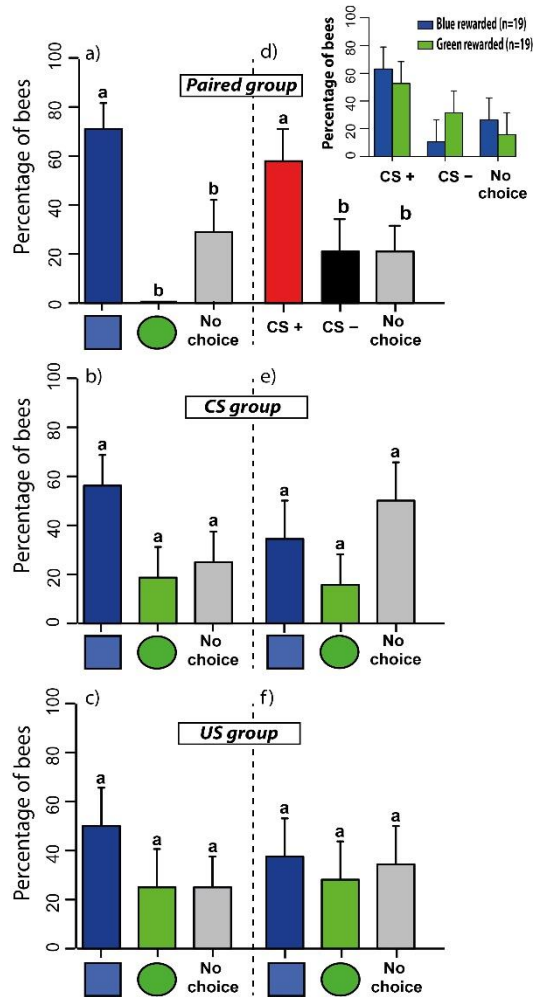
reinforcement. Bees in the *US group* experienced a sequence of 12 reinforcements (6 S, sucrose, and 6 Q, quinine) matching that of the *paired group*.

**Figure 3**

**Figure 3. Visual learning of freely moving bees at the miniature maze [Experiment 1]. Left panels:** Choice performance (percentage of bees choosing a given stimulus or not making a choice + 95% confidence interval, based on the first choice made by the bees upon maze entrance) during the pre-test (a) and the post-test (b) at the mini Y-maze (n=21). (a) Bars show the percentage of bees choosing spontaneously the blue square (blue bar), the green disc (green bar) or not choosing any stimulus (grey bar) during the pre-test. (b) Bars show the percentage of bees choosing the CS+ (red bar), the CS- (black bar) or not choosing (grey bar) after conditioning. Perfect learning was attained after conditioning as all bees chose the CS+ in the post-test. Different lower-case letters above bars indicate significant differences within each panel ( $p < 0.05$ ). **Middle panels:** Time spent (median, quartiles and outliers) in each arm of the maze in the pre-test (white boxplot) and in the post-test (grey boxplot) during the 30 s of stimulus presentation. (c) Time spent in the CS+ arm. (d) Time spent in the CS- arm. Following conditioning, bees increased the time spent in the CS+ arm and concomitantly decreased the time spent in the CS- arm. \*\*  $p < 0.001$ \*\*\*;  $p < 0.0001$ . **Right Panel (e):** Acquisition performance (percentage of learners choosing the CS+, red curve, and the CS-, black curve; first choices) during the 12 conditioning trials (6 for each CS alternative) in the mini Y-maze. Bees (n=21) decreased CS- responses while keeping CS+ responses high. Discrimination was significant. The 95% confidence interval is shown for each curve (dashed lines; in pink for the CS+ curve, and in grey for the CS- curve). \*  $p < 0.05$



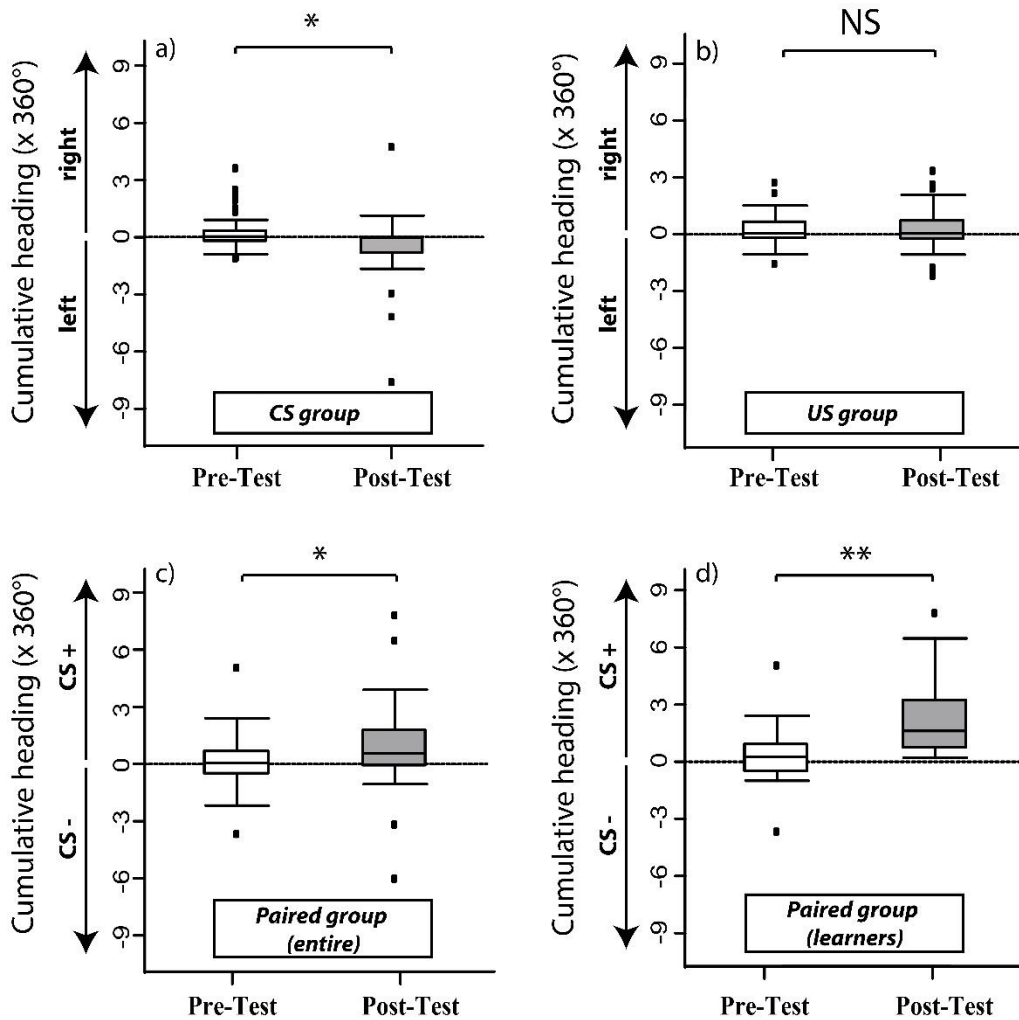
Figure 4



**Figure 4. Spontaneous preferences (pre-test) and learning-induced preferences (post-test) at the spherical treadmill [Experiment 2].** Choice performance (percentage of bees choosing a given stimulus or not making a choice + 95% confidence interval) during the pre-test (left panels) and the post-test (right panels). Upper graph: *paired group* (n=38); middle graph: *CS group* (n=32); lower graph: *US group* (n=32). Bars in (a), (b) and (c) show the percentage of bees choosing spontaneously the blue square (blue bar), the green disc (green bar) or not choosing (grey bar) in the pre-test. The *paired group* showed a spontaneous preference for the blue square. Bars in (d) show the percentage of bees choosing the CS+ (red bar), the CS- (black bar) or not choosing (grey bar) after conditioning (*paired group*). Bars in (e) and (f) show the percentage of bees choosing the blue square, the green disc or not choosing in the *CS group* and the *US group*, respectively (during training, there were no reinforcements in the *CS group* and no visual stimuli in the *US group*). Only the *paired group* (d) exhibited a significant variation in the percentage of bees choosing between

stimulus alternatives. In this case, the percentage of bees choosing the CS+ was significantly higher. The inset in **(d)** shows that this higher proportion was independent of the nature of the stimulus chosen as CS+ (blue square,  $n = 19$ , or green disc,  $n = 19$ ). Different lower-case letters above bars indicate significant differences ( $p < 0.05$ ).

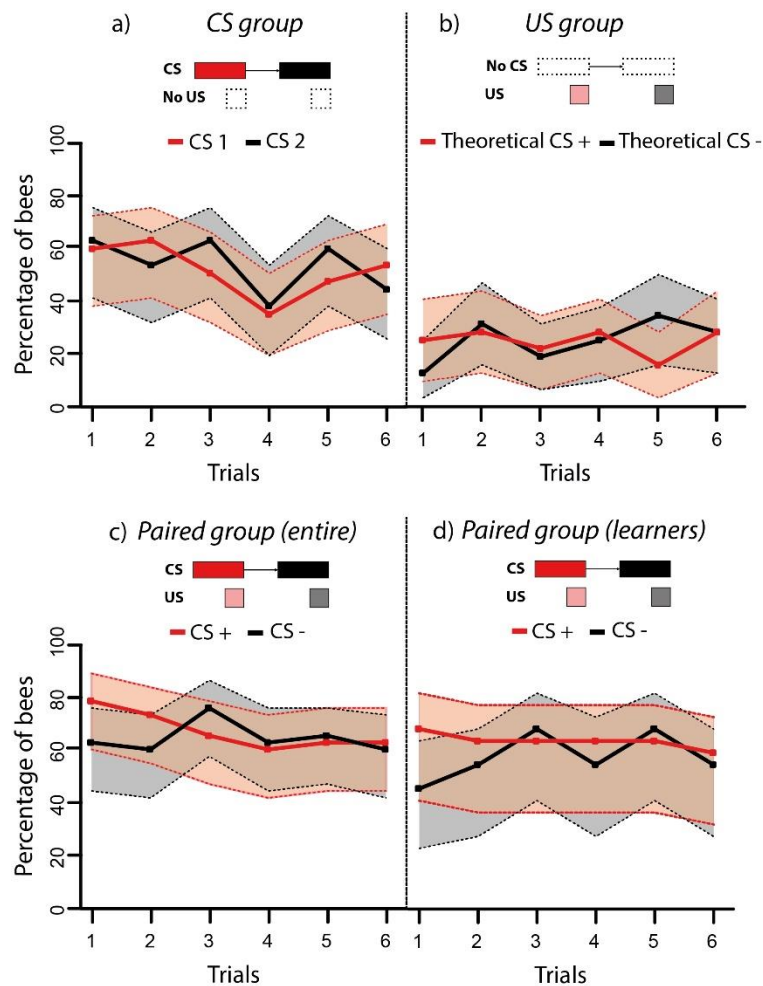
Figure 5



**Figure 5. Cumulative-heading performance (in degrees; median, quartiles and outliers) during the pre-test (white boxplots, left) and the post-test (grey boxplots, right) at the spherical treadmill [Experiment 2].** Values represented correspond to multiples of a 360° rotation. **(a)** *CS group* (n=32); **(b)** *US group* (n=32); **(c)** *entire paired group* (n=38); **(d)** *Learners of the paired group* (n=22). In **(a)** and **(b)**, as there was neither a CS+ nor a CS-, the convention is that the more positive was the cumulative heading, the more bees went to the right. In **(c)** and **(d)** the convention is that the more positive was this heading, the more bees chose the CS+. No variation in heading between pre-test and post-test was found in the *US group*; in the *CS group*, a

tendency to turn to the left was visible after training. The paired group (entire and learners) oriented more towards the CS+ following conditioning. \*  $p < 0.05$ ; \*\*  $p < 0.001$ ; NS: non-significant.

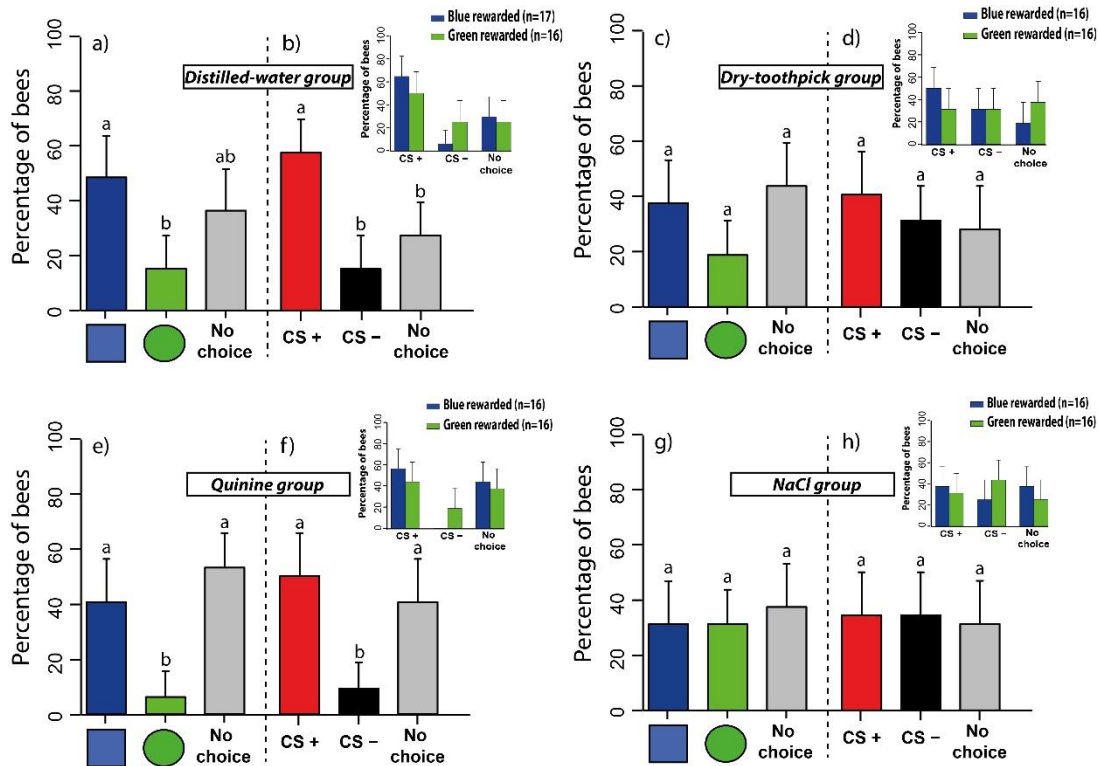
Figure 6



**Figure 6. Acquisition performance (percentage of bees choosing the stimulus alternatives offered) during the 12 conditioning trials (6 for each alternative) at the spherical treadmill [Experiment 2]. (a) CS group (n=32);** this group received only visual stimulation without reinforcement (see inset) so that performance is represented in terms of bees choosing the CS1 (red curve) and the CS2 (black curve), which were presented in a succession matching that of the *paired group*. **(b) US group (n=32);** this group did not receive visual stimulations (see inset) so that it is not possible to represent performance in terms of a CS response. The graph shows, therefore, the responses occurring in the interval of time prior to US delivery (red curve: responses to a ‘theoretical CS+’, prior to sucrose delivery; black curve: responses to a ‘theoretical CS-’, prior to quinine delivery). Reinforcement succession was matched to that of the *paired group*. **(c) Entire paired group (n=38) and (d) Learners of the paired group (n=22);** red curve: choice of the CS+ (paired with sucrose solution); black curve: choice of the CS- (paired with quinine solution). For

all four groups, the 95% confidence interval is shown (dashed lines; in pink for the CS+, CS1 or theoretical CS+ curve, and in grey for the CS-, CS2 or theoretical CS- curve). No group showed significant discrimination between alternative stimuli.

Figure 7



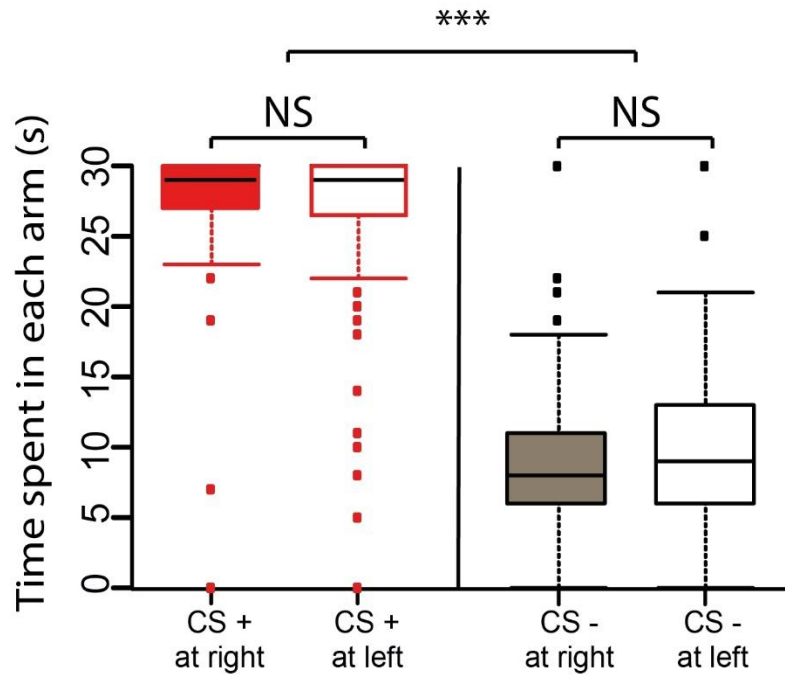
**Figure 7. Spontaneous preferences and learning-induced preferences at the spherical treadmill for groups, which got different negative unconditioned stimuli (US) on the CS- [Experiment 3].** The graph shows the choice performance (percentage of bees choosing a given stimulus or not making a choice + 95% confidence interval) during the pre-test (left panel in each graph) and the post-test (right panel in each graph). **(a, b)** *distilled-water group* (n=33); **(c, d)** *dry-toothpick group* (n=32); **(e, f)** *quinine group* (n=32); **(g, h)** *NaCl group* (n=32). Bars in **(a, c, e, g)** show the percentage of bees choosing spontaneously the blue square (blue bar), the green disc (green bar) or not choosing (grey bar) in the pre-test. The *quinine group* and the *distilled-water group* had a higher proportion of bees preferring the blue square compared to bees preferring the green disc. Bars in **(b, d, f, g)** show the percentage of bees choosing the CS+ (red bar), the CS- (black bar) or not choosing (grey bar) after conditioning. In both the *quinine group* and the *distilled-water group* a higher proportion of bees preferred the CS+ compared to bees preferring the CS-. The insets in **(b)** and **(f)** show that this higher proportion was independent of the nature of the

stimulus chosen as CS+ (blue square or green disc). Different lower-case letters above bars indicate significant differences ( $p < 0.05$ ).



## Supplementary information

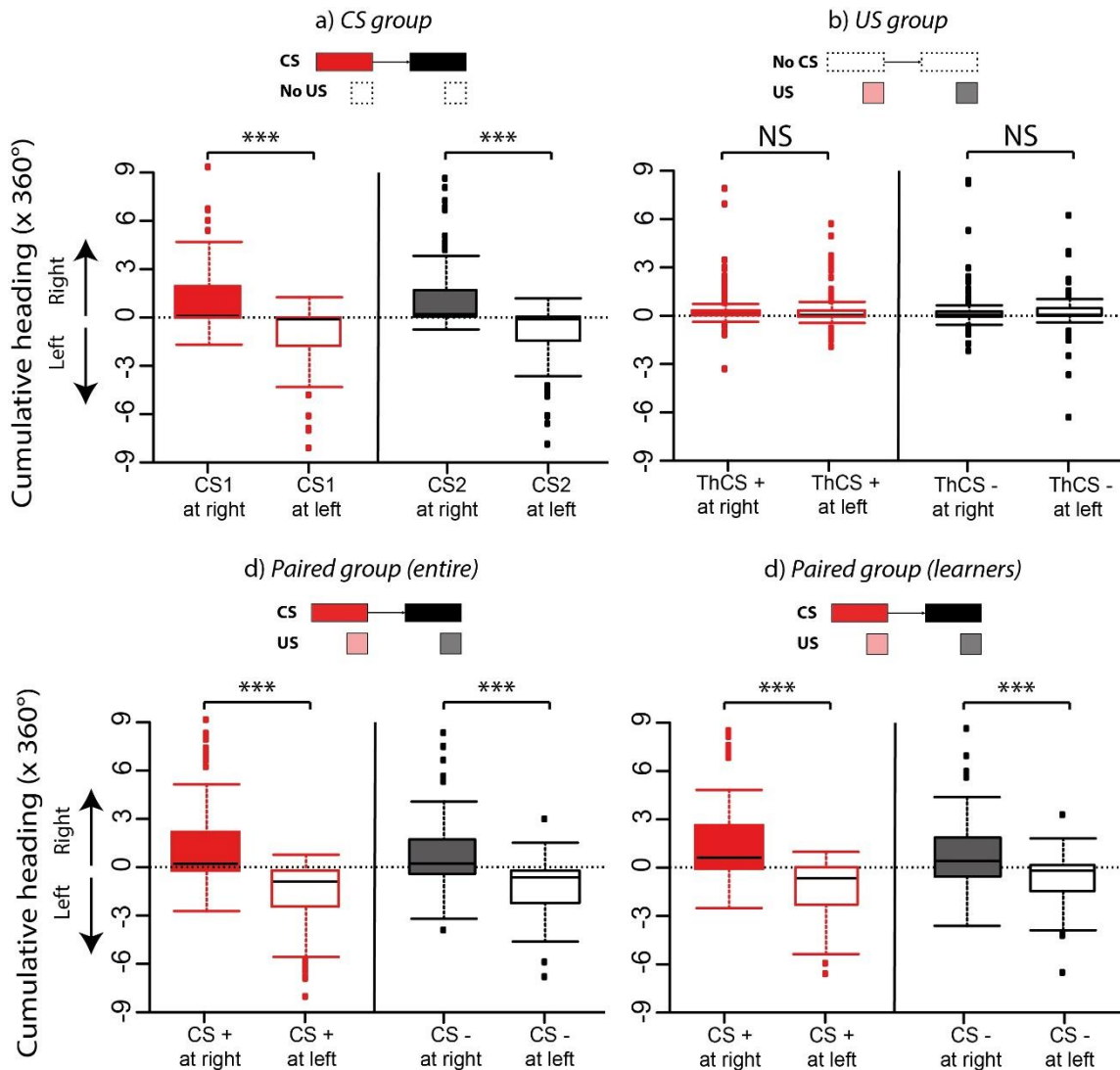
### Figure S1



**Figure S1. Experiment 1 (miniature maze).** Time spent in each arm of the miniature maze (in seconds; median, quartiles and extreme values) during the 30 s of CS+ or CS- presentation in the 12 conditioning trials (6 for each alternative). For each stimulus type, there were three presentations on the right and three presentations on the left side of the screen. The graphs show the response of the bees ( $n = 21$ ) to each stimulus alternative when it was on the right (pooled data of the 3 presentations) vs. when it was on the left (pooled data of the 3 presentations). Red boxplot, full: CS+ at right; red boxplot, empty: CS+ at left. Dark-grey boxplot, full: CS- at right; dark-grey boxplot, empty: CS- at left. In all four cases, the time spent in each arm was significantly different from zero (one-sample Mann-Whitney test; CS+<sub>right</sub>:  $U = 1891$ ,  $p < 0.0001$ ; CS+<sub>left</sub>:  $U = 1891$ ,  $p < 0.001$ ; CS-<sub>right</sub>:  $U = 1830$ ,  $p < 0.0001$ ; CS-<sub>left</sub>:  $U = 1891$ ,  $p < 0.0001$ ). Bees spent the same time in the left and right arms when they saw the CS+ (Wilcoxon rank test;  $U = 392$ ,  $p = 0.11$ ) and the CS- ( $U = 681$ ,  $p = 0.11$ ). Nevertheless, this time was significantly longer in the case of the CS+ (Wilcoxon rank test;  $U = 148$ ,  $p < 0.0001$ ). Indeed, bees spent most of the CS+ stimulation time (30

s) in the CS+ arm and did practically not visit the empty arm. On the contrary, they spent only between 5 and 10 s in the CS- arm during the CS- stimulation.

Figure S2

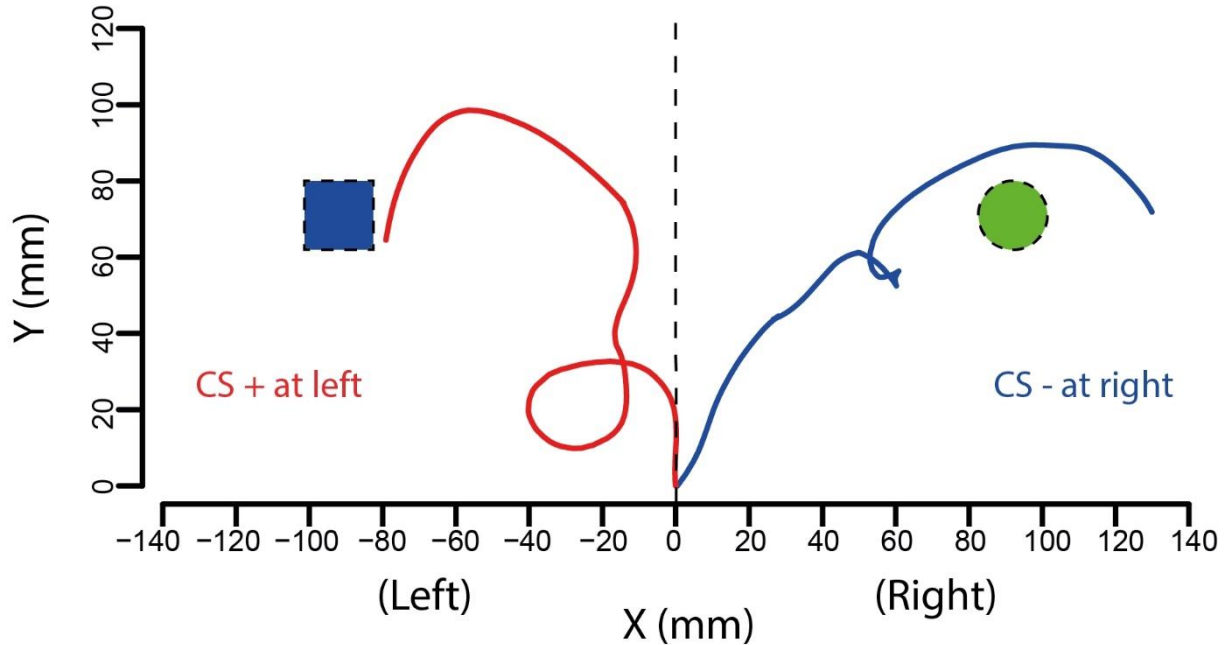


**Figure S2. Experiment 2 (spherical treadmill).** Cumulative heading (in degrees, median, quartiles and extreme values) for the two stimuli offered to the bees during the 12 conditioning trials (6 for each alternative). The values reported correspond to multiples of a 360° rotation. They were quantified during the 30 s of stimulus presentation. For each stimulus type (e.g. CS+ and CS- in the *paired group*), there were 3 presentations on the right and three presentations on the left side of the screen. The graphs show the response of the bees to each stimulus alternative when it was on the right (pooled data of the 3 presentations; median and quartiles) vs. when it was on the left (pooled data of the 3 presentations; median and quartiles). **(a) CS group** (n = 32). For these bees, there were only visual stimulations (CS1, CS2) without US. Red boxplot, full: CS1 at right; red

boxplot, empty: CS1 at left. Dark-grey boxplot, full: CS2 at right; dark-grey boxplot, empty: CS2 at left. Bees of the *CS group* always oriented towards the stimulus that was visible on the screen, be it CS1 or CS2. In all cases, the cumulative heading was significantly different from a theoretical orientation of  $0^\circ$  (one-sample Mann-Whitney test; CS1<sub>right</sub>:  $U = 3834$ ,  $p < 0.0001$ ; CS1<sub>left</sub>:  $U = 691$ ,  $p < 0.001$ ; CS2<sub>right</sub>:  $U = 3883$ ,  $p < 0.0001$ ; CS2<sub>left</sub>:  $U = 809$ ,  $p < 0.001$ ). The change in heading depending on stimulus side was significant for the CS1 (Wilcoxon rank test;  $U = 4137$ ,  $p < 0.0001$ ) and for the CS2 ( $U = 4041$ ,  $p < 0.0001$ ). In other words, when bees saw a stimulus on the right side of the screen, they headed towards the right and when they saw it on the left side they headed towards the left. **(b)** *US group* ( $n = 32$ ). Bees in this group maintained a rather straightforward trajectory irrespectively of the US received just after performance recording (sucrose or quinine solution). The period before sucrose delivery is termed ‘Theoretical CS+’ (ThCS+) and that before quinine delivery is termed ‘Theoretical CS-’ (ThCS-). The cumulative heading was significantly different from a theoretical orientation of  $0^\circ$  only for the ThCS<sub>right</sub>, i.e. prior to sucrose delivery on the right antenna ( $U = 3017$ ,  $p = 0.01$ ) and for the ThCS<sub>left</sub>, i.e. prior to quinine delivery on the left antenna ( $U = 2954$ ,  $p = 0.023$ ). The other two situations, ThCS<sub>left</sub> and ThCS<sub>right</sub>, i.e. prior to sucrose delivery on the left antenna, and prior to quinine delivery on the right antenna, resulted in headings that did not differ from  $0^\circ$  (ThCS<sub>left</sub>:  $U = 2670$ ,  $p = 0.21$ ; ThCS<sub>right</sub>:  $U = 2586$ ,  $p = 0.35$ ). The cumulative heading did not differ between situations (ThCS<sub>right</sub> vs. ThCS<sub>left</sub>:  $U = 2748$ ,  $p = 0.12$ ; ThCS<sub>right</sub> vs. ThCS<sub>left</sub>:  $U = 2286$ ,  $p = 0.88$ ). **(c)** Entire *Paired group* ( $n = 38$ ). Red boxplot, full: CS+ at right; red boxplot, empty: CS+ at left. Dark-grey boxplot, full: CS- at right; dark-grey boxplot, empty: CS- at left. Bees of the paired group always oriented towards the stimulus that was visible on the screen, be it CS+ or CS-. Thus, in all four cases, the cumulative heading was significantly different from a theoretical orientation of  $0^\circ$  (one-sample Mann-Whitney test; CS<sub>right</sub>:  $U = 5367$ ,  $p < 0.0001$ ; CS<sub>left</sub>:  $U = 844$ ,  $p < 0.001$ ; CS<sub>right</sub>:  $U = 4743$ ,  $p < 0.0001$ ; CS<sub>left</sub>:  $U = 1138$ ,  $p < 0.0001$ ). Moreover, the change in heading depending on the side of presentation was significant both for the CS+ (Wilcoxon rank test;  $U = 5797$ ,  $p < 0.0001$ ) and for the CS- ( $U = 5425$ ,  $p < 0.0001$ ). In other words, when a stimulus was displayed on the right side of the screen, bees headed towards the right, and when it was shown on the left side, they headed towards the left. **(d)** Learners of the *paired group* ( $n = 22$ ). Red boxplot, full: CS+ at right; red boxplot, empty: CS+ at left. Dark-grey boxplot, full: CS- at right; dark-grey boxplot, empty: CS- at left. These bees always oriented towards the stimulus that was visible on the screen, be it CS+ or CS-. In all four cases, the

cumulative heading was significantly different from a theoretical orientation of  $0^\circ$  (one-sample Mann-Whitney test; CS<sub>+right</sub>:  $U = 1758$ ,  $p < 0.0001$ ; CS<sub>+left</sub>:  $U = 349$ ,  $p < 0.001$ ; CS<sub>-right</sub>:  $U = 1455$ ,  $p < 0.03$ ; CS<sub>-left</sub>:  $U = 523$ ,  $p < 0.001$ ). The change in heading depending on the side of presentation was significant both for the CS+ (Wilcoxon rank test;  $U = 1878$ ,  $p < 0.0001$ ) and for the CS- ( $U = 1707$ ,  $p < 0.0001$ ). Thus, learners behaved like the entire *paired group*: they simply headed towards the visible stimulus be it on the right or on the left. \*\*\*  $p < 0.0001$ ; NS: non-significant

Figure S3



**Figure S3. Experiment 2 (spherical treadmill).** Example of two trajectories of a bee in the *paired* group, which correspond to two conditioning trials, one in which the CS+ was shown at the left (red trajectory), and another in which the CS- was shown at the right (blue trajectory). The dashed square and disc on the left and right, respectively, indicate the fictive position of the stimuli (one shown at a time during training). In both cases, the bee headed towards the visual stimulus presented, irrespectively of the US associated with the visual stimulus, thus showing either a phototactic effect or an object-fixation effect. Trajectories were calculated following Seelig et al.<sup>1</sup>.

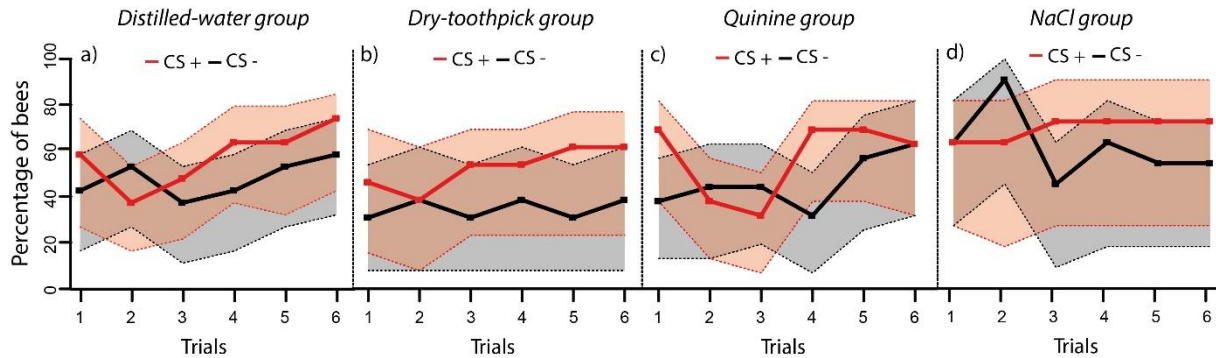
For the walking displacements on the X-axis ( $dX$ ; right – left), we used the following equation:

$$dX = (Y1 + Y2) * \cos(45^\circ) * \sin(\text{heading}) - (Y1 - Y2) * \sin(45^\circ) * \cos(\text{heading})$$

For the displacements on the Y-axis ( $dY$ ; forward), we used the following equation:

$$dY = -(Y1 + Y2) * \cos(45^\circ) * \cos(\text{heading}) - (Y1 - Y2) * \sin(45^\circ) * \sin(\text{heading})$$

with  $Y1$  and  $Y2$  being the vertical movements detected by the sensors 1 and 2, respectively. See the main text to see how the bee's heading was calculated.

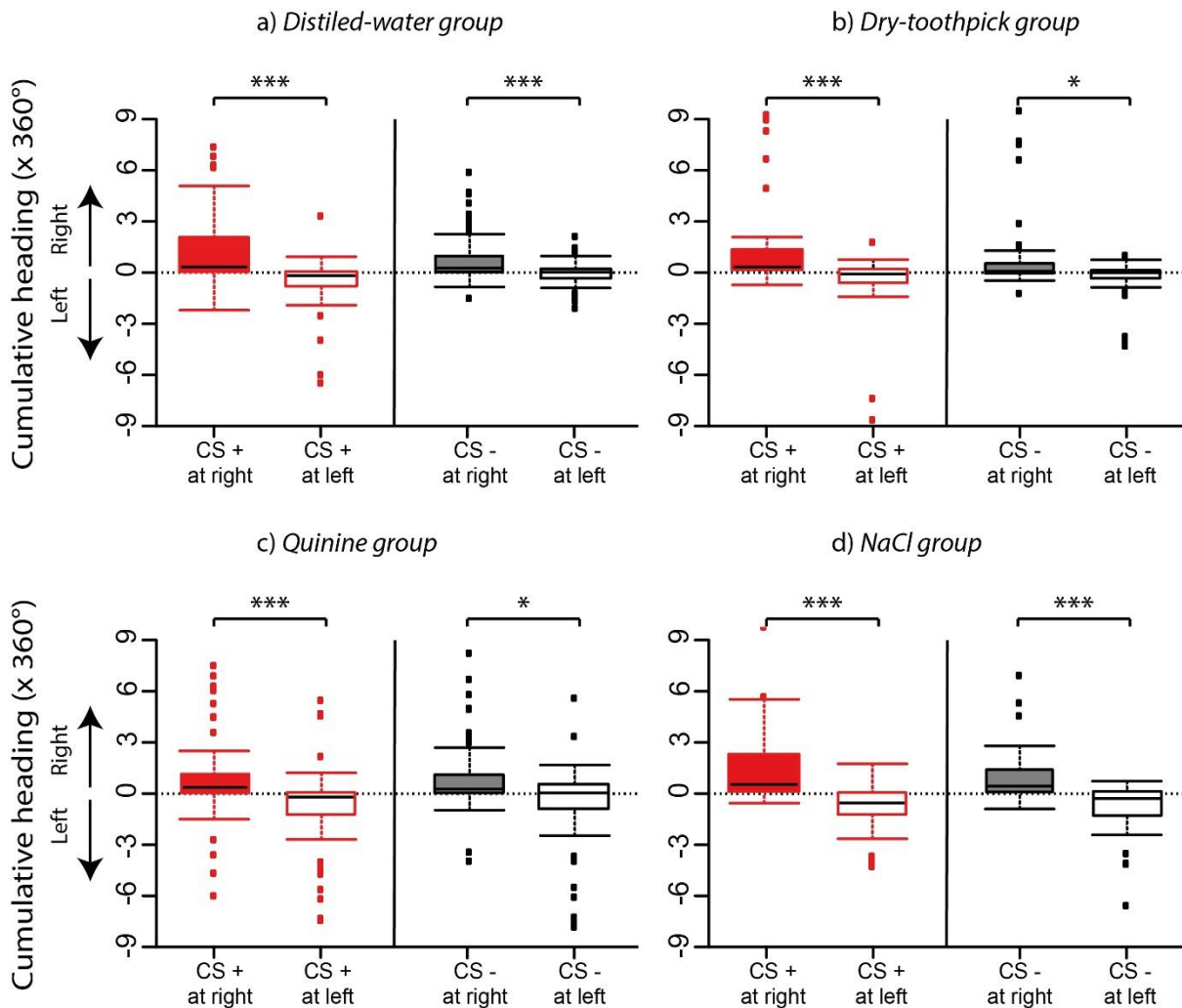
**Figure S4**

**Figure S4. Experiment 3 (spherical treadmill).** Acquisition performance (percentage of learners choosing the CS+, red curve, and the CS-, black curve) during the 12 conditioning trials (6 for each CS alternative). **(a)** Learners of the *distilled-water group* (n=19). **(b)** Learners of the *dry-toothpick group* (n=13). **(c)** Learners of the *quinine group* (n=16). **(d)** Learners of the *NaCl group* (n=11). In all four groups, the 95% confidence interval is shown (dashed lines; in pink for the CS+ curve and in grey for the CS- curve). Learners in all groups exhibited a strong tendency to choose the single visual stimulus presented, be it CS+ or CS-, from the very first trial (Figs. 8 a, b, c, d) and kept doing so during the 12 trials. Consequently, the bees' performance did not show any variation along trials (trial effect; *distilled water group*:  $\eta^2 = 3.57$ , df: 5,  $p = 0.61$ ; *dry-toothpick group*:  $\eta^2 = 5.75$ , df: 5,  $p = 0.33$ ; *quinine group*:  $\eta^2 = 7.40$ , df: 5,  $p = 0.19$ ; *NaCl group*:  $\eta^2 = 3.88$ , df: 5,  $p = 0.57$ ). For the same reason, the learning curves did not show any significant evidence of CS discrimination (CS effect; *distilled water group*:  $\eta^2 = 0.09$ , df: 1,  $p = 0.77$ ; *dry-toothpick group*:  $\eta^2 = 0.36$ , df: 1,  $p = 0.55$ ; *quinine group*:  $\eta^2 = 0.81$ , df: 1,  $p = 0.37$ ; *NaCl group*:  $\eta^2 = 0.38$ , df: 1,  $p = 0.54$ ). In all four groups, the interaction CS x trial was also not significant (*distilled water group*:  $\eta^2 = 4.21$ , df: 5,  $p = 0.52$ ; *dry-toothpick group*:  $\eta^2 = 4.40$ , df: 5,  $p = 0.49$ ; *quinine group*:  $\eta^2 = 4.75$ , df: 5,  $p = 0.45$ ; *NaCl group*:  $\eta^2 = 4.43$ , df: 5,  $p = 0.49$ ). This indicates that in all groups there was an apparent absence of learning and that the responses to both CS types followed the same dynamics. A global analysis including all four groups confirmed that performance was homogeneous irrespective of the US associated with the CS- (group effect; df: 3,  $\eta^2 = 2.65$ ,  $p = 0.44$ ; group x trial x CS effect;

df: 35,  $t^2 = 24.7$ ,  $p = 0.91$ ). These results can be explained based on positive phototaxis or on an object-fixation response, which drove the bees towards the single visual stimulus displayed on the screen during a training trial.



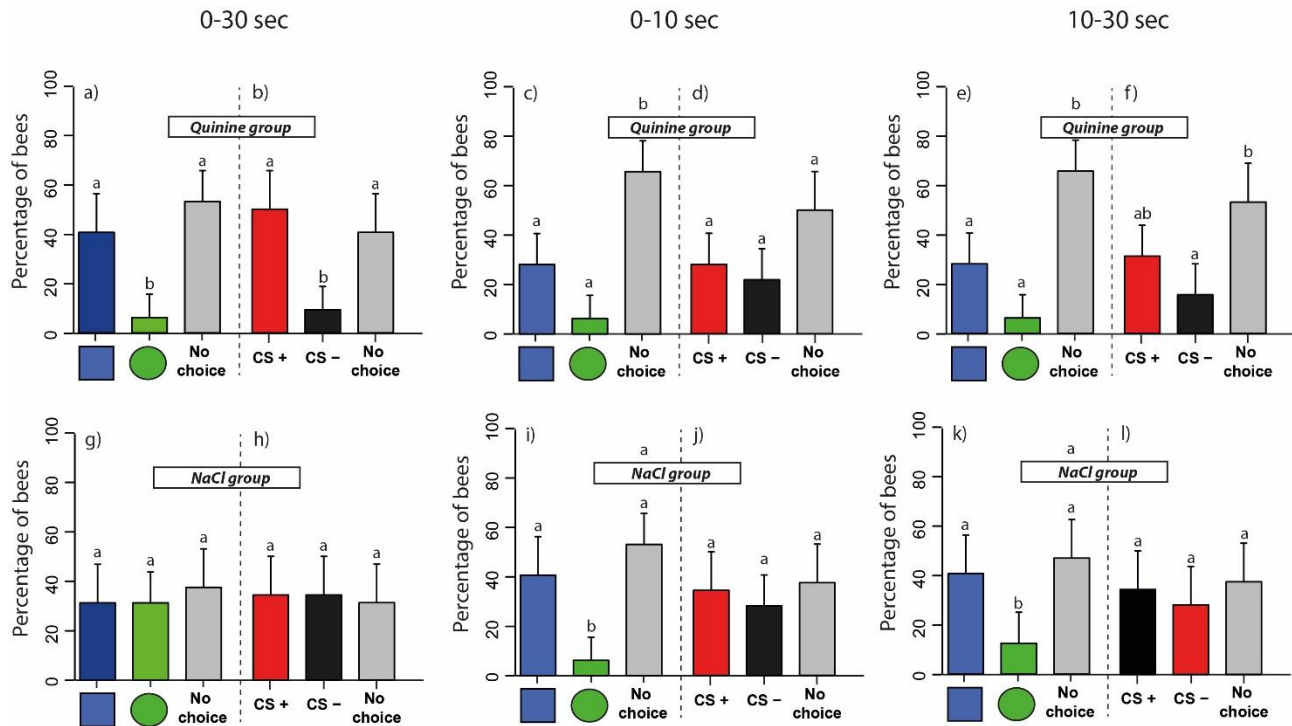
Figure S5



**Figure S5. Experiment 3 (spherical treadmill).** Cumulative heading (in degrees, median, quartiles and extreme values) for the CS+ and the CS- offered to the bees during the 12 conditioning trials (6 for each alternative). The values reported correspond to multiples of a 360° rotation. They were quantified during the 30 s of stimulus presentation. For each CS type, there were three presentations on the right and three presentations on the left side of the screen. The graphs show the response of learner bees to each CS when it was on the right (pooled data of the 3 presentations; median and quartiles) vs. when it was on the left (pooled data of the 3 presentations; median and quartiles). Red boxplot, full: CS+ at right; red boxplot, empty: CS+ at left. Dark-grey boxplot, full: CS- at right; dark-grey boxplot, empty: CS- at left. (a) Learners of the *distilled-water group* ( $n = 19$ ). Bees of this group headed towards the right when a visual target was shown on the right of the

screen and towards the left when it was shown on the left of the screen, irrespectively of the CS type. Thus, in three of four possible situations, the cumulative heading was significantly different from a theoretical orientation of  $0^\circ$  (one-sample Mann-Whitney test; CS<sub>+right</sub>: U = 1296, p < 0.0001; CS<sub>+left</sub>: U = 286, p < 0.0001; CS<sub>-right</sub>: U = 1287, p < 0.001; CS<sub>-left</sub>: U = 681, p = 0.25). The change in heading depending on stimulus side was significant for the CS<sub>+</sub> (Wilcoxon rank test; U = 1344, p < 0.0001) and for the CS<sub>-</sub> (U = 1320, p < 0.0001). **(b)** Learners of the *dry-toothpick group* (n=13). Bees of this group also headed towards the right when the CS<sub>+</sub> was shown on the right of the screen and towards the left when it was shown on the left of the screen. No significant heading was found for the CS<sub>-</sub> (CS<sub>+right</sub>: U = 645, p < 0.001; CS<sub>+left</sub>: U = 205, p < 0.01; CS<sub>-right</sub>: U = 507, p = 0.10; CS<sub>-left</sub>: U = 268, p = 0.09). The change in heading depending on stimulus side was significant for the CS<sub>+</sub> (U = 646, p < 0.0001) and for the CS<sub>-</sub> (U = 583, p < 0.01). **(c)** Learners of the *quinine group* (n = 16). Bees of this group exhibited a cumulative heading that was significantly different from a theoretical orientation of  $0^\circ$  in three of four possible situations (CS<sub>+right</sub>: U = 881, p < 0.001; CS<sub>+left</sub>: U = 264, p < 0.001; CS<sub>-right</sub>: U = 924, p < 0.001; CS<sub>-left</sub>: U = 506, p = 0.41). The change in heading depending on stimulus side was significant for the CS<sub>+</sub> (U = 993, p < 0.0001) and for the CS<sub>-</sub> (U = 857, p < 0.01). **(e)** Learners of the *NaCl group* (n=11). Bees headed toward the CS regardless of its position and its associated US (CS<sub>+right</sub>: U = 506, p < 0.0001; CS<sub>+left</sub>: U = 78, p < 0.0001; CS<sub>-right</sub>: U = 494, p < 0.0001; CS<sub>-left</sub>: U = 119, p < 0.01). The change in heading depending on stimulus side was significant for the CS<sub>+</sub> (U = 525, p < 0.0001) and for the CS<sub>-</sub> (U = 520, p < 0.0001). \*\*\* p < 0.0001; \* p < 0.01; NS: non-significant.

Figure S6



**Figure S6. Pre- and post-test performances under shorter visual-stimulus periods (0-10 s, middle panels, and 10-30 s, right panels). Left panels show performances computed using the entire stimulus period of 30 s.** Choice performance (percentage of bees choosing a given stimulus or not making a choice + 95% confidence interval) during the pre-test. **(a,b)** Pre- and post-tests performances of the *quinine* group computed over the entire 30 s of stimulus duration (as in Fig. 7, main text). **(g,h)** Same for the *NaCl* group (as in Fig. 7, main text). **(c,d)** Pre- and post-tests performances of the *quinine* group computed over the first 10 s of stimulus duration. Pre-test: blue square vs. green disc:  $z_{1161} = -2.13$ ,  $p = 0.08$ , blue square vs. no choice:  $z_{1161} = -2.93$ ,  $p = 0.009$ , green disc vs. no choice:  $z_{1161} = -4.09$ ,  $p = 0.0001$ . Post-test: CS+ vs. CS-:  $z_{1161} = -0.58$ ,  $p = 0.83$ , CS+ vs. no choice:  $z_{1161} = -1.77$ ,  $p = 0.18$ , CS- vs. no choice:  $z_{1161} = -2.29$ ,  $p = 0.06$ . The use of a shorter period for computing the bee choices did not result in significant differences between choice categories **(i,j)** Pre- and post-tests performances of the *NaCl* group computed over the first 10 s stimulus duration. Pre-test: blue square vs. green disc:  $z_{1161} = -2.86$ ,  $p = 0.01$ , blue square vs. no choice:  $z_{1161} = -0.99$ ,  $p = 0.58$ , green disc vs. no choice:  $z_{1161} = -3.49$ ,  $p = 0.001$ . Post-test: CS+ vs. CS-:  $z_{1161} = -0.53$ ,  $p = 0.85$ , CS+ vs. no choice:  $z_{1161} = -0.26$ ,  $p = 0.96$ , CS- vs. no choice:  $z_{1161} = -$

0.79,  $p = 0.70$ . As for the *quinine group*, the use of a shorter stimulus duration did not result in significant differences between choice categories in the post-test. **(e,f)** Pre- and post-tests performances of the *quinine group* computed over the subsequent 10 - 30 s stimulus duration. Pre-test: blue square vs. green disc:  $z_{1161} = -2.13$ ,  $p = 0.08$ , blue square vs. no choice:  $z_{1161} = -2.93$ ,  $p = 0.01$ , green disc vs. no choice:  $z_{1161} = -4.09$ ,  $p = 0.0001$ . Post-test: CS+ vs. CS-:  $z_{1161} = -1.45$ ,  $p = 0.31$ , CS+ vs. no choice:  $z_{1161} = -1.75$ ,  $p = 0.18$ , CS- vs. no choice:  $z_{1161} = -3.01$ ,  $p = 0.007$ . No clear significant difference between choice categories was visible in the post-test when restricting the analysis to this period. **(k,l)** Pre- and post-tests performances of the *NaCl group* computed over the subsequent 10 - 30 s stimulus duration. Pre-test: blue square vs. green disc:  $z_{1161} = -2.43$ ,  $p = 0.04$ , blue square vs. no choice:  $z_{1161} = -0.5$ ,  $p = 0.87$ , green disc vs. no choice:  $z_{1161} = -2.84$ ,  $p = 0.013$ . Post-test: CS+ vs. CS-:  $z_{1161} = -0.54$ ,  $p = 0.85$ , CS+ vs. no choice:  $z_{1161} = -0.26$ ,  $p = 0.96$ , CS- vs. no choice:  $z_{1161} = -0.79$ ,  $p = 0.7$ . Again, no significant difference between the proportions of bees choosing the CS+ or the CS- was visible during the Post-test when restricting the analysis to this stimulus period. These results illustrate well why the 30 s period was appropriate for computing preferences based on a cumulative proportion of bees choosing either stimulus or not performing any choice.

## References

- 1 Seelig, J. D. *et al.* Two-photon calcium imaging from head-fixed *Drosophila* during optomotor walking behavior. *Nat Meth* **8**, 184-184, doi:10.1038/nmeth0211-184b (2011).

## References

- 1 Giurfa, M. Cognition with few neurons: higher-order learning in insects. *Trends Neurosci* **36**, 285-294, doi:10.1016/j.tins.2012.12.011 (2013).
- 2 Giurfa, M. & Sandoz, J. C. Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn Mem* **19**, 54-66, doi:10.1101/lm.024711.111 (2012).
- 3 Guven-Ozkan, T. & Davis, R. L. Functional neuroanatomy of *Drosophila* olfactory memory formation. *Learn Mem* **21**, 519-526, doi: 10.1101/lm.034363.114 (2014).
- 4 Keene, A. C. & Waddell, S. *Drosophila* olfactory memory: single genes to complex neural circuits. *Nat Rev Neurosci* **8**, 341-354, doi:10.1038/nrn2098 (2007).
- 5 Heisenberg, M. Mushroom body memoir: from maps to models. *Nat Rev Neurosci* **4**, 266-275, doi:10.1038/nrn1074 (2003).
- 6 Mizunami, M. & Matsumoto, Y. Roles of aminergic neurons in formation and recall of associative memory in crickets. *Front Behav Neurosci* **4**, doi:10.3389/fnbeh.2010.00172 (2010).
- 7 Josens, R., Eschbach, C. & Giurfa, M. Differential conditioning and long-term olfactory memory in individual *Camponotus fellah* ants. *J Exp Biol* **212**, 1904-1911, doi:10.1242/jeb.030080 (2009).
- 8 Giurfa, M. Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *J Comp Physiol A* **193**, 801-824, doi:10.1007/s00359-007-0235-9 (2007).
- 9 Menzel, R. Memory dynamics in the honeybee. *J Comp Physiol A* **185**, 323-340 (1999).
- 10 Avarguès-Weber, A., Deisig, N. & Giurfa, M. Visual cognition in social insects. *Annu Rev Entomol* **56**, 423-443, doi:10.1146/annurev-ento-120709-144855 (2011).
- 11 Takeda, K. Classical conditioned response in the honey bee. *J Insect Physiol* **6**, 168-179, doi:10.1016/0022-1910(61)90060-9 (1961).
- 12 Bitterman, M. E., Menzel, R., Fietz, A. & Schäfer, S. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J Comp Psychol* **97**, 107-119, doi:10.1037/0735-7036.97.2.107 (1983).
- 13 Hammer, M. An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* **366**, 59-63, doi:10.1038/366059a0 (1993).

- 14 Menzel, R. Searching for the memory trace in a mini-brain, the honeybee. *Learn Mem* **8**, 53-62, doi:10.1101/lm.38801 (2001).
- 15 Giurfa, M. Cognitive neuroethology: dissecting non-elemental learning in a honeybee brain. *Curr Opin Neurobiol* **13**, 726-735 doi:10.1016/j.conb.2003.10.015 (2003).
- 16 von Frisch, K. *The Dance Language and Orientation of Bees*. (Harvard University Press, 1967).
- 17 Srinivasan, M. V. Honey bees as a model for vision, perception, and cognition. *Annu Rev Entomol* **55**, 267-284, doi:10.1146/annurev.ento.010908.164537 (2010).
- 18 Giurfa, M. & Menzel, R. Insect visual perception: complex abilities of simple nervous systems. *Curr Opin Neurobiol* **7**, 505-513, doi:10.1016/S0959-4388(97)80030-X (1997).
- 19 Avarguès-Weber, A., Dyer, A. G. & Giurfa, M. Conceptualization of above and below relationships by an insect. *Proc Biol Sci* **278**, 898-905, doi:10.1098/rspb.2010.1891 (2011).
- 20 Avarguès-Weber, A., Portelli, G., Benard, J., Dyer, A. & Giurfa, M. Configural processing enables discrimination and categorization of face-like stimuli in honeybees. *J Exp Biol* **213**, 593-601, doi:10.1242/jeb.039263 (2010).
- 21 Giurfa, M., Eichmann, B. & Menzel, R. Symmetry perception in an insect. *Nature* **382**, 458-461, doi:10.1038/382458a0 (1996).
- 22 Giurfa, M., Zhang, S., Jenett, A., Menzel, R. & Srinivasan, M. V. The concepts of 'sameness' and 'difference' in an insect. *Nature* **410**, 930-933, doi:10.1038/35073582 (2001).
- 23 Stach, S., Benard, J. & Giurfa, M. Local-feature assembling in visual pattern recognition and generalization in honeybees. *Nature* **429**, 758-761, doi:10.1038/nature02594 (2004).
- 24 Srinivasan, M. V. & Zhang, S. Visual motor computations in insects. *Annu Rev Neurosci* **27**, 679-696, doi:10.1146/annurev.neuro.27.070203.144343 (2004).
- 25 Menzel, R. & Giurfa, M. Cognitive architecture of a mini-brain: the honeybee. *Trends Cognit Sci* **5**, 62-71, doi:10.1016/S1364-6613(00)01601-6 (2001).
- 26 Avarguès-Weber, A., Dyer, A. G., Combe, M. & Giurfa, M. Simultaneous mastering of two abstract concepts by the miniature brain of bees. *Proc Natl Acad Sci U.S.A.* **109**, 7481-7486, doi:10.1073/pnas.1202576109 (2012).
- 27 Benard, J., Stach, S. & Giurfa, M. Categorization of visual stimuli in the honeybee *Apis mellifera*. *Anim Cogn* **9**, 257-270, doi:10.1007/s10071-006-0032-9 (2006).

- 28 Chittka, L. & Geiger, K. Can honey-bees count landmarks? *Anim Behav* **49**, 159-164, doi:10.1016/0003-3472(95)80163-4 (1995).
- 29 Dacke, M. & Srinivasan, M. V. Evidence for counting in insects. *Anim Cogn* **11**, 683-689, doi:10.1007/s10071-008-0159-y (2008).
- 30 Gross, H. J. *et al.* Number-based visual generalisation in the honeybee. *Plos One* **4**, doi:10.1371/journal.pone.0004263 (2009).
- 31 Perry, C. J. & Barron, A. B. Honey bees selectively avoid difficult choices. *Proc Natl Acad Sci USA* **110**, 19155-19159, doi:10.1073/pnas.1314571110 (2013).
- 32 Avarguès-Weber, A. & Mota, T. Advances and limitations of visual conditioning protocols in harnessed bees. *Journal of physiology, Paris*, doi:10.1016/j.jphysparis.2016.12.006 (2016).
- 33 Kramer, E. The orientation of walking honeybees in odour fields with small concentration gradients. *Physiol Entomol* **1**, 27-37, doi:10.1111/j.1365-3032.1976.tb00883.x (1976).
- 34 Paulk, A. C. *et al.* Selective attention in the honeybee optic lobes precedes behavioral choices. *Proc Natl Acad Sci U S A* **111**, 5006-5011, doi:10.1073/pnas.1323297111 (2014).
- 35 Becher, P. G. & Guerin, P. M. Oriented responses of grapevine moth larvae *Lobesia botrana* to volatiles from host plants and an artificial diet on a locomotion compensator. *J Insect Physiol* **55**, 384-393, doi:10.1016/j.jinsphys.2009.01.006 (2009).
- 36 Party, V., Hanot, C., Busser, D. S., Rochat, D. & Renou, M. Changes in odor background affect the locomotory response to pheromone in moths. *PLoS One* **8**, e52897, doi:10.1371/journal.pone.0052897 (2013).
- 37 Brandstaetter, A. S., Bastin, F. & Sandoz, J. C. Honeybee drones are attracted by groups of conspecifics in a walking simulator. *J Exp Biol* **217**, 1278-1285, doi:10.1242/jeb.094292 (2014).
- 38 Vinauger, C., Buratti, L. & Lazzari, C. R. Learning the way to blood: first evidence of dual olfactory conditioning in a blood-sucking insect, *Rhodnius prolixus*. II. Aversive learning. *J Exp Biol* **214**, 3039-3045, doi:10.1242/jeb.057075 (2011).
- 39 Arnold, S. E., Stevenson, P. C. & Belmain, S. R. Shades of yellow: interactive effects of visual and odour cues in a pest beetle. *PeerJ* **4**, e2219, doi:10.7717/peerj.2219 (2016).

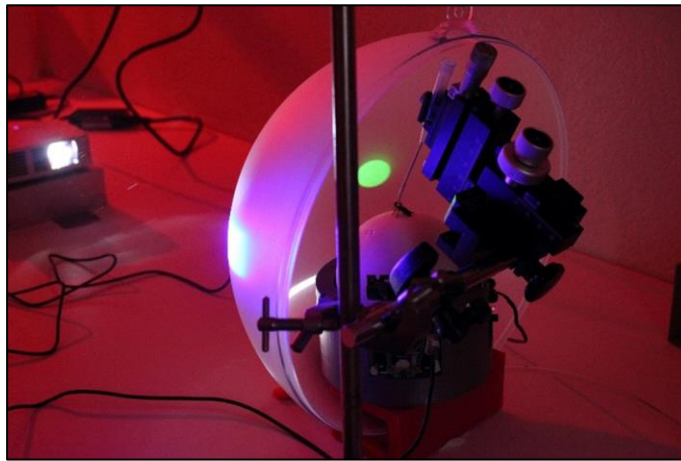
- 40 Peckmezian, T. & Taylor, P. W. A virtual reality paradigm for the study of visually mediated behaviour and cognition in spiders. *Anim Behav* **107**, 87-95, doi:10.1016/j.anbehav.2015.06.018 (2015).
- 41 Van De Poll, M. N., Zajackowski, E. L., Taylor, G. J., Srinivasan, M. V. & van Swinderen, B. Using an abstract geometry in virtual reality to explore choice behaviour: visual flicker preferences in honeybees. *J Exp Biol* **218**, 3448-3460, doi:10.1242/jeb.125138 (2015).
- 42 Taylor, G. J. *et al.* Insects modify their behaviour depending on the feedback sensor used when walking on a trackball in virtual reality. *J Exp Biol* **218**, 3118-3127, doi:10.1242/jeb.125617 (2015).
- 43 Yamashita, T., Haupt, S. S., Ikeno, H. & Ai, H. Walking patterns induced by learned odors in the honeybee, *Apis mellifera* L. *J Exp Biol* **219**, 12-16, doi:10.1242/jeb.123356 (2016).
- 44 Zeil, J., Kelber, A. & Voss, R. Structure and function of learning flights in bees and wasps. *J Exp Biol* **199**, 245-252 (1996).
- 45 Zeil, J. Orientation flights of solitary wasps (Cerceris; Sphecidae; Hymenoptera) 1. Description of flight. *J Comp Physiol A* **172**, 189-205, doi:10.1007/BF00189396 (1993).
- 46 Zeil, J. Orientation flights of solitary wasps (Cerceris; Sphecidae; Hymenoptera) 2. Similarities between orientation and return flights and the use of motion parallax. *J Comp Physiol A* **172**, 207-222, doi:10.1007/BF00189397 (1993).
- 47 Zeil, J. The control of optic flow during learning flights. *J Comp Physiol A* **180**, 25-37 (1997).
- 48 Hempel de Ibarra, N. & Giurfa, M. Discrimination of closed coloured shapes requires only contrast to the long wavelength receptor. *Anim Behav* **66**, 903-910, doi:10.1006/anbe.2003.2269 (2003).
- 49 Lehrer, M. & Srinivasan, M. V. Object detection by honeybees - Why do they land on edges? *J Comp Physiol A* **173**, 23-32 (1993).
- 50 Giurfa, M., Vorobyev, M., Kevan, P. & Menzel, R. Detection of coloured stimuli by honeybees: minimum visual angles and receptor specific contrasts. *J Comp Physiol A* **178**, 699-709 (1996).
- 51 Giurfa, M., Vorobyev, M., Brandt, R., Posner, B. & Menzel, R. Discrimination of coloured stimuli by honeybees: alternative use of achromatic and chromatic signals. *J Comp Physiol A* **180**, 235-243, doi:10.1007/s003590050044 (1997).



- 52 Avarguès-Weber, A., de Brito Sanchez, M. G., Giurfa, M. & Dyer, A. G. Aversive reinforcement improves visual discrimination learning in free-flying honeybees. *PLoS One* **5**, e15370, doi:10.1371/journal.pone.0015370 (2010).
- 53 Rescorla, R. A. & Wagner, A. R. in *Classical conditioning II: Current research and theory* (eds A. H. Black & W. F. Prokasy) 64-99 (Appleton-Century-Crofts, 1972).
- 54 de Brito Sanchez, M. G., Serre, M., Avarguès-Weber, A., Dyer, A. G. & Giurfa, M. Learning context modulates aversive taste strength in honey bees. *J Exp Biol* **218**, 949-959, doi:10.1242/jeb.117333 (2015).
- 55 Hori, S. *et al.* Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera* L. *J Comp Physiol A* **192**, 691-700, doi:10.1007/s00359-005-0091-4 (2006).
- 56 Dobrin, S. E. & Fahrbach, S. E. Visual associative learning in restrained honey bees with intact antennae. *Plos One* **7**, e37666, doi:10.1371/journal.pone.0037666 (2012).
- 57 Mota, T., Giurfa, M. & Sandoz, J. C. Color modulates olfactory learning in honeybees by an occasion-setting mechanism. *Learn Mem* **18**, 144-155, doi:10.1101/lm.2073511 (2011).
- 58 Balamurali, G. S., Somanathan, H. & Hempel de Ibarra, N. Motion cues improve the performance of harnessed bees in a colour learning task. *J Comp Physiol A* **201**, 505-511, doi:10.1007/s00359-015-0994-7 (2015).
- 59 Spaethe, J., Tautz, J. & Chittka, L. Do honeybees detect colour targets using serial or parallel visual search? *J Exp Biol* **209**, 987-993, doi:10.1242/jeb.02124 (2006).
- 60 Nityananda, V., Skorupski, P. & Chittka, L. Can bees see at a glance? *J Exp Biol* **217**, 1933-1939, doi:10.1242/jeb.101394 (2014).
- 61 Egelhaaf, M. & Kern, R. Vision in flying insects. *Curr Opin Neurobiol* **12**, 699-706, doi:10.1016/S0959-4388(02)00390-2 (2002).
- 62 Egelhaaf, M., Boeddeker, N., Kern, R., Kurtz, R. & Lindemann, J. P. Spatial vision in insects is facilitated by shaping the dynamics of visual input through behavioral action. *Front Neural Circuits* **6**, 108, doi:10.3389/fncir.2012.00108 (2012).
- 63 Lehrer, M., Srinivasan, M. V. & Zhang, S. W. Visual edge detection in the honeybee and its chromatic properties. *Proc Biol Sci* **238**, 321-330, doi:10.1098/rspb.1990.0002 (1990).
- 64 Skinner, B. F. *The Behavior of Organisms. An Experimental Analysis.* (Appleton-Century-Crofts, 1938).

- 65 Chittka, L., Dyer, A. G., Bock, F. & Dornhaus, A. Psychophysics: bees trade off foraging speed for accuracy. *Nature* **424**, 388-388, doi:10.1038/424388a (2003).
- 66 Avarguès-Weber, A. & Giurfa, M. Cognitive components of color vision in honey bees: how conditioning variables modulate color learning and discrimination. *J Comp Physiol A* **200**, 449-461, doi:10.1007/s00359-014-0909-z (2014).
- 67 de Brito Sanchez, M. G., Giurfa, M., de Paula Mota, T. R. & Gauthier, M. Electrophysiological and behavioural characterization of gustatory responses to antennal 'bitter' taste in honeybees. *Eur J Neurosci* **22**, 3161-3170, doi:10.1111/j.1460-9568.2005.04516.x (2005).
- 68 Robertson, H. M. & Wanner, K. W. The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res* **16**, 1395-1403, doi:10.1101/gr.5057506 (2006).
- 69 Ayestarán, A., Giurfa, M. & de Brito Sanchez, M. G. Toxic but drank: gustatory aversive compounds induce post-ingestional malaise in harnessed honeybees. *PLoS One* **5**, e15000, doi:10.1371/journal.pone.0015000 (2010).
- 70 Hurst, V., Stevenson, P. C. & Wright, G. A. Toxins induce 'malaise' behaviour in the honeybee (*Apis mellifera*). *J Comp Physiol A* **200**, 881-890, doi:10.1007/s00359-014-0932-0 (2014).
- 71 de Brito Sanchez, M. G. Taste perception in honey bees. *Chem Senses* **36**, 675-692, doi:10.1093/chemse/bjr040 (2011).
- 72 Carcaud, J., Roussel, E., Giurfa, M. & Sandoz, J. C. Odour aversion after olfactory conditioning of the sting extension reflex in honeybees. *J Exp Biol* **212**, 620-626, doi:10.1242/jeb.026641 (2009).





## Chapitre 2



Transfer of visual learning between a virtual and a real environment in honey bees: the role of active vision.

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Le premier chapitre nous a permis de soulever les limites d'un système en circuit ouvert. Un nouveau dispositif a donc été créé permettant de tester les abeilles en circuit fermé, avec un mouvement des stimuli à l'écran en réponse à leurs mouvements sur la boule. En parallèle, nous avons développé un labyrinthe en Y de marche avec des boîtes interchangeable, qui permettaient de replacer une abeille de manière répétée au départ du labyrinthe afin d'observer des décisions répétées. En utilisant des expériences de conditionnement et de transfert entre le système de réalité virtuelle (RV) et le labyrinthe, nous avons étudié comment les libertés de mouvement et la vision active sont cruciales pour l'apprentissage discriminatif de couleur. Environ 57% des abeilles apprennent la discrimination dans les deux contextes. Le transfert de la RV vers le labyrinthe améliore les performances des abeilles. En effet, 75% des abeilles qui ont choisi le SC+ continue de le choisir, alors que 100 % des abeilles qui ont choisi le SC- changent leur choix pour le CS+. En revanche, aucune amélioration n'a été observée dans le transfert opposé. Le transfert asymétrique entre les contextes indique que les informations apprises dans chaque environnement sont peut-être différentes malgré les performances similaires. De plus, la réduction des possibilités de vision active et de liberté de mouvement au moment du passage dans la RV réduit l'expression de l'apprentissage visuel alors qu'augmenter ces possibilités dans le transfert inverse l'améliore. Nos résultats soulignent la nature des processus visuels et l'importance de développer des systèmes de RV immersifs.



# Transfer of visual learning between a virtual and a real environment in honey bees: the role of active vision

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## **ABSTRACT**

To study visual learning in honey bees, we developed a virtual reality (VR) system in which the movements of a tethered bee walking stationary on a spherical treadmill update the visual panorama presented in front of it (closed-loop conditions), thus creating an experience of immersion within a virtual environment. In parallel, we developed a small Y-maze with interchangeable end-boxes, which allowed replacing repeatedly a freely walking bee into the starting point of the maze for repeated decision recording. Using conditioning and transfer experiments between the VR setup and the Y-maze, we studied the extent to which movement freedom and active vision are crucial for learning a simple color discrimination. Approximately 57% of the bees learned the visual discrimination in both conditions. Transfer from VR to the maze improved significantly the bees' performances: 75% of bees having chosen the CS+ continued doing so and 100% of bees having chosen the CS- reverted their choice in favor of the CS+. In contrast, no improvement was seen for these two groups of bees during the reciprocal transfer from the Y-maze to VR. In this case, bees exhibited inconsistent choices in the VR setup. The asymmetric transfer between contexts indicates that the information learned in each environment may be different despite the similar learning success. Moreover, it shows that reducing the possibility of active vision and movement freedom in the passage from the maze to the VR impairs the expression of visual learning while increasing them in the reciprocal transfer improves it. Our results underline the active nature of visual processing in bees and allow discussing the developments required for immersive VR experiences in insects.

**Keywords: Visual conditioning, Transfer of Learning, Virtual Reality, Y-maze, Honey Bees, Insect Cognition**

## INTRODUCTION

The visual capacities of honey bees have been intensively investigated for more than a century. Since the pioneer experiments by Karl von Frisch (von Frisch, 1914) and Mathilde Hertz (e.g. Hertz, 1935) on honey bee color and pattern vision, respectively, many scientists have used simple behavioral protocols to access different aspects of bee vision. These protocols rely on the fact that free-flying bees learn rapidly to choose and land on visual targets that have been associated with a reward of sucrose solution (Giurfa and Menzel, 1997; Srinivasan and Zhang, 2004; Srinivasan, 2010; Avarguès-Weber et al., 2011).

In the last decades, many experiments on bee visual perception and learning have been performed in Y-mazes as this type of setup allows a proper control of the distance at which a decision based on visual information is made. It is thus possible to determine the visual cues accessible to the bees upon decision (Hateren et al., 1990; Giurfa et al., 1996). Free-flying bees can be easily trained to enter such mazes to collect sucrose solution upon appropriate choice of trained colors and patterns in simple or complex learning sets (e.g. Giurfa et al., 1996; Giurfa et al., 2001; Avarguès-Weber et al., 2010; Avarguès-Weber et al., 2011; Avarguès-Weber et al., 2012). These experiments are also possible in the case of walking bees that are presented with visual discriminations within mazes of small size in which flight is precluded, thus adding further possibilities for behavioral control (e.g. Chittka, 1998; Zhang et al., 1998; Buatois et al., 2017). Using such controlled conditions, researchers showed that bees do not only learn simple discriminations between colors and/or shapes associated with different reinforcements but also learn higher-order discriminations in conceptual and categorization problems (see reviews in Srinivasan and Zhang, 2004; Benard et al., 2006; Srinivasan, 2010; Avarguès-Weber et al., 2011; Dyer, 2012; Giurfa, 2013).

The neural underpinnings of these capacities, both for simple and higher-order learning, remain, however, elusive. On the one hand, the use of free-flying or walking bees precludes the use of invasive methods to obtain more in-depth information about the neural mechanisms involved in visual learning. On the other hand, attempts to train harnessed bees to associate visual stimuli with sucrose reward have been mostly unsatisfactory, at least when using the proboscis extension response (PER) as the behavioral readout of visual learning and memory formation. While

harnessed bees easily learn odor-sucrose associations and extend their proboscis to odors previously rewarded (Takeda, 1961; Bitterman et al., 1983; Giurfa and Sandoz, 2012), their learning of colors in the same conditions is usually poor, even for simple color discrimination tasks (Hori et al., 2006; Mota et al., 2011; Dobrin and Fahrbach, 2012; Balamurali et al., 2015; Avarguès-Weber and Mota, 2016). Similarly, when movements of striped patterns are associated with sucrose reward in order to condition PER to a forward or backward movement, learning is typically slow and deficient (Hori et al., 2007). Attempts to condition antennal movements to visual stimuli rather than PER were also disappointing: while bees exhibit stereotyped and specific antennal movements to the ventro-dorsal movement of a striped pattern (Erber et al., 1993), enhancing these responses via pairing with sucrose yielded only partial success: improvement occurred only for certain directions of stripe-pattern movement and in no case bees could learn to discriminate between opposite directions (Erber and Schildberger, 1980).

A potential explanation for the deficit resulting from preparations in which bees are fully immobilized, as required by PER conditioning protocols, is the absence or limitation of active vision, which might be essential to learn visual targets. In active vision, an observer varies its viewpoint to investigate the environment and extract more information from it. This strategy is used for example by flying bees and wasps (Zeil, 1993a; b; Zeil et al., 1996; Zeil, 1997; Srinivasan and Zhang, 2004) to extract the borders of objects for better recognition (Lehrer and Srinivasan, 1993; Hempel de Ibarra and Giurfa, 2003) via a series of flight maneuvers. Addressing this hypothesis requires manipulating the possibilities of active vision, i.e. the freedom of movement of a bee solving visual discriminations.

Studying the visual performances of tethered insects offers the possibility of controlling both their visual environment and their freedom of movement. For instance, in the so-called ‘flight simulator’ an insect glued to a small hook of copper wire and attached to a torque meter flies stationary in the middle of a cylindrical arena displaying different visual patterns. In this device, originally conceived for fruit flies (Götz, 1964; Heisenberg and Wolf, 1988; Wolf and Heisenberg, 1991), the rotational speed of the arena is proportional to the fly's yaw torque around its vertical body axis under closed loop conditions. This allows the fly to stabilize the rotational movements of the panorama and to establish flight directions with respect to visual patterns displayed on the cylinder. The flight simulator allowed to study visual landmark learning in several neurogenetic

*Drosophila* mutants, thus uncovering the neural and molecular bases of some forms of visual learning and memory (Liu et al., 1999; Brembs and Heisenberg, 2000; Tang and Guo, 2001; Liu et al., 2006; Xi et al., 2008; Pan et al., 2009).

The study of visual learning and memory in a flight simulator has not been possible until now in the case of honey bees. The closest attempt consisted of an analysis of the body posture of a tethered bee flying stationary in the middle of a visual arena made up of four LCD monitors disposed in a diamond-like arrangement and displaying a moving panorama (Luu et al., 2011). The monitors provided a simulation of image variation as the insect flies. It was shown that the bee raised its abdomen progressively higher as the simulated speed of the image increased, and tilted it down when the visual motion stimulus stopped. This behavior termed ‘streamlining response’ is a spontaneous response to motion cues ‘*en route*’ to the goal. It does not involve the learning of visual cues and occurs in a context different from the close-up recognition of visual targets learned in association with food reward, when the animal is about to land.

A better solution for the study of learning of visual targets in tethered bees is provided by the use of treadmills onto which bees walk stationary while being exposed to visual targets paired with food reward or with punishment (Buatois et al., 2017; Rusch et al., 2017; Schultheiss et al., 2017). In this kind of setup, closed loop conditions allowed creating a virtual environment in which the bee’s responses are tracked and used to update the virtual environment in real time, thus creating an experience of immersion within this virtual reality (Buatois et al., 2017; Rusch et al., 2017; Schultheiss et al., 2017).

Comparison of performances between this kind of device and Y-mazes offers the possibility of addressing the role of active vision in visual learning. In a Y-maze, full freedom is granted during visual learning while in the treadmill, movements are constrained by tethering the bee to avoid its escape from the setup. Although a tethered bee may walk in any intended direction, as a bee walking in a Y-maze, the physical presence of the tether creates a higher resistance against movements. Thus, additional forces are needed for the animal to achieve a displacement towards a goal (Catton et al., 2007). Moreover, slight asymmetries in the positioning of the tether with respect to the longitudinal axis of the body may favor movements on the side opposite to the tether (Catton et al., 2007), thus affecting the possibility of symmetrical active vision.

Here we performed a comparative analysis of visual learning in honey bees placed in these two experimental conditions. We used a small Y-maze, where freely walking bees experienced visual stimuli projected onto its back walls, and a virtual reality (VR) setup, where tethered bees walking stationary on a treadmill experienced the same visual stimuli projected onto a semi-circular screen placed in front of them. In the latter case, the bee movements constantly updated the visual panorama accordingly (closed-loop conditions). We conditioned independent groups of bees in parallel, either in the VR setup or in the maze, and compared their learning of a color discrimination. After training, each group was transferred to the alternative condition to determine whether VR and maze learning are robust to a change in context. In doing this, we analyzed if restricting movement freedom (from the maze to VR) or enhancing it (from VR to the maze) affected transfer performances and thus discrimination success. Our results show that bees mastered equally well the visual discrimination in both the Y-maze and the VR setup despite obvious differences in movement freedom and in the possibility of performing active vision. Transfer between both contexts affected the expression of learning in an asymmetric way: granting the bees with a greater opportunity for active vision improved visual performances while diminishing it impaired them. We discuss the learning strategies employed by the bees in both contexts and how to achieve better immersive VR experiences in the case of insects.

## **MATERIALS AND METHODS**

### **Animals**

Honey bees (*Apis mellifera*) were obtained from the apiary located at the campus of the University of Toulouse. Only non-fed foragers caught upon landing on a gravity feeder filled with a 0.9 M sucrose solution were used in our experiments to ensure high appetitive motivation. Once caught, each bee was anesthetized by cooling it on ice during 3 min. The thorax was then shaved to improve the fixation of a custom-built tag with UV-cured dentine (Fig. 1a), which allows to tether the bee during the VR experiment. Bees were fed with 4  $\mu$ l of 0.9 M sucrose solution and kept for 3 h in the laboratory before starting the experiments in order to homogenize their appetitive motivation. Feeding was achieved by means of a toothpick in the case of bees assigned to VR training, while it was done using an Eppendorf in the case of bees assigned to Y-maze training. During the 3 h period, bees assigned to VR training were placed individually on miniature treadmills while bees

assigned to Y-maze training were placed individually in the starting box of the maze (see below) to allow familiarization with their respective setup.

### Virtual reality apparatus

The apparatus (Figs.1 b,c) is composed of a spherical treadmill on which a tethered bee walked stationary, and a video projection system displaying visual stimuli in front of the bee. The treadmill consists of a polystyrene ball (diameter: 10cm, weight: 8g, Fig. 1b) positioned on a 3D-printed support (Fig. 1b) and floating on a constant air flow produced by an air pump (air flow: 555ml/s; Aqua Oxy CWS 2000, Oase, Wasquehal, France). The treadmill was placed in front of a semi-spherical semi-transparent plastic screen (diameter: 29 cm, distance to the bee: 10 cm, Ballkit, Varennes, France, Fig. 1b) coated with matt picture varnish (Pébéo, Gemenos, Italy). Visual stimuli were projected onto the screen from behind using a video projector (Acer k135i, Roissy, France).

All VR experiments were done under closed-loop conditions, i.e. rotations of the ball generated by the walking activity of the tethered bee displaced the visual stimuli accordingly on the screen. To this end, the movements of the ball were recorded by two infrared optic-mouse sensors (Logitech M500, 1000 dpi, Logitech, Lausanne, Switzerland, Fig. 1b), which were placed on the ball support, at 90° from each other. The rotational speed of the ball around the vertical axis was calculated with a LabVIEW (National Instruments, Austin, USA) custom software to account for the directional walking movements of the bee (“instant heading”; one data point every 250 ms) using the following equation:

$$\text{Instant heading} = - \left( \frac{X1 + X2}{5700} \right) * \left( \frac{25.4 * 180}{R\pi} \right)$$

X1 and X2 are, respectively, the translational movement in dots recorded in the horizontal axis of each sensor and 5700 is the sampling rate of the sensors in dots/inch. Multiplying the obtained value by 25.4 allows conversion into millimeters while dividing it by  $2\pi R$  (with R being the radius

of the ball) converts the measured distance from millimeters into radians. Finally, multiplying by  $180/\pi$  converts radians to degrees.

These values were used by the software to rotate the angular position of the stimuli on the screen proportionally to the movement of the bee ( $0^\circ$  being the initial position,  $-180^\circ$  the left extremity and  $180^\circ$  the right extremity). In order to decrease the speed of image movement and achieve a proper gain control, the software was configured in such a way that  $2^\circ$  of ball rotation correspond to  $1^\circ$  of stimulus rotation. Thus, in our graphic representations of the bees' turning activity, a vector pointing towards  $+90^\circ$  (circular plot) represents a bee oriented towards a visual stimulus located at  $45^\circ$  to the right of the central axis of the bee body.

### **Y-maze apparatus**

The maze (Fig. 1d,e) consisted of three PVC arms defining a Y. Each arm had a length of 10 cm, a height of 4 cm and a width of 5.5 cm. At the end of each arm, detachable boxes with the same section (4 x 5.5 cm) allowed replacing the bee at the starting position of the maze after each choice, thus facilitating further data collection and a better control of the experimental time parameters. The same visual cues used in the VR setup were projected onto the back walls of the detachable boxes (Fig 1f). The back walls were made of transparent paper. Both the roof and the floor of the maze were made of thin transparent plastic to allow the passage of light. The setup was placed on an infrared light-emitting platform. Experiments were recorded with an infrared camera (acA1300-60gm, 60fps, 1.3 MP, Basler, Ahrensburg, Germany) equipped with light filters to remove the light wavelengths of the stimuli in the videorecordings and facilitate the tracking of the bee. Video recordings were afterwards analyzed using the EthoVision 12 software (Noldus, Wageningen, The Netherlands).

### **Visual Stimuli**

The visual stimuli that bees had to discriminate in both setups were a blue disc (RGB: 0, 0, 255, with dominant wavelength at 450 nm) and a green disc (RGB: 0, 51, 0, with dominant wavelength at 530 nm) displayed on a black background (RGB: 0, 0, 0). Their spectral curves, and their

chromatic and achromatic properties are shown in Fig. S1 and Table S1, respectively. Their intensity, measured at the level of the bee eye, was  $3363 \mu\text{Watt}/\text{cm}^2$  and  $2950 \mu\text{Watt}/\text{cm}^2$ , respectively. These values were chosen to suppress an original attraction of the bees towards the green light detected in preliminary assays. The two discs were 2 cm in diameter. They displayed the same total area ( $3.14 \text{ cm}^2$ ) and subtended the same visual angle to the bee eyes ( $11.42^\circ$ ) in both setups (VR and Y-maze).

## Reinforcements

The positive reinforcement was a 0.9 M sucrose solution while the negative reinforcement was a 60 mM quinine solution (Buatois et al., 2017). During VR experiments, reinforcements were delivered by means of a toothpick to the antennae and then to the proboscis (see below for more details). In the Y-maze, reinforcements were provided in small Eppendorf tube covers (Eppendorf tube 3810x, Hamburg, Germany) located at the end of the arms, in association with the projected color discs. They contained  $4\mu\text{l}$  of solution, a volume that was chosen to correspond to the amount delivered by the toothpick during the VR experiments according to a preliminary quantification.

## Training and testing protocol

**Experiment 1: from VR to the Y-maze.** Conditioning was performed in the VR setup (Fig. 2a). Bees were then transferred to the Y-maze to determine whether the change in context, with its associated increase in movement freedom and possibility of active vision, changed the bees' performance.

The experiment started in the VR setup with a 'pre-test' performed to determine the spontaneous stimulus preference of each bee. During this pre-test, the stimuli to be discriminated were presented simultaneously at  $45^\circ$  on each side of the bee's body axis for 30 s and without reinforcement. The position (left or right) of the blue and green discs was randomized from bee to bee. During the 30 s, the position of the stimuli was constantly updated by the movements of the bee on the treadmill. In order to define which stimulus would act as CS+ and as CS- in the subsequent training, we recorded the time spent by each bee fixating either the blue or the green



disc. The stimulus that was fixated longer became the CS- and was reinforced negatively with quinine solution. The stimulus that was less fixated became the CS+ and was reinforced positively with sucrose solution. If the bee did not make any choice during the pre-test, CS+ and CS- were assigned randomly. Besides the fixation time, we also recorded the first choice made by each bee when facing both stimuli (see below, Statistical Analysis). When the pre-test was concluded, a black screen was displayed for one minute before starting the conditioning protocol.

Each tethered bee was trained in closed-loop conditions to discriminate the blue from the green disc based on their different reinforcement outcome (differential conditioning). Training consisted of a succession of twelve trials, each 30 s in duration, separated by an intertrial interval of one minute. During each trial, the bee was presented simultaneously with both stimuli, the CS+ paired with 0.9 M sucrose solution and the CS- with 60 mM quinine solution. Stimuli were displayed during at most 30s and appeared at the start of the trial at  $-45^\circ$  (left) and  $+45^\circ$  (right) from the bee's body axis. The stimulus side of CS+ and CS- was varied from trial to trial and the side sequence was the same from bee to bee ( $G_R/G_L/G_L/G_R/G_L/G_R/G_L/G_R/G_L/G_L/G_R$ ; with G: green, R: right and L: left;  $G_R$  means green disc displayed on the right, i.e. blue disc displayed simultaneously on the left). When the bee oriented towards a CS and centered it on the screen due to the closed loop conditions ( $0^\circ$  from the bee's body axis), the CS remained stationary at this position during 8 seconds to facilitate reinforcement delivery and its association with a plain frontal view of the CS. Sucrose solution was then provided on the antennae using a toothpick. This stimulation triggered proboscis extension, which allowed us to feed the bee. Quinine was provided directly to the proboscis. After the end of the 8-s period, the stimulus was turned off and was replaced by the black background, which was displayed to the bee during intertrial intervals. The bees never moved while the CS was stationary in front of them or while being reinforced. This procedure ensured that all bees experienced the same reinforcement duration.

One minute after the end of the last conditioning trial, the trained bee was subjected to a 'post-test' during which it was again presented during 30 s with the CS+ and the CS- simultaneously, but in the absence of reinforcement. As in the pre-test, during the 30 s, the position of the stimuli was constantly updated by the movements of the bee on the treadmill. We recorded the first choice of each bee and the time spent fixating each CS. This post-test allowed verifying if

the bee's original stimulus preference recorded during the pre-test was modified because of learning.

Once the post-test was finished, each bee was subjected to two refreshment trials to avoid extinction and then to a transfer test in the Y-maze. To this end, the bee was taken away from the VR setup, and after removing its tether, it was placed in the departure box of the Y-maze for one minute. The bee was now free to move and the transfer-test started when both stimuli, CS+ and CS-, were displayed simultaneously, each one in one arm of the maze. The bee was free to move during 30 seconds during which it could choose the stimuli presented without reinforcement. The left/right position of the stimuli was varied within the maze. The choice behavior of the bee was then recorded.

**Experiment 2: from the Y-maze to VR.** In the Y-maze, the bees underwent the same conditioning protocol as in the VR setup, i.e. preference testing in a pre-test, subsequent CS assignment, conditioning during twelve trials and learning assessment in a post-test were performed following the same schedules and timing (Experiment 1). Both visual stimuli were projected simultaneously onto the paper walls at the end of the maze arms. The main difference with the previous experiment is that bees were free to walk within the maze during trials.

The experiment started with a 'pre-test' performed to determine the spontaneous stimulus preference of each bee. During this pre-test, the bee could freely walk between the arms displaying the blue and the green discs without reinforcement during 30 s. The position (left or right) of the blue and green discs was randomized from bee to bee. We recorded the time spent by each bee within each arm. The stimulus that was more attractive to the bee (i.e. more time spent in its associated arm) became the CS- and was reinforced negatively with quinine solution during the subsequent training. The stimulus that was less attractive (i.e. less time spent in its associated arm) became the CS+ and was reinforced positively with sucrose solution during the training. If the bee did not make any choice during the pre-test, CS+ and CS- were assigned randomly. Besides the fixation time, we also recorded the first choice made by each bee when facing both stimuli (see below, Statistical Analysis). When the pre-test was concluded, the conditioning protocol started.

During training, the bee was subjected to 12 trials during which both the CS+ and the CS- were made available. In each trial, the bee left the starting arm to enter one of the boxes displaying either the CS+ or the CS-. As the bee was familiar with the Eppendorf containing the sucrose solution, which was used to feed it at the beginning of the experiment (see above, ‘Animals’), it found the reinforcement rapidly. When the bee touched the Eppendorf cover, a sliding door trapped it the compartment. The bee was left in this detachable compartment during 8 s, a period that was enough to consume the 4  $\mu$ l of sucrose solution associated with the rewarded color. Between trials, the bee was kept in the dark within the detachable box in which it was trapped. The box was then translocated to the starting point of the maze where the bee could be released to reinitiate a new stimulus choice.

After completing the training, a post-test was performed in which the first choice and the time spent within each CS arm was recorded during 30 s (see below, Statistical Analysis). At the end of the post-test, the bee was subjected to two refreshment trials in the maze to avoid extinction. Afterwards, it was captured, attached to the tether and placed in the VR setup for the transfer-test. The bee was then presented with the CS+ and the CS-, which were displayed at  $\pm 45^\circ$  of its body axis in the absence of reinforcement and in open-loop conditions. Presentation lasted 30 s and the choice behavior of the bee was then recorded.

## Statistical Analysis

**Experiment 1: from VR to the Y-maze.** During the pre-test, the first choice of the bee (i.e. the first image centered on the screen, aligned with the axis of the bee’s body) was categorized as “choice of the green disc” or “choice of the blue disc”. If the bee did not fixate a CS, its performance was categorized as “no-choice”. During the conditioning trials, post-test and transfer test, the first choice of the bee was categorized as “choice of the CS+” (i.e. choice of the sucrose-reinforced stimulus) or “choice of the CS-” (i.e. choice of the quinine-reinforced stimulus). If the bee did not fixate either stimulus, its performance was recorded as a “no-choice”. In the transfer test, the first choice, which occurred in the Y-maze, corresponded to the first arm displaying a visual stimulus visited by a bee.

Individual data were converted into a binomial format (0 or 1) to calculate the proportions of bees that chose the CS+, the CS- or made no choice. Each bee was assigned to a unique category. For instance, a bee choosing the CS+ was quantified as (1, 0, 0) for choice of the CS+, choice of the CS- and no-choice, respectively. Data were bootstrapped to plot these proportions  $\pm$  their corresponding 95% confidence interval. Proportions were compared by means of a generalized linear mixed model (GLMM) for binomial family in which the individual identity (Bee) was considered as a random factor (individual effect). The Tukey method was used for multiple comparisons; z values with corresponding degrees of freedom are reported throughout for this kind of analysis.

The angular positions of the stimulus on the screen were recorded during the pre- and post-test, and their distribution was analyzed using circular statistics. To test whether angular positions were uniformly distributed, we used a Rayleigh Test of Uniformity for General Unimodal Alternative (Rayleigh test), which assesses the significance of the mean resultant length. A Rayleigh Test of Uniformity for Specified Mean Direction (V-test) was used to assess the departure of our data from the specific directions defined by the angular position of the stimuli ( $\pm 45^\circ$  on the screen, which translated into ideal angular orientations of  $\pm 90^\circ$ ; see above, ‘Virtual Reality Apparatus’ section). Finally, to compare the angular means obtained in the pre- and the post-test, a Watson-Wheeler test was performed.

We also quantified the time spent fixating the stimuli during the pre- and the post-test. Mean values were compared to a theoretical fixation time of 0 s using a one-sample Mann Whitney test. The fixation times of CS+ and CS- were compared using a Wilcoxon U rank test.

**Experiment 2: from the Y-maze to VR.** First-choice categorization was similar as in Experiment 1: during the pre- test, the first choice of an arm displaying a visual stimulus was categorized as “choice of the CS1” or “choice of the CS2”. The absence of choice was recorded as “no-choice”. During the conditioning trials, post-test and transfer test, the first choice of the bee was categorized as “choice of the CS+” (i.e. choice of the sucrose-reinforced stimulus) or “choice of the CS-” (i.e. choice of the quinine-reinforced stimulus). If the bee did not make any choice, its performance was categorized as “no-choice”. In the transfer test, the first choice, which occurred in the VR setup, corresponded to the first CS fixated by a bee.

Individual data were converted into a binomial format (0 or 1) to calculate the proportions of bees, which chose the CS+, the CS- or made no choice (see above). Data were treated and analyzed as described for Experiment 1.

Choice performance in the Y-maze could be further described using a heat map, which represents the normalized mean time spent in a given region of the maze during the pre-test and the post-test. Heat maps were obtained using the EthoVision XT tracking system (Noldus, Wageningen - The Netherlands). Moreover, the times spent in the arms displaying the CS+ and the CS- during the pre- and post-test were compared against a theoretical time of 0 s by means of a one-sample Mann Whitney test. The times spent within each arm were compared by means of a Wilcoxon U rank test.

**Experiments 1 and 2: Transfer-test performances.** To evaluate transfer-test performances, we focused exclusively on learners and non-learners, based on post-test performances (i.e. bees that chose the CS+ and bees that chose the CS-, respectively). Bees that did not make a choice were not included in this analysis. We determined whether the proportions of learners and non-learners changed in the transfer-test with respect of those obtained in the post-test. To evaluate the significance of change, we used a McNemar test.

All statistical analyses were done using the R 3.2.3 software (R Core Team 2016; <https://www.r-project.org/>). Packages lme4 and lsmeans were used for GLMMs, with Tukey's method for multiple comparisons. Circular and CircStats were used for circular graphics and circular statistics.

## RESULTS

### Experiment 1: From VR to the Y-maze

**Performance in VR.** Figure 3 shows the performance of bees in the VR setup under closed-loop conditions. In the *pre-test*, all bees (n=30) made a decision: the proportion of bees spontaneously choosing the blue disc was 40% while that choosing the green disc was 60%. These proportions did not differ significantly from each other (Fig. 3a; GLM binomial family; blue disc vs green disc:  $z_{59}=1.87$ ,  $p=0.06$ ). Yet, when choices were analyzed in terms of a side bias, more

bees oriented spontaneously towards the right than to the left side ( $z_{59}=-2.28$ ,  $p=0.02$ ). This was overcome during training, as the side of the rewarding stimulus was randomized from bee to bee.

During *training*, bees were rewarded with sucrose solution on the stimulus they did not prefer in the pre-test (which became the CS+) and punished with quinine solution on the stimulus they preferred (which became the CS-). Due to this criterion, the distribution of orientation vectors exhibited by bees during the pre-test was biased towards the subsequent CS- (Fig. 3b; V test,  $p<0.0001$ ) and more time was spent fixating it than the CS+ (Fig. 3c; Wilcoxon U test,  $U=477$ ,  $p<0.0001$ ).

After the 12 conditioning trials, a *post-test* allowed determining whether bees ( $n=30$ ) reverted their color preference because of learning. During this post-test (Fig. 3d), the proportions of bees choosing first the CS+, the CS- or not choosing any stimulus were 56.6%, 30% and 13.4% respectively. The former was significantly higher than the two latter (Fig. 3d; CS+ vs. CS- :  $z_{88}=2.13$ ,  $p=0.02$ ; CS+ vs. no choice:  $z_{88}=-2.99$ ,  $p=0.003$ ; CS- vs. no choice:  $z_{88}=-0.87$ ,  $p=0.38$ ). Discriminating learning success according to which color was rewarded (Fig. 3g) showed that when the blue disc was the CS+, the proportions of bees choosing the CS+, the CS- or not choosing any stimulus were 61.1%, 16.7% and 22.2% respectively. The former was significantly higher than the two other (CS+ vs. CS- :  $z_{53}=2.59$ ,  $p=0.01$ ; CS+ vs. no choice:  $z_{53}=-2.28$ ,  $p=0.02$ ; CS- vs. no choice:  $z_{53}=0.42$ ,  $p=0.67$ ). When the green disc was the CS+, the proportions of bees choosing the CS+, the CS- or not choosing any stimulus were 50%, 41.7% and 8.3%. In this case, the CS+ proportion was not different from the CS- one, but differed from the no-choice proportion (CS+ vs. CS- :  $z_{35}=0.41$ ,  $p=0.68$ ; CS+ vs. no choice:  $z_{35}=-2.01$ ,  $p=0.04$ ; CS- vs. no choice:  $z_{35}=-1.72$ ,  $p=0.08$ ). Thus, learning was more effective for the blue color than for the green color. In other words, reverting the pre-test color preference from green to blue was more effective than from blue to green.

The distribution of orientation vectors during the post-test was not significantly directed towards the CS+ (Fig. 3e, Rayleigh test,  $p=0.18$ ) irrespectively of the nature of the positively reinforced stimulus (blue rewarded:  $p=0.11$ , green rewarded:  $p=0.51$ ). Furthermore, bees did not spend more time fixating the CS+ (Fig. 3f;  $U=131$ ,  $p=0.26$ ). Both results seem to contradict a learning effect; yet, the mean direction of trained bees changed significantly between pre- and post-

test as it was no longer oriented towards the CS- (Figs. 3b,e; Watson-Wheeler test,  $F=18.02$ ,  $p<0.0001$ ), thus revealing a training-induced change also at this level.

As these post-test analyses included performances from learners (i.e. bees that chose the CS+ in the post-test) and non-learners (i.e. bees that chose the CS- in the post-test), and this could have obscured the significance of the learning effects resulting from the training, we focused on the post-test performances of learners ( $n=17$ ). Learners were significantly oriented toward the CS+ (Fig. 3h, Rayleigh test,  $p<0.0001$ ) irrespectively of the nature of the positively reinforced stimulus (blue rewarded:  $p=0.003$ , green rewarded:  $p=0.002$ ), and spent significantly more time fixating the CS+ (Fig.3i;  $U=510$ ,  $p<0.0001$ ).

These results confirm that under closed-loop conditions, a significant proportion of bees learned to revert their original color preferences and oriented towards the CS+, which they fixated longer during the post-test.

**Transfer test in the Y-maze.** Following two refreshment trials in the VR setup (see Fig. 2a), bees experienced a *transfer test* inside the Y-maze where they recovered complete freedom of movement. They had to choose between the color discs previously trained, now projected on the back walls of the maze (Fig. 2a). We focused our analyses on learners ( $n=17$ ) and on non-learners (bees that chose the CS- in the post-test;  $n=9$ ) to determine how the transfer test affected their performance. From the 17 learners, 5 were excluded from the analysis as they did not make any choice during the transfer test. Figure 4a shows that from the remaining bees, 75% ( $n=9$ ) chose again the CS+ and 25% ( $n=3$ ) chose the CS- erroneously. Interestingly, 100% of the non-learners ( $n=9$ ) reverted their incorrect choice and chose the CS+ in the transfer test. A McNemar test including both learners and non-learners showed that the change in performance was significant ( $\chi^2=7.11$ ,  $df:1$ ,  $p<0.01$ ).

Thus, performance was improved following the change of context between VR and Y-maze, in particular because the transfer revealed that the bees originally labelled as non-learners had learned the visual discrimination. The two refreshment trials performed after the post-test cannot account for this change as we observed in parallel a 25% reduction in the proportion of learners

following these refreshment trials. We conclude that granting bees with the possibility of free movement and enhanced active vision improved the expression of visual learning.

## Experiment 2: from the Y-maze to VR

**Performance in the Y-maze.** Figure 5 shows the performance of bees in the Y-maze. During the *pre-test* (Fig. 5a), 33.3% and 53.3% of the bees preferred the blue disc and the green disc, respectively; 13.3% of the bees did not choose any stimulus. Despite the apparent preference for the green disc, no difference was found between the percentages of bees choosing the blue vs. the green disc (Fig. 5a; blue vs. green:  $z_{89}=1.55$ ,  $p=0.12$ ; blue vs. no choice:  $z_{89}=-1.78$ ,  $p=0.07$ ; green vs. no choice:  $z_{89}=-3.08$ ,  $p=0.002$ ). An analysis of trajectories within the Y-maze allowed establishing a heat map representing the normalized mean time spent within the maze during the pre-test (Fig. 5b). The pooled heat map showed that bees spent more time in the arm displaying the color that was subsequently designated as CS- during training. Bees also spent significantly more time fixating this stimulus during the pre-test (Fig. 5c;  $U=29$ ,  $p=0.0001$ ).

During *training*, bees were rewarded with sucrose solution on the stimulus (CS+) they did not prefer in the pre-test and punished with quinine solution on the stimulus (CS-) they preferred. After the 12 conditioning trials, a *post-test* allowed determining whether bees reverted their color preference because of learning. In this post-test, no bee remained undecided. The proportion of bees choosing first the CS+ was 57% while that of bees choosing the CS- was 43% (Fig. 5d; CS+ vs CS-:  $z_{59}=1.03$ ,  $p=0.3$ ). Note that the percentage of bees choosing the CS+ was similar to that observed in the post-test in the VR setup (56.6%; see Fig. 3d). A main difference between that post-test and the one in the Y-maze resides in the absence of bees not making any decision in the maze. The maze seems to have increased the percentage of bees choosing the CS-. This pattern of results was common to bees rewarded on green and on blue colors (Fig. 5g). We also analyzed the time spent in the maze arms. The pooled heat map showed that during the post-test bees tended to spend more time in the CS+ arm (Fig. 5e), thus confirming that despite the high percentage of bees labeled as ‘CS- choosers’ (43%, see above), learning occurred in the Y-maze. A comparison of the time spent in the CS+ and in the CS- arms was marginally non-significant (Fig. 5f;  $U=306$ ,  $p=0.06$ ).



To confirm that learning indeed occurred in the maze, at least for the bees having chosen the CS+ as their first choice in the post-test, we focused on the performance of these bees ('learners', n=17). Their pooled heat map indicated that they preferred to stay in the CS+ arm (Fig. 5h) and the quantitative analysis of the time spent in each arm of the maze revealed a highly significant preference for the CS+ arm (Fig.5i; U=148, p<0.001). Thus, 57% of the bees did indeed learn to choose the rewarded color in the Y-maze.

**Transfer test in the VR setup.** After two refreshment trials in the Y-maze, bees were fixed to the tether and placed, one by one, in the VR setup for a *transfer test*. They could see in front of them the two colored discs that were trained in the Y-maze with the difference that the degrees of freedom were reduced by the tether. We focused on learners (n =17) and on non-learners (n= 13) to determine how the transfer test affected their performance. From the 17 learners, 4 were excluded from the analysis as they did not make any choice during the transfer test. From the 13 non-learners, 2 were excluded for the same reason. Figure 4b shows that from the remaining learners 61.54% (n=8) chose again the CS+ and 38.46% (n=5) chose the CS- erroneously. In the case of the non-learners, 45.45% (n=5) reverted their choice and chose the CS+ while 54.54% (n=6) persisted in choosing the CS- incorrectly. A McNemar test including both learners and non-learners showed that there was no significant change in performance between the post-test and the transfer test ( $\chi^2=0.07$ , df:1, p=0.79).

Thus, the transfer from the Y-maze to the VR setup induced unpredictable performances in both groups of bees; i.e. a learner could become a non-learner with practically the same probability of remaining a learner, and the same occurred with a non-learner. This shows that constraining movements and impairing active vision influence the expression of visual learning.

## **Comparison of acquisition performances in Experiments 1 and 2**

The results of both transfer experiments indicate that the expression of learning was improved (Experiment 1) or impaired (Experiment 2) depending on the direction of transfer but that acquisition success was similar, at least when the % of bees choosing the CS+ in the post-test of

both experiments was considered. Yet, to conclude that this was the case, an analysis of the dynamics of acquisition during the learning trials is necessary.

In both experiments, bees experienced twelve successive conditioning trials. Figures 6a,b show the acquisition performances of all bees trained in Experiments 1 and 2, respectively. Tables S2 and S3 show the individual performances of the bees in terms of their responses to the CS+, the CS-, and the absence of choice along trials of Experiments 1 and 2, respectively. Performances were not significantly different between experiments (Experiment effect:  $\eta^2=0.07$ ,  $df:1$ ,  $p=0.79$ ). There were neither differences according to the CS (CS effect:  $\eta^2=0.07$ ,  $df:1$ ,  $p=0.79$ ) nor to trial (Trial effect:  $\eta^2=4.80$ ,  $df:11$ ,  $p=0.94$ ). Accordingly, the global dynamics of both experiments did not differ (Interaction Experiment\*CS\*Trial:  $\eta^2=45.37$ ,  $df:34$ ,  $p=0.09$ ). Interestingly, the learning curves of both experiments show that discrimination was apparently reached in trial 5, but that with further trials it was no longer visible, even if the post-tests showed that a significant percentage of bees learned the discrimination. Focusing on individual performances (Tables S2 and S3) did not allow detecting particular strategies followed by bees. For instance, bees categorized as learners for their correct choice of the CS+ in the post-test did not necessarily performed correctly in the last acquisition trial.

Restraining the analysis to the bees whose performances were analyzed in the transfer tests (i.e. bees that chose either the CS+ or the CS- in the respective post-tests) showed again that learning did not differ between experiments (Figs. 6c,d: Experiment effect:  $\eta^2=0.52$ ,  $df:1$ ,  $p=0.47$ ). There were neither significant difference according to the CS effect ( $\eta^2=0.07$ ,  $df:1$ ,  $p=0.79$ ) nor to the trial effect ( $\eta^2=9.44$ ,  $df:11$ ,  $p=0.58$ ). The global dynamics of both experiments was, therefore, not different (Interaction Experiment\*CS\*Trial:  $\eta^2=40.15$ ,  $df:34$ ,  $p=0.22$ ). A similar result was obtained if the analysis was restrained only to learners (Fig. S2a) or to non-learners (Fig. S2b). In the case of learners, no difference in acquisition were found between experiments (Experiment effect:  $\eta^2=0.05$ ,  $df:1$ ,  $p=0.82$ ) but again, discrimination in trial 5 was maximal. There were neither differences according to the CS (CS effect:  $\eta^2=0$ ,  $df:1$ ,  $p=1$ ) nor to trial (Trial effect:  $\eta^2=6.03$ ,  $df:11$ ,  $p=0.87$ ). The global dynamics of both experiments did not differ in the case of learners (Experiment\*CS\*Trial:  $\eta^2=29.16$ ,  $df:34$ ,  $p=0.7$ ). In the case of non-learners, acquisition did also not differ between experiments (Experiment effect:  $\eta^2=0.49$ ,  $df:1$ ,  $p=0.49$ ) and there were neither differences according to the CS (CS effect:  $\eta^2=0.39$ ,  $df:1$ ,  $p=0.53$ ) nor to trial (Trial

effect:  $\eta^2=4.61$ ,  $df:11$ ,  $p=0.95$ ). For non-learners, the global dynamics of both experiments did not differ (Interaction Experiment\*CS\*Trial:  $\eta^2=30.14$ ,  $df:34$ ,  $p=0.66$ ).

These results show that despite the differences in movement freedom and access to active vision between the different contexts of Experiments 1 and 2, performances during training were similar. The acquisition curves did not provide clear evidence of learning, even if in both experiments 57% of bees learned the discrimination, as revealed by the post-tests following training. In any case, the fact that transfer performances differed depending on transfer direction (Fig. 4) indicates that bees may have learned the visual discrimination differently, as revealed by the different sensitivity to a change in context.

## DISCUSSION

We found that acquisition of a simple color discrimination was possible both in a VR and in a real environment. Even if learning curves did not provide clear evidence of discrimination learning, bees learned the task with a similar success in both contexts. There were more bees choosing the CS- in the post-test of Experiment 2 but this increase was at the expense of bees not making any decision in the post-test of Experiment 1; otherwise, the percentage of bees choosing the CS+ (57%) was the same in both experiments (compare figs 3d and 5d). Yet, the transfer of information learned between contexts was asymmetric: bees trained in the VR setup improved their performances when moved to the Y-maze, while transfer from the Y-maze to the VR setup induced inconsistent performances. These results reveal that despite apparent similarities in acquisition, bees may have learned different visual cues in VR and in the Y-maze. They also underline the importance of free movements and active vision while performing a visual discrimination.

Active vision is the capacity to vary the observer's viewpoint to scan the environment and extract better information from it. Motor processes are necessary for achieving this task (Findlay and Gilchrist, 2003) and they are therefore important for visual identification and location of objects in a scene. Motion detection is necessary to interpret the spatiotemporal flow of information that arises when an animal moves within a complex environment and that is detected by its visual system (Borst and Egelhaaf, 1989; Srinivasan et al., 1999; Egelhaaf et al., 2012). Flying insects face the challenge of extracting adaptive information from this continuous visual input occurring

at the high speed imposed by flight performances (Zeil et al., 1996; Egelhaaf and Kern, 2002; Egelhaaf et al., 2012). Bees, flies and other flying insects actively shape the dynamics of the image flow on their eyes, a strategy that facilitates the solving of spatial vision tasks (Zeil, 1993a; b; Zeil et al., 1996; Zeil, 1997; Srinivasan, 1998; Srinivasan and Zhang, 2004). In this context, active vision is crucial to segregate rotational from translational optic flow components. In the case of object detection, motion contrast is particularly relevant at the object borders. When an object protrudes from its background, motion-parallax cues are detectable at its borders, thus differentiating it from the background in terms of retinal speed: the object seems to move faster than the background (or slower, if it is located on a plane behind that of a foreground). Many insect species, including honey bees, use this relative motion to detect objects and to infer their distance (Lehrer et al., 1988; Srinivasan et al., 1989; Lehrer et al., 1990). In bees, relative motion is processed via the L-receptor (“green” receptor) channel, which provides an achromatic pathway for motion detection (Lehrer et al., 1990). In our experiments, the colored stimuli to be learned were projected onto the screens of our setups, thus lying flat on their respective backgrounds. For such stimuli, L-receptor contrast with respect to the background is also important for edge detection and shape discrimination (Lehrer et al., 1990; Lehrer and Srinivasan, 1993; Hempel de Ibarra and Giurfa, 2003). Thus, to better perceive and learn our color discs, bees need to detect their contrasting borders via their L-receptor contrast. Our stimuli provided such a contrast relative to the background, the semi-transparent screen onto which they were projected (blue disc: 0.33; green disc: 0.90; see Table S1), for a bee to scan the stimuli and their edges.

The task to which the bees were trained could therefore rely on two main cues. One is the chromatic contrast (color difference) between the green and the blue disc. These two colors can be easily differentiated by bees as they occupy different distant loci in the bee color space (14.7 COC units; Menzel and Backhaus, 1991). Bees could thus learn the chromatic difference by focusing on the colored area of the discs. The other cue is the L-receptor contrast, which has been shown to contribute to color-stimulus discrimination and detectability (Giurfa et al., 1996; Giurfa et al., 1997). As mentioned above (see also Table S1), it facilitates edge detection and thus a better perception of the stimulus global shape (Hempel de Ibarra and Giurfa, 2003). Typically, bees scan edges actively in order to apprehend stimulus shape (Lehrer et al., 1990). Total stimulus intensity also differed between the blue and the green disc (3363  $\mu\text{Watt}/\text{cm}^2$  and 2950  $\mu\text{Watt}/\text{cm}^2$ , respectively); yet, numerous works have shown that total intensity is not taken into account by bees

during color discrimination tasks (Backhaus, 1991; Chittka et al., 1992; Brandt and Vorobyev, 1997).

In the Y-maze, where bees could freely move and actively scan stimulus edges, discrimination learning could have thus relied on both the chromatic and the L-receptor contrast, which together could contribute to efficient stimulus differentiation. In the VR setup, both cues were in principle available, but bees were limited in their capacity to scan edges due to the tethering situation and the fact that it creates a higher resistance against movements (Catton et al., 2007). The closed-loop situation allowed updating of stimulus position but it may not have induced the same flow of visual information as the one derived from free active scanning. Thus, learning in the VR setup may have relied essentially on chromatic contrast and to a minor extent on achromatic contrast. On the contrary, in the Y-maze, bees may have used actively both cues and to a similar and complementary extent. In both scenarios, learning the discrimination was possible making use of the cues that were available. Yet, transferring them from the Y-maze to the VR setup may have implied a loss or a decrease of L-receptor contrast information, thus resulting in inconsistent performances, depending on the extent to which bees could retrieve both cues in the VR setup. By opposition, the reverse transfer may have implied a gain of this information, thus resulting in an improvement of performance in the Y-maze. This hypothesis could account for the asymmetric transfer of learning observed in our experiments. Experiments testing transfer of visual learning between the VR setup and free-flight conditions could help to gain a clearer understanding of the strategies used by bees in these different scenarios. Yet, achieving a proper transfer between free-flight and tethering conditions is difficult because when a tethered bee is moved to free-flight conditions, it may simply not return to the experimental place if the tethering is considered as a negative experience.

The acquisition curves of both experiments did not provide clear evidence of discrimination learning (Fig. 6) even if the post-tests showed that at least 57% of the bees learned the discrimination (in Experiment 1 it may have been even more given the transfer performance of bees having chosen the CS- in the post-test). It thus seems that bees learned the difference between CS+ and CS- but their performance in the two setups did not reflect such learning. In other words, this deficit was common to both setups and it cannot be ascribed to particular constraints imposed by one of them. A possible explanation for this result could be the amount of reward delivered along the training procedure. As 4  $\mu$ l of sucrose solution were delivered per rewarding trial (see

Materials and Methods) and bees experienced 12 CS+ trials in both setups, they would reach the end of training with an almost filled crop (48  $\mu$ l for 50~60  $\mu$ l of crop capacity; (Núñez, 1966)). This could result in a progressive loss of appetitive motivation and thus in a loss of conditioned responses. The fact that bees reached a high stimulus differentiation on trial 5 but showed afterwards less or no evidence of discrimination agrees with this hypothesis. In the post-test, this effect could have been overcome by the absence of reward following stimulus choice. Diminishing the amount of reward provided per trial, or the number of trials, could help solving this problem.

The limited visual information available in the dark environment in which the experiments were performed cannot fully account for the fact that the level of learning reached at the end of training (57% in both experiments) was lower than that typically obtained in experiments with free-flying bees trained and tested in daylight conditions (80-100%)(Giurfa et al., 1996; Giurfa et al., 1997). In a previous work, we characterized learning of free-flying bees trained to discriminate a green disc from a blue square in a maze similar to the one used in this work and set under similar illumination conditions (Buatois et al., 2017). In this case, bees flying freely between the hive and the maze showed 100% discrimination learning in the post-test and learning curves that were clearly segregated for the CS+ and the CS-. A first difference with the present work resides in the possibility to return to the hive to deliver the food gathered after each conditioning trial, which was not granted in our present work. In our case, bees did not return to the hive after collecting the food in each trial, thus filling progressively more their crop. As mentioned above, this could induce a loss of appetitive motivation. Note that in another conditioning protocol widely used in honey bees, the olfactory conditioning of the proboscis extension response (Bitterman et al., 1983; Giurfa and Sandoz, 2012), harnessed bees learn efficiently to associate an odorant with sucrose reward without having the possibility to return to the hive between trials. Thus, the loss of social contact is probably not the main factor affecting the bees' performance during training. Instead, the loss of appetitive motivation may be more important for the manifestation of learning.

Does tethering just affect the possibility of active vision or may it additionally induce undesirable levels of stress in the bees trained and tested in the VR setup, responsible for deficits in performances? Under VR conditions, 56.6 % of the bees learned the discrimination, i.e. chose the CS+ in the post test. Notably, 100 % of those that chose the CS- in the same conditions, chose the CS+ when granted with movement freedom. Thus, tethering may affect the expression of learning but not learning in itself. Furthermore, in the olfactory conditioning of the proboscis

extension response (Bitterman et al., 1983; Giurfa and Sandoz, 2012), which does not require the use of active vision, total immobilization does not affect at all the capacity to learn odor-sucrose associations. In a visual variant of this protocol in which visual stimuli paired with sucrose solution are used to condition the proboscis extension response of harnessed bees, learning is facilitated when movement is added to the visual stimulation (Balamurali et al., 2015). Thus, when active vision is available it may help overcoming the potential stress of the harnessing situation. If bees are motivated enough to obtain food, which may be controlled by prolonging the starvation period before training and testing and by delivering minute rewards during training (Matsumoto et al., 2012), they will learn appetitive associations like the ones conditioned in the VR setup.

Several aspects of the VR setup may be improved to overcome some of the limitations mentioned above. For instance, the updating of the visual panorama following the bee's decisions could be made more realistic. Indeed, only rotational stimulus movement was allowed but no stimulus looming/receding was provided, thus suppressing a translational component that may be essential for learning in bees. In the absence of a depth dimension for stimulus variation, efferent copies generated by the bee's motor decisions on the treadmill were only collated incompletely with the reafferent sensory input that resulted from its movements (von Holst and Mittelstaedt, 1950; von Holst, 1954; Webb, 2004). Therefore, the bee would never attain a desired goal, thus making efferent copies useless to predict the effect of its actions. This situation could create a situation akin to 'frustration' or 'learned helplessness', conspiring against learning success (Yang et al., 2013; de Brito Sanchez et al., 2015; Dinges et al., 2017). We are consequently improving our setup to include looming/receding cues in correspondence with the bee's forward or backward movements.

Training and testing tethered bees with visual discriminations in a VR environment should solve the problem of coupling behavioral analyses with invasive recordings of neural activity in the bee brain (Schultheiss et al., 2017). This was not possible until now because visual behavior was only accessible in free flying bees. Although several attempts have been done to develop visual PER conditioning (Avarguès-Weber and Mota, 2016), our results provide a more realistic and at the same time controlled scenario for studying such learning at multiple levels, from a behavioral to a molecular level. Thus, at least for the simple task trained in our work, the goal of accessing visual-neuropile activity, for instance, via electrophysiological procedures, is realistic. Multielectrodes that record local-field potentials could be implanted in visual areas such as the

medulla, the lobula or the central complex, or in central integration regions such as the mushroom bodies to characterize the neural signature of this task. Future challenges should focus also on developing higher-order learning protocols to reproduce in a controlled VR environment the cognitive feats of bees which have firmly established their reputation as a model for cognition.

## **ACKNOWLEDGEMENTS**

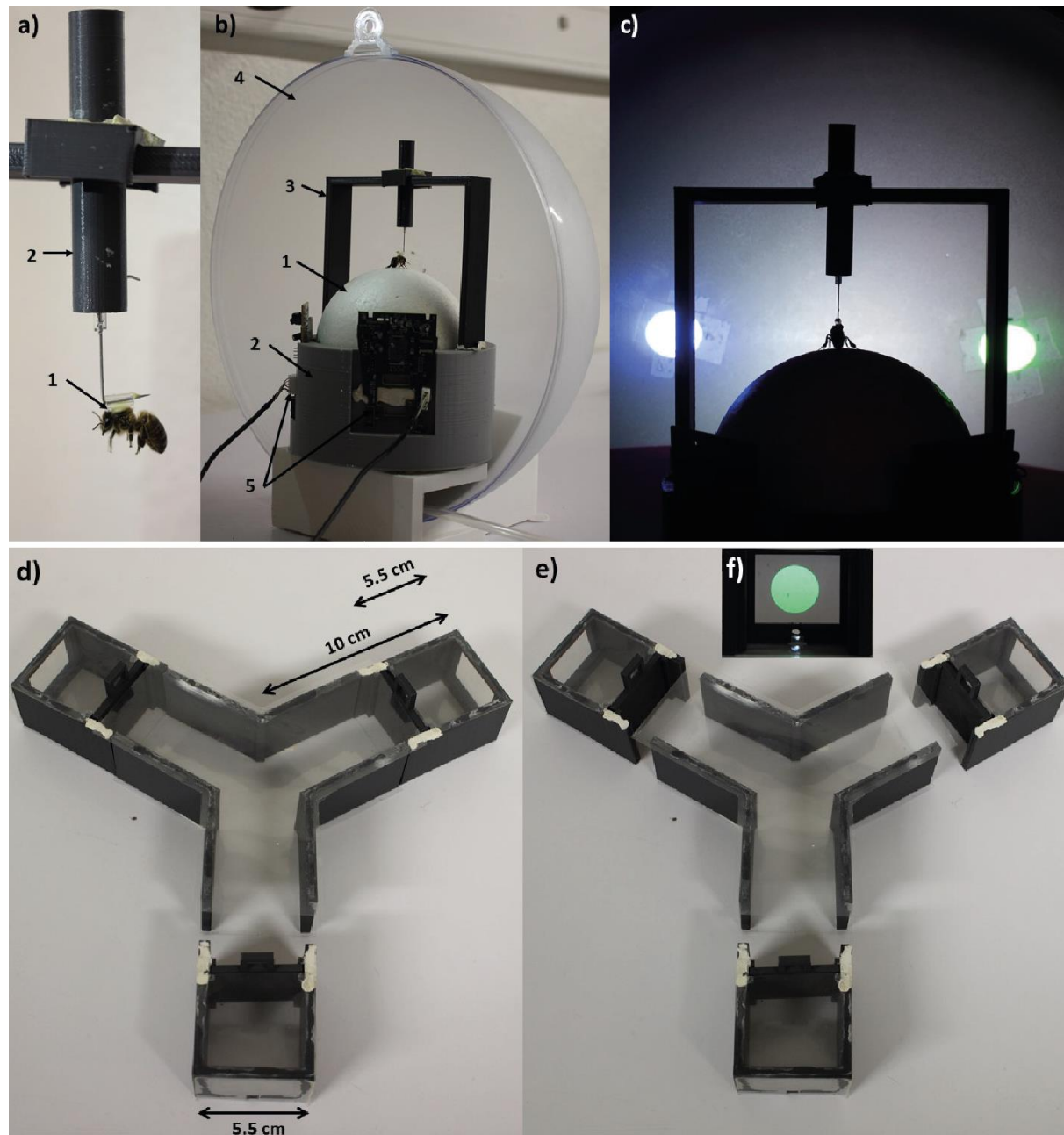
Our work was supported by the Human Frontier Science Program (grant RGP0022/2014), the French National Research Agency (grant ANR-13-BSV4-0004-0), the French National Research Center (CNRS) and the University Paul Sabatier of Toulouse. Lucie Hotier provided help in terms of beekeeping activities. Special thanks are due to Sébastien Weber for his technical assistance in designing and implementing the software for visual stimulus control and for his regular support throughout this project.

## **AUTHOR CONTRIBUTIONS**

A.B., A.A.-W. and M.G. designed the experiments. The experiments were conducted by A.B. and C.F. A.B. analyzed the results and prepared figures and tables. A.B., A.A.-W., P.S. and M.G. wrote the manuscript. All authors reviewed and approved the final manuscript.



Figure 1

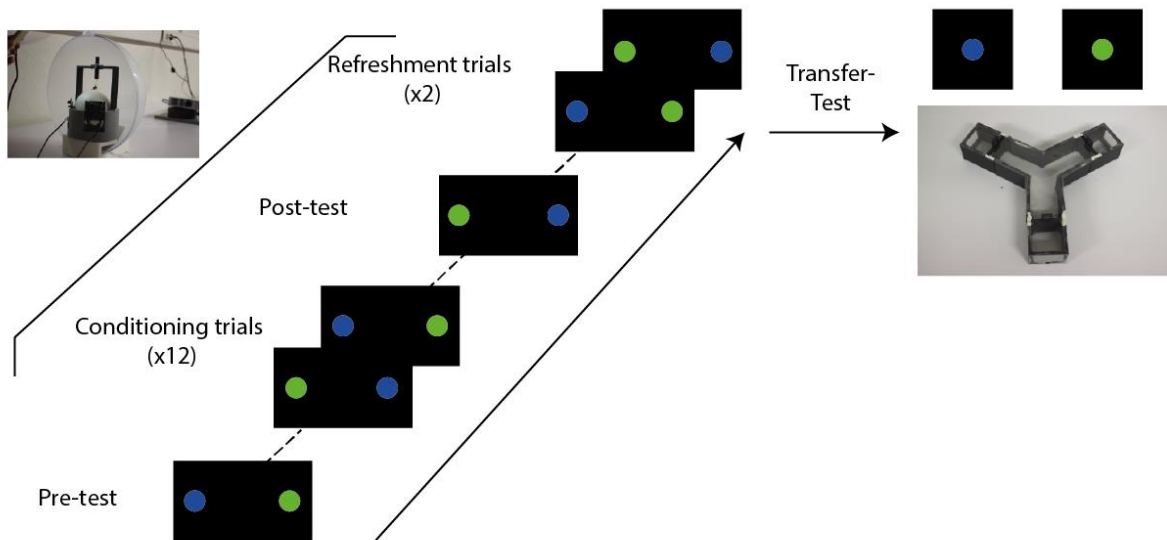


**Figure 1. Experimental setups. The virtual reality setup (a-c) and the Y-maze (d-f).** (a) A bee tethered by the thorax by means of a vertical attachment (1) made of a custom-built tag (1) and an

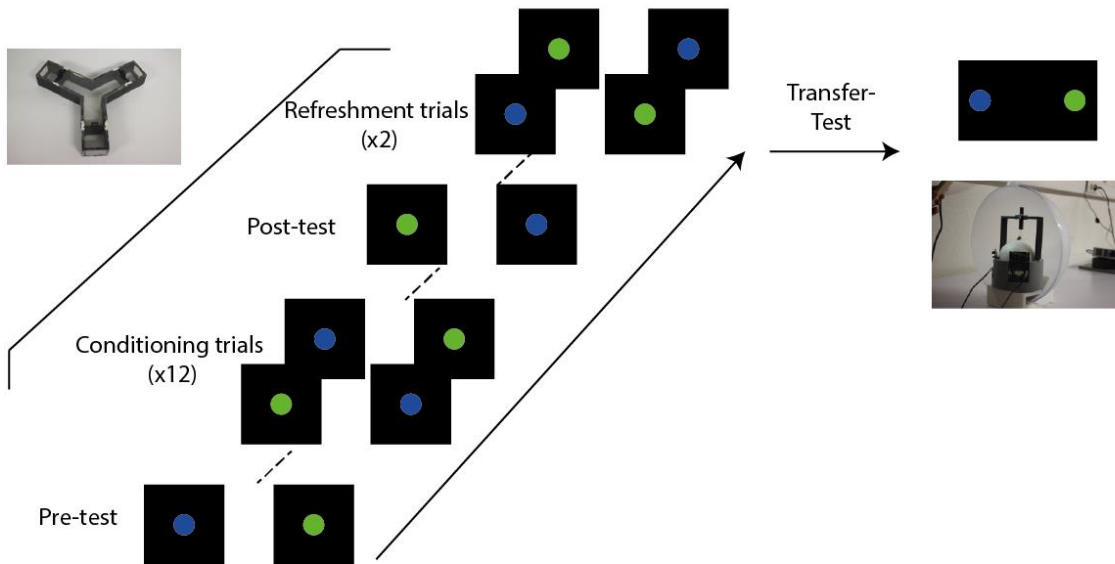
L-shaped metal piece glued to the thorax. The metal piece was enclosed in a plastic cylinder (2), which allowed its vertical displacement and thus the accommodation of the bee on the surface of the treadmill. (2). **(b)** Global view of the virtual reality system. The polystyrene ball (1) floated on a constant airflow provided at the basis of a ball support (2). The tethered bee was placed on the ball thanks to a holding support (3). The apparatus is placed behind a semi-spherical opaque screen (4) on which visual stimuli were projected. Two optic-mouse sensors (5) were placed on the ball support, at  $90^\circ$  of each other to record the ball movements. The setup translates the movements of the walking bee into rotations of the ball. **(c)** Front view of the setup during a conditioning trial. The tethered bee walking stationary faced the two-colored discs presented at  $-45^\circ$  and  $+45^\circ$  of its longitudinal axis. **(d)** Top view of the Y-maze. Each arm was connected to a removable box with a sliding door, which allowed displacing an enclosed bee from an arm to another. Arms were 10 cm in length, 4 cm in height and 5.5 cm in width. Each box had a length of 5.5 cm. **(e)** Top view of the maze showing the disconnected boxes and how they could be interchanged between arms of the maze **(f)** Front view of the inside of a box. A color disc was projected by the video projector onto the paper screen placed at the end of each box.

Figure 2

## a) Experiment 1: from virtual reality to a real-world Y-maze



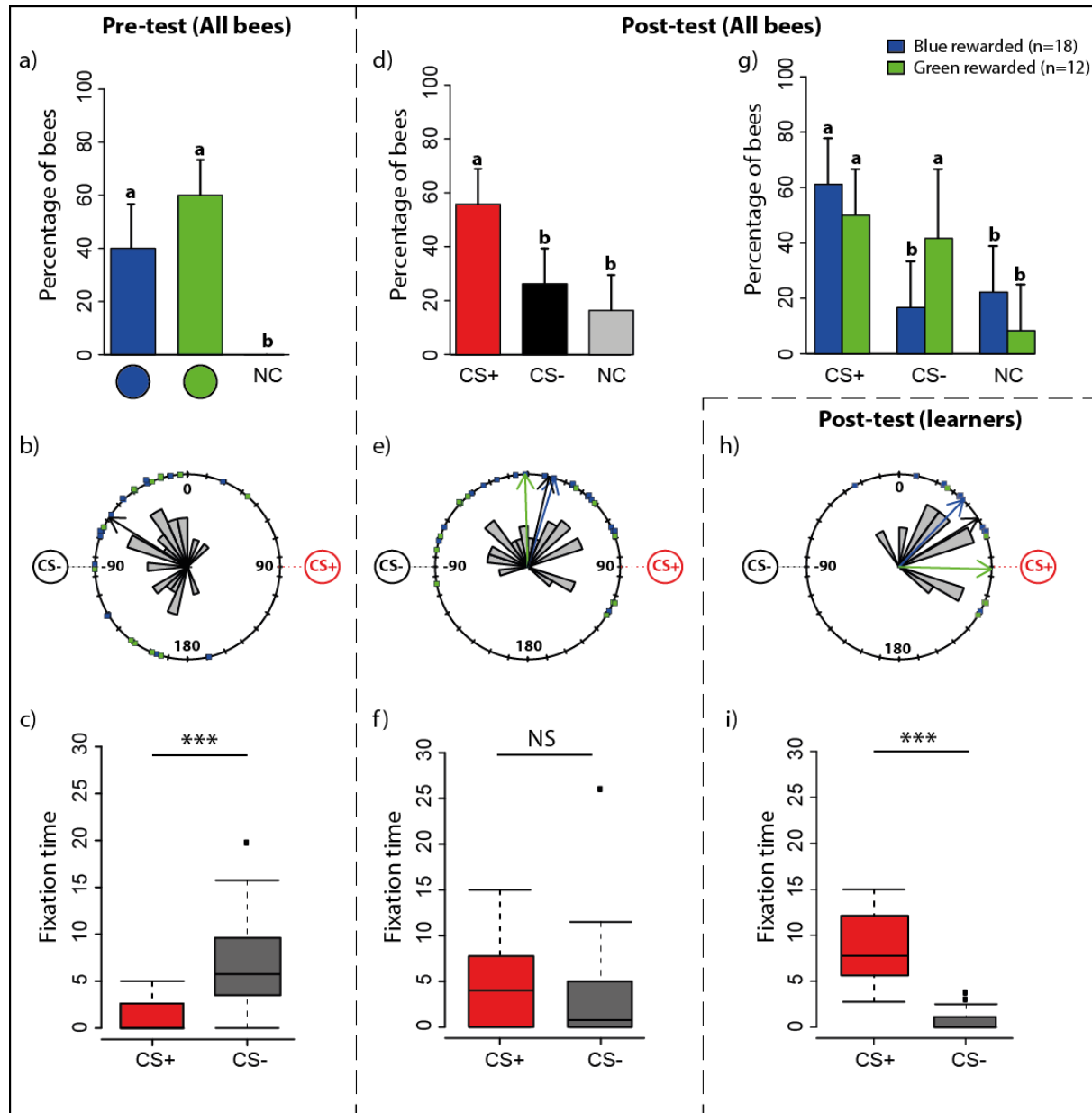
## b) Experiment 2: from a real-world Y-maze to virtual reality



**Figure 2. Experimental schedule at the VR setup (a) and at the Y-maze (b).** (a) Experimental sequence of Experiment 1. Bees started with a pre-test in which both colored discs were shown in the absence of reinforcement to check for spontaneous preferences and determine the CS+ (the

color less preferred) and the CS- (the color more preferred) for the conditioning procedure. The pre-test was followed by 12 conditioning trials in which both colored discs were shown simultaneously and associated with sucrose solution (CS+) or quinine solution (CS-). After conditioning, a post-test in which both stimuli were shown simultaneously without reinforcement allowed to determine whether bees learned the visual discrimination. Two refreshment trials with reinforcement were performed after the post-test and before the transfer to the Y-maze to avoid extinction of the learned information. Pre-test, conditioning and post-test were performed in closed-loop conditions, i.e. the movements of the bee controlled the visual cues displayed on the screen in front of it. After the post-test, bees were transferred to the Y-maze in which both stimuli were presented in different arms of the maze. Bees were tested for transfer of discrimination learning to this new context. **(b)** Experimental sequence of Experiment 2. The schedule was similar to that of Experiment 1 with the difference that pre-test, training and post-test took place in the Y-maze where the bee movements were not constrained. Colored discs were presented on the paper walls at the end of the maze arms. After the post-test, bees were transferred to the VR setup where they could see both colored discs simultaneously.

Figure 3

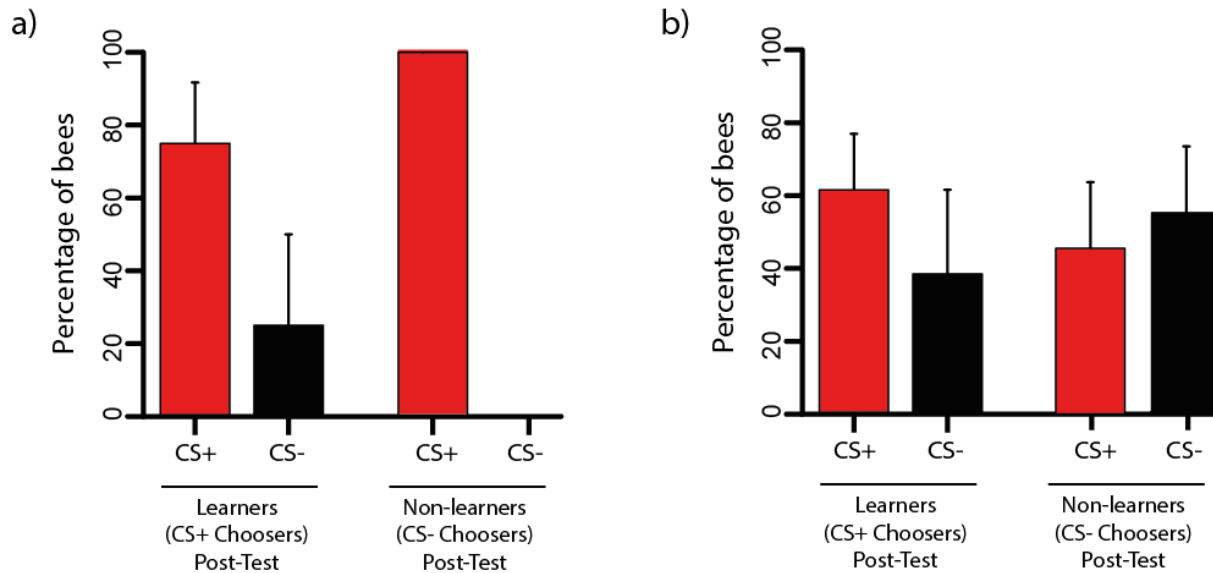


**Figure 3. Experiment 1: spontaneous preferences (pre-test) and learning-induced preferences (post-test) at the VR setup** (a) Percentage of bees (n=30) choosing first either the green disc, the blue disc or not making a choice (NC) during the pre-test. The 95% confidence interval is shown in each case. (b) Circular distribution of orientation vectors of bees during the pre-test. Note that

while the stimuli appeared at  $45^\circ$  to the left and right of the main axis of the bee body, they appear at  $90^\circ$  to the left and right in these circular plots because  $2^\circ$  of ball rotation corresponded to  $1^\circ$  of stimulus rotation. Thus, a vector pointing towards  $90^\circ$  represents a bee oriented towards a visual stimulus located at  $45^\circ$  to the right of the central axis of the bee body. The black arrow inside the distribution is the mean resultant vector of the group of bees ( $n=30$ ,  $p<0.0001$ ). The blue arrow shows the mean resultant vector of bees preferring the blue disc ( $n=12$ ,  $p<0.0001$ ); the green arrow shows the same for bees preferring the green disc ( $n = 18$ ,  $p<0.0001$ ) (here blue and green arrows fully coincide with the black arrow and cannot be seen). Although at this stage of the experiment there is neither a CS+ nor a CS-, we use these terms to indicate the stimuli that will become CS+ and CS- during the subsequent training. **(c)** Fixation time (sec; median, quartiles and outliers) of the CS+ (red boxplot) and of the CS- (grey boxplot) during the pre-test. The terms CS+ and CS- are used here in the sense indicated above. **(d)** Percentage of bees ( $n=30$ ) choosing first either the CS+, the CS- disc or not making a choice (NC) during the post-test. The 95% confidence interval is shown in each case. **(e)** Circular distribution of orientation vectors of bees during the post-test. The black arrow inside the distribution is the mean resultant vector of the entire group of bees ( $n=30$ ,  $p=0.18$ ). The blue arrow shows the mean resultant vector of bees rewarded on the blue disc ( $n=18$ ,  $p=0.11$ ); the green arrow shows the same for bees rewarded on the green disc ( $n = 12$ ,  $p=0.51$ ) **(f)** Fixation time (sec; median, quartiles and outliers) of the CS+ (red boxplot) and of the CS- (grey boxplot) during the post-test. **(g)** Percentage of bees choosing first either the CS+, the CS- disc or not making a choice (NC) according to the color onto which they were rewarded. The 95% confidence interval is shown in each case. **(h)** Circular distribution of orientation vectors of learners (i.e. bees that chose first the CS+ in the post-test) during the post-test. The black arrow inside the distribution is the mean resultant vector of the entire groups of learners ( $n=17$ ,  $<0.0001$ ). The blue arrow shows the mean resultant vector of learners rewarded on the blue disc ( $n=11$ ,  $p=0.003$ ); the green arrow shows the same for learners rewarded on the green disc ( $n = 6$ ,  $p=0.002$ ) **(i)** Fixation time (sec; median, quartiles and outliers) of the CS+ (red boxplot) and of the CS- (grey boxplot) by learners during the post-test. **(a)**, **(d)**, **(g)**: different lower-case letters above bars indicate significant differences ( $p<0.05$ ). The position of the stimuli was varied between bees during the pre-test; data were normalized to display always the CS + on the left side and the CS- on the right side. **(c)**, **(f)** **(i)**, bees were considered as fixating a stimulus when they were at  $90^\circ \pm 20^\circ$  for the CS+ and  $-90^\circ \pm 20^\circ$  for the CS-. \*:  $p < 0.05$ ; \*\*:  $p < 0.001$ ; NS: non-significant.



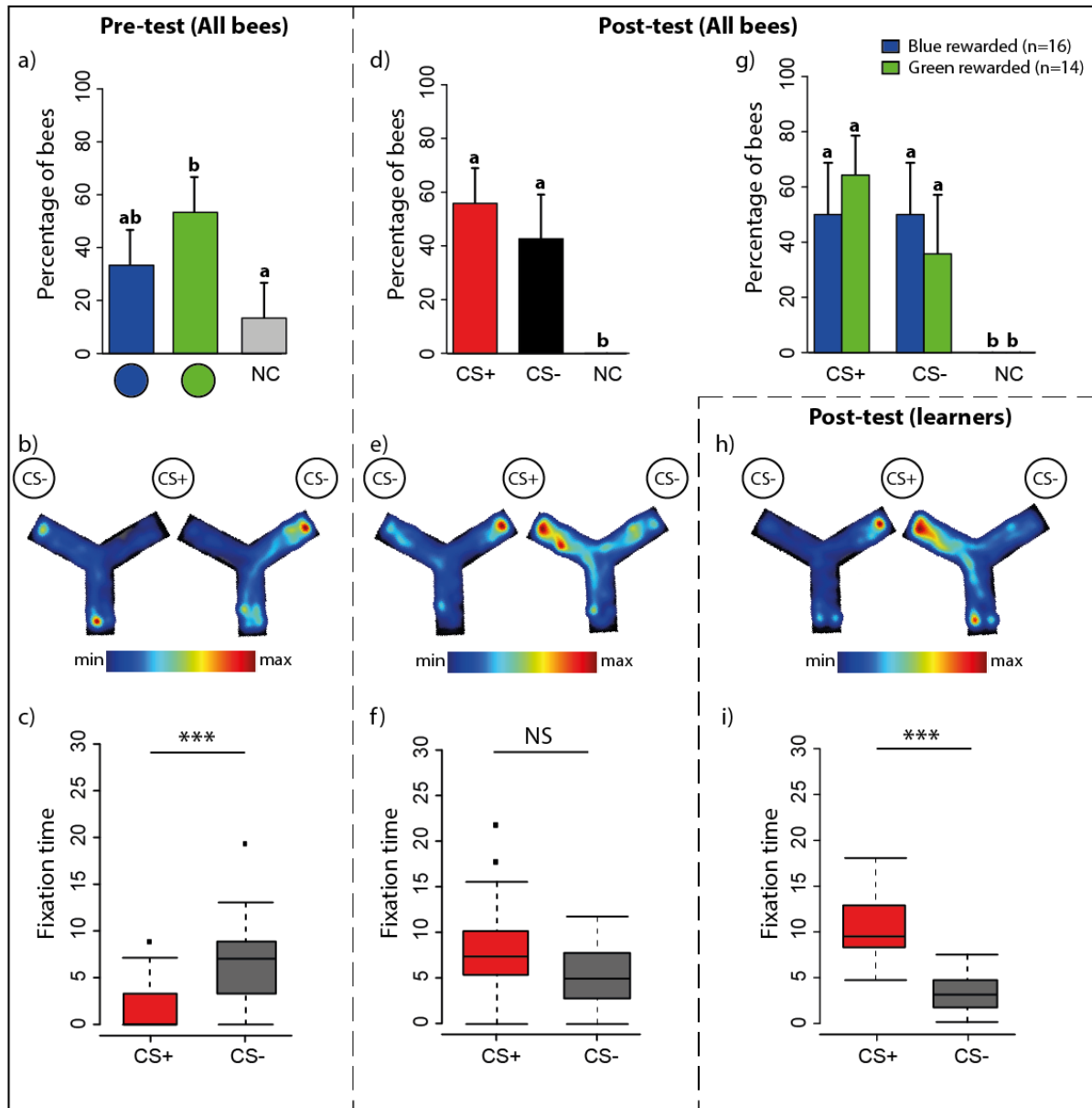
Figure 4



**Figure 4. Performance during the transfer test of Experiments 1 and 2** (a) Transfer test from Experiment 1. Bees trained in the VR setup were tested in the Y-maze. Bees having chosen the CS+ (learners;  $n=12$ ) or the CS- (non-learners;  $n=9$ ) in the post-test were considered for the analysis. The bars represent their choice of the CS+ or the CS- during the transfer test in terms of the % of bees within each choice category. The change in performance from the post-test to the transfer test was highly significant ( $p<0.01$ ) as more bees chose the CS+ in the transfer test. (b) Transfer test from Experiment 2. Bees trained in the Y-maze were tested in the VR setup. Bees having chosen the CS+ (learners;  $n=13$ ) or the CS- (non-learners;  $n=11$ ) in the post-test were considered for the analysis. The bars represent their choice of the CS+ or the CS- during the transfer test in terms of the percentage of bees within each choice category. There was no significant change of performance between the post-test and the transfer test as both the percentage of bees choosing the CS+ and that of bees choosing the CS- were halved. The 95% confidence interval is shown in each case.



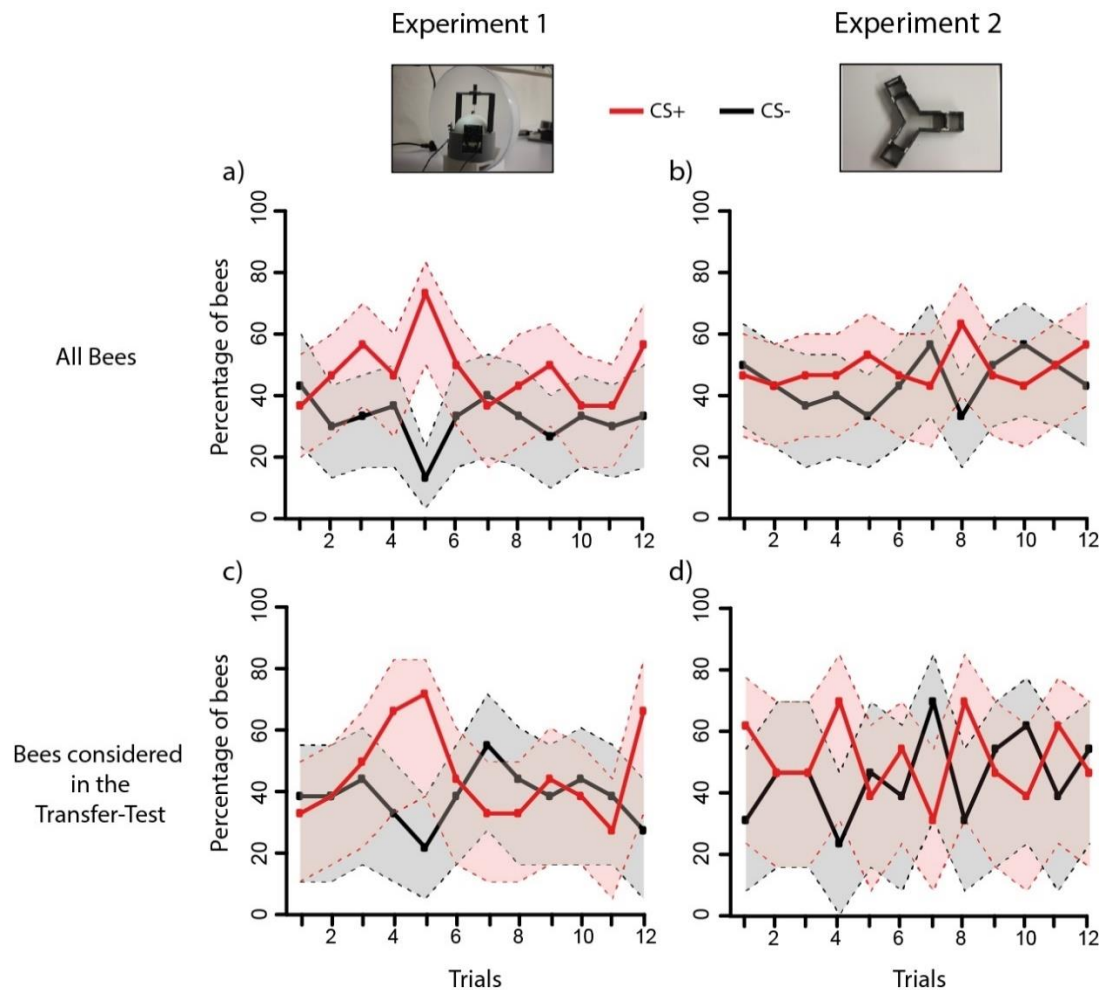
Figure 5



**Figure 5. Experiment 2: spontaneous preferences (pre-test) and learning-induced preferences (post-test) at the Y-maze.** (a) Percentage of bees (n=30) either choosing first the green disc, the blue disc or not making a choice (NC) during the pre-test in the maze. (b) Pooled heatmap representing the normalized mean time spent within the maze during the pre-test. Although at this stage of the experiment there is neither a CS+ nor a CS-, we use these terms to indicate the stimuli that will become CS+ and CS- during the subsequent training. The stimulus that was more attractive

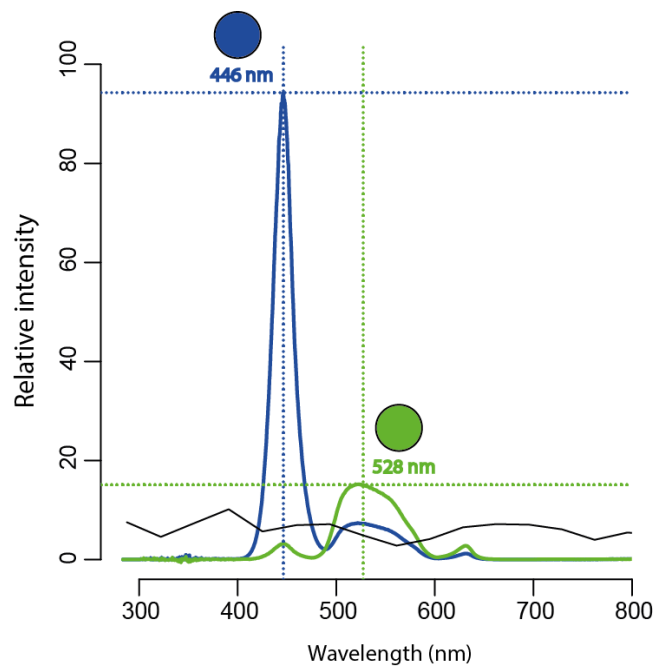
to bees became the CS- and the less attractive became the CS+. **(c)** Time spent (sec; median, quartiles and outliers) in the arm of the CS+ (red boxplot) and of the CS- (grey boxplot) during the pre-test. **(d)** Percentage of bees (n=30) choosing first either the CS+, the CS- disc or not making a choice (NC) during the post-test. The 95% confidence interval is shown in each case. **(e)** Pooled heatmap representing the normalized mean time spent within the maze during the post-test. The position of the CS+ and CS- is indicated. **(f)** Time spent (sec; median, quartiles and outliers) in the arm of the CS+ (red boxplot) and of the CS- (grey boxplot) during the post-test. **(g)** Percentage of bees choosing first either the CS+, the CS- disc or not making a choice (NC) according to the color onto which they were rewarded. The 95% confidence interval is shown in each case. **(h)** Pooled heatmap representing the normalized mean time spent by learners within the maze during the post-test (n=17). **(i)** Time spent (sec; median, quartiles and outliers) in the arm of the CS+ (red boxplot) and of the CS- (grey boxplot) by learners (n=17) during the post-test. **(a), (d), (g):** different lower-case letters above bars indicate significant differences ( $p < 0.05$ ). **(b), (e), (h):** The position of the CS+ and the CS- was varied during training; the left maze corresponds to a trial in which the CS+ was presented on the right and the CS- on the left; the right maze shows the reversed situation. **(c), (f), (i),** \*:  $p < 0.05$ ; \*\*:  $p < 0.001$ ; NS: non-significant.

Figure 6

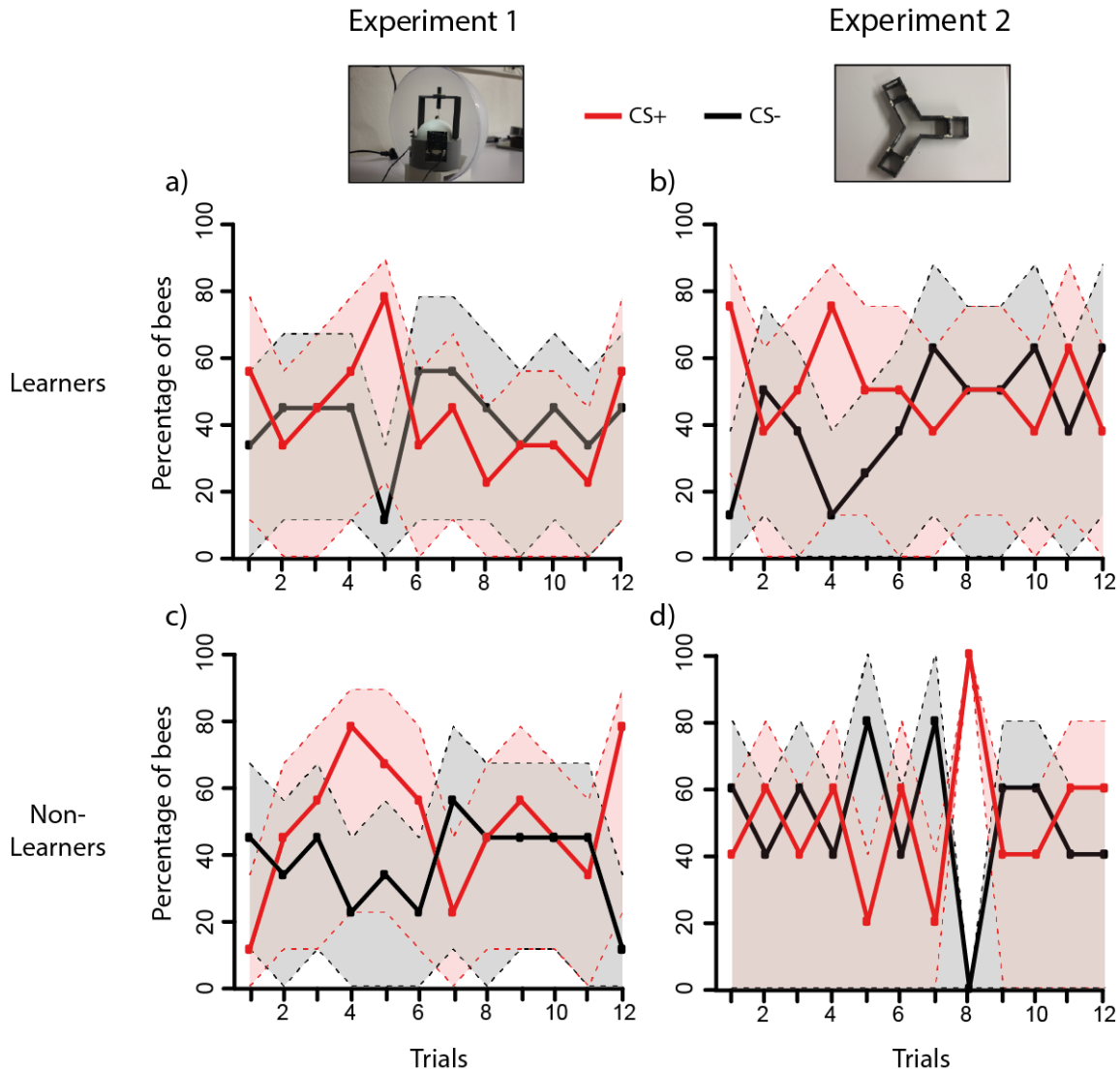


**Figure 6. Acquisition performance of bees during Experiments 1 and 2.** The graphs show the proportion of bees choosing first the CS+ (red curve) or the CS- (black curve) during the twelve conditioning trials. **(a)** Acquisition performance of all bees trained in Experiment 1 in the VR setup ( $n=30$ ); **(b)** acquisition performance of all bees trained in Experiment 2 in the Y-maze ( $n=30$ ). **(c)** Same as in **(a)** but only for bees that made a choice in the post-test and whose performances were consequently analyzed in the transfer test ( $n = 21$ ; see Fig. 4a). **(d)** Same as in **(b)** but only for bees that made a choice in the post-test and whose performances were analyzed in the transfer test ( $n = 24$ ; see Fig. 4b). The grey and pink areas around the curves represent the 95% confidence interval of CS+ and CS- choices, respectively.

## Supplementary information



**Figure S1.** Spectral curves of the green (dominant wavelength 528 nm) and the blue disc (446 nm). The curve of the black background surrounding the colored discs is also shown (black curve). This curve slightly differs from a flat curve with a null intensity due to minimum amount of light produced by the videoprojector.



**Figure S2. Acquisition performance of bees (learners and non-learners) during Experiments 1 and 2.** The graphs show the proportion of bees choosing first the CS+ (red curve) or the CS- (black curve) during the twelve conditioning trials. **(a)** Acquisition performance of bees categorized as learners in the post-test of Experiment 1 in the VR setup (i.e. bees that chose the CS+ in the post-test of Experiment 1); **(b)** acquisition performance of bees categorized as learners in the post-test of Experiment 2 in the Y-maze (i.e. bees that chose the CS+ in the post-test of Experiment 2). **(c)** Same as in **(a)** but for bees categorized as non-learners (i.e. bees that chose the CS- in the post-test of Experiment 1). **(d)** Same as in **(b)** but for bees categorized as non-learners (i.e. bees that chose the CS- in the post-test of Experiment 2). Grey and pink areas around the curves represent the 95% confidence interval of CS+ and CS- choices, respectively.

**Table S1:** Chromatic and achromatic properties of the trained stimuli.

Color stimulus	Color Distance from the background (COC units)	Chromatic Contrast to the alternative stimulus (COC units)	Receptor Contrast (absorbed quanta relative to the background)			Intensity (sum of absorbed quanta relative to the background)
			UV	B	G	
Background (Plastic screen)	--	--	1	1	1	3
Blue	1.75	14.7 (to green)	0.02	0.64	0.33	0.99
Green	7.1	14.7 (to blue)	0.002	0.10	0.90	1.002

Contrasts are evaluated with respect to the black background. The illumination light considered was the one provided by the videoprojector lamp.

Receptor-specific contrasts, i.e. the relative number of absorbed quanta  $q$  with respect to the black background, were calculated as:

$$q_i = \frac{\int_0^{\infty} I(\lambda) R(\lambda) S_i(\lambda) d\lambda}{\int_0^{\infty} I(\lambda) B(\lambda) S_i(\lambda) d\lambda}, \quad i = uv, \text{ blue, green receptor}; \quad (1)$$

with  $I(\lambda)$  being the intensity distribution of the illuminating light of the videoprojector,  $R(\lambda)$  the spectral curve of the stimulus considered (blue or green disc; see Fig. S1),  $B(\lambda)$  the spectral curve

of the black background (see Fig. S1) and  $S_i(\lambda)$  the spectral sensitivity of the receptor with index  $i$  (Menzel and Backhaus, 1991).

Intensity contrast of a stimulus against the black background was calculated as the sum of the absorbed quanta in the three types of photoreceptors, relative to the background (see Eq. 1).

To quantify chromatic differences, the color opponent coding space proposed specifically for the honey bee (Backhaus, 1991) was used. In such a space, chromatic coordinates A and B of the stimuli were determined as:

$$A = \sum_{i=u,b,g} a_i \frac{q_i}{q_{i+1}}, \quad B = \sum_{i=u,b,g} b_i \frac{q_i}{q_{i+1}} \quad (2)$$

with  $a_i = \{-9.86, 7.70, 2.16\}$  and  $b_i = \{-5.17, +20.25, -15.08\}$ ;  $i = uv$ , blue, green receptor.

The perceptual colour distance  $D$  between two stimuli  $S_1$  and  $S_2$  was calculated as the sum of the absolute differences in chromatic coordinates A and B (city block metric; Backhaus, 1991):

$$D(S_1, S_2) = |A_{S1} - A_{S2}| + |B_{S1} - B_{S2}| \quad (3)$$

Chromatic contrast is defined as the perceptual color distance  $D$  of a stimulus ( $S$ ) to the black background (Back.). Because the background coordinates in the color space used are (0,0) (Backhaus, 1991), chromatic contrast  $D$  is calculated as:

$$D(S, \text{Back.}) = |A_S| + |B_S|$$

**Table S2.** Individual performances during the acquisition phase of Experiment 1.

	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	Trial 11	Trial 12
Bee1	CS+	CS+	CS+	CS+	CS+	CS-	CS-	NC	NC	NC	CS-	CS-
Bee2	CS-	CS+	CS-	NC	CS+	CS+	NC	NC	NC	NC	CS+	CS-
Bee3	CS-	CS+	CS-	CS-	CS+	CS-	CS-	CS-	CS-	CS+	CS+	CS+
Bee4	CS-	CS-	CS+	CS+	CS-	NC	NC	CS+	CS+	CS-	NC	CS+
Bee5	CS+	NC	NC	CS+	CS+	CS+	CS+	NC	NC	CS+	NC	CS+
Bee6	CS-	CS+	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Bee7	CS-	NC	CS+	CS+	CS-	CS+	NC	CS+	CS+	CS-	CS-	CS+
Bee8	CS+	CS+	CS+	NC	NC	CS+	CS+	CS+	CS+	CS+	CS+	CS+
Bee9	CS+	CS+	CS+	CS-	CS+	CS+	CS-	CS-	CS+	NC	CS+	NC
Bee10	CS+	CS-	CS-	CS-	CS-	CS-	CS+	NC	CS+	NC	NC	CS-
Bee11	NC	CS-	CS-	CS+	CS+	CS+	CS+	CS+	CS+	CS+	NC	CS+
Bee12	CS+	CS+	CS+	NC	CS+	CS+	NC	CS-	NC	CS+	CS+	CS-
Bee13	CS-	CS+	CS+	CS-	CS+	CS-	CS+	NC	NC	NC	CS-	CS-
Bee14	CS+	CS-	CS-	CS+	CS+	CS-	CS+	CS-	CS-	CS-	CS-	CS+
Bee15	CS-	CS-	CS+	CS+	CS+	CS+	CS-	CS-	CS-	CS-	NC	CS+
Bee16	NC	NC	CS+	CS-	CS+	CS+	CS+	CS+	CS+	CS-	CS-	CS+
Bee17	NC	CS+	CS-	CS+	CS+	CS+	NC	NC	CS+	CS-	CS-	CS+
Bee18	CS+	CS-	CS+	CS-	CS+	CS+	CS+	CS+	CS-	CS+	CS+	CS+
Bee19	CS+	CS-	CS+	CS-	CS+	CS-	CS+	CS+	CS+	CS-	CS-	CS+
Bee20	CS+	NC	NC	CS+	CS+	CS-	CS-	CS-	CS+	CS+	CS+	CS+
Bee21	NC	NC	CS-	CS+	CS+	CS+	CS+	CS+	CS+	CS-	CS+	CS+
Bee22	CS-	CS-	CS+	CS+	CS-	CS+	CS-	CS+	CS+	NC	CS-	CS+
Bee23	CS-	NC	CS+	CS+	CS+	CS+	CS-	CS-	CS-	CS+	CS+	CS+
Bee24	CS-	CS+	CS+	CS+	NC	NC	CS-	CS-	NC	CS-	CS-	CS-
Bee25	NC	NC	CS-	NC	NC	NC	NC	CS+	CS+	NC	NC	NC
Bee26	CS-	CS+	CS+	CS-	CS+	CS-	CS-	CS+	CS-	NC	NC	CS-
Bee27	CS+	CS-	CS-	CS-	CS+	CS-	CS-	CS-	CS-	CS+	NC	CS-
Bee28	CS-	CS+	CS+	CS-	CS+	CS-	CS-	CS-	CS-	CS+	CS+	NC
Bee29	NC	CS+	CS-	CS+	CS+	CS+	CS-	CS+	CS+	CS+	NC	CS-
Bee30	CS-	CS+	CS+	CS-	CS+	NC	CS+	CS+	CS+	CS-	CS+	CS-
CS+	36,67%	46,67%	56,67%	46,67%	73,33%	50,00%	36,67%	43,33%	50,00%	36,67%	36,67%	53,33%
CS-	43,33%	30,00%	33,33%	36,67%	13,33%	33,33%	40,00%	33,33%	26,67%	33,33%	30,00%	33,33%



The data correspond to the learning curves (CS+ vs CS- choices) shown in Fig. 6a. For each trial, the choice of the bee in the VR setup was recorded (see Materials and Methods for details) and categorized as CS+ choice (in green), CS- choice (in red) or no choice (NC, in grey). Each row corresponds to an individual bee, thus showing the succession of choices made by this individual. The bottom rows show the percentages of CS+ and CS- choices obtained from these performances (see Fig. 6a). Learners (bees that chose the CS+ in the post-test of Experiment 1) are highlighted in yellow.

**Table S3:** Individual performances during the acquisition phase of Experiment 2.

	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	Trial 11	Trial 12
Bee1	CS-	CS-	CS-	CS+	CS-	CS+	CS-	CS+	CS+	CS-	CS+	CS+
Bee2	CS+	CS+	CS+	CS+	CS+	CS-	CS-	CS+	CS+	CS+	CS-	CS+
Bee3	CS+	CS+	CS-	CS+	CS+	CS-	CS-	CS-	CS+	CS-	CS+	CS+
Bee4	CS+	NC	NC	NC	NC	NC	CS+	CS-	CS+	CS-	CS-	CS-
Bee5	CS+	CS-	CS+	CS-	CS-	CS+	CS+	CS-	CS+	CS-	CS-	CS+
Bee6	CS+	CS+	CS+	CS-	CS+	CS-	CS-	CS+	CS-	CS+	CS+	CS+
Bee7	CS+	CS-	CS+	CS-	NC	CS+	CS-	CS+	CS-	CS-	CS+	CS-
Bee8	CS-	CS-	CS+	CS-	CS+	CS+	CS+	CS+	CS-	CS+	CS-	CS+
Bee9	CS-	CS-	CS+	CS-	CS+	CS-	CS-	CS+	CS-	CS+	CS-	CS-
Bee10	CS-	CS-	NC	CS-	CS+	CS+	CS-	CS+	CS-	CS-	CS+	CS-
Bee11	CS-	CS-	CS-	CS+	CS-	CS+	CS+	CS-	CS+	CS-	CS-	CS-
Bee12	NC	CS-	CS+	CS+	CS+	CS-	CS-	CS+	CS-	CS+	CS-	CS-
Bee13	CS-	CS-	CS-	CS+	CS-	CS+	CS-	CS+	CS-	CS-	CS-	CS-
Bee14	CS+	CS+	CS+	CS+	CS+	CS+	CS-	CS-	CS-	CS+	CS+	CS+
Bee15	CS-	CS+	CS+	CS-	CS-	CS-	CS-	CS-	CS-	CS-	CS-	CS-
Bee16	CS-	CS-	CS-	CS-	CS-	CS-	CS-	CS+	CS-	CS+	CS+	CS+
Bee17	CS-	CS-	CS-	CS+	CS+	CS-	CS-	NC	CS+	CS-	CS-	CS+
Bee18	CS+	NC	NC	NC	NC	NC	CS+	CS+	CS+	CS-	CS+	CS-
Bee19	CS+	NC	NC	NC	CS+	CS-	CS+	CS+	CS+	CS+	CS-	CS+
Bee20	CS-	CS-	CS-	CS+	CS+	CS-	CS+	CS-	CS-	CS+	CS-	CS+
Bee21	CS-	CS+	CS-	CS-	CS+	CS+	CS-	CS+	CS-	CS-	CS+	CS+
Bee22	CS+	NC	NC	NC	NC	CS+	CS+	CS+	NC	CS-	CS-	CS+
Bee23	CS-	CS+	CS+	CS-	CS+	CS+	CS+	CS-	CS+	CS-	CS+	CS-
Bee24	CS+	CS+	CS-	CS-	CS-	CS-	CS-	CS+	CS-	CS-	CS-	CS-
Bee25	CS-	CS+	CS+	CS+	CS-	CS+	CS+	CS+	CS+	CS+	CS+	CS+
Bee26	CS+	CS-	CS-	CS+	CS-	CS-	CS+	CS-	CS-	CS+	CS-	CS+
Bee27	CS+	CS+	CS+	CS+	CS+	CS+	CS-	CS+	CS+	CS-	CS+	CS-
Bee28	CS-	CS+	CS+	CS-	CS+	NC	CS+	CS+	CS+	CS+	CS+	CS-
Bee29	CS-	CS+	CS+	CS+	CS+	CS-	CS+	CS+	CS-	CS+	CS+	CS+
Bee30	CS+	CS+	CS-	CS+	CS-	CS+	CS-	CS-	CS+	CS-	CS+	CS+
CS+	46,67%	43,33%	46,67%	46,67%	53,33%	46,67%	43,33%	63,33%	46,67%	43,33%	50,00%	56,67%
CS-	50,00%	43,33%	36,67%	40,00%	33,33%	43,33%	56,67%	33,33%	50,00%	56,67%	50,00%	43,33%

The data correspond to the learning curves (CS+ vs CS- choices) shown in Fig. 6b. For each trial, the choice of the bee in the Y-maze was recorded (see Materials and Methods for details) and categorized as CS+ choice (in green), CS- choice (in red) or no choice (NC, in grey). Each row corresponds to an individual bee, thus showing the succession of choices made by this individual. The bottom rows show the percentages of CS+ and CS- choices obtained from these performances (see Fig. 6b). Learners (bees that chose the CS+ in the post-test of Experiment 2) are highlighted in yellow.

## REFERENCES

- Avarguès-Weber, A., Deisig, N., and Giurfa, M. (2011). Visual cognition in social insects. *Annu Rev Entomol* 56, 423-443. doi: 10.1146/annurev-ento-120709-144855.
- Avarguès-Weber, A., Dyer, A.G., Combe, M., and Giurfa, M. (2012). Simultaneous mastering of two abstract concepts by the miniature brain of bees. *Proc Natl Acad Sci U.S.A.* 109(19), 7481-7486. doi: 10.1073/pnas.1202576109.
- Avarguès-Weber, A., and Mota, T. (2016). Advances and limitations of visual conditioning protocols in harnessed bees. *J Physiol Paris* 110(3 Pt A), 107-118. doi: 10.1016/j.jphysparis.2016.12.006.
- Avarguès-Weber, A., Portelli, G., Benard, J., Dyer, A., and Giurfa, M. (2010). Configural processing enables discrimination and categorization of face-like stimuli in honeybees. *J Exp Biol* 213(4), 593-601. doi: 10.1242/jeb.039263.
- Backhaus, W. (1991). Color opponent coding in the visual system of the honeybee. *Vision Res.* 31, 1381-1397.
- Balamurali, G.S., Somanathan, H., and Hempel de Ibarra, N. (2015). Motion cues improve the performance of harnessed bees in a colour learning task. *J Comp Physiol A* 201(5), 505-511. doi: 10.1007/s00359-015-0994-7.
- Benard, J., Stach, S., and Giurfa, M. (2006). Categorization of visual stimuli in the honeybee *Apis mellifera*. *Anim Cogn* 9(4), 257-270. doi: 10.1007/s10071-006-0032-9.
- Bitterman, M.E., Menzel, R., Fietz, A., and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J Comp Psychol* 97(2), 107-119. doi: 10.1037/0735-7036.97.2.107.
- Borst, A., and Egelhaaf, M. (1989). Principles of visual motion detection. *Trends Neurosci* 12(8), 297-306.
- Brandt, R., and Vorobyev, M. (1997). Metric analysis of threshold spectral sensitivity in the honeybee. *Vision Res.* 37(4), 425-439.
- Brembs, B., and Heisenberg, M. (2000). The operant and the classical in conditioned orientation of *Drosophila melanogaster* at the flight simulator. *Learn Mem* 7(2), 104-115.
- Buatois, A., Pichot, M.C., Schultheiss, P., Sandoz, J.C., Lazzari, C., Chittka, L., et al. (2017). Associative visual learning by tethered bees in a controlled visual environment. *Sci Reports* 7(1), 127903.

- Catton, K.B., Webster, D.R., Brown, J., and Yen, J. (2007). Quantitative analysis of tethered and free-swimming copepodid flow fields. *J Exp Biol* 210(2), 299-310. doi: 10.1242/jeb.02633.
- Chittka, L. (1998). Sensorimotor learning in bumblebees: long-term retention and reversal training. *J.exp.Biol.* 201, 515-524.
- Chittka, L., Beier, W., Hertel, H., Steinmann, E., and Menzel, R. (1992). Opponent colour coding is a universal strategy to evaluate the photoreceptor inputs in Hymenoptera. *J Comp Physiol A* 170, 545-563.
- de Brito Sanchez, M.G., Serre, M., Avargues-Weber, A., Dyer, A.G., and Giurfa, M. (2015). Learning context modulates aversive taste strength in honey bees. *J Exp Biol* 218(Pt 6), 949-959. doi: 10.1242/jeb.117333.
- Dinges, C.W., Varnon, C.A., Cota, L.D., Slykerman, S., and Abramson, C.I. (2017). Studies of learned helplessness in honey bees (*Apis mellifera ligustica*). *J Exp Psychol Anim Learn Cogn* 43(2), 147-158. doi: 10.1037/xan0000133.
- Dobrin, S.E., and Fahrbach, S.E. (2012). Visual associative learning in restrained honey bees with intact antennae. *Plos One* 7(6), e37666. doi: 10.1371/journal.pone.0037666.
- Dyer, A.G. (2012). The mysterious cognitive abilities of bees: why models of visual processing need to consider experience and individual differences in animal performance. *J Exp Biol* 215(3), 387-395. doi: 10.1242/jeb.038190.
- Egelhaaf, M., Boeddeker, N., Kern, R., Kurtz, R., and Lindemann, J.P. (2012). Spatial vision in insects is facilitated by shaping the dynamics of visual input through behavioral action. *Front Neural Circuits* 6, 108. doi: 10.3389/fncir.2012.00108.
- Egelhaaf, M., and Kern, R. (2002). Vision in flying insects. *Curr Opin Neurobiol* 12(6), 699-706. doi: 10.1016/S0959-4388(02)00390-2.
- Erber, J., Pribbenow, B., Bauer, A., and Kloppenburg, P. (1993). Antennal reflexes in the honeybee - tools for studying the nervous-system. *Apidologie* 24(3), 283-296. doi: 10.1051/apido:19930308.
- Erber, J., and Schildberger, K. (1980). Conditioning of an antennal reflex to visual-stimuli in bees (*Apis-mellifera* L.). *J Comp Physiol* 135(3), 217-225. doi: 10.1007/bf00657249.
- Findlay, J.M., and Gilchrist, I.D. (2003). "Active Vision: The Psychology of Looking and Seeing". Oxford University Press).

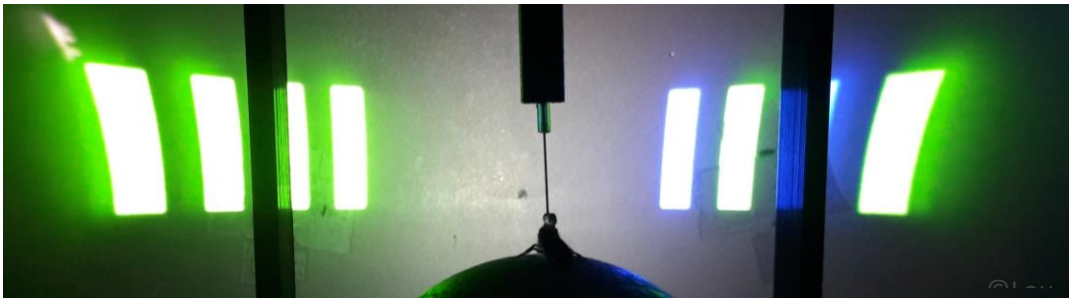
- Giurfa, M. (2013). Cognition with few neurons: higher-order learning in insects. *Trends Neurosci* 36(5), 285-294. doi: 10.1016/j.tins.2012.12.011.
- Giurfa, M., and Menzel, R. (1997). Insect visual perception: complex abilities of simple nervous systems. *Curr Opin Neurobiol* 7(4), 505-513. doi: 10.1016/S0959-4388(97)80030-X.
- Giurfa, M., and Sandoz, J.C. (2012). Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn Mem* 19(2), 54-66. doi: 10.1101/lm.024711.111.
- Giurfa, M., Vorobyev, M., Brandt, R., Posner, B., and Menzel, R. (1997). Discrimination of coloured stimuli by honeybees: alternative use of achromatic and chromatic signals. *J Comp Physiol A* 180, 235-243. doi: 10.1007/s003590050044.
- Giurfa, M., Vorobyev, M., Kevan, P., and Menzel, R. (1996). Detection of coloured stimuli by honeybees: minimum visual angles and receptor specific contrasts. *J Comp Physiol A* 178, 699-709.
- Giurfa, M., Zhang, S., Jenett, A., Menzel, R., and Srinivasan, M.V. (2001). The concepts of 'sameness' and 'difference' in an insect. *Nature* 410(19 April 2001), 930-933. doi: 10.1038/35073582.
- Götz, K.G. (1964). Optomotorische Untersuchung des visuellen Systems einiger Augenmutanten der Fruchtfliege *Drosophila*. *Kybernetik* 2, 77-92.
- Hateren, J.H.v., Srinivasan, M.V., and Wait, P.B. (1990). Pattern recognition in bees; orientation discrimination. *J Comp Physiol A* 167, 649-654.
- Heisenberg, M., and Wolf, R. (1988). Reafferent control of optomotor yaw torque in *Drosophila melanogaster*. *J Comp Physiol A* 163(3), 373-388.
- Hempel de Ibarra, N., and Giurfa, M. (2003). Discrimination of closed coloured shapes requires only contrast to the long wavelength receptor. *Anim Behav* 66, 903-910. doi: 10.1006/anbe.2003.2269.
- Hertz, M. (1935). Die Untersuchungen ueber den Formensinn der Honigbiene. *Naturwissenschaften* 36, 618-624.
- Hori, S., Takeuchi, H., Arikawa, K., Kinoshita, M., Ichikawa, N., Sasaki, M., et al. (2006). Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera* L. *J Comp Physiol A* 192(7), 691-700. doi: 10.1007/s00359-005-0091-4.

- Hori, S., Takeuchi, H., and Kubo, T. (2007). Associative learning and discrimination of motion cues in the harnessed honeybee *Apis mellifera* L. *J Comp Physiol A* 193(8), 825-833. doi: 10.1007/s00359-007-0234-x.
- Lehrer, M., and Srinivasan, M.V. (1993). Object detection by honeybees - Why do they land on edges? *J Comp Physiol A* 173, 23-32.
- Lehrer, M., Srinivasan, M.V., and Zhang, S.W. (1990). Visual edge detection in the honeybee and its chromatic properties. *Proc Biol Sci* 238, 321-330. doi: 10.1098/rspb.1990.0002.
- Lehrer, M., Srinivasan, M.V., Zhang, S.W., and Horridge, G.A. (1988). Motion cues provide the bee's visual world with a third dimension. *Nature* 332, 356-357.
- Liu, G., Seiler, H., Wen, A., Zars, T., Ito, K., Wolf, R., et al. (2006). Distinct memory traces for two visual features in the *Drosophila* brain. *Nature* 439(7076), 551-556. doi: 10.1038/nature04381.
- Liu, L., Wolf, R., Ernst, R., and Heisenberg, M. (1999). Context generalization in *Drosophila* visual learning requires the mushroom bodies. *Nature* 400, 753-756. doi: 10.1038/23456.
- Luu, T., Cheung, A., Ball, D., and Srinivasan, M.V. (2011). Honeybee flight: a novel 'streamlining' response. *J Exp Biol* 214(13), 2215-2225. doi: 10.1242/jeb.050310.
- Matsumoto, Y., Menzel, R., Sandoz, J.C., and Giurfa, M. (2012). Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: a step towards standardized procedures. *J Neurosci Meths* 211(1), 159-167. doi: 10.1016/j.jneumeth.2012.08.018.
- Menzel, R., and Backhaus, W. (1991). "Colour Vision in Insects," in *Vision and Visual Dysfunction. The Perception of Colour.*, ed. P. Gouras. (London: MacMillan Press), 262-288.
- Mota, T., Giurfa, M., and Sandoz, J.C. (2011). Color modulates olfactory learning in honeybees by an occasion-setting mechanism. *Learn Mem* 18(3), 144-155. doi: 10.1101/lm.2073511.
- Núñez, J.A. (1966). Quantitative Beziehungen zwischen den Eigenschaften von Futterquellen und dem Verhalten von Sammelbienen. *Z vergl Physiol* 53, 142-164.
- Pan, Y., Zhou, Y., Guo, C., Gong, H., Gong, Z., and Liu, L. (2009). Differential roles of the fan-shaped body and the ellipsoid body in *Drosophila* visual pattern memory. *Learn Mem* 16(5), 289-295. doi: 10.1101/lm.1331809.

- Rusch, C., Roth, E., Vinauger, C., and Riffell, J.A. (2017). Honeybees in a virtual reality environment learn unique combinations of colour and shape. *J Exp Biol* 220(Pt 19), 3478-3487. doi: 10.1242/jeb.164731.
- Schultheiss, P., Buatois, A., Avarguès-Weber, A., and Giurfa, M. (2017). Using virtual reality to study visual performances of honeybees. *Curr Opin Insect Sci* 24, 43–50.
- Srinivasan, M.V. (1998). Insects as Gibsonian animals. *Ecol Psychol* 10(3-4), 251-270.
- Srinivasan, M.V. (2010). Honey bees as a model for vision, perception, and cognition. *Annu Rev Entomol* 55, 267-284. doi: 10.1146/annurev.ento.010908.164537.
- Srinivasan, M.V., Lehrer, M., Zhang, S.W., and Horridge, G.A. (1989). How honeybees measure their distance from objects of unknown size. *J Comp Physiol A* 165, 605-613.
- Srinivasan, M.V., Poteser, M., and Kral, K. (1999). Motion detection in insect orientation and navigation. *Vision Res.* 39, 2749-2766.
- Srinivasan, M.V., and Zhang, S. (2004). Visual motor computations in insects. *Annu Rev Neurosci* 27, 679-696. doi: 10.1146/annurev.neuro.27.070203.144343.
- Takeda, K. (1961). Classical conditioned response in the honey bee. *Journal of Insect Physiology* 6, 168-179.
- Tang, S.M., and Guo, A. (2001). Choice behavior of *Drosophila* facing contradictory visual cues. *Science* 294(5546), 1543-1547. doi: 10.1126/science.1058237.
- von Frisch, K. (1914). Der Farbensinn und Formensinn der Biene. *Zool Jahrb Abt Allg Zool Physiol Tiere* 37, 1-238.
- von Holst, E. (1954). Relations between the central nervous system and the peripheral organs. *Brit J Anim Behav* 2(3), 89–86.
- von Holst, E., and Mittelstaedt, H. (1950). "The reafference principle. Interaction between the central nervous system and the periphery.," in *Selected Papers of Erich von Holst: The Behavioural Physiology of Animals and Man*. (London: Methuen ), 139-173.
- Webb, B. (2004). Neural mechanisms for prediction: do insects have forward models? *Trends in Neurosciences* 27(5), 278-282. doi: 10.1016/j.tins.2004.03.004.
- Wolf, R., and Heisenberg, M. (1991). Basic organization of operant-behavior as revealed in *Drosophila* flight orientation. *J Comp Physiol A* 169(6), 699-705.



- Xi, W., Peng, Y.Q., Guo, J.Z., Ye, Y.Z., Zhang, K., Yu, F., et al. (2008). Mushroom bodies modulate salience-based selective fixation behavior in *Drosophila*. *Eur J Neurosci* 27(6), 1441-1451. doi: 10.1111/j.1460-9568.2008.06114.x.
- Yang, Z., Bertolucci, F., Wolf, R., and Heisenberg, M. (2013). Flies cope with uncontrollable stress by learned helplessness. *Curr Biol* 23(9), 799-803. doi: 10.1016/j.cub.2013.03.054.
- Zeil, J. (1993a). Orientation flights of solitary wasps (*Cerceris*; Sphecidae; Hymenoptera) 1. Description of flight. *J Comp Physiol A* 172, 189-205. doi: 10.1007/BF00189396.
- Zeil, J. (1993b). Orientation flights of solitary wasps (*Cerceris*; Sphecidae; Hymenoptera) 2. Similarities between orientation and return flights and the use of motion parallax. *J Comp Physiol A* 172, 207-222. doi: 10.1007/BF00189397.
- Zeil, J. (1997). The control of optic flow during learning flights. *J Comp Physiol A* 180, 25-37.
- Zeil, J., Kelber, A., and Voss, R. (1996). Structure and function of learning flights in bees and wasps. *J Exp Biol* 199, 245-252.
- Zhang, S.W., Lehrer, M., and Srinivasan, M.V. (1998). Eye-specific learning of routes and "signposts" by walking honeybees. *J Comp Physiol* 182, 747-754.



# Chapitre 3



## Non-elemental visual learning of honey bees in virtual reality

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Les abeilles sont connues pour leurs impressionnantes capacités d'apprentissage visuel. Malheureusement, malgré un siècle de recherche dans ce domaine, il est toujours impossible de caractériser les corrélats neuronaux à cause de la difficulté d'enregistrer l'activité du cerveau en conditions de libre vol. Un nouveau dispositif basé sur la réalité virtuelle (RV) a été proposé ces dix dernières années pour résoudre ce problème. Dans ce dispositif, il est possible d'étudier l'apprentissage visuel simple sur des abeilles attachées placées sur un compensateur de locomotion derrière un écran semi-cylindrique. Ceci a constitué une première avance, mais ne fournissant une solution que pour l'apprentissage visuel élémentaire. En effet, en conditions naturelles et face à de nombreux stimuli, les abeilles sont capables d'utiliser des processus non élémentaires pour résoudre des tâches plus complexes comme le patterning négatif. Dans ce paradigme, les abeilles doivent apprendre A+, B+ et AB-, tâche impossible à résoudre en utilisant uniquement des liens élémentaires car A et B sont présentés autant de fois récompensés que non récompensés. En utilisant un système de RV, des abeilles attachées étaient testées sur une tâche de patterning négatif visuel. Pendant le conditionnement, une grille bleue (A) et une grille verte (B) étaient présentées récompensées, alors qu'une grille bleue et verte (AB) ne l'était pas. Ensuite, les abeilles étaient confrontées à A vs AB et B vs AB pour tester leur apprentissage. Pendant le test, les abeilles étaient capables de discriminer A, B et AB démontrant leur habileté à résoudre une tâche non-élémentaire en RV. Cette étude fournit un paradigme non élémentaire visuel en RV et la possibilité d'aller plus loin dans la compréhension des bases neurales de l'apprentissage visuel chez l'abeille.



# Non-elemental visual learning of honey bees in virtual reality

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## ABSTRACT

Bees are known for their impressive visual learning abilities. Unfortunately, despite a century of research on this area, it is still impossible to characterize the underlying neural correlates because of the difficulty to record brain activity in free flight conditions. Novel setup based on virtual reality (VR) have been proposed in the last decade to overcome this problem. In these setups, it was thus possible to study simple visual learning on tethered honeybees placed on a treadmill behind a semi-cylindrical screen. This was a first advance but providing a solution only for elemental visual learning. Indeed, in natural conditions and faced to numerous stimuli, bees are also able to use non-elemental processes to resolve more complex tasks as the negative patterning. In this paradigm, bees have to learn A+, B+ and AB- which is not resolvable using only elemental link because A and B are presented as much rewarded than not rewarded. Using a VR system, tethered bees was therefore tackled to a visual negative patterning protocol. During the conditioning, a blue grating (A) and a green grating (B) were presented as rewarded, whereas the blue and green grating (AB) was not. Then, bees were faced to A vs AB and B vs AB to test their learning. During the test, bees were able to discriminate A, B and AB demonstrating the ability of bees to resolve a non-elemental task in VR. This study provides therefore a solid non-elemental paradigm in VR and the possibility to go further in the understanding of neural bases of visual learning in honeybees.

### Keywords

*Apis mellifera*; Negative patterning; Complex learning; Configural learning; Absolute conditioning; Visual cognition; Trackball

## Introduction

Learning is a fundamental capacity for individual survival as it renders a complex environment predictable. Several types of learning have been defined depending on the nature of the information learned and the way the animal interacts with its environment. For instance, elemental forms of learning revolve around apprehending simple associative links between events in animal's world. In Pavlovian conditioning, a neutral stimulus (conditioned stimulus, CS) is associated with a biologically relevant stimulus (unconditioned stimulus, US) so that a simple, predictive link is established between them (Pavlov, 1941). In natural conditions, however, animals are rarely exposed to a single stimulus. Yet, in nature, animals are usually confronted to situations that are more complex. This complexity has inspired the study of non-elemental forms of learning in which animals cannot rely on simple associative links to solve the problem presented to them (Pearce, 2002; Rescorla, 1972, 1973). In this learning category, the events to be discriminated have usually an ambiguous valence, being as often reinforced as non-reinforced. It is thus impossible to rely on a simple, unambiguous link to decide on which response is appropriate when facing a given stimulus. Typically, stimulus compounds are part of the discrimination problems proposed to animals in this field; the underlying idea is that animals have to learn that a compound made of two elements is not the simple sum of its elements; solving the problem thus requires adopting a non-linear strategy and inhibit linear summation (Alvarado and Rudy, 1992; Pearce, 2002).

Negative patterning constitutes a recurrent protocol to study non-elemental learning (Whitlow and Wagner, 1972). In this task, the animal has to learn that two different stimulations A and B are rewarded when presented in isolation but not when presented together as a compound (A+, B+ / AB-). A and B are therefore both reinforced and non-reinforced depending on whether they are presented alone or in compound. Animals have to learn to respond to A and B alone, and to inhibit their response to AB, which in normal conditions should be twice as higher as their response to A or B alone. Vertebrates are well known for their abilities to resolve this task (Buckley and Gaffan, 1998; Rudy and Sutherland, 1989; Shanks et al., 1998), and offered by many ways to enrich our knowledges about neural correlates underlying to the configural learning. This is especially true for rodents for which the hippocampus has been highlighted as a high-order structure involved in the treatment of these contradictory information (Rudy and Sutherland, 1989; Sutherland and Rudy, 1989). Indeed, rat deprived of their hippocampus were able to resolve a simple discrimination task but not a negative patterning task. However, vertebrates possess a very



structured brain composed by many neurons (200 million for the rat) which could explain their abilities to resolve such complex task. We could therefore wonder if the insects with their simple brains could be able to perform as well as the vertebrates.

Honeybees constitute an excellent model to study non-elemental learning due to their sophisticated learning abilities (Giurfa, 2007). Indeed, bees demonstrate impressive learning abilities in a variety of tasks, from elemental associative learning (Giurfa, 2004, 2013; Giurfa and Sandoz, 2012) to non-elemental discriminations such as negative patterning (Deisig et al., 2001a, 2003; Devaud et al., 2015; Schubert et al., 2002), categorization (Avargues-Weber et al., 2010; Benard et al., 2006) and even concept learning abilities (Avargues-Weber et al., 2011; Giurfa et al., 2001). In the context of the olfactory conditioning of the proboscis extension reflex, a protocol in which harnessed bees learn to associate odorants with sucrose reward (Giurfa and Sandoz, 2012), bees can solve negative patterning discriminations, responding with an extension of the proboscis to single rewarded odorants and not to their compound (Deisig et al., 2001b, 2002, 2003). Similarly to the vertebrate case, specific circuits of a higher-order brain structure, the mushroom bodies, are required to solve this task (Devaud et al., 2015). Interestingly, these circuits are dispensable for solving elemental olfactory learning discriminations, thus reaffirming the principle that specific neural architectures are devoted to non-linear discrimination solving in brains with different evolutionary histories.

By opposition to the olfactory domain, non-linear discriminations in the visual domain have been scarcely studied in bees. Using free-flying bees trained to discriminate single colors from two-color checkerboards, Schubert et al (2002) showed that bees trained to go towards a yellow or a violet checkerboard were able to learn to avoid the association of both (violet and yellow checkerboard) and thus that bees were able to inhibit the generalization to A and B in the direction of AB.

Although these results are interesting in the sense that they indicate that free-flying bees can achieve non-linear discriminations in the visual modality, they exhibit the typical limitation of these experiments, namely the impossibility of ensuring a full control of the animal behavior (such as in PER experiments). This precludes, therefore, the coupling with invasive methods aimed at unravelling the mechanisms of these performances. The use of virtual reality (VR), in which tethered bees walk stationary, while learning simple visual discriminations (see chapters II and III), provides an appealing context to study the bees' capacity to solve non-linear discriminations in the

visual domain. The use of closed-loop conditions allows the tethered bee to control its visual environment with its movement and choices, thus creating a sensation of immersion within this virtual environment. Yet, as shown in chapter III, the tethering imposes limitations to active vision, which may not affect the solving of simple discriminations (as described in Chapter III) but could impair the learning of higher-order problems such as a negative patterning discrimination.

Here we studied the capacity of tethered bees to solve a negative patterning discrimination in a VR context. We aimed therefore at reproducing the bees' ability to solve higher-order problems in a fully controlled environment, which could be coupled with the use of invasive techniques to understand the neural bases of visual non-elemental learning.

## **Materials and methods**

### **Animals preparation**

Honey bee foragers (*Apis mellifera*) were caught each morning at the apiary of the University Paul Sabatier, Toulouse III on a gravity feeder before they started collecting sucrose. Once in the lab, bees were anesthetized on ice for 3 min. The wings were then cut and the thorax shaved before attaching thanks to UV-cured dentine with a home-made tether on the thorax allowing positional adjustment in high while preventing side movements (Buatois et al., 2018). Once attached, bees received 4 $\mu$ l of 0.9M sucrose solution and then were placed on a miniature treadmill during 3h to allow familiarization with the tethering condition and the treadmill. This period also allowed inducing a starvation state. (Figure 1a). Finally, bees were placed on the experimental treadmill 1 minutes before the start of the training procedure.

### **VR setup**

The same VR setup than in (Buatois et al., 2018) was used (Figure 1b): a treadmill composed of a polystyrene ball (diameter: 10cm, weight: 8g) raised by and air flow (air flow: 555ml/s; Aqua Oxy CWS 2000, Oase, Wasquehal, France) on a home-made 3D printing support, with a semi spherical screen and a video projection system (Acer k135i, Roissy, France). The walking movements of bees were recorded via to two infrared optic-mouse sensors (Logitech M500, 1000 dpi, Logitech, Lausanne, Switzerland). The

rotations of the ball induced by the movements of the bees triggered corresponding transversal movements of the visual stimuli on the screen.

### **Visual stimuli**

Visual stimuli (Figure 1c, Figure S1, Table S1) were a blue grating "A" (RGB: 0, 0, 255; irradiance = 161 000 uW.cm<sup>2</sup>; dominant wavelength = 450 nm), a green grating "B" (RGB: 0, 100, 0; irradiance = 24 370 uW.cm<sup>2</sup>; dominant wavelength= 530nm) and a mix of both "AB" (blue/green grating, irradiance = 116 347 uW.cm<sup>2</sup>). The irradiance of green stimuli is relatively lower to reduce attraction of naïve bees toward the green stimulus (Buatois et al., 2018; Kirkerud et al., 2017). The gratings were composed of 4 stripes each measuring 1 by 4 cm (width\*height) corresponding to a visual angle of 5.7° thus allowing resolution of each stripe.

### **Control Experiment: Are the elemental stimuli perceived within the composed stimuli?**

Negative patterning paradigm requires that animals perceive both A and B in AB without overshadowing of one stimulus on the other nor perception of AB as a novel stimulus C. Bees had been consequently conditioned to learn the association between A, B or AB with a sucrose solution during a classical absolute conditioning protocol (Figure 2) performed in the VR setup and then tested for generalization with the other stimulations. Three groups of tethered bees were tested, A+ (n=20), B+ (n=20) and AB+ (n=20). The experiment was performed under complete darkness.

The absolute conditioning phase lasted 10 trials. The conditioned visual stimulus was presented pseudo randomly at right or at left (L, R, R, L, R, L, L, R, L, R) on the screen (+/- 50° respectively from the bee's body axis) at the beginning of each trial. When the bees centered the stimuli (0° from the body axis), which indicates that the bee tried to orientate toward it, the stimulus remained its position for 8 seconds and a sucrose solution was provided with an imbibed toothpick on their antennae first and then on the proboscis for 5 seconds. A trial lasted 30 seconds maximum, and the reward delivery indicated the end of the trial even if the 30 seconds was not over. A black background appeared then for 60 seconds before the start of a new trial. The trials lasted a maximum of 30 seconds in case the bee did not center the stimulus. In this case, bees didn't receive any reinforcement. Fortunately, and thanks to the phototaxy this is not frequent, thus a trial lasted in average  $20 \pm 3$  seconds. After the conditioning phase was completed, non-reinforced tests were performed a minute later, in which bees from the A+ group were confronted to two tests (A vs B

and A vs AB) that lasted 30 seconds. Bees from the B+ group faced the tests A vs B and B vs AB while the AB+ conditioned bees were tested for three pairs of stimuli: A vs. AB, B vs. AB and A vs. B). At the beginning of each test, both stimuli were pseudo randomly placed either on the right or on the left of the bee ( $50^\circ$  and  $-50^\circ$  from the body axis). The tests' order was randomized between bees. Each test was separated by a refreshment trial of 30 seconds during which the CS+ was presented and associated with sucrose to avoid extinction of the learnt association. Tests and refreshments trials were separated by 60 seconds. Contrary to the conditioning trials, even if the bees centered the stimulus, there were no blockade for 8 seconds. Thus, bees were able to switch from one stimulus to the other. The experiment was performed under complete darkness.

### **Main experiment: Can bees solve a visual negative patterning task in a VR setup?**

The negative patterning protocol (Figure 3, Table S2) was composed of 3 absolute conditioning phases with a total of 32 trials (two phases of 10 trials and one of 11 trials, order of the phase was random). As for the control experiment, bees ( $n=20$ ) were faced to one stimulus at time, pseudo randomly presented at right or at left ( $\pm 50^\circ$  from the body axis; 10 trials: L, R, R, L, R, L, L, R, L, R; 11 trials: L, R, R, L, R, L, L, R, L, R, R) at the beginning of each conditioning trial. When A and B were presented (16 trials), bees received a sucrose solution when the stimulation was centered by the bee (A+; B+). However, when AB was presented (16 trials) the same principle was remained, with the difference that no reward was delivered (AB-). The choice of the stimulation shown at each trial (A, B or AB) was pseudo-randomized in order to get a total of 16 trials by stimulation and no more than twice the same stimulation in a row (Table S2). Once centered, the stimulus remained its position for 8 seconds irrespective of the stimulus nature in order to provide the reinforcement. Each phase was separated by one resting hour on a miniature treadmill. After the three conditioning phases were completed and one resting hour, the bees faced two non-reinforced tests in a random order, A vs AB and B vs AB, separated by three refreshment trials (one per stimulation). The tests lasted 30 seconds and were separated by 60 seconds. The relative position of the stimuli (right or left) at the beginning of the tests was pseudo randomly chosen. Contrary to the conditioning trials, even if the bees centered the stimulus, there were no blockade for 8 seconds. Thus, bees were able to switch from one stimulus to the other. The experiment was performed under complete darkness.

## Statistical analyses

The data consisted in the individual first choice (first stimulation centered) and the time spent to fixate each stimulus during the tests. The proportions of bees that did not make a choice during the duration of the test or choosing each stimulus were then calculated and data were bootstrapped to plot these proportions and their corresponding 95% confidence interval.

To compare the proportion of bees choosing the different stimuli during the test, generalized linear mixed models (GLMMs) in a binomial family were used. For each model, the subjects were considered as a random factor to account for the repetitive measurement design. Wilcoxon U rank tests were used to compare the time spent to fixate each stimulus during the tests.

All statistical analyses were done using the R 3.2.3 software (R Development Core team, 2018). Packages lme4 was used for GLMMs.

## Results

### Control Experiment: Are the elemental stimuli perceived within the composed stimuli?

In a first experiment, we verified that bees process a compound AB as the sum of A and B, thus revealing a linear processing. This condition is necessary to investigate afterwards if such a linear processing can be inhibited in the framework of a negative-patterning discrimination. Three different groups of bees were conditioned with either the single-color gratings (A+, B+) or with the compound grating (AB+) and tested for their learning, discrimination and generalization abilities of these stimuli.

The groups trained with A+ or B+ were tested with their respective CS+, which was opposed to the alternative single-colored grating not using during the training and to the compound grating as novel stimuli. The group trained with AB+ experienced its CS+ vs. both single-colored gratings as novel stimuli.

Regarding the group A+ and B+, performances were not significantly different according to the group (1<sup>st</sup> choice analysis: GLMM; A vs B: Group\*Choice effect:  $\chi^2=2.37$ ,  $df=2$ ,  $p=0.30$ ; A/B vs AB: Group\*Choice effect:  $\chi^2=4.40$ ,  $df=2$ ,  $p=0.11$ ; Fixation time: Man-Whitney:  $U=200$ ,  $p=1$ ) and

were therefore pooled for further analysis and shown in figure 4. Bees trained with a single-colored grating, be it green or blue, preferred their known grating to the novel one (test A vs B, Figure 4a, b). The proportion of the bees choosing at first the previously rewarded grating was significantly higher than the proportion of bees choosing the novel alternative grating (CS+: 67.5 %; NS: 22.5 %), or not making any choice (NC: 10 %) (Figure 4a, GLMM: CS+ vs NS:  $z_{357}=-3.68$ ,  $p<0.001$ ; CS+ vs NC:  $z_{357}=-4.68$ ,  $p<0.0001$ ; NS vs NC:  $z_{357}=-1.48$ ,  $p=0.14$ ). Bees thus clearly recognized the previously rewarded single-colored grating and oriented preferentially towards it. Accordingly, they spent significantly more time fixating it than the novel grating during the test (Figure 4b, Wilcoxon test:  $U=548$ ,  $p<0.001$ ).

In a further test, bees trained with the single colored grating experienced this stimulus against the compound grating (A or B vs. AB, Figure 4c, d). Interestingly, bees exhibited a high generalization between their respective CS and the compound grating, thus showing that they perceived the rewarded element in it. Indeed, no significant preference was observed for the CS+ (42.5 % of bees) when opposed to the compound stimulus (47.5 % of bees) ( $z_{357}=0.45$ ,  $p=0.65$ ). In this case, the number of bees that did not make a choice was significantly lower (NC, 10 %) (Figure 4c, CS+ vs NC:  $z_{357}=-3.07$ ,  $p<0.01$ ; AB vs NC:  $z_{357}=-3.41$ ,  $p<0.001$ ). Consistently, bees did not spend significantly more time fixating the CS than the compound grating (Figure 4d,  $U=285.5$ ,  $p=0.46$ ).

Bees trained with the compound grating (AB+; Figure 5) were confronted to three different tests: AB vs. A, AB vs. B and A vs. B. In the test A vs. B, there was no significant difference between the proportion of bees choosing at first A (36.8 %) or B (52,6 %) (A vs B:  $z_{118}=0.95$ ,  $p=0.34$ ) but there were significantly less bees which did not make any (NC, 10.5 %) than bees choosing B (Figure 5a, , A vs NC:  $z_{118}=-1.79$ ,  $p=0.07$ ; B vs NC:  $z_{118}=-2.53$ ,  $p=0.01$ ). The bees did not spend significantly more time fixating A or B (Figure 5b,  $U= 86.5$ ,  $p=0.65$ ).

There was no significant difference between the results of the test A vs AB and B vs AB so that the data were pooled for analysis and graphical display (1<sup>st</sup> choice: Test\*Choice:  $\chi^2=3.69$ ,  $p=0.72$ ; Fixation time:  $U=159$ ,  $p=0.27$ ). In these tests, bees did choose equally the trained compound grating and the novel single-colored training, be it A or B. The proportion of bees choosing at first the CS+ (45%) and the NS (Novel Stimulus, A or B) (42.5%) were not significantly different (CS+ vs NS:  $z_{357}=-0.67$ ,  $p=0.50$ ). The proportion of bees that did not make any choice

was low. (NC, 5%) (Figure 5c, CS+ vs NC :  $z_{357}=-3.22$ ,  $p<0.01$ ; NS vs NC :  $z_{357}=-2.67$ ,  $p<0.01$ ). Accordingly, bees did not spend significantly more time fixating the CS+ compared to the NS (Figure 5d,  $U=336$ ,  $p=0.74$ ). These results indicate that bees trained to AB generalize towards A and B thus showing an elemental processing of the compound grating. Bees perceive these two components A and B within AB. Also, our results show that there was no overshadowing of one elemental stimulus over the other. These three stimuli can therefore be used in the negative patterning experiment in which bees are requested to inhibit their elemental processing of the compound AB.

### **Main experiment: Can bees solve a visual negative patterning task in a VR setup?**

During the visual negative patterning protocol, bees were rewarded on the single-colored gratings A and B, but not on the two-colored grating AB (A+, B+, AB-). The task consists therefore in learning that AB is not the linear sum of the elemental properties of A and B. Afterwards, the bees were subjected to two non-reinforced tests (figure 3): A vs AB and B vs AB.

As there was no significant difference between performances of both tests (Test\*Choix:  $\chi^2=5.68$ ,  $df=2$ ,  $p=0.06$ ), data were pooled for analysis and graphical display (the term CS+ corresponds to responses to A and B pooled while the term CS- to responses to AB). In these tests, the proportion of the bees choosing the CS+ at first (55%) was significantly higher than the proportion of the bees choosing the CS- (30%) or making no choice (NC, 15%) (Figure 6a, CS+ vs CS-:  $z_{238}=-2.23$ ,  $p=0.025$ ; CS+ vs NC:  $z_{238}=3.55$ ,  $p<0.001$ , CS- vs NC:  $z_{238}=-1.58$ ,  $p=0.11$ ), thus revealing a capacity to solve the negative patterning discrimination. However, bees did not spend significantly more time fixating the CS+ than the CS- (Figure 6b,  $U=44.5$ ,  $p=0.13$ ). This contradiction could be due to the relatively high proportion of non-learners in this experiment, which may have biased the results. Indeed, when restricting the analysis of the fixation time to the learner bees (i.e. bees that chose the CS+ at first), a significant difference was observed (Figure 6c,  $U=21$ ,  $p<0.001$ ). However, regarding the fixation time, it seems that performances were significantly different between tests ( $W=108$ ,  $p=0.01$ ). Indeed, bees spent significantly more time to fixate the CS+ when this one was B, contrary to when CS+ was A (Figure 7, A vs AB:  $U=175.5$ ,  $p=0.51$ ; B vs AB:  $U=75.5$ ,  $p<0.001$ ). Over the 20 bees trained in the negative patterning task, 5

bees were successful in both tests, 12 bees made a correct choice in one of the test while the remaining 3 bees consistently made an incorrect choice.

Taken together, these results show that bees solve a visual negative patterning problem in VR conditions, and that to this end, they are capable of inhibiting their otherwise elemental processing of the compound grating. However, it seems to be easier for the bees to discriminate B with AB than A with AB.

## Discussion

This study demonstrates for the first time that tethered bees can solve a non-elemental task in the visual domain under VR conditions. We therefore provide here the first case of non-elemental learning in a virtual reality context, showing that the well-known cognitive abilities evinced by free-flying bees in mazes and other similar setups can be reproduced under controlled conditions in the case of bees walking stationary on a treadmill. This finding opens new doors for the study of the neurobiological mechanisms underlying these capacities.

Negative patterning relies on the ability to discriminate A+, B+ from AB- (Whitlow and Wagner, 1972), i.e. on the suppression of elemental processing treating the compound AB as the simple sum of A and B. Our control experiment showed that bees perceive both A and B in AB and without that non element dominated over the other (Figure 4,5). This experiment showed generalization from A and B towards AB and vice versa, a performance that reflects the elemental processing of the compound AB in which bees recognized the presence of the single elements. The spectral analyses of the stimuli and the color perceptual distances calculation (Backhaus, 1991; Menzel and Backhaus, 1991) confirmed that the bees' performance consisted in true generalization and was not based on a lack of discrimination as the colors chosen, green and blue, were well spaced and thus well distinguishable in the bee color opponent coding space, a psychophysical representation of the bee color vision (14.7 COC units separating A and B, which yield a high discrimination level above 90%, Table S1). Furthermore, the stripes of the gratings were wide enough to allow their resolution (visual angle: 5.7°) while the global stimulus was large enough to facilitate color discrimination (38.6°). (Giurfa et al., 1996, 1997). These colored gratings used in



our experiments were thus well adapted to test the capacity of bees to achieve a non-elemental discrimination.

Our experiments showed that bees successfully mastered the negative patterning discrimination, responding more to the elements (single-colored gratings) than to the compound (two-colored grating). From an elemental perspective, this problem resolution would be impossible as A and B having been rewarded, the predictions for AB are that it should be twice as rewarded. Thus, the double of responses should be expected for AB from this perspective. This was not the case and bees clearly reduced their responding to AB at the end of the conditioning period. Thus, the bees demonstrated non-elemental learning abilities despite movement restriction in a virtual reality environment.

The performance level of the bees was relatively low compared to that obtained under free flight conditions (Schubert et al., 2002). In free flight, almost 80% of the bees responded correctly when faced to the single elements against the compound, whereas 60% did it during our experiment. The reduced performances could be due to a large difference in training length. Indeed, contrary to the free-flight experiment, our device doesn't yet provide the possibility to spread the conditioning on several days, forcing us to do 32 trials while the free-flying bees experienced 60 trials over two days (Schubert et al., 2002). In addition, our protocol was based on absolute conditioning, i.e. a single stimulus was presented at a time. This kind of procedure tends to decrease discrimination success compared to differential conditioning in which two stimuli are presented at a time with different reinforcement outcomes (Giurfa, 2004). Nevertheless, Schubert et al used the same process, this should therefore not have an impact on the performance. Finally, the inter-trial interval (ITI) was longer in free-flight conditions (in average  $5 \pm 2$  min). This parameter has an impact on negative patterning learning in olfactory PER conditions (Deisig et al., 2007). Indeed, conditioned with an ITI lower than 8min, bees were not able to resolve an olfactory negative patterning task. Accordingly, and knowing that the ITI during our experiment was 1min, this parameter could explain the reduced performance. However, regarding the number of trial, our experiment was closer in its design to the olfactory negative patterning protocol performed using harnessed honeybees (Deisig et al., 2001b, 2002).

In addition, with these protocol differences, better performance in free flight experiments may result from the freedom of the bees reducing stress level and allowing them to come back to the hive to deliver the collected sucrose between each trial. Consequently, the bee's motivation for

the sucrose reward remains intact between trials while, in our setup, the bees may be satiated at the end of the conditioning phase. The bees received on average 32  $\mu$ l of sucrose solution during one experiment which represents half of their crop capacity (Núñez, 1966) Moreover, the tethering and our virtual reality constraints induce the impossibility of bees to scan the stimuli in all dimension. Active vision is important for object recognition and is based on the scan of stimuli edges by the individual thanks to motor processes (Findlay and Gilchrist, 2003). However, a previous study has shown that bees tested in parallel for visual discrimination in our virtual reality conditions and a Y-maze allowing active vision showed similar performance (Buatois et al., 2018). The Y-maze experiment was designed to replicate most of the virtual reality protocol constraints: the Inter-trial interval, the stimuli, the stimuli presentation duration and impossibility to come back to the hive. Importantly, another experiment has shown that when the bees were free to return to the hive between Y-maze trials, color learning was drastically improved thus allowing to discard that the nature of our visual stimulations used and the walking situation imposed to the bees had a negative effect on the bees' performance (Buatois et al., 2017).

Results from the negative patterning experiment show that it seems more difficult for the bees to discriminate A+ from AB- than B+ from AB- (Figure 7, Figure S2). This difficulty could be explained when looking at the brightness of the stimuli. The brightness was highly similar between A and AB while B had a far lower brightness (A: 126 542  $\mu$ W.cm<sup>2</sup>, B: 16 959  $\mu$ W.cm<sup>2</sup>, AB: 116 347  $\mu$ W.cm<sup>2</sup>). Brightness is generally not considered as a parameter in color perceptual spaces models (Backhaus, 1991; Chittka, 1992; Menzel and Backhaus, 1991), although our results suggest that it had an impact on the bees' performance. Furthermore, several studies using colored light as stimuli showed the importance of modulating stimulus brightness to avoid spontaneous biases in naïve bees (Buatois et al., 2017, 2018; Kirkerud et al., 2017; Rusch et al., 2017). A novel model of color perception in bees taking into account stimulus intensity as a third dimension besides hue and saturation would be of high value to the analysis of virtual reality experiments.

According to (Devaud et al., 2015), mushroom bodies, and more specifically the GABAergic feedback circuits from the vertical lobes to the calyces are required for olfactory non-elemental learning such as negative and positive (A-,B- vs AB+) patterning. It is likely that a similar circuit is involved in the inhibition of the response towards AB, if feedback GABAergic neurons reach not only the lip, to modulate olfactory processing, but also the collar to modulate

visual processing of information coming from the optic lobes. Neuroanatomical studies should unravel the fine connectivity of these neurons at the level of the collar to test the validity of this hypothesis. By coupling the conditioning procedure developed in our work with local injection of a neuronal activity inhibitor (e.g. procaine) or picrotoxin (as a blocker of GABA receptors) in the mushroom body calyces (in particular in the collar) is a necessary step to test this idea. This is only one example among the numerous possibilities offered by this setup to start studying the neural mechanisms underlying complex visual learning abilities. Further development should include the reproduction of other visual complex-problem solving (e.g. categorization or concept formation) in order to access the neural substrates of these capacities in our VR setup.

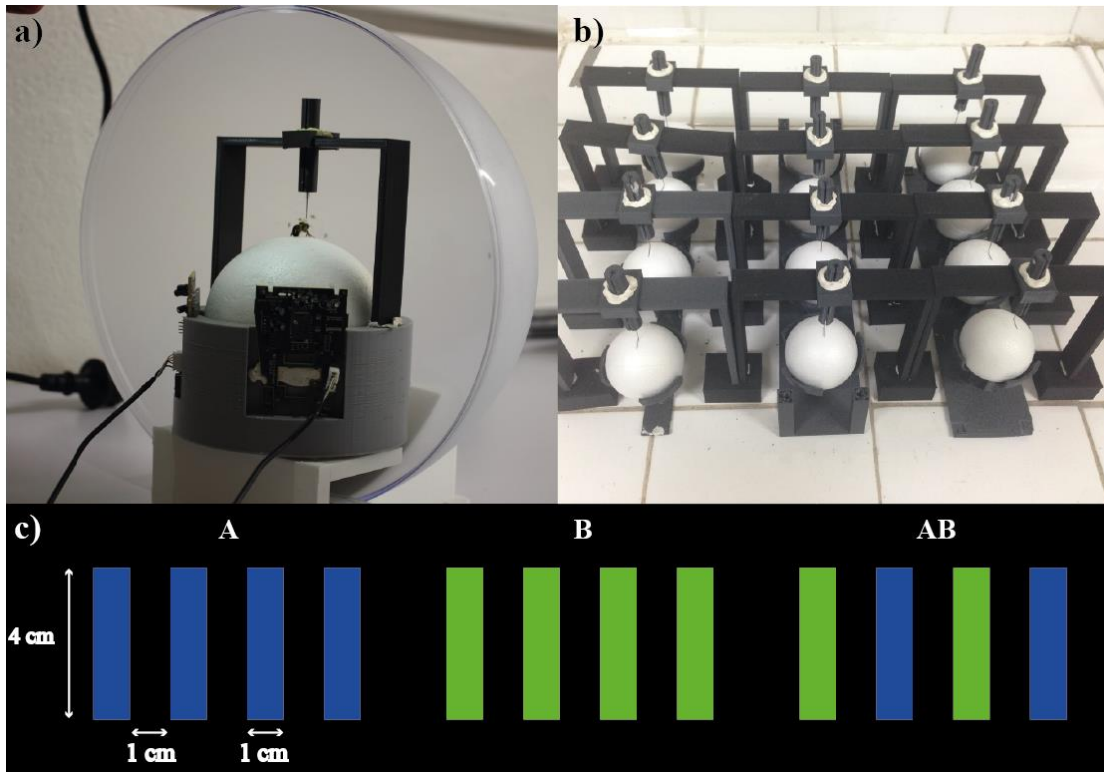
### **Acknowledgments**

Our work was supported by the Human Frontier Science Program (grant RGP0022/2014), the French National Research Agency (grant ANR-13-BSV4-0004-0), the French National Research Center (CNRS) and the University Paul Sabatier- Toulouse III. Jean-Pierre Papy provided help in terms of beekeeping activities. Special thanks are due to Sébastien Weber for his technical assistance in designing and implementing the software for visual stimulus control and for his regular support throughout this project.

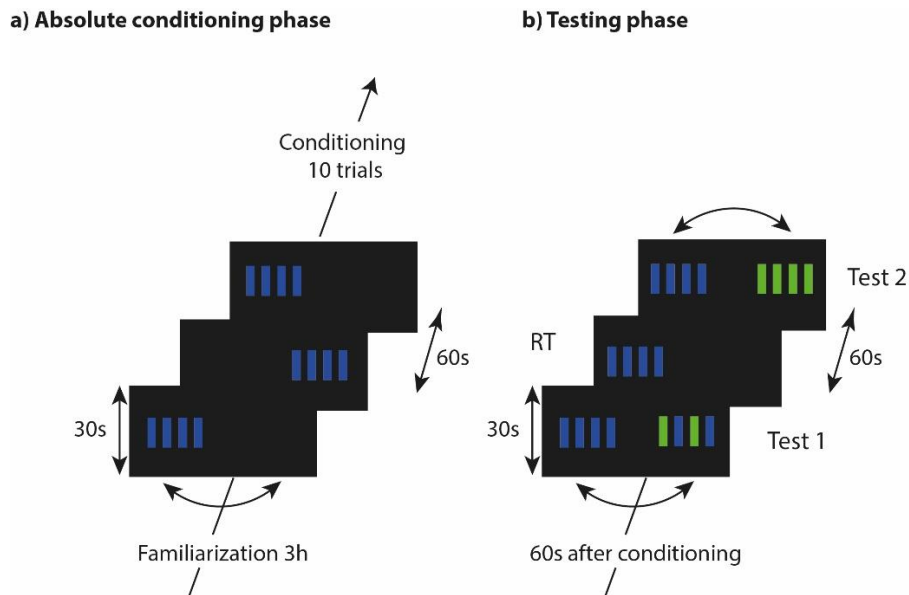
### **Author contributions**

A.B., A.A.-W. and M.G designed the experiments. The experiments were conducted by A.B and L.L. A.B. and L.L analyzed the results and prepared figures and tables. A.B., A.A.-W. and M.G. wrote the manuscript. All authors reviewed and approved the final manuscript.

**Figure 1**

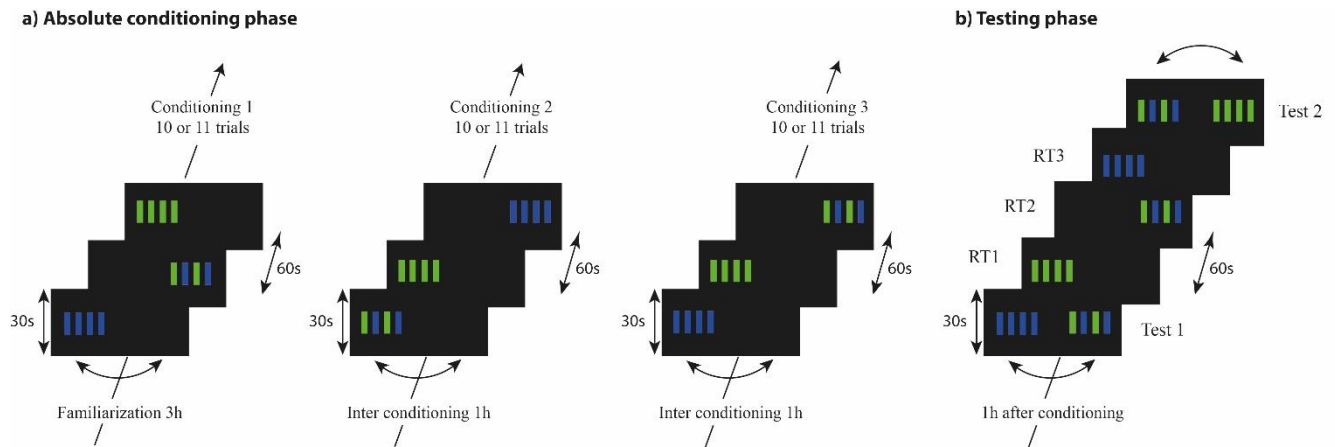


**Figure 1. Virtual reality setup and visual stimulations.** (a) Global view of the virtual reality system. (b) Miniature treadmills used for the familiarization phase. A succession of small balls over individual support. Bees were placed for 3 h on one miniature treadmill for familiarization after the dorsal tether was attached. (c) A blue grating (A), a green grating (B) and a blue and green grating (AB) were used as visual stimuli in the experiments.

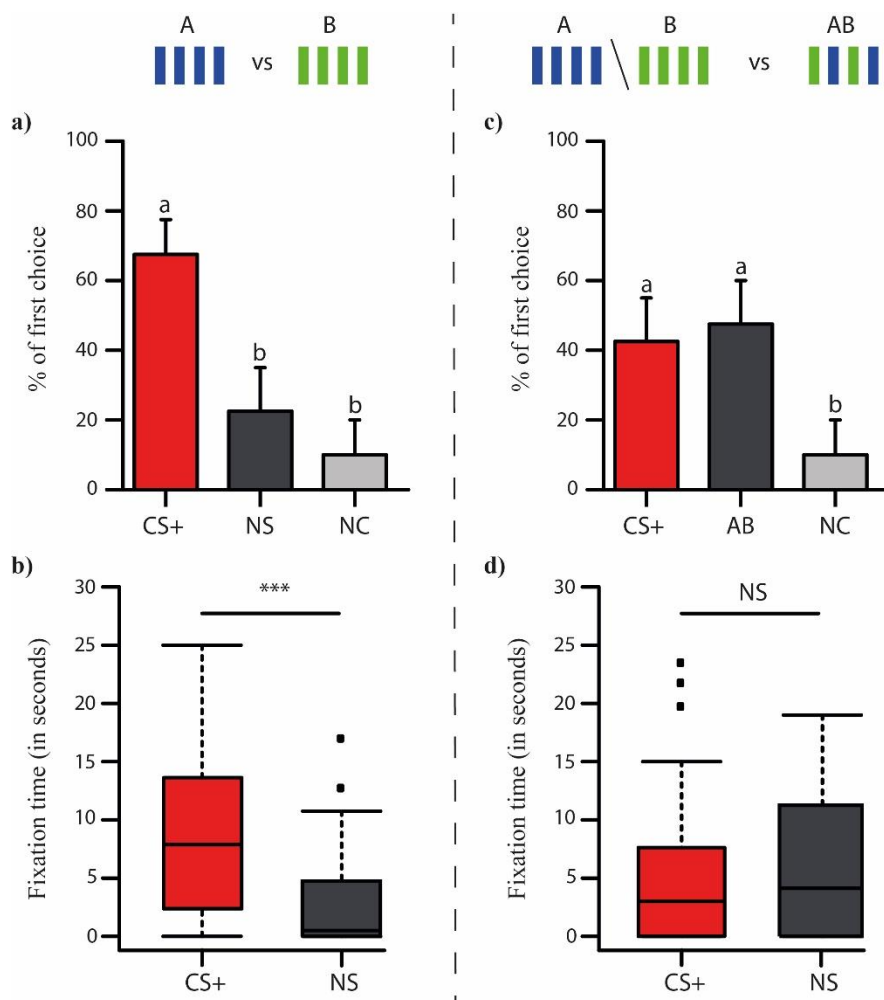
**Figure 2**

**Figure 2. Experimental schedule of the control experiment.** Bees started with a familiarization phase of 3 hours in the absence of visual stimulation. Then, the bees were conditioned for 10 trials to associate either A, B or AB with a sucrose solution. Only the case where A was rewarded is presented on this diagram. The stimulus was pseudo-randomly presented alone on the right or one the left ( $\pm 50^\circ$  from the body axis) and rewarded with sucrose solution when centered on the screen by the bee's walking activity ( $0^\circ$  from the body axis). Trials lasted 30 seconds maximum (according to if bees made a choice) and were separated from each other by 60 seconds. After conditioning was completed, the bees faced non-reinforced tests in which the rewarded stimulus was opposed to new stimuli. In this example, the blue grating A was rewarded and was therefore opposed to AB during the first test and to B during the second test. The order of the tests was random. a refreshment trial was performed between tests to avoid extinction. Regarding the group rewarded for AB, three tests were performed.

**Figure 3**



**Figure 3. Experimental schedule of the negative patterning experiment.** Bees started with a familiarization phase for 3 hours. The experiment was then divided into 3 conditioning phases. Each phase was composed by 10 or 11 absolute conditioning trials in which either A, B or AB was pseudo-randomly presented alone on the right or on the left ( $\pm 50^\circ$  from the body axis). A and B were rewarded with a sucrose solution when they were centered on the screen by the bee's walking movements ( $0^\circ$  from body axis), while AB was not rewarded. Trials lasted 30 seconds maximum (according to if the bees made a choice) and were separated from each other by 60 seconds. The presentation of A, B and AB was pseudo-randomized during the three conditioning phases. Each phase was separated by 1h. One hour after the last conditioning phase, bees faced to two non-reinforced tests in which the rewarded stimuli (A and B) were respectively opposed to the non-rewarded stimulus (AB). The order of the tests was randomized. , three refreshment trials were performed between test to avoid extinction.

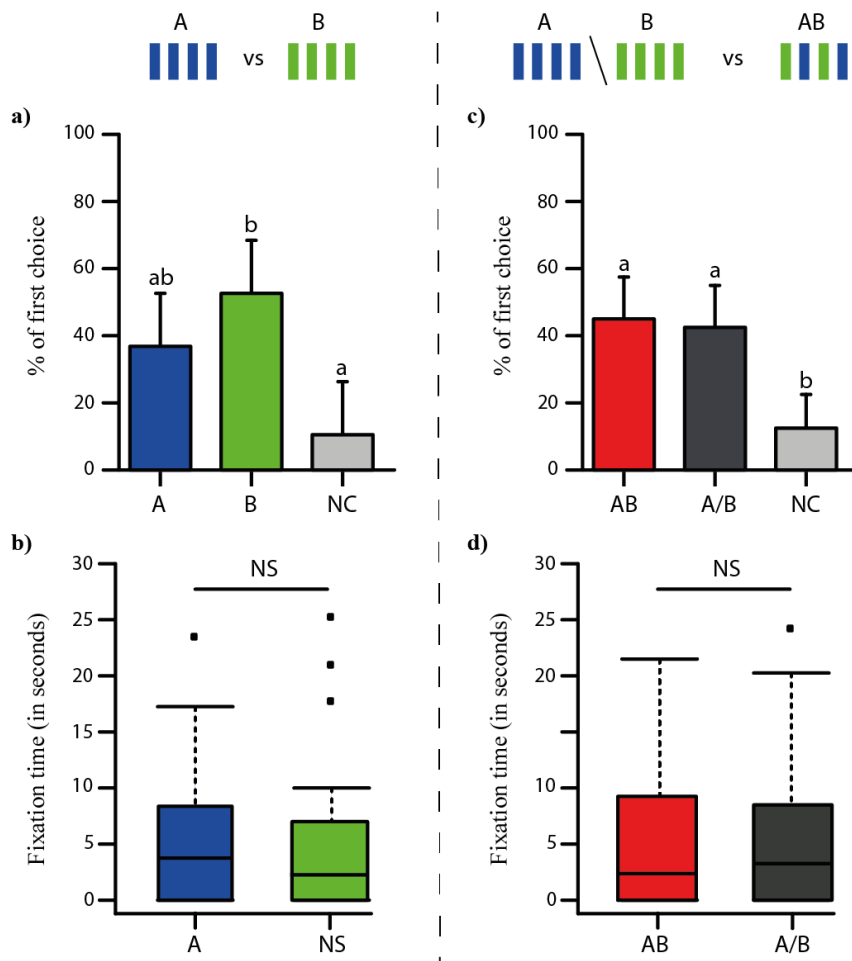
**Figure 4**

**Figure 4. Control experiment: Performance in the tests for bees rewarded for A or for B.** (a) Percentage of bees ( $n=40$ ) ( $\pm$  95% confidence intervals) choosing at first the CS+, the novel stimulus (NS) or not making choice (NC) during the test in which A and B were opposed. CS+ and NS were either A or B depending on the stimulus rewarded during conditioning (20 bees rewarded for A; 20 bees rewarded for B). (b) Fixation time (sec; median, quartiles and outliers) of the CS+ (red boxplot) and of the NS (grey boxplot) during the test in which A and B were opposed. (c) Percentage of bees ( $n=40$ ) ( $\pm$  95% confidence intervals) choosing at first the CS+, the novel stimulus (AB) or not making choice (NC) during the test in which A or B were opposed to AB. CS+ was either A or B depending on the stimulus rewarded during conditioning. (d) Fixation time

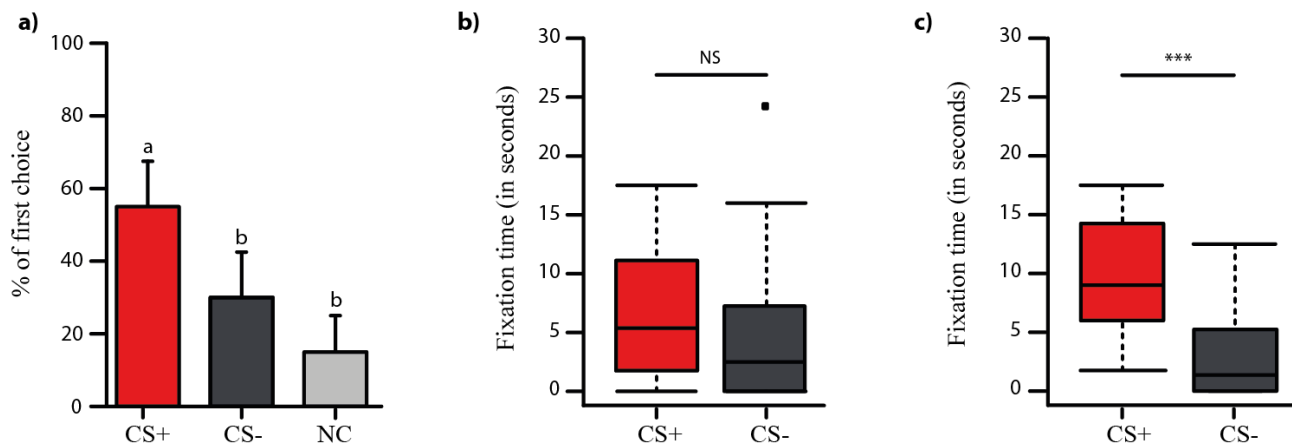
(sec; median, quartiles and outliers) of the CS+ (red boxplot) and of the NS (grey boxplot) during the test in which A and B were opposed to AB. **(a)**, **(c)**: different lower-case letters indicate significant differences ( $p < 0.05$ ). **(b)**, **(d)**: \*:  $p < 0.05$ , \*\*:  $p < 0.001$ , \*\*\*:  $p < 0.0001$ , NS: non-significant,  $p > 0.05$ .



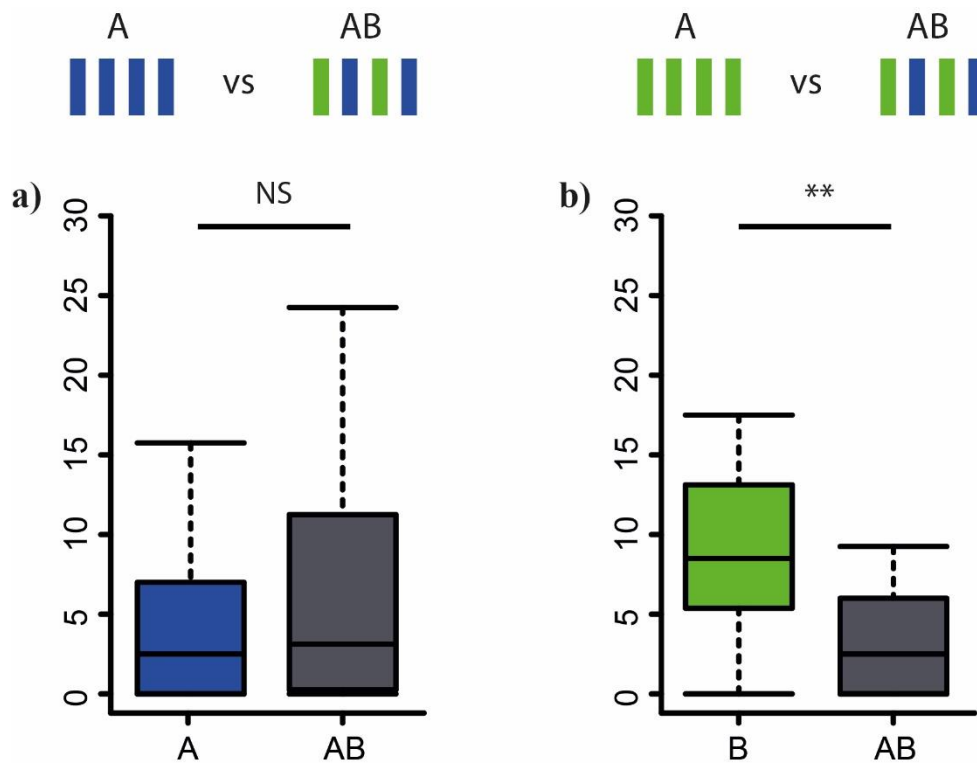
Figure 5



**Figure 5. Control experiment: Performance in the tests for bees rewarded for AB.** (a) Percentage of bees ( $n=20$ ) ( $\pm 95\%$  confidence intervals) choosing at first A, B or not making choice (NC) during the test in which A and B were opposed. (b) Fixation time (sec; median, quartiles and outliers) of A (blue boxplot) and of B (green boxplot) during the test in which A and B were opposed. (c) Percentage of bees ( $n=20$ ) ( $\pm 95\%$  confidence intervals) choosing at first AB, A/B and not making choice (NC) during the test in which A and B were opposed to AB. (d) Fixation time (sec; median, quartiles and outliers) of AB (red boxplot) and of A/B (grey boxplot) during the test in which A and B were opposed to AB. (a), (c): different lower-case letters above bars indicate significant differences ( $p < 0.05$ ). (b), (d): \*:  $p < 0.05$ , \*\*:  $p < 0.001$ , \*\*\*:  $p < 0.0001$ , NS: non-significant.

**Figure 6**

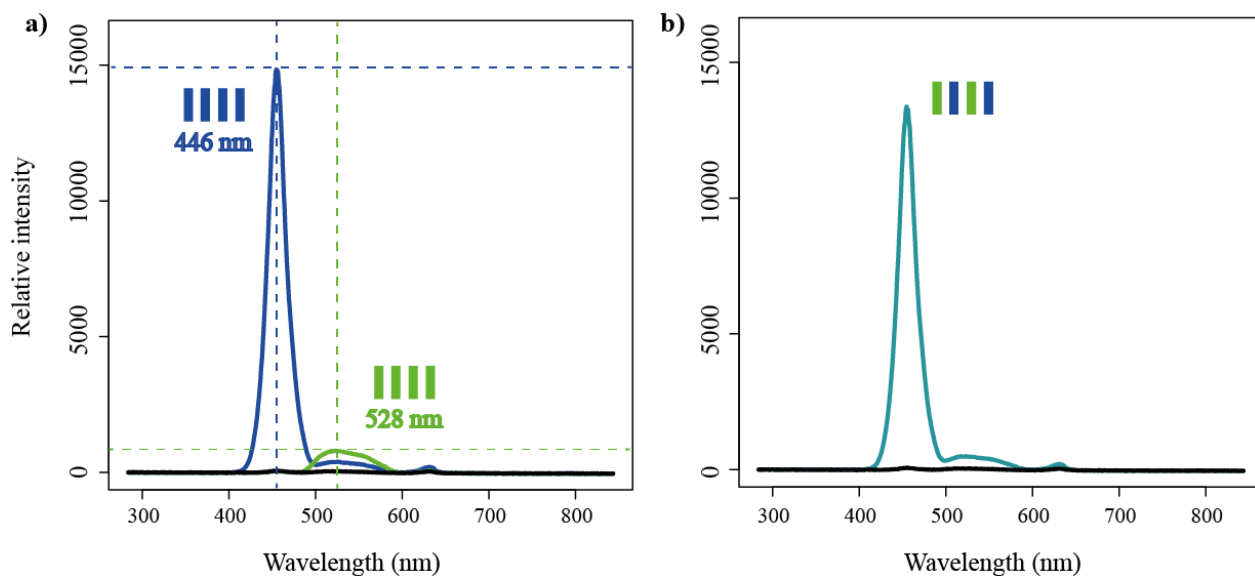
**Figure 6. Negative patterning experiment: Performance during test for bees tested on a negative patterning protocol.** (a) Percentage of bees ( $n=20$ ) choosing at first CS+, CS- and not making choice (NC) during the test in which A and B were opposed to AB. CS+ correspond to pooled data for A and B and CS- correspond to AB. The 95% confidence interval is shown in each case. (b) Fixation time (sec; median, quartiles and outliers) of the CS+ (red boxplot) and of the CS- (grey boxplot) during the test in which A and B were opposed to AB for all bees ( $n=20$ ). CS+ correspond to the pooled data for A and B and CS- correspond to AB (c) Fixation time (sec; median, quartiles and outliers) of the CS+ (red boxplot) and of the CS- (grey boxplot) during the test in which A and B were opposed to AB for bees making a good first choice at a test ( $n=17$ ). CS+ correspond to the pooled data for A and B and CS- correspond to AB (a): different lower-case letters above bars indicate significant differences ( $p<0.05$ ). (b), (c): \*:  $p<0.05$ , \*\*:  $p<0.001$ , \*\*\*:  $p<0.0001$ , NS: non-significant.

**Figure 7**

**Figure 7. Negative patterning experiment: Fixation time during test on a negative patterning protocol (a)** Fixation time (sec; median, quartiles and outliers) of A (blue boxplot) and of AB (grey boxplot) during the test in which A were opposed to AB for all bees (n=20). **(b)** Fixation time (sec; median, quartiles and outliers) of B (green boxplot) and of AB (grey boxplot) during the test in which B were opposed to AB for all bees (n=17). \*: p<0.05, \*\*: p<0.001, \*\*\*: p<0.0001, NS: non-significant.

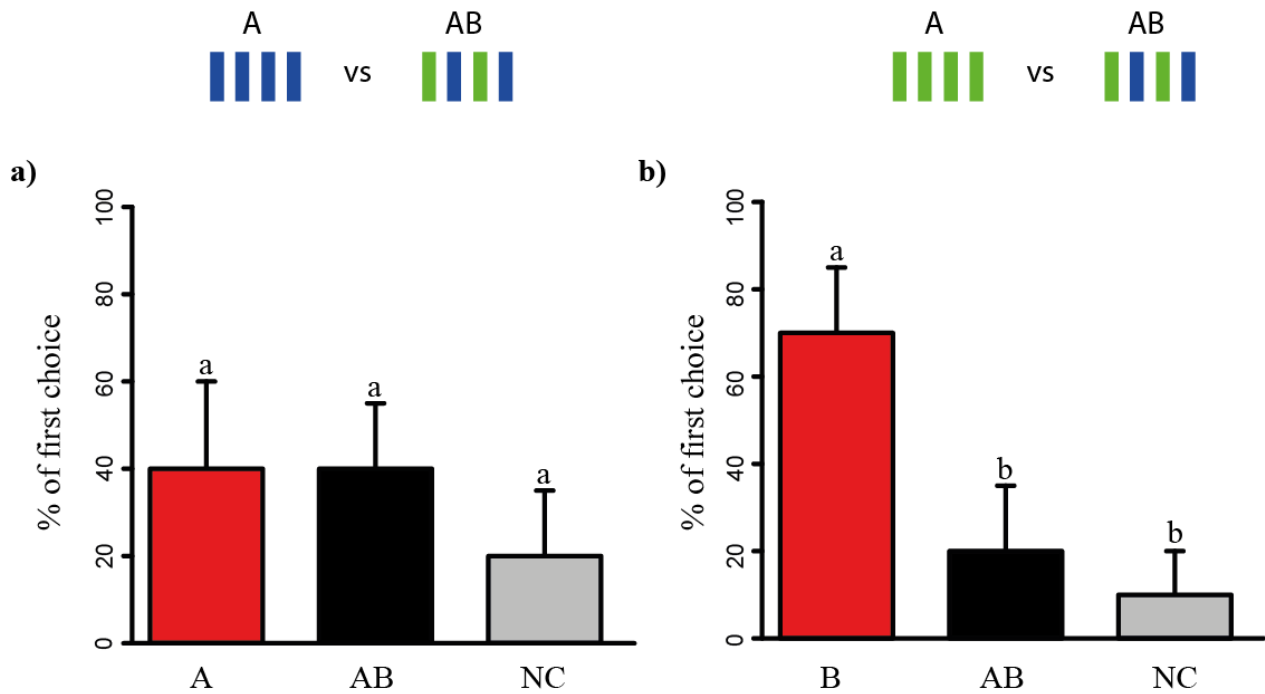
## Supplementary information

Figure S1



**Figure S1.** (a) Spectral curves of the blue grid (dominant wavelength 528nm) and the blue grid (446 nm). (b) Spectral curves of the blue and green grid. The curve of the black background surrounding the colored grid is also shown (black curve, a, b).

Figure S2



**Figure S2:** (a) Percentage of bees ( $n=20$ ) choosing at first A, AB and not making choice (NC) during the test in which A and AB were opposed. The 95% confidence interval is shown in each case. There were not significantly more bees choosing A or AB (A vs AB:  $z_{56}=0$ ,  $p=1$ ; A vs NC:  $z_{56}=-1.36$ ,  $p=0.17$ ; AB vs NC:  $z_{56}=-1.36$ ,  $p=0.17$ ). (b) Percentage of bees ( $n=20$ ) choosing at first B, AB and not making choice (NC) during the test in which B and AB were opposed. The 95% confidence interval is shown in each case. There were significantly more bees choosing B or AB (B vs AB:  $z_{56}=3.01$ ,  $p=0.003$ ; B vs NC:  $z_{56}=0.89$ ,  $p<0.001$ ; AB vs NC:  $z_{56}=-0.87$ ,  $p=0.38$ ). (a), (b): different lower-case letters above bars indicate significant differences ( $p<0.05$ ).

**Table S1**

Color stimulus	Color distance from the background (COC units)	Chromatic contrast to the alternative stimulus (COC units)	Recetor Contrast (absorbed quanta relative to the background)			Intensity (sum of absorbed quanta relative to the background)
			UV	B	G	
Background	--	--	1	1	1	3
Blue	1.75	14.7 (to green)	0.02	0.64	0.33	0.99
Green	7.1	14.7 (to blue)	0.002	0.10	0.90	1.002

**Table S1.** Chromatic and achromatic properties of the trained stimuli.

Contrasts are evaluated with respect to the black background. The illumination light considered was the one provided by the videoprojector lamp.

Receptor-specific contrasts, i.e. the relative number of absorbed quanta  $q$  with respect to the black background, were calculated as:

$$q_i = \frac{\int_0^\infty I(\lambda)R(\lambda)S_i(\lambda)d\lambda}{\int_0^\infty I(\lambda)B(\lambda)S_i(\lambda)d\lambda}, \quad i = uv, \text{ blue, green receptor}; \quad (1)$$

with  $I(\lambda)$  being the intensity distribution of the illuminating light of the videoprojector,  $R(\lambda)$  the spectral curve of the stimulus considered (blue or green disc; see Figure S1),  $B(\lambda)$  the spectral curve of the black background (see Figure S1) and  $S_i(\lambda)$  the spectral sensitivity of the receptor with index  $i$  (Menzel and Backhaus, 1991).

Intensity contrast of a stimulus against the black background was calculated as the sum of the absorbed quanta in the three types of photoreceptors, relative to the background (eq 1).

To quantify chromatic differences, the color opponent coding space proposed specifically for the honey bee (Backhaus, 1991) was used. In such a space, chromatic coordinates A and B of the stimuli were determined as:

$$A = \sum_{i=u,b,g} a_i \frac{q_i}{q_{i+1}}, \quad B = \sum_{i=u,b,g} b_i \frac{q_i}{q_{i+1}} \quad (2)$$

With  $a_i = \{-9.86, 7.70, 2.16\}$  and  $b_i = \{-5.17, 20.25, -15.08\}$ ;  $i=uv$ , blue, green receptor.

The perceptual colour distance D between two stimuli S1 and S2 was calculated as the sum of the absolute differences in chromatic coordinates A and B (Backhaus, 1991):

$$D(S_1, S_2) = |A_{S1} - A_{S2}| + |B_{S1} - B_{S2}| \quad (3)$$

Chromatic contrast is defined as the perceptual color distance D of a stimulus (S) to the black background (Back.). Because the background coordinates in the color space used are (0,0) (Backhaus, 1991), chromatic contrast D is calculated as:

$$D(S, \text{Back.}) = |A_S| + |B_S| \quad (4)$$

Table S2

a)

Trial	1	2	3	4	5	6	7	8	9	10	11
Phase A	A	B	AB	AB	A	AB	AB	A	AB	B	
Phase B	B	AB	B	B	AB	AB	A	AB	AB	A	AB
Phase C	AB	AB	A	A	AB	B	A	AB	B	AB	B

b)

Bee N°	Phases			Test 1	RT			Test 2
	1	2	3		1	2	3	
1	A	B	C	A/AB	B	A	AB	B/AB
2	B	C	A	AB/A	B	AB	A	AB/B
3	A	C	B	A/AB	A	B	AB	AB/B
4	C	B	A	AB/A	A	AB	B	B/AB
5	C	A	B	B/AB	AB	B	A	A/AB
6	B	A	C	AB/B	AB	A	B	AB/A
7	A	C	B	B/AB	A	AB	B	AB/A
8	C	B	A	AB/B	B	AB	A	A/AB
9	B	C	A	A/AB	AB	B	A	B/AB
10	C	B	A	AB/A	A	B	AB	AB/B
11	B	C	A	A/AB	A	AB	B	AB/B
12	A	C	B	AB/A	AB	A	B	B/AB
13	B	A	C	B/AB	B	A	AB	A/AB
14	C	A	B	AB/B	AB	A	B	AB/A
15	B	A	C	B/AB	A	B	AB	AB/A



<b>16</b>	C	A	B	AB/B	B	A	AB	A/AB
<b>17</b>	A	B	C	B/AB	AB	B	A	A/AB
<b>18</b>	A	B	C	AB/B	B	AB	A	AB/A
<b>19</b>	C	B	A	A/AB	B	AB	A	B/AB
<b>20</b>	B	A	C	AB/A	AB	A	B	AB/B

**Table S2.** Sequences of conditioning.

**(a)** Order of stimuli presentation during the different conditioning phases. Only the phase A was composed by 10 trials. **(b)** Conditioning sequences, tests and refreshment trials (RT) for each bee

## References

- Alvarado, M. C., and Rudy, J. W. (1992). Some Properties of Configural Learning: An Investigation of the Transverse-Patterning Problem. *J. Exp. Psychol. Anim. Behav. Process.* 18, 145–153. doi:10.1037/0097-7403.18.2.145.
- Avargues-Weber, A., Dyer, A. G., and Giurfa, M. (2011). Conceptualization of above and below relationships by an insect. *Proc. R. Soc. B Biol. Sci.* 278, 898–905. doi:10.1098/rspb.2010.1891.
- Avargues-Weber, A., Portelli, G., Benard, J., Dyer, A., and Giurfa, M. (2010). Configural processing enables discrimination and categorization of face-like stimuli in honeybees. *J. Exp. Biol.* 213, 593–601. doi:10.1242/jeb.039263.
- Backhaus, W. (1991). Color opponent coding in the visual system of the honeybee. *Vision Res.* 31, 1381–1397. doi:10.1016/0042-6989(91)90059-E.
- Benard, J., Stach, S., and Giurfa, M. (2006). Categorization of visual stimuli in the honeybee *Apis mellifera*. *Anim. Cogn.* 9, 257–270. doi:10.1007/s10071-006-0032-9.
- Buatois, A., Flumian, C., Schultheiss, P., Avargues-Weber, A., and Giurfa, M. (2018). Transfer of visual learning between a virtual and a real environment in honey bees: the role of active vision. *Front. Behav. Neurosci.*
- Buatois, A., Pichot, C., Schultheiss, P., Sandoz, J.-C., Lazzari, C. R., Chittka, L., et al. (2017). Associative visual learning by tethered bees in a controlled visual environment. *Sci. Rep.* 7, 12903. doi:10.1038/s41598-017-12631-w.
- Buckley, M. J., and Gaffan, D. (1998). Perirhinal cortex ablation impairs configural learning and paired-associate learning equally. *Neuropsychologia* 36, 535–546. doi:10.1016/S0028-3932(97)00120-6.
- Chittka, L. (1992). The colour hexagon: A chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi:10.1007/BF00199331.
- Deisig, N., Lachnit, H., and Giurfa, M. (2002). The Effect of Similarity between Elemental Stimuli and Compounds in Olfactory Patterning Discriminations. *Learn. Mem.* 9, 112–121.

doi:10.1101/lm.41002.

Deisig, N., Lachnit, H., Giurfa, M., and Hellstern, F. (2001a). Configural Olfactory Learning in Honeybees : Negative and Positive Patterning Discrimination Configural Olfactory Learning in Honeybees : Negative and Positive Patterning Discrimination. 70–78. doi:10.1101/lm.38301.

Deisig, N., Lachnit, H., Giurfa, M., and Hellstern, F. (2001b). Configural olfactory learning in honeybees: negative and positive patterning discrimination. *Learn. Mem.* 8, 70–78. doi:10.1101/lm.38301.

Deisig, N., Lachnit, H., Sandoz, J.-C., Lober, K., and Giurfa, M. (2003). A Modified Version of the Unique Cue Theory Accounts for Olfactory Compound Processing in Honeybees. *Learn. Mem.* 10, 199–208. doi:10.1101/lm.55803.

Deisig, N., Sandoz, J. C., Giurfa, M., and Lachnit, H. (2007). The trial-spacing effect in olfactory patterning discriminations in honeybees. *Behav. Brain Res.* 176, 314–322. doi:10.1016/j.bbr.2006.10.019.

Devaud, J.-M., Papouin, T., Carcaud, J., Sandoz, J.-C., Grünewald, B., and Giurfa, M. (2015). Neural substrate for higher-order learning in an insect: Mushroom bodies are necessary for configural discriminations. *Proc. Natl. Acad. Sci.* 112, E5854–E5862. doi:10.1073/pnas.1508422112.

Findlay, J. M., and Gilchrist, I. D. (2003). *Active vision: The psychology of looking and seeing*. Oxford.

Giurfa, M. (2004). Conditioning procedure and color discrimination in the honeybee *Apis mellifera*. *Naturwissenschaften* 91, 228–231. doi:10.1007/s00114-004-0530-z.

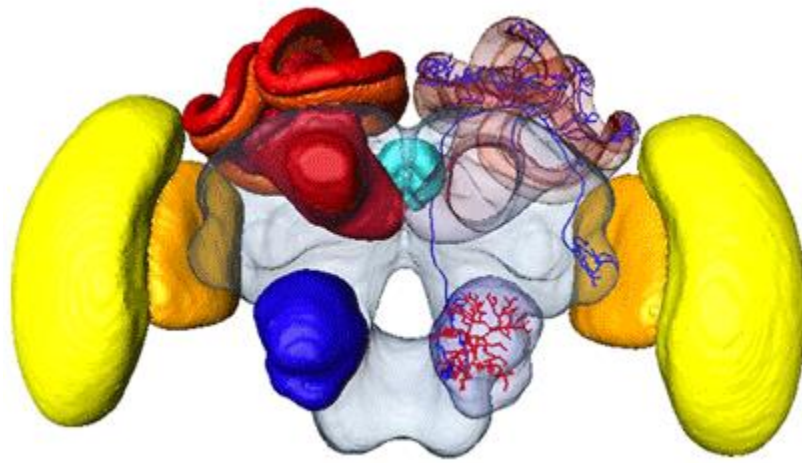
Giurfa, M. (2007). Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *J. Comp. Physiol. A* 193, 801–824. doi:10.1007/s00359-007-0235-9.

Giurfa, M. (2013). Cognition with few neurons: higher order learning in insects. *Trends Neurosci.* 35, 285–294.

Giurfa, M., and Sandoz, J.-C. (2012). Invertebrate learning and memory: Fifty years of olfactory

- conditioning of the proboscis extension response in honeybees. *Learn. Mem.* 19, 54–66. doi:10.1101/lm.024711.111.
- Giurfa, M., Vorobyev, M., Brandt, R., Posner, B., and Menzel, R. (1997). Discrimination of coloured stimuli by honeybees: Alternative use of achromatic and chromatic signals. *J. Comp. Physiol. - A Sensory, Neural, Behav. Physiol.* 180, 235–243. doi:10.1007/s003590050044.
- Giurfa, M., Vorobyev, M., Kevan, P., and Menzel, R. (1996). Detection of coloured stimuli by honeybees: minimum visual angles and receptor specific contrasts. *J. Comp. Physiol. A* 178. doi:10.1007/BF00227381.
- Giurfa, M., Zhang, S., Jenett, a, Menzel, R., and Srinivasan, M. V (2001). The concepts of “sameness” and “difference” in an insect. *Nature* 410, 930–933. doi:10.1038/35073582.
- Kirkerud, N. H., Schlegel, U., and Giovanni Galizia, C. (2017). Aversive Learning of Colored Lights in Walking Honeybees. *Front. Behav. Neurosci.* 11, 1–17. doi:10.3389/fnbeh.2017.00094.
- Menzel, R., and Backhaus, W. (1991). Colour vision in insects. *Vis. Vis. Dysfunct.* 6, 262–293.
- Núñez, J. A. (1966). Quantitative beziehungen zwischen den eigenschaften von futterquellen und dem verhalten von sammelbienen. 53, 142–164.
- Pavlov, I. P. (1941). Lectures on Conditioned Reflexes. Volume II: Conditioned Reflexes and Psychiatry. *JAMA J. Am. Med. Assoc.* 117, 1749–1749. doi:10.1001/jama.1941.02820460087037.
- Pearce, J. M. (2002). Evaluation and development of a connectionist theory of configural learning. *Anim. Learn. Behav.* 30, 73–95. doi:10.3758/BF03192911.
- R Development Core team (2018). R: A language and environment for statistical computing.
- Rescorla, R. A. (1972). “Configural” conditioning in discrete-trial bar pressing. *J. Comp. Physiol. Psychol.* 79, 307–317. doi:10.1037/h0032553.
- Rescorla, R. A. (1973). Evidence for “unique stimulus” account of configural conditioning. *J. Comp. Physiol. Psychol.* 85, 331–338. doi:10.1037/h0035046.
- Rudy, J. W., and Sutherland, R. J. (1989). The hippocampal formation is necessary for rats to learn

- and remember configural discriminations. *Behav. Brain Res.* 34, 97–109. doi:10.1016/S0166-4328(89)80093-2.
- Rusch, C., Roth, E., Vinauger, C., and Riffell, J. A. (2017). Honeybees in a virtual reality environment learn unique combinations of colour and shape. *J. Exp. Biol.*, jeb.164731. doi:10.1242/jeb.164731.
- Schubert, M., Lachnit, H., Francucci, S., and Giurfa, M. (2002). Nonelemental visual learning in honeybees. *Anim. Behav.* 64, 175–184. doi:10.1006/anbe.2002.3055.
- Shanks, D. R., Charles, D., Darby, R. J., and Azmi, A. (1998). Configural processes in human associative learning. *J. Exp. Psychol. Learn. Mem. Cogn.* 24, 1353–1378. doi:10.1037/0278-7393.24.6.1353.
- Sutherland, R. J., and Rudy, J. W. (1989). Configural association theory: The role of the hippocampal formation in learning, memory, and association. 17, 129–144.
- Whitlow, J. W., and Wagner, A. R. (1972). Negative patterning in classical conditioning: Summation of response tendencies to isolable and configurai components. *Psychon. Sci.* 27, 299–301. doi:10.3758/BF03328970.



# Chapitre 4



## Immediate early genes as a tool to identify the neural bases of color memory in honey bees

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Les chapitres précédents ont constitué une base solide pour fournir un paradigme d'apprentissage de couleur en réalité virtuelle. L'enjeu majeur de ce dispositif est de pouvoir explorer les mécanismes du cerveau, ce dernier chapitre sera donc la première étude préliminaire au cours de laquelle l'activation des aires cérébrales est observée lors d'une rétention de mémoire de couleur en réalité virtuelle. De nombreuses approches ont été utilisées en passant des injections aux quantifications génétiques et ont fait leur preuve pour l'exploration de l'apprentissage et de la mémoire. Des gènes précoces immédiats (GPIs) tel que *kakusei* et *egr* sont considérés comme des marqueurs de l'activité neurale dans le cerveau des abeilles, permettant d'observer des cartes d'activation du cerveau pendant une tâche d'apprentissage. En combinant une tâche de discrimination de couleur en réalité virtuelle et une quantification de ces deux gènes à postériori dans différentes aires cérébrales, nous avons exploré comment le cerveau était activé pendant une rétention de mémoire de couleur. Aucune régulation des deux gènes n'a été observée entre les groupes ou les aires, suggérant que ni les corps pédonculés, ni le reste du cerveau n'étaient spécifiquement impliqués dans ce type de mémoire.





# Immediate early genes as a tool to identify the neural bases of color memory in honey bees

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## Abstract

Our knowledge on the impressive visual learning abilities of bees contrasts drastically with the quasi absence of studies on their neural correlates. The main limitation lies on the difficulty to combine invasive methods to explore brain with free flight experiments used to study visual learning in honey bees. Nevertheless, numerous approaches from injection to genetic quantification appeared and won its spurs to explore brain mechanisms of learning and memory. Some immediate early genes as *kakusei* or *egr* are considered as direct neural markers in honey bees brain, allowing to map the brain activation during a learning task. Combining a color discrimination task in virtual reality and a retrospective quantification of these two genes in different brain areas, we explored how the brain was activated during a color memory recall. No upregulation of the two genes has been observed between groups and areas, suggesting that neither the MBs nor the rest was specifically involved in this kind of memory.

## Keywords

*Apis mellifera*, Color discrimination, Virtual reality, IEGs, Mushroom bodies

## Introduction

Bees are known for their impressive visual learning abilities, which have been explored in numerous behavioral experiments in free-flight conditions (Averages-Weber et al., 2010, 2011; Giurfa, 2004; Giurfa et al., 1996, 2001). Unfortunately, despite a century of research in this area, few advances had been done regarding the characterization and understanding of their underlying neural correlates. Indeed, invasive methods such as electrophysiological recordings or neuropharmacology interference are not compatible with a free-flight experiment. In addition, the absence of control of the visual experience of bees before and during training may induce too much noise when analyzing brain modification *a posteriori* (Sommerlandt et al., 2016, 2017). Recently, conditioning protocols were finally made available to train harnessed bees to respond to visual cues (Averages-weber and Mota, 2016; Buatois et al., 2017, 2018; Paulk et al., 2015; Rusch et al., 2017). In particular, virtual reality has been developed to study visual learning in honey bees thus allowing controlled experimental conditions and the possibility for bees to make choice between stimuli. Tethered bees walking stationary on a treadmill (see chapter 2,3) are subjected to simple colored stimuli video-projected whose lateral displacements could be linked to the bees' locomotion activity (Buatois et al., 2017, 2018; Rusch et al., 2017). Such a setup provides consequently the opportunity to study visual learning by associating the visual stimuli with reinforcement while fully controlling the visual experience of the bees. Among the neurobiological analysis techniques that can now be coupled with the virtual reality setup, the observation of the pattern of expression of immediate early genes (IEGs) offers the opportunity to get access to the neural activity induced by visual learning in different brain areas without interfering with the bee's behavior during the learning task. The genes quantification is indeed done on brains collected after the task of interest.

Neuronal activity is thus known to be associated with an increase of the expression of IEGs, for which the transcription is activated rapidly and transiently within minutes of stimulation (Bahrami and Drabløs, 2016). The expression of IEGs is independent of *de novo* protein synthesis and is considered as the first genomic response after a sensory input (Terleph and Tremere, 2006). Furthermore, IEGs products seems to contribute to neuronal synaptic plasticity (Clayton, 2000; Terleph and Tremere, 2006). IEGs are consequently currently used as neural activity markers, allowing to visualize activation maps in the vertebrates' brain (Guzowski et al., 2005). The characterization of neural activity in bees via the measure of the transcription of immediate early genes has also been evidenced (Kiya et al., 2012; Ugajin et al., 2013, 2017). Two IEGs, *kakusei* and *egr*, have proved to be good candidates to visualize neural activation during a simple learning

task such as homing behaviour (Kiya et al., 2007; Ugajin et al., 2017). *Kakusei* functions as a nuclear ncRNA, whereas *egr* is a homolog of *egr-1* in vertebrates and encodes a transcription factor with a DNA-binding domain in honey bees. The expression levels of these two genes were indeed upregulated during this task.

The honey bee brain is composed of different neuropils, among which the mushroom bodies (MBs) and the central complex (CC) are the ideal candidates for the treatment and storage of the visual information. The collar region of the MB receives direct visual input from the optical lobe in honey bees (Ehmer and Gronenberg, 2002; Gronenberg and López-Riquelme, 2004). Furthermore, the output of this collar region terminates in an inner layer of the vertical lobes in honey bees (Strausfeld, 2002), which has been already identified as a crucial structure for olfactory learning and memory (Giurfa, 2013). Consequently, MB could be an important area regarding the treatment and the storage of the visual information. The CC, as for it, is known for his role in walking, turning and climbing behavior in fruit flies (Triphan et al., 2010) and crickets (Kai and Okada, 2013). Furthermore, several studies in fruit flies have already shown the importance of this structure in visual learning and memory (Kuntz et al., 2012, 2017; Liu et al., 2006; Ofstad et al., 2011). Unfortunately, and because of the limitations described above, the involvement of these two structures in visual learning and memory of bees remains unexplored with a single exception (Plath et al., 2017).

This recent study has indeed confirmed the implication of these two structures in an aversive visual learning task in which walking bees had to avoid a colored area punished with an electric shock within a two-compartment device (Plath et al., 2017). The behavioral analyses on color learning were coupled with pharmacological blockade of neural activity via local injections of an anesthetic in the bee brain. Plath et al showed thus that the performance of bees was deteriorated when inactivating either MBs or CC. However, the question of the brain areas involved in appetitive learning and memory remain unresolved as different reinforcements may lead to different neuronal circuits involved (Mizunami et al., 2009; Suver et al., 2012; Ye et al., 2004).

In this study, we used the recently developed virtual reality setup to condition honey bees in an appetitive visual learning task combined with a quantification of immediate early genes expression induced. We thus explored whether an upregulation of *kakusei* and/or *egr* in the MB and the CC could be observed as a marker of neuronal activity during visual-memory recall

following a visual discrimination learning task in which bees had to learn to discriminate a blue disc from a green disc.

## **Materials and methods**

### **Animals**

Honey bee (*Apis mellifera*) foragers were caught before collecting sucrose solution on a feeder placed at the CRCA's apiary in the University Paul Sabatier of Toulouse. Bees were then fed with 4 $\mu$ l of a 1M sucrose solution and prepared for the behavioral experiments. Based on (Buatois et al., 2018), bees were anesthetized on ice for 3min. The thorax was then shaved to improve the fixation of the tethering used during the experiment. Finally, bees were placed for 3h on a miniature treadmill to allow familiarization with the device and control the satiety level of all bees tested.

### **Behavioral Assay**

Bees were placed in a virtual reality setup composed of a spherical treadmill combined with a video projection system (Buatois et al., 2018). We used the same conditioning protocol described in Chapter 3 in which bees were trained to discriminate a blue disc and a green disc one being rewarded with a 1M sucrose solution, while the other was punished with a 60mM quinine solution (Figure 1). Bees were first tested during a pre-test which lasted 30 second to identify their naïve preference for either the blue or the green disc. The non-preferred stimulus was then set as the rewarded stimulus (CS+) for the subsequent conditioning phase while the alternative color was set as the non-rewarded stimulus (CS-). The conditioning protocol was composed of 12 trials. At the beginning of each trial, both stimuli were presented first on the screen respectively on the right or on the left of the bees' body axis. When the bee has centered one of the stimulation indicating that it orientated toward it, the stimulus remained at its centered position during 8 second and the corresponding reinforcement was delivered (sucrose or quinine) for 5 seconds. Then a resting period in the dark of 60 seconds was proposed to the bee before the start of the next trial. A trial lasted a maximum of 30 seconds if the bee did not make a choice. Each trial lasted in average  $22 \pm 2$  seconds according to if the bees made a choice. Finally, one hour after the last conditioning trial, bees were tested during a non-reinforced post-test for 30 seconds to observe whether their initial preference has been modified after being conditioned against it. Three groups of bees were run in

parallel: a *paired group* (n=20), a *CS group* (n=20) and a *US group* (n=20). In the paired group, bees experienced the colors paired with their respective reinforcement. In the *CS group*, bees only experienced the visual stimuli in the same number and order as the paired group, but never received reinforcement on them. Bees of the *US group* only experienced the reinforcement in the same number as bees of the paired group but in a random order, but they were never exposed to a visual stimulus in the setup. Bees of the paired group were classified as *Learners* and *Non-learners* according to whether they changed their preference between the two tests. The *CS group* and the *US group* were used as control for neuronal activation induced respectively by the visual stimulation or reinforcement in the absence of learning.

Forty-five minutes after the post-test, the bees were decapitated, and their heads were placed into a  $-80^{\circ}$  liquid-nitrogen container to fix the brains before dissection.

### **Dissection**

Each frozen brain was dissected on dry ice under a binocular microscope. The cuticle was first removed, as well as all superficial glands. Then, brains were separated in four parts, the antennal lobes as the lower region (AL), the optical lobes as the lateral regions (OL), the mushroom bodies (MBs) as the upper region, and the remaining central part (RP) including the central complex and the suboesophageal ganglion. Brain samples were stored at  $-80^{\circ}\text{C}$  until RNA extractions.

### **RNA extraction**

Total RNA samples were extracted using a RNA-Quick Microkit (Zym research/Ozyme). Quantifications were performed using a Nanodrop spectrophotometer ND-1000. Each sample concentration was adjusted to 3 ng/  $\mu\text{l}$ .

### **Reverse Transcription**

Reverse Transcriptions (RT) were performed in 20  $\mu\text{l}$  with 300 ng of total RNA using RevertAid First Strand cDNA Synthesis (from ThermoFischer Scientific) using the standard protocol provided by the manufacturer. The RT minus reaction has been used as a control (mix of reaction buffer with no RNA template) in order to test genomic DNA contamination.

### **qPCR**

Quantification of IEGs *Egr* and *Kakusei* was done using quantitative real time PCR (Polymerase Chain Reaction) after dilution one in third of the cDNA product and using Sso Advanced Syber Green from Biorad. The real time PCR rests on the introduction of a fluorochrome inside the amplicon. It is therefore possible to quantify the fluorescence over the time with a camera and thus indirectly to quantify relatively the target gene expression. The genes *Rps8* and *Efla* were used as reference for the quantification. These two housekeeping genes offer an absence of variability of their expression between brain areas and between groups (linear model, Group effect:  $F=0.52$ ,  $df=2$ ,  $p=0.76$ , Area effect:  $F=2.02$ ,  $df=3$ ,  $p=0.15$ , Group\*Area effect:  $F=1.01$ ,  $df=6$ ,  $p=0.42$ ). Primers (*Egr* 131nt sense: 5'TATATGTCGCCTCTACTC and anti-sense: 5'GAGAGAAAGAGAGGAAGA), (*Kakusei* 167nt sense: 5'TTCATTCAAGCGTTCGTA and anti-sense: 5'AGAATGAAGTTGAAGGAGAA), (*Rps8* 176nt sense: ACGAGGTGCGAACTGACTGA and anti-sense 5'GCACTGTCCAGGTCTACTCGA) and (*Efla* 143nt sense: GCACTGTCCAGGTCTACTCGA 5' and anti-sense: TGTGGGCGTCATTAA) were used at a final concentration of 250 nM. The reaction was run in triplicate and incubated at 95°C 30s, followed by 40 cycles at 94°C 5 sec, 55°C 30 sec. The specificity of the PCR products was assessed by generating a dissociation curve (95°C for 10 sec, 55°C for 1 sec, 95°C 5 sec and increases in temperatures of 0.5°C from 55°C to 95°C). Analysis of the dissociation curves of the different amplification products revealed a single melting peak. We also checked for the absence of amplification in the negative control RT minus, confirming the efficiency of the DNase treatment during RNA extraction. The relative quantity of target genes *Egr* and *Kakusei* was quantified using the  $\Delta\Delta C_t$ -method.

### Statistical analyses

Because of the difficulty to dissect and extract the RNA, we noted the loss of several brains before the qPCR. Thus, only 10 brains in each group have been analyzed, and more specifically 4 learners brains and 6 non-learners brains in the *Paired group*.

Because of the organization of the qPCR plates, only two bees were analyzed on a same plate. To compare the brain area within and between groups, we normalized the expression of *kakusei* and *egr* by two different ways. A first one was the normalization of the IEGs expression in each area according to the expression in the other area. The second one was the normalization of expression in each group according to the expression in the other group. These normalizations allowed to



provide two series of data, one to compare brain areas in each group, and a second one to compare each group within the different brain areas. In order to know if these two comparisons were correlated and provided the same conclusions, we did a principal component analysis (PCA). The axe 1 and 2 of the PCA explained 61.37% of the *egr* expression variance (Figure 4a) and 60.92% of the *kakusei* expression variance (Figure 4b). Moreover, the two kinds of analyze seems to be graphically correlated either on the axe 1 or 2 irrespective of the IEGs nature (Figure 4a, b). The results of the two comparisons are therefore complementary.

Thus, the relative expression levels of *egr* and *kakusei* are presented within each group by comparing brain areas (1), and by comparing groups within each area (2). A linear model was used to make the comparison between area or between groups. Because of the dependency of the data (1), bees were considered as a random factor and the model included the relative expression in function of the brain areas. However, for the comparison (2), data were completely independent, allowing to use a model only including the relative expression in function of the group. To overcome the non-normality of the data, the linear model was combined with a permutation test.

Moreover, for each comparison and to know if there was a regulation of gene expression compared to the expression of the reference genes, relative expression was compared to a theoretical value of 1 thanks to a Mann-Whitney test.

All graphics and analyses were done with R 3.4.1 (RCoreTeam, Vienna, Austria) using the packages lme4 and ade4 for the linear model and the ACP respectively.

## Results

Following the learning of a color discrimination task in virtual reality, the relative expression of the two IEGs, *kakusei* and *egr*, was quantified in the brain of bees of the three experimental groups, paired, CS and US.

### **Is there difference of *egr* and *kakusei* relative expression between brain areas within each group?**

Within each of the three groups (CS, US and paired, the latter divided in Learners and Non-Learners), we quantified the relative expression of *egr* and *kakusei* and compared brain areas for this variable.

Regarding the normalized levels of expression of *egr*, no up-regulation according to the reference genes expression was observed in any brain areas irrespective of the group considered (Table 1, Figure 2) except in the MB for the US group (Figure 2, Table 1,  $W=47$ ,  $p=0.05$ ). Moreover, no significant differences were found for *egr* normalized levels between the brain areas of the CS group (Figure 2, linear model,  $F=0.53$ ,  $df=3$ ,  $p=0.66$ ), the Learners (Figure 2,  $F=0.77$ ,  $df=3$ ,  $p=0.53$ ) or the Non-Learners (Figure 2,  $F=0.56$ ,  $df=3$ ,  $p=0.63$ ) as well as of the US group despite the up-regulation observed for the MB (Figure 2,  $F=1.07$ ,  $df=3$ ,  $p=0.37$ ),

The results were almost similar for the *kakusei* relative expression (Table 1, Figure 2), except for the US group in which a downregulation according to the reference genes expression was observed in the MB (Figure 2, Table 1,  $W=1$ ,  $p=0.004$ ) whereas an upregulation was observed in the RP (Figure 2, Table S1,  $W=5$ ,  $p=0.02$ ). Furthermore, a significant difference was found between areas regarding relative expression levels of *kakusei* (Figure 2,  $F=8.24$ ,  $df=3$ ,  $p<0.001$ ). The expression in the AL and the RP was significantly higher than in the OL and the MB for the US group (Figure 2, AL vs MB:  $F=6.53$ ,  $df=1$ ,  $p=0.02$ ; AL vs CC:  $F=1.36$ ,  $df=1$ ,  $p=0.26$ ; OL vs MB:  $F=1.36$ ,  $df=1$ ,  $p=0.26$ ; OL vs CC:  $F=9.17$ ,  $df=1$ ,  $p=0.004$ ; MB vs CC:  $F=6.53$ ,  $df=1$ ,  $p=0.02$ ). Nevertheless, no significant difference was found when comparing *kakusei* relative expression between brain areas in the case of the CS group (Figure 2,  $F=0.03$ ,  $df=3$ ,  $p=0.99$ ), the Learners (Figure 2,  $F=1.46$ ,  $df=3$ ,  $p=0.27$ ) or the Non-Learners (Figure 2,  $F=0.93$ ,  $df=3$ ,  $p=0.44$ ).

### **Is there a difference of *egr* and *kakusei* relative expression between groups for a given brain area?**

As for the previous section, the analysis was done for the CS, the US, and the paired group divided in Learners and Non-Learners. The relative expression of *egr* and *kakusei* was compared between groups for each brain area.

Regarding *egr* relative expression, no regulation was observed according to the reference genes expression within brain areas irrespective of the group considered (Table 2) except for a downregulation of *egr* expression was detected in the CS group for the optic lobes (OL) (Figure 3, Table 2,  $W=7$ ,  $p=0.03$ ). Moreover, no significant differences in *egr* relative expression were found between groups at the level of the AL (Figure 3,  $F=1.1$ ,  $df=3$ ,  $p=0.37$ ), the OL (Figure 3,  $F=1.1$ ,  $df=3$ ,  $p=0.37$ ), the MB (Figure 3,  $F=0.28$ ,  $df=3$ ,  $p=0.84$ ) or the RP (Figure 3,  $F=1.06$ ,  $df=3$ ,  $p=0.38$ ).

In the case of *kakusei*, a downregulation of expression according to the reference genes expression was found in the MBs of the US group (Figure 3, Table 2,  $W=6$ ,  $p=0.03$ ). However, and as for *egr* expression, no significant differences were found between groups when *kakusei* expression was compared between brain areas such as the AL (Figure 3,  $F=2.81$ ,  $df=3$ ,  $p=0.07$ ), the OL (Figure 2,  $F=0.80$ ,  $df=3$ ,  $p=0.51$ ), the MB (Figure 2,  $F=2.34$ ,  $df=3$ ,  $p=0.07$ ) or the RP (Figure 3,  $F=1.1$ ,  $df=3$ ,  $p=0.34$ ).

These results suggest therefore that despite several up or downregulation of *egr* and *kakusei* expression within areas of the bee brain, no clear effect resulting from learning or retention could be detected.

## Discussion

Although the ability of bees to learn and memorize color discriminations in free-flight conditions has been intensively studied over 100 years (Avarguès-Weber and Giurfa, 2014; von Frisch, 1914) and more recently in virtual reality conditions (Buatois et al., 2017; Rusch et al., 2017), the neural correlates underlying these capacities are poorly understood despite the fact that the localization of visual memory traces has occupied neuroscientists during decades (ref ?). Previous studies on the brain areas involved in visual learning used either local injection of anesthetics into the bee brain (Plath et al., 2017) or staining of synaptic connectivity at the level of the mushroom bodies (Sommerlandt et al., 2016). Yet, the lack of control of the individual variability might have introduced too much noise on the results for a clear interpretation.

Our studies combined a color discrimination protocol in virtual reality with the quantification of the relative expression of two immediate early genes considered as markers of neural activity, *egr* and *kakusei* (Kiya et al., 2007, 2012; Ugajin et al., 2013). During this study, no clear upregulation of either *egr* or *kakusei* was observed between groups or between brain areas. This absence of effect could reflect several possibilities; firstly, the time delay chosen for analyzing quantitative changes in expression levels of IEGs (i.e. 45 min following the post test) does not allow visualizing such changes. But this first hypothesis seems to not be consistent with studies in rodents or bees showing that this time delay is enough to achieve the maximum of IEGs expression (Cullinan et al., 1995; Ugajin et al., 2017). Secondly, it is possible that no brain area considered has a specific role in memory recall. Nevertheless, this second hypothesis can be rejected. Indeed,

for instance, mushroom bodies have been already identified as the localization of the olfactory short-term and the olfactory long-term memory in *Drosophila* (McBride et al., 1999; McGuire et al., 2001; Pascual and Preat, 2001; Zars et al., 2000) and in bees (Erber et al., 1980; Hourcade et al., 2010; Liu et al., 1999). This assumption is less clear regarding the visual memory in bees and our results will unfortunately not provide more elements to highlight the role of the MBs. Indeed, until now, only two studies using different approaches have been explored the importance of the MB and the central complex (CC) during visual learning and memory in honey bees. The first one used an aversive conditioning (Kirkerud et al., 2017) combined with localized injections of the local anesthetic procaine and showed that the vertical lobe of the MBs and the CC were involved in visual aversive learning, contrary to the MBs collar (Plath et al., 2017). Yet, in these experiments only one of four mushroom-body collars were injected which means that three remained active. It is therefore difficult to take their results on collar for granted. Furthermore, results obtained injecting procaine in the ventral lobe of the MBs and in the CCS are also questionable. Bees injected with vehicle or procaine showed the same decrease of learning and memory performances so that the pretended effect of the silencing of brain areas by the anesthetic could be in fact a general effect of the injection.

In the second study, using specific staining of the microglomeruli, a synaptic complex formed by the axonal terminal of one projection neuron coming from a sensory brain area, connecting multiple dendritic spines of MB neurons, researchers showed that there were no variation of microglomeruli number and density in the collar, the visual region of the calyces, after a color discrimination task (Sommerlandt et al., 2016). These parameters contradict findings on synaptic plasticity and the building of long-term memories following olfactory learning in honeybees (Hourcade et al., 2010). After this learning, a long-term (72h post conditioning) odor-specific increase in the density of microglomeruli was observed in the lip, the olfactory region of the calyces, but not in the collar, not required for the olfactory learning task. In the case of bees tested after a color learning task, no test was performed to verify the presence of long-term memory in the bees whose mushroom bodies were analyzed. Thus, the absence of changes resulting from learning may simply reflect the absence of memory.

The *Drosophila* literature remains so far, the most documented source about the role of the MBs and the CC in visual learning in insects. In *Drosophila*, the role of the MBs is also largely discussed according to studies showing that it is dispensable for some forms of visual memory

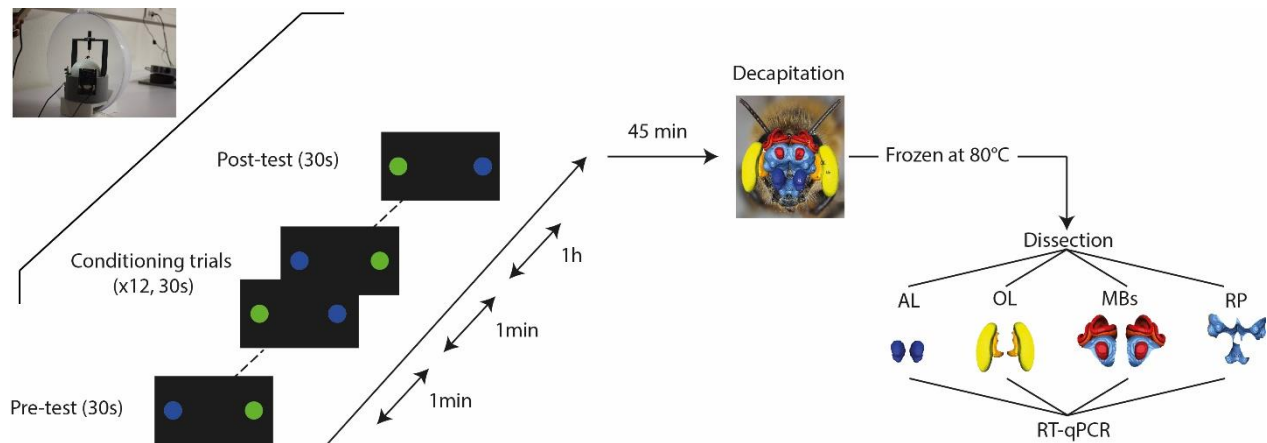
tested in flight simulator (Ofstad et al., 2011; Wolf et al., 1998) and to be required only when the learning context change between the conditioning and the memory test (Liu et al., 1999; Peng et al., 2007). However, it seems that focusing only on a complete area does not offer a sufficient resolution, as some specific subset of intrinsic neurons in the MBs are required for specific visual memories (Vogt et al., 2014). Nevertheless, the MBs are not the only important neuropil for visual learning and memories in drosophila. Indeed, the CC seems to also have a main role, allowing the feature detection (Seelig and Jayaraman, 2013) and the visual pattern memory (Liu et al., 2006; Pan et al., 2009; Wang et al., 2008). According to these results, the logical hypothesis would be to not have a specific activation of the MBs and the CC during a memory recall succeeding a color discrimination task either in drosophila or in honey bee. The distribution and the properties of the neurons coming from the optical lobe in bees suggests however another hypothesis. Indeed, it seems that all the MBs input neurons coming from the OL are color sensitive (Paulk and Gronenberg, 2008) attesting to the involvement of the MBs in color treatment. Moreover, perception of a light stimulus induces a distinct pattern of activation in output interneurons of the anterior optic tubercle (Mota et al., 2013), which could suggest that the trace of color memory could be localized inside the optic tubercle.

Our results, although negative, are in agreement with (Ofstad et al., 2011; Pan et al., 2009; Seelig and Jayaraman, 2013; Sommerlandt et al., 2016; Wang et al., 2008; Wolf et al., 1998), regarding the non-specificity of MBs and CC during a color discrimination memory recall not including any features. Furthermore, we didn't observe a specific activation of the optical lobe according to the group, meaning that there was not any inhibition of input neurons activity in the optical lobe regardless of the number of presentation of the stimuli. This result is in accordance with Paulk and Gronenberg (2008), who showed that a when a bumblebee was excited with a blue or a green light, there was no inhibition of the input neurons activity in the optical lobe.

However, the dissection was not enough accurate to study highly localized areas of the brain. It was therefore impossible to verify the activation of specific subset of intrinsic neurons (Vogt et al., 2014) for example, certainly covered by the activation of other neurons inside the MBs. To bypass this, it would be possible to either perform an *in situ* hybridization of *kakusei* and *egr* on brain slices to localize the activation of neurons, or to repeat quantification on smallest areas thanks to the microlaser dissection method. All these suggestions are also valuable regarding the

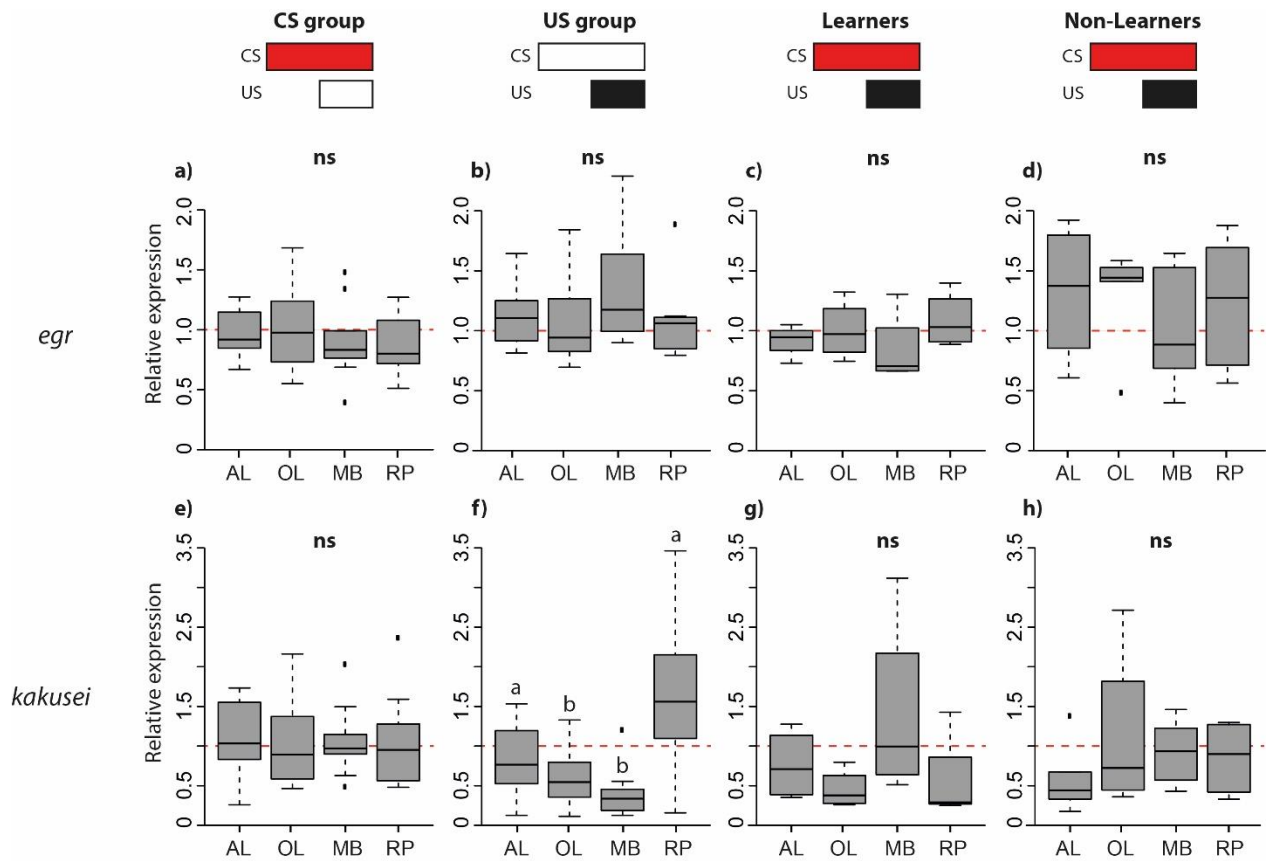
role of the CC for which it is difficult to conclude on with our results as mixed with other structures the RP such as the suboesophageal ganglion for instance.

Our approach to characterize the activation of the brains area during visual memory recall provides advantages as well as limits. Indeed, explore the brain retrospectively provides a possibility to avoid interacting with the learning process. The limit is, obviously, the lack of accuracy of the methods, not allowing to observe a local area inside the MB for instance. It is still difficult to conclude something from a negative result, but according to all the points discussed above, it seems that this experiment allows us to suggest the non-specificity of the MBs for color memory. However, in light of our results we can't conclude about the real involvement of the MBs, it just seems that it is not involve more than another area. It is, however, important to keep in mind the small sample size in the Learners group which should be increase. Moreover, a new non-linear paradigm had been presented in the chapter 4: the visual negative patterning in virtual reality. The next step would be to combine this new approach with this paradigm in order to explore the difference of activation between the simple learning (the present study) and the complex learning.

**Figure 1****Figure 1. Experimental procedure.**

As in (Buatois et al., 2018), bees were tested in a color discrimination task in virtual reality. During a pre-test (30s), bees were faced to two colored stimuli, a blue disc and a green disc without any reinforcement. The preference during this pre-test was determinant to choose what stimuli would be the CS- (punished stimulus). Then, during 12 trials (30s), bees were once again faced to the two stimuli. When one was centered by the bee, it remained at its position allowing to provide the reinforcement. Finally, one hour after the conditioning, as in the pre-test, bees were face to the two stimuli without reinforcement in order to observe if the bee changed its initial preference. This test allowed us to determine the *Learners* and the *Non-learners* in the *Paired group*. Then, after 45 min of rest time, bees were decapitated, and heads were maintained at  $-80^{\circ}\text{C}$ . Finally, brains were dissected to isolate the AL, the OL, the MBs and the remaining part in order to perform RT-qPCR on it. This procedure has been done on the *paired group*, the *US group* and the *CS group*.

Figure 2

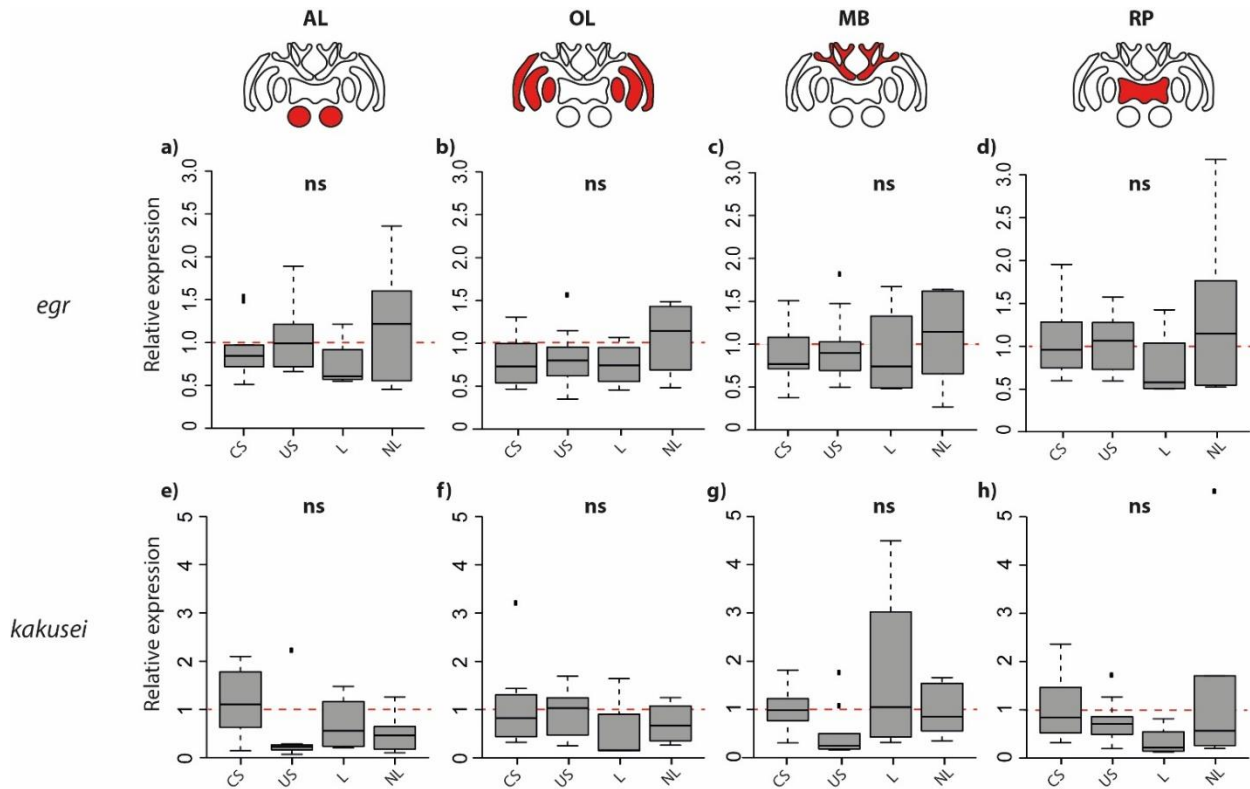


**Figure 2. Relative expression of *egr* and *kakusei* in each brain area for each group.**

The first line represents the relative expression of *egr* (median, quartiles and outliers) for the CS group (**a**, **n=10**), the US group (**b**, **n=10**), the Learners (**c**, **n=4**) and the Non-Learners (**d**, **n=6**). The second line represents the relative expression of *kakusei* (median, quartiles and outliers) for the CS group (**e**), the US group (**f**), the Learners (**g**) and the Non-Learners (**h**). The red dot line corresponding to 1 represents a relative equivalence between the expression of the target genes (*egr*, *kakusei*) and the expression of the reference genes (). Above this line, the relative expression was saying upregulated, whereas under the line it was saying downregulated. Different lower-case letters above bars indicate significant difference ( $p < 0.05$ ).



Figure 3



**Figure 3. Relative expression of *egr* and *kakusei* in each group for each brain area.**

The first line represents the relative expression of *egr* (median, quartiles and outliers) for the AL (a), the OL (b), the MB (c) and the RP (d). The second line represents the relative expression of *kakusei* (median, quartiles and outliers) for the AL (e), the OL (f), the MB (g) and the RP (h). The red dot line corresponding to 1 represents a relative equivalence between the expression of the target genes (*egr*, *kakusei*) and the expression of the reference genes. Above this line, the relative expression was saying upregulated, whereas under the line it was saying downregulated. Different lower-case letters above bars indicate significant difference (p < 0.05). CS correspond to the CS group, US to the US group, L to the Learners and NL to the Non-Learners.

Figure 4

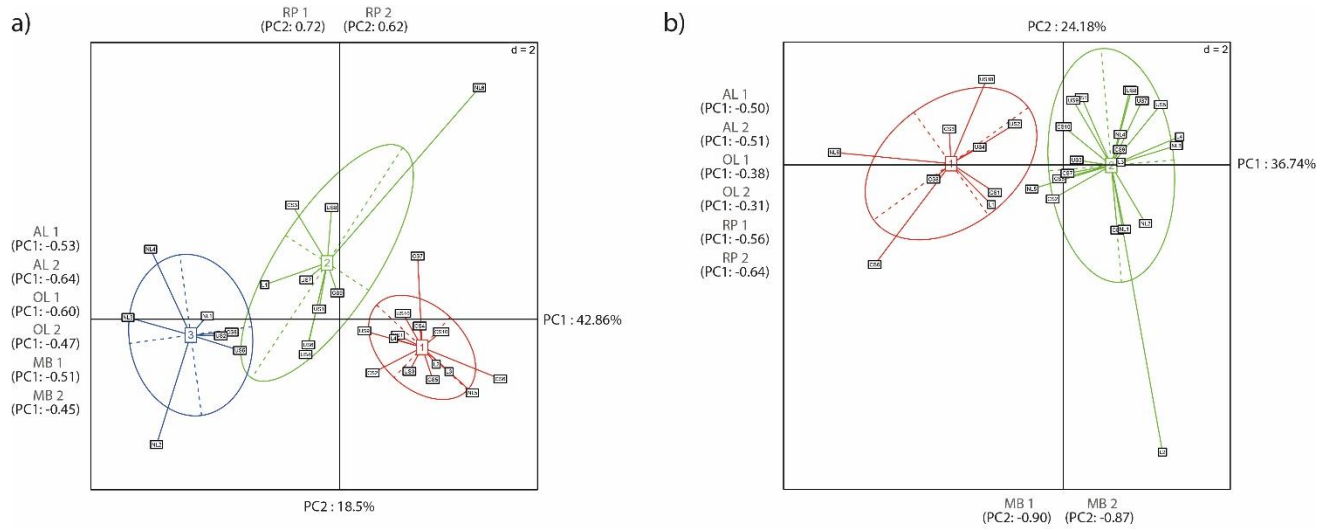


Figure 4. PCA of the relative expression

PCA of the relative expression normalized according to the first or the second normalization way for (a) *egr* and (b) *kakusei*. AL: Antennal Lobe, OL: Optical Lobe, MB: Mushroom body, RP: Remaining Part. The number 1 and 2 succeeding the area correspond to the normalization way. The two main axes explain 61.36% of the variance of *egr* (a) and 60.92% for *kakusei* (b).

**Table 1**

Group	CS group				US group				Learners				Non-Learners			
Brain area	AL	OL	MB	RP	AL	OL	MB	RP	AL	OL	MB	RP	AL	OL	MB	RP
<i>egr</i>	W=20 P=0.49	W=10 P=0.08	W=17 P=0.32	W=25 P=0.84	W=41 P=0.19	W=25 P=0.85	W=47 P=0.05	W=27 P=1	W=2 P=0.37	W=7 P=0.62	W=2 P=0.37	W=5 P=1	W=17 P=0.22	W=10 P=0.62	W=8 P=1	W=12 P=0.31
<i>kakusei</i>	W=32 P=0.69	W=27 P=1	W=30 P=0.85	W=25 P=0.85	W=16 P=0.27	W=45 P=0.08	W=1 P=0.004	W=5 P=0.02	W=2 P=0.37	W=1 P=0.25	W=5 P=1	W=0 P=0.12	W=2 P=0.09	W=5 P=0.62	W=6 P=0.81	W=9 P=0.81

**Table 1. Regulation of *egr* and *kakusei* in each brain area for each group.**

The relative expression of *egr* and *kakusei* were compared with the theoretical mean of 1 thanks to a Mann-Whitney test. The results of the tests are sum up in this table, in the first line for *egr* and the second line for *kakusei*. Significant results are highlighted in grey.

**Table 2**

Brain area	AL				OL				MB				RP			
Group	CS	US	L	NL	CS	US	L	NL	CS	US	L	NL	CS	US	L	NL
<i>egr</i>	W=18 P=0.37	W=27 P=1	W=1 P=0.25	W=14 P=0.56	W=7 P=0.03	W=11 P=0.10	W=1 P=0.25	W=12 P=0.84	W=16 P=0.27	W=18 P=0.37	W=4 P=0.87	W=12 P=0.84	W=19 P=0.43	W=26 P=0.92	W=1 P=0.25	W=14 P=0.56
<i>kakusei</i>	W=33 P=0.62	W=10 P=0.08	W=2 P=0.37	W=1 P=0.06	W=22 P=0.62	W=26 P=0.92	W=1 P=0.25	W=5 P=0.31	W=26 P=0.92	W=6 P=0.03	W=6 P=0.87	W=11 P=1	W=27 P=1	W=13 P=0.16	W=0 P=0.12	W=9 P=0.84

**Table 2. Regulation of *egr* and *kakusei* in each group for brain area.**

The relative expression of *egr* and *kakusei* were compared with the theoretical mean of 1 thanks to a Mann-Whitney test. The results of the tests are sum up in this table, in the first line for *egr* and the second line for *kakusei*. Significant results are highlighted in grey.

## References

- Avargues-Weber, A., Dyer, A. G., and Giurfa, M. (2011). Conceptualization of above and below relationships by an insect. *Proc. Biol. Sci.* 278, 898–905. doi:10.1098/rspb.2010.1891.
- Avarguès-Weber, A., and Giurfa, M. (2014). Cognitive components of color vision in honey bees: How conditioning variables modulate color learning and discrimination. *J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol.* 200, 449–461. doi:10.1007/s00359-014-0909-z.
- Avarguès-weber, A., and Mota, T. (2016). Advances and limitations of visual conditioning protocols in harnessed bees. *J. Physiol. - Paris*. doi:10.1016/j.jphysparis.2016.12.006.
- Avargues-Weber, A., Portelli, G., Benard, J., Dyer, A., and Giurfa, M. (2010). Configural processing enables discrimination and categorization of face-like stimuli in honeybees. *J. Exp. Biol.* 213, 593–601. doi:10.1242/jeb.039263.
- Bahrami, S., and Drabløs, F. (2016). Gene regulation in the immediate-early response process. *Adv. Biol. Regul.* 62, 37–49. doi:10.1016/j.jbior.2016.05.001.
- Buatois, A., Flumian, C., Schultheiss, P., Avarguès-Weber, A., and Giurfa, M. (2018). Transfer of visual learning between a virtual and a real environment in honey bees: the role of active vision. *Front. Behav. Neurosci.*
- Buatois, A., Pichot, C., Schultheiss, P., Sandoz, J.-C., Lazzari, C. R., Chittka, L., et al. (2017). Associative visual learning by tethered bees in a controlled visual environment. *Sci. Rep.* 7, 12903. doi:10.1038/s41598-017-12631-w.
- Clayton, D. F. (2000). The genomic action potential. *Neurobiol. Learn. Mem.* 74, 185–216. doi:10.1006/nlme.2000.3967.
- Cullinan, W. E., Herman, J. P., Battaglia, D. F., Akil, H., and Watson, S. J. (1995). Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 64, 477–505. doi:10.1016/0306-4522(94)00355-9.
- Ehmer, B., and Gronenberg, W. (2002). Segregation of visual input to the mushroom bodies in the honeybee (*Apis mellifera*). *J. Comp. Neurol.* 451, 362–373. doi:10.1002/cne.10355.

- Erber, J., Masuhr, T., and Menzel, R. (1980). Localization of short-term memory in the brain of the bee, *Apis mellifera*. *Physiol. Entomol.* 5, 343–358. doi:10.1111/j.1365-3032.1980.tb00244.x.
- Giurfa, M. (2004). Conditioning procedure and color discrimination in the honeybee *Apis mellifera*. *Naturwissenschaften* 91, 228–231. doi:10.1007/s00114-004-0530-z.
- Giurfa, M. (2013). Cognition with few neurons: higher order learning in insects. *Trends Neurosci.* 35, 285–294.
- Giurfa, M., Vorobyev, M., Kevan, P., and Menzel, R. (1996). Detection of coloured stimuli by honeybees: minimum visual angles and receptor specific contrasts. *J. Comp. Physiol. A* 178. doi:10.1007/BF00227381.
- Giurfa, M., Zhang, S., Jenett, A., Menzel, R., and Srinivasan, M. (2001). The concepts of “sameness” and ‘difference’ in an insect. *Nature* 410, 2–5. Available at: <http://www.nature.com/nature/journal/v410/n6831/abs/410930a0.html>.
- Gronenberg, W., and López-Riquelme, G. O. (2004). Multisensory convergence in the mushroom bodies of ants and bees. *Acta Biol. Hung.* 55, 31–37. doi:10.1556/ABiol.55.2004.1-4.5.
- Guzowski, J. F., Timlin, J. A., Roysam, B., McNaughton, B. L., Worley, P. F., and Barnes, C. A. (2005). Mapping behaviorally relevant neural circuits with immediate-early gene expression. *Curr. Opin. Neurobiol.* 15, 599–606. doi:10.1016/j.conb.2005.08.018.
- Hourcade, B., Muenz, T. S., Sandoz, J.-C., Rössler, W., and Devaud, J.-M. (2010). Long-term memory leads to synaptic reorganization in the mushroom bodies: a memory trace in the insect brain? *J. Neurosci.* 30, 6461–6465. doi:10.1523/JNEUROSCI.0841-10.2010.
- Kai, K., and Okada, J. (2013). Characterization of Locomotor-Related Spike Activity in Protocerebrum of Freely Walking Cricket. *Zoolog. Sci.* 30, 591–601. doi:10.2108/zsj.30.591.
- Kirkerud, N. H., Schlegel, U., and Giovanni Galizia, C. (2017). Aversive Learning of Colored Lights in Walking Honeybees. *Front. Behav. Neurosci.* 11, 1–17. doi:10.3389/fnbeh.2017.00094.
- Kiya, T., Kunieda, T., and Kubo, T. (2007). Increased neural activity of a mushroom body neuron

- subtype in the brains of forager honeybees. *PLoS One* 2. doi:10.1371/journal.pone.0000371.
- Kiya, T., Ugajin, A., Kunieda, T., and Kubo, T. (2012). Identification of kakusei, a nuclear non-coding RNA, as an immediate early gene from the honeybee, and its application for neuroethological study. *Int. J. Mol. Sci.* 13, 15496–15509. doi:10.3390/ijms131215496.
- Kuntz, S., Poeck, B., Sokolowski, M. B., and Strauss, R. (2012). The visual orientation memory of *Drosophila* requires Foraging (PKG) upstream of Ignorant (RSK2) in ring neurons of the central complex. *Learn. Mem.* 19, 337–340. doi:10.1101/lm.026369.112.
- Kuntz, S., Poeck, B., and Strauss, R. (2017). Visual Working Memory Requires Permissive and Instructive NO/cGMP Signaling at Presynapses in the *Drosophila* Central Brain. *Curr. Biol.* 27, 613–623. doi:10.1016/j.cub.2016.12.056.
- Liu, G., Seiler, H., Wen, A., Zars, T., Ito, K., Wolf, R., et al. (2006). Distinct memory traces for two visual features in the *Drosophila* brain. *Nature* 439, 551–556. doi:10.1038/nature04381.
- Liu, L., Wolf, R., Ernst, R., and Heisenberg, M. (1999). Context generalization in *Drosophila* visual learning requires the mushroom bodies. *Nature* 400, 753–756. doi:10.1038/23456.
- McBride, S. M. J., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., et al. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in *Drosophila melanogaster*. *Neuron* 24, 967–977. doi:10.1016/S0896-6273(00)81043-0.
- McGuire, S. E., Le, P. T., and Davis, R. L. (2001). The Role of *Drosophila* Mushroom Body Signaling in Olfactory Memory. *Science* (80-. ). 293, 1330 LP-1333. Available at: <http://science.sciencemag.org/content/293/5533/1330.abstract>.
- Mizunami, M., Unoki, S., Mori, Y., Hirashima, D., Hatano, A., and Matsumoto, Y. (2009). Roles of octopaminergic and dopaminergic neurons in appetitive and aversive memory recall in an insect. *BMC Biol.* 7, 1–16. doi:10.1186/1741-7007-7-46.
- Mota, T., Gronenberg, W., Giurfa, M., and Sandoz, J.-C. (2013). Chromatic Processing in the Anterior Optic Tubercle of the Honey Bee Brain. *J. Neurosci.* 33, 4–16. doi:10.1523/JNEUROSCI.1412-12.2013.

- Ofstad, T. A., Zuker, C. S., and Reiser, M. B. (2011). Visual place learning in *Drosophila melanogaster*. *Nature* 474, 204–209. doi:10.1038/nature10131.
- Pan, Y., Zhou, Y., Guo, C., Gong, H., Gong, Z., and Liu, L. (2009). Differential roles of the fan-shaped body and the ellipsoid body in *Drosophila* visual pattern memory. *Learn. Mem.* 16, 289–295. doi:10.1101/lm.1331809.
- Pascual, A., and Preat, T. (2001). Localization of long-term memory within the *Drosophila* mushroom body. 294, 1115–1118.
- Paulk, A. C., and Gronenberg, W. (2008). Higher order visual input to the mushroom bodies in the bee, *Bombus impatiens*. *Arthropod Struct. Dev.* 37, 443–458. doi:10.1097/MPG.0b013e3181a15ae8.Screening.
- Paulk, A. C., Kirszenblat, L., Zhou, Y., and van Swinderen, B. (2015). Closed-Loop Behavioral Control Increases Coherence in the Fly Brain. *J. Neurosci.* 35, 10304–10315. doi:10.1523/JNEUROSCI.0691-15.2015.
- Peng, Y., Xi, W., Zhang, W., Zhang, K., and Guo, A. (2007). Experience Improves Feature Extraction in *Drosophila*. *J. Neurosci.* 27, 5139–5145. doi:10.1523/JNEUROSCI.0472-07.2007.
- Plath, J. A., Entler, B. V., Kirkerud, N. H., Schlegel, U., Galizia, C. G., and Barron, A. B. (2017). Different Roles for Honey Bee Mushroom Bodies and Central Complex in Visual Learning of Colored Lights in an Aversive Conditioning Assay. *Front. Behav. Neurosci.* 11. doi:10.3389/fnbeh.2017.00098.
- Rusch, C., Roth, E., Vinauger, C., and Riffell, J. A. (2017). Honeybees in a virtual reality environment learn unique combinations of colour and shape. *J. Exp. Biol.*, jeb.164731. doi:10.1242/jeb.164731.
- Seelig, J. D., and Jayaraman, V. (2013). Feature detection and orientation tuning in the *Drosophila* central complex. *Nature* 503, 262–266. doi:10.1038/nature12601.
- Sommerlandt, F. M. J., Rössler, W., and Spaethe, J. (2017). Impact of light and alarm pheromone on immediate early gene expression in the European honeybee, *Apis mellifera*. *Entomol. Sci.* 20, 122–126. doi:10.1111/ens.12234.



- Sommerlandt, F. M. J., Spaethe, J., Rössler, W., and Dyer, A. G. (2016). Does fine color discrimination learning in free-flying honeybees change mushroom-body calyx neuroarchitecture? *PLoS One* 11, 1–17. doi:10.1371/journal.pone.0164386.
- Strausfeld, N. J. (2002). Organization of the honey bee mushroom body: Representation of the calyx within the vertical and gamma lobes. *J. Comp. Neurol.* 450, 4–33. doi:10.1002/cne.10285.
- Suver, M. P., Mamiya, A., and Dickinson, M. H. (2012). Octopamine neurons mediate flight-induced modulation of visual processing in drosophila. *Curr. Biol.* 22, 2294–2302. doi:10.1016/j.cub.2012.10.034.
- Terleph, T. A., and Tremere, L. A. (2006). “The Use of Immediate Early Genes as Mapping Tools for Neuronal Activation: Concepts and Methods,” in *Immediate Early Genes in Sensory Processing, Cognitive Performance and Neurological Disorders*, eds. R. Pinaud and L. A. Tremere (Boston, MA: Springer US), 1–10. doi:10.1007/978-0-387-33604-6\_1.
- Triphan, T., Poeck, B., Neuser, K., and Strauss, R. (2010). Visual Targeting of Motor Actions in Climbing *Drosophila*. *Curr. Biol.* 20, 663–668. doi:10.1016/j.cub.2010.02.055.
- Ugajin, A., Kunieda, T., and Kubo, T. (2013). Identification and characterization of an Egr ortholog as a neural immediate early gene in the European honeybee (*Apis mellifera* L.). *FEBS Lett.* 587, 3224–3230. doi:10.1016/j.febslet.2013.08.014.
- Ugajin, A., Uchiyama, H., Miyata, T., Sasaki, T., Yajima, S., and Ono, M. (2017). Identification and initial characterization of novel neural immediate early genes possibly differentially contributing to foraging-related learning and memory processes in the honeybee. *Insect Mol. Biol.* 27, 154–165. doi:10.1111/imb.12355.
- Vogt, K., Schnaitmann, C., Dylla, K. V., Knapek, S., Aso, Y., Rubin, G. M., et al. (2014). Shared mushroom body circuits underlie visual and olfactory memories in *Drosophila*. *Elife* 3, e02395. doi:10.7554/eLife.02395.
- von Frisch, K. (1914). Der Farbensinn und Formensinn der Bienen. *Zool. Jahrbucher Physiol.*, 1–128.
- Wang, Z., Pan, Y., Li, W., Wang, Z., Pan, Y., Li, W., et al. (2008). complex of *Drosophila* Visual

pattern memory requires foraging function in the central complex of *Drosophila*. 133–142. doi:10.1101/lm.873008.

Wolf, R., Wittig, T., Liu, L., Wustmann, G., Eydin, D., and Heisenberg, M. (1998). *Drosophila* Mushroom Bodies Are Dispensable for Visual, Tactile, and Motor Learning. *Learn. Mem.* 5, 166–178. doi:10.1101/lm.5.1.166.

Ye, Y., Xi, W., Peng, Y., Wang, Y., and Guo, A. (2004). Long-term but not short-term blockade of dopamine release in *Drosophila* impairs orientation during flight in a visual attention paradigm. *Eur. J. Neurosci.* 20, 1001–1007. doi:10.1111/j.1460-9568.2004.03575.x.

Zars, T., Fischer, M., Schulz, R., and Heisenberg, M. (2000). Localization of a short-term memory in *Drosophila*. *Science (80-. )*. 288, 672–675. doi:10.1126/science.288.5466.672.





# Discussion



Ma thèse a eu pour objectif d'améliorer nos connaissances sur les bases neurales sous-jacentes aux apprentissages visuels chez l'abeille. L'enjeu majeur a été de trouver une alternative aux expériences de libre-vol afin de conditionner des abeilles fixées à répondre à des stimuli visuels. Cette réflexion est à l'origine d'une revue bibliographique (Schultheiss et al., 2017, voir Annexe 1), dans laquelle toutes les technologies déjà existantes pour l'insecte ont été répertoriées ainsi que leurs avantages et inconvénients. Nous avons donc choisi de mettre en place un dispositif de réalité virtuelle permettant d'observer tout type d'apprentissage visuel, qu'il soit élémentaire ou non, chez des abeilles fixées. La réussite des conditionnements des abeilles dans un système de réalité virtuelle était la condition *sine qua non* afin d'ensuite explorer les mécanismes cérébraux sous-jacents à l'apprentissage ou à la mémoire visuelle.

Au cours de notre première étude (Chapitre I), nous avons mis en évidence la capacité des abeilles à apprendre à discriminer des stimulations virtuelles émises par un vidéoprojecteur. Ce résultat est loin d'être une formalité de par les nombreux changements et contraintes apportées par un tel dispositif par rapport aux expériences en libre-vol. Ainsi, les abeilles ont pu être conditionnées visuellement dans un environnement visuel contrôlé. Il était néanmoins alors impossible de parler encore de réalité virtuelle, car notre environnement était dit en « boucle ouverte ». En effet, le mouvement de l'abeille n'entraînait pas de mouvement des stimulations visuelles, l'abeille était alors conditionnée à discriminer deux stimulations colorées de manière classique et sa direction de marche était uniquement utilisée pour analyser de quel côté elle s'orientait préférentiellement. Cette expérience a été conclue par la mise en évidence des capacités d'apprentissage discriminatif de couleur dans un environnement visuel contrôlé, se traduisant par le choix du stimulus récompensé après le conditionnement pour 60% des abeilles. Ces performances étaient loin de celles obtenues en libre vol, mais étaient plus que prometteuses au regard de la simplicité du système utilisé et des contraintes qu'il impliquait. Enfin, cette étude a aussi été l'occasion de mettre en évidence l'impact de différents renforcements négatifs sur les performances de discrimination. À l'issue de ces expériences, la quinine est ressortie comme le stimulus inconditionnel le plus pertinent. Son utilisation a donc été validée pour l'ensemble des expériences de ma thèse.

Ma seconde étude (Chapitre II), a débuté par la mise en place d'un nouveau dispositif permettant cette fois-ci de travailler avec un environnement visuel en « boucle fermée », dans

lequel le déplacement des stimulations à l'écran étaient corrélés avec les mouvements de l'abeille. Ce nouveau dispositif peut donc être considéré comme un système de réalité virtuelle. Améliorer le dispositif était indispensable mais il restera toujours la contrainte de garder les abeilles attachées pour notre objectif. Nous nous sommes alors demandé si le système d'attache pouvait être limitant pour l'apprentissage des abeilles. Pour répondre à cette question, nous avons réalisé en parallèle un protocole de conditionnement de discrimination de couleur sur des abeilles dans le système de réalité virtuelle (RV) et dans un labyrinthe en Y. Pour aller plus loin, ces abeilles ont ensuite été transférées dans le dispositif opposé afin de tester leur capacité à transférer l'apprentissage de la RV vers le labyrinthe, ou l'inverse. Cette étude nous a permis de montrer que les abeilles testées en RV ou en labyrinthe, présentaient les mêmes performances lors du conditionnement. En effet, 60% des abeilles choisissaient la stimulation récompensée dans les deux contextes. En revanche, au cours des tests de transfert, les abeilles conditionnées en RV ont vu leurs performances s'améliorer au moment du passage dans le labyrinthe, contrairement aux abeilles transférées dans le sens inverse. Ces résultats suggèrent que les stratégies de traitement des stimuli utilisées dans notre système de RV et dans un labyrinthe habituellement utilisé en libre marche sont différentes, et que les indices visuels offerts au sein du labyrinthe sont certainement plus nombreux que ceux fournis dans la RV.

Les deux chapitres précédents ont permis d'explorer les capacités des abeilles à apprendre à discriminer des stimulations colorées simples, dans un apprentissage dit élémentaire car chaque stimulation est soit récompensée soit punie de façon constante et non ambiguë. Les capacités cognitives des abeilles ne s'arrêtent pas à ce type d'association, elles sont aussi capables de résoudre des problèmes plus complexes dits non élémentaires. Afin de déterminer comment le cerveau code les informations au cours des apprentissages visuels, il est indispensable de pouvoir étudier son fonctionnement au cours d'apprentissages simples comme décrits dans les deux premiers chapitres, mais aussi pendant des apprentissages complexes. L'objectif au cours de cette troisième étude (Chapitre III), a donc été de mettre en place un paradigme de « patterning négatif » dans notre système de réalité virtuelle. Dans ce paradigme, les abeilles vont être récompensées pour un stimulus visuel A et B, mais non récompensées pour un stimulus composé de A et B à la fois. Cet apprentissage est particulièrement ambigu du fait que A et B vont être autant récompensés que non récompensés. Les abeilles vont alors devoir inhiber la généralisation vers AB et ne plus le

considérer comme une somme de A et B. 60% des individus ont réussi à résoudre cette tâche en réalité virtuelle, le paradigme de « patterning négatif » est donc validé en RV.

Pour finir, et fort de notre paradigme de discrimination de couleur, cette expérience en réalité virtuelle a été combinée avec une analyse a posteriori des cerveaux par RT-qPCR de l'expression de gènes précoces immédiats (GPIs) considérés comme des marqueurs de l'activité neurale. Cette expérience est l'objet du chapitre IV. La question ici était de savoir si une zone cérébrale particulière était activée spécifiquement au cours d'une rétention de mémoire de couleurs. Plusieurs groupes ont été testés, un groupe apprentissage et deux groupes contrôles pour lesquels il était impossible que les abeilles apprennent. Après l'expérience, les cerveaux ont été récupérés, puis disséqués afin de séparer les parties importantes (LA, LO, CPs, CC). Par la suite, l'expression de *kakusei* et *egr* (deux GPIs) a été quantifiée et comparée entre les différentes zones et les différents groupes. Il semblerait qu'aucune activation spécifique d'une zone n'ait lieu au cours de la rétention de mémoire de couleur.

L'ensemble de ces études s'est conclue par la mise en place d'un système de RV permettant d'étudier l'apprentissage visuel chez des abeilles attachées. Ceci constitue une grande avancée qui permettra l'utilisation de méthodes invasives et ainsi la caractérisation des bases neurales sous-jacentes. Dans un premier temps, la validité de la réalité virtuelle pour étudier l'apprentissage visuel sera discutée. Les avantages de cette méthode seront abordés, ainsi que ses limites au regard de nos résultats. Des perspectives seront ainsi proposées. Par la suite, des hypothèses quant aux bases neurales de l'apprentissage visuel seront proposées, en s'appuyant sur les résultats du dernier chapitre.

## I. La réalité virtuelle : une bonne alternative au libre vol ?

### a) Les stimuli virtuels sont perçus différemment des stimuli réels.

Les systèmes de réalité virtuelle sont variés et utilisent différents procédés afin d'afficher l'environnement visuel. Chez l'abeille, on peut ainsi trouver des écrans LCD (Taylor et al., 2013),



des écrans composés de LEDs (Paulk et al., 2015) ou encore des écrans en plastique sur lesquels les environnements sont projetés (Buatois et al., 2017; Rusch et al., 2017). C'est cette dernière solution qui a été choisie au cours de ma thèse, bien qu'elle implique quelques limites. Les vidéoprojecteurs utilisés projettent à une fréquence de 120 Hz (soit 120 images par seconde) alors que les abeilles possèdent une vision à 200 Hz (Srinivasan and Lehrer, 1984). De ce fait, les abeilles ont une vision saccadée des stimuli à l'écran. Ceci aurait pu être un problème, mais au cours du premier chapitre, il a été montré que 100% des abeilles testées en libre vol étaient capables d'apprendre à discriminer deux stimuli colorés générés par les vidéoprojecteurs. Cette expérience a ainsi permis de valider l'utilisation de stimuli colorés simples avec un système de vidéoprojection.

Cependant, il est important de noter la limite de la vidéoprojection, ainsi que des autres systèmes cités précédemment. En effet, le fonctionnement de ces systèmes est basé sur l'utilisation du système RVB (Rouge-Vert-Bleu). Ce codage informatique va permettre de reconstituer une couleur par synthèse additive à partir de trois couleurs primaires en formant sur l'écran une mosaïque imperceptible à l'œil nu (**Figure 1**). Par exemple, pour créer du violet, une mosaïque composée de succession de bandes verticales et horizontales va être créée. La position et l'ordre de



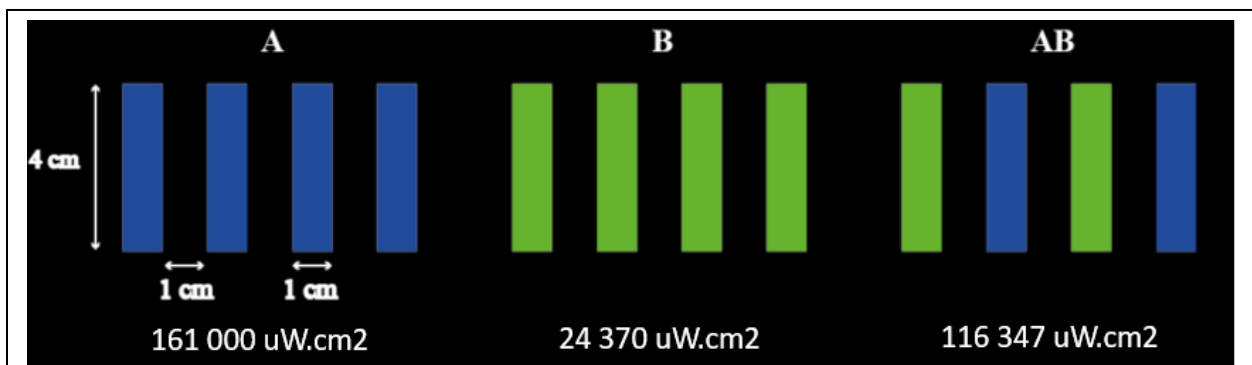
**Figure 1. Mosaïque RVB**

La mosaïque des lumières rouges, vertes et bleues vue lorsque l'on zoom au maximum sur un écran.

ces bande va varier aléatoirement selon le taux de rafraîchissement de ces bandes, qui pour rappel est de 120Hz pour notre vidéoprojecteur. Cela signifie qu'une abeille pourrait voir nettement l'alternance entre les bandes bleues et rouges. Cela pose souci, car comme décrit dans l'introduction générale, les abeilles ne possèdent pas de récepteur pour le rouge (Menzel and

Blakers, 1976). De ce fait, il est tout à fait envisageable que lorsqu'on projette une stimulation violette, les abeilles ne perçoivent qu'une stimulation bleue. La solution consisterait à trouver des écrans ou des vidéoprojecteurs dont le taux de rafraîchissement est supérieur à 200Hz, tout en utilisant des couleurs qui n'impliquent jamais de mélange avec du rouge. Ici, nous n'avons à faire qu'à un problème mineur qui va trouver sa solution dans l'évolution des technologies. Ainsi, on voit déjà apparaître de nouveaux écrans ou projecteurs permettant d'obtenir ce résultat.

Dans le cadre de notre étude, nous avons ainsi pu démontrer que l'utilisation de stimuli visuels projetés n'était pas un problème, et que les abeilles étaient capables de les discriminer que ce soit en semi-liberté ou au sein de notre système de réalité virtuelle (Chapitre I et II). Nous avons d'ailleurs basé nos études sur la capacité des abeilles à discriminer ces stimuli en fonction de leurs caractéristiques chromatiques et de la distance colorimétrique qui les séparait. Ces distances avaient été calculées sur la base des différents modèles de distance de couleur : le COC (« Color Opponent Coding ») (Backhaus, 1991) et l'hexagone des couleurs (Chittka, 1992). Basé sur les valeurs excitatrices des stimulations par rapport à chaque photorécepteur, ces modèles permettent de prédire la probabilité qu'une abeille réussisse à discriminer deux couleurs en fonction de la



**Figure 2 : Stimuli utilisés lors de l'expérience de « Patterning négatif » du chapitre 3.**

Grilles de couleurs bleue (A), verte (B) et bleue et verte (AB). L'intensité lumineuse est indiquée en dessous de chaque stimulus.

distance chromatique qui les sépare. Ces modèles ont été construits en se référant aux performances comportementales d'abeilles testées en libre vol à discriminer des stimulations colorées en papier. Ces données, ont ainsi pu révéler que les abeilles n'utilisaient pas l'information de luminosité pour réaliser ces tâches de discrimination. Le facteur luminosité n'est de ce fait jamais pris en compte dans ces modèles, ce qui pourrait être problématique dans nos études. En effet, les stimuli papiers

réfléchissent la lumière, alors que dans le cas de nos expériences, le stimulus est la lumière. Les résultats du chapitre III soulèvent d'autant plus cette problématique. Une première expérience de conditionnement absolu a permis de mettre en évidence la capacité des abeilles à discriminer une grille bleue (A) et une grille verte (B) (**Figure 2**). En revanche, en suivant le même protocole, les abeilles étaient incapables de discriminer A de AB ou B de AB malgré les différences d'intensité lumineuse, ce qui s'est traduit par une généralisation des abeilles vers AB. Ces résultats ont validé l'utilisation de ces stimuli pour réaliser un protocole de « Patterning négatif ». Cependant, au cours de cette expérience dite non élémentaire, les abeilles sont censées inhiber leur comportement de généralisation vers AB. Ainsi, nous avons pu observer ce comportement lorsque B était opposé à AB, mais par lorsque A l'était. Du fait de l'utilisation des mêmes stimulations dans le protocole d'apprentissage absolu et de « Patterning négatif », nous sommes certains de la capacité des abeilles à pouvoir discriminer A et B. Dans le modèle COC (Backhaus, 1991), la distance chromatique entre nos deux couleurs est égale à 14,7, valeur pour laquelle les abeilles ne devraient avoir aucun mal à discriminer deux couleurs. De plus, chaque bande représentait un angle visuel de 5,7°, de même que les bandes noires entre chaque bande colorée. Cet angle visuel est plus que suffisant pour permettre aux abeilles de les distinguer les unes des autres (Giurfa et al., 1996, 1997). En s'appuyant sur les mêmes principes, la stimulation entière représentait 38,6°, permettant ainsi facilement à l'abeille de discriminer les deux stimuli. Au regard de ces différents éléments, le seul paramètre pouvant être incriminé pour la chute des performances des abeilles au cours des tests A vs AB est donc l'intensité lumineuse des stimuli. En effet, l'intensité lumineuse entre A et AB était très proche (**Figure 2**), à l'inverse de celle entre B et AB. Il était alors plus facile pour l'abeille de discriminer B et AB en se basant sur ce paramètre.

Force est de constater que ce problème est récurrent dans chacun de mes chapitres, où les performances paraissent toujours moindres lorsque le stimulus vert est récompensé et ce malgré la diminution de la luminosité initiale de celui-ci. En effet, à son niveau maximal en RVB, le vert atteint une luminosité bien plus importante que le bleu, entraînant une réponse quasi systématique des abeilles pour celle-ci. Ce comportement a aussi été observé dans le cadre des expériences visuelles aversives dans le système APIS (Kirkerud et al., 2017) au cours desquelles il est très simple d'entraîner une abeille à éviter une zone bleue électriée, mais beaucoup moins lorsque la zone verte est électriée. Le plus étonnant est que l'on ne retrouve pas ce comportement dans les expériences en libre vol décrites dans le chapitre I. Les expériences contrôlées dans lesquelles

l'abeille est bloquée pourraient donc être la cause de cette attraction pour le vert qui ne correspond en rien à la littérature. En effet, la préférence naïve des abeilles pour une couleur lorsqu'elles sont en libre vol se traduit normalement par un choix pour le violet ou le bleu face à d'autres couleurs comme le vert ou le jaune (Giurfa et al., 1995). Il semblerait que dans nos conditions, ces préférences naïves ne sont pas systématiquement retrouvées, avec au contraire une préférence pour le vert. Il semblerait que tous les types de photorécepteurs de l'abeille participent au contrôle de la phototaxie (Kaiser et al., 1977; Menzel and Greggers, 1985). La répartition et le nombre de chacun de ces récepteurs n'est pas la même et on retrouve en grande majorité des photorécepteurs au vert dans l'oeil (Wakakuwa et al., 2005). Ces observations anatomiques suggèrent que malgré une implication de tous les photorécepteurs, les récepteurs au vert en plus grand nombre ont un rôle majoritaire dans ces comportements. À cet effet, ceci pourrait expliquer les différences de comportement pour les stimuli lumineux verts.

Pour finir, les courbes d'acquisition observées dans le chapitre I, valident une fois de plus l'importance de la luminosité dans notre dispositif. En effet, quelle que soit la stimulation, récompensée ou punie, les individus vont se diriger vers ce stimulus et adopter un comportement phototactique ou de fixation. Ces comportements ont été largement étudiés en utilisant le même type de dispositif chez l'abeille en s'appuyant sur le comportement naturel des abeilles à fixer une stimulation lumineuse (Paulk et al., 2014, 2015).

**L'utilisation de la RV présente des intérêts, mais il est important de garder à l'esprit que les abeilles ne traitent peut-être pas l'information visuelle comme elles le feraient dans la nature. Cette première partie a permis de mettre en avant les limites de l'utilisation de la vidéoprojection tant en termes de couleurs utilisables, qu'en termes de luminosité. Ce dernier point semble être déterminant et mériterait une attention particulière. Les modèles sont à ce jour basés sur l'utilisation de stimulations papiers, et ne sont peut-être pas adaptés à l'utilisation de ces nouvelles technologies. Fort d'un dispositif fonctionnel, il serait alors intéressant de répéter les expériences de discrimination en variant les luminosités afin d'enrichir nos connaissances sur le traitement des images numériques par les abeilles et ainsi d'implémenter la notion de luminosité dans les modèles de distance chromatique déjà existants.**

## **b) La vision active est dispensable dans certains contextes**

Dans le premier chapitre, la totalité des expériences ont été réalisées dans un système dit en « circuit ouvert ». Dans ce type de système, l'individu n'a aucun retour sur son comportement, le fait de bouger ne va donc induire aucune modification de son environnement. Lors de ces expériences, 60% des abeilles ont montré une capacité à discriminer deux stimuli simples colorés. Ces résultats, bien qu'encourageants, étaient loin d'égaliser ceux obtenus dans les expériences en libre vol au cours desquelles 100% des individus ont montré de l'apprentissage. Ces différences de performances sont peut-être dues aux limitations induites par le système et à l'impossibilité des abeilles d'accéder à la vision active. Le principe de vision active veut qu'un individu observateur peut varier son point de vue pour explorer son environnement et extraire plus d'informations. Ainsi, la vision active est particulièrement importante dans le vol des abeilles ou des guêpes par exemple (Srinivasan and Zhang, 2004; Zeil, 1993, 1997), ou encore pour extraire les bordures d'un objet (Hempel de Ibarra and Giurfa, 2003). Ainsi, face à ce constat, nous avons mis en place un nouveau dispositif qui, lui, était en « circuit fermé » et permettait à l'individu de contrôler son environnement virtuel. Cependant, ce contrôle n'était pas total, le mouvement des stimuli n'étant que transversal. En parallèle, un labyrinthe en Y a été développé, permettant de tester une abeille dans les mêmes conditions que la RV mais sans aucune attache. Grâce à ces deux dispositifs, dans le chapitre 2, nous avons pu mettre en évidence que la RV n'était pas limitante et que des abeilles testées dans les deux contextes démontraient les mêmes performances d'apprentissage. Pourtant, la détection de mouvement est nécessaire à l'individu pour interpréter le flux spatio-temporel de l'information en fonction de ses mouvements (Borst and Egelhaaf, 1989; Egelhaaf et al., 2012; Srinivasan et al., 1999) et les possibilités de vision active dans le labyrinthe étaient plus importantes grâce au mouvement en profondeur absent dans la RV. Ces résultats suggèrent que pour une tâche simple telle que la discrimination de couleur, les abeilles dépourvues de la totalité de leur liberté visuelle restent néanmoins capables d'apprendre et qu'un mouvement transversal est suffisant malgré le fait que jusqu'à maintenant il était montré que le mouvement de profondeur était important, sans quoi la décision motrice de l'individu était basée sur une information visuelle incomplète (Webb, 2004).

Dans ce même chapitre et même expérience, les abeilles ont aussi été transférées dans le labyrinthe après avoir été conditionnées dans la RV, ou dans la RV après conditionnement dans le

labyrinthe. Ce transfert a permis de démontrer que les abeilles n'utilisaient pas la même stratégie visuelle pour apprendre la discrimination dans la RV ou dans le labyrinthe. En effet, les abeilles transférées de la RV vers le labyrinthe ont démontré une amélioration des performances, traduit par l'augmentation du nombre d'individus choisissant la bonne stimulation. En revanche, le transfert dans la direction opposée a eu l'effet inverse. Il semblerait donc qu'il est plus simple d'exprimer un apprentissage dans un contexte riche en indices visuels pour un individu conditionné dans un contexte pauvre en indices visuels que l'inverse. Ces résultats ont récemment été confirmés dans (Rodrigues Vieira et al., 2018) et avaient aussi été observés dans une expérience olfactive (de Brito Sanchez et al., 2015) au cours de laquelle des abeilles conditionnées en REP étaient capables de transférer leur apprentissage dans un labyrinthe, alors que l'inverse était impossible. Nos résultats démontrent que malgré la similarité des performances, les abeilles conditionnées dans le labyrinthe, ont utilisé une stratégie visuelle différente permise par un nombre d'indices visuels plus important que dans la RV.

Ces résultats démontrent la capacité des abeilles à discriminer deux stimuli colorés simples malgré une vision active incomplète. Il est toutefois possible qu'un apprentissage complexe nécessitant une analyse plus poussée des stimuli soit handicapé par ces limitations de vision active. En effet, dans le chapitre III, les abeilles testées dans un protocole de « Patterning négatif » sont moins performantes que des abeilles testées pour la même tâche en libre vol avec une vision active intacte (Schubert et al., 2002). A ceci s'ajoute que les abeilles testées en absolu dans la RV pour les mêmes stimuli n'ont aucun souci de discrimination. La complexité de la tâche pourrait alors nécessiter l'utilisation d'une RV permettant une expression complète de la vision active. En effet, sans une vision active complète, il devient très difficile, voire impossible de reconnaître des stimuli composés, constituant une limite considérable pour la mise en place de protocole plus complexe.

**Aux vues de nos résultats, nous pourrions penser qu'un dispositif ne permettant pas une vision active complète n'est pas limitant pour permettre l'apprentissage des abeilles. En revanche, des différences de stratégies ont été observées. Ainsi, il est évident que des abeilles qui en ont la possibilité vont utiliser la totalité des indices visuels offerts. Ces indices pourraient même permettre de résoudre certaines tâches, alors qu'ils sont inutiles pour d'autres. De ce fait, il serait pertinent pour la suite que le dispositif soit amélioré afin d'offrir**

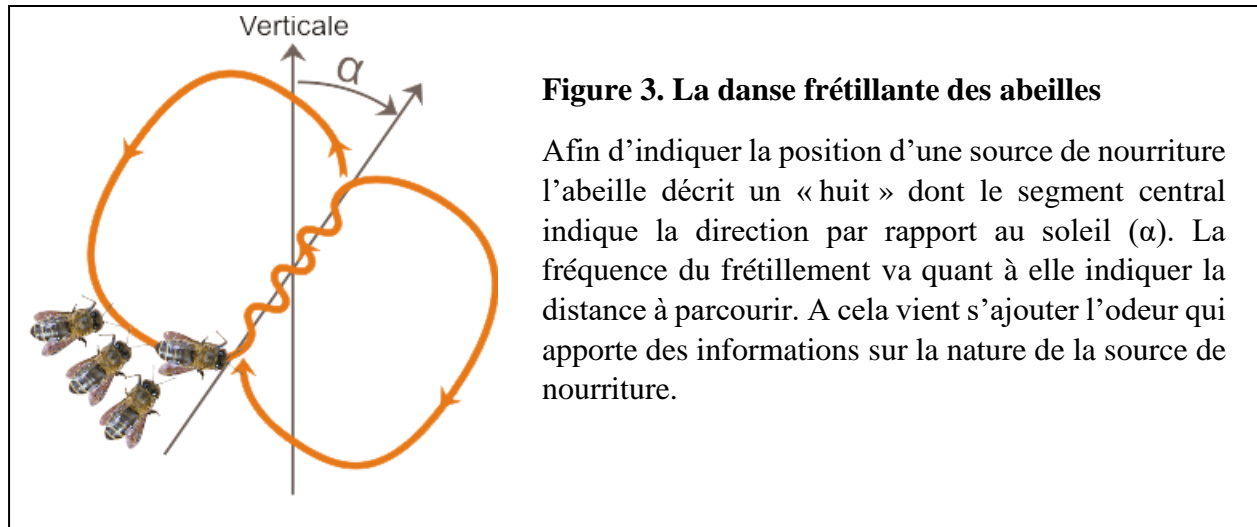
**un déplacement transversal et longitudinal et ainsi permettre aux abeilles de développer une vision active complète.**

**c) Se rapprocher des conditions de libre vol pour améliorer les performances en RV**

Au cours des deux premiers chapitres, nous avons pu montrer que les performances des abeilles testées dans un système en « circuit ouvert » ou « circuit fermé » étaient très similaires. En effet, dans les deux cas, environ 60 % des abeilles ont réussi à discriminer les stimuli en fonction de leur renforcement. De plus, les performances durant l'acquisition n'étaient pas non plus différentes alors que dans le chapitre II nous présentions deux stimuli à chaque essai afin d'éviter tout comportement phototactique. Les expériences parallèles du chapitre II dans la RV et dans le labyrinthe ont permis de montrer que le dispositif n'était pas le problème et qu'il n'était pas limitant pour les performances des abeilles. La limite des informations visuelles disponibles dans notre dispositif ne peut donc pas être le seul facteur influençant le niveau d'apprentissage des abeilles (environ 60%) qui est très en-dessous de celui des abeilles testées en libre vol avec des stimuli papiers (Giurfa, 2004; Giurfa et al., 1997). De plus, pour des abeilles testées en libre vol avec des stimuli virtuels dans le chapitre I, nous avons obtenu 100% de discrimination et la courbe d'acquisition démontrait clairement une augmentation de choix pour le stimulus récompensé. La principale différence entre une expérience en RV et une expérience en libre-vol est la possibilité pour l'abeille de revenir à la ruche entre deux essais de conditionnement. Ce retour à la ruche n'est donc pas négligeable et va impliquer de nombreux paramètres qui peuvent agir sur l'apprentissage.

Le premier est le paramètre de satiété, qui va largement être régulé grâce à de nombreux retours à la ruche. En effet, en libre vol, l'abeille va se nourrir *ad libitum* à chaque essai et revenir à la ruche pour déposer le nectar collecté. Dans notre système de RV, l'abeille n'a pas la possibilité de revenir à la ruche. Elle va donc être récompensée avec 4µl de solution sucrée afin d'éviter de remplir son jabot avant la fin des 12 essais. Le jabot d'une abeille peut contenir jusqu'à 60µl (Núñez, 1966), mais une abeille peut rentrer à la ruche avant d'atteindre cette limite. Ceci pourrait donc entraîner une perte progressive de motivation à obtenir du sucre et ainsi une diminution du comportement d'apprentissage. Lors du dépôt de nectar à la ruche, l'abeille entre en contact avec d'autres abeilles de la colonie afin de leur transmettre ce qu'elle a collecté (Winston, 1991). Ce contact dit direct va se caractériser par un comportement de trophallaxie qui consiste à transférer le nectar par contact

du proboscis (la langue) entre deux individus. Ce n'est pas le seul contact que la butineuse peut avoir dans la ruche à son retour. En effet, cette espèce est connue pour sa collaboration lors du butinage, mise en évidence par l'existence de la danse frétillante (von Frisch, 1967) qui va



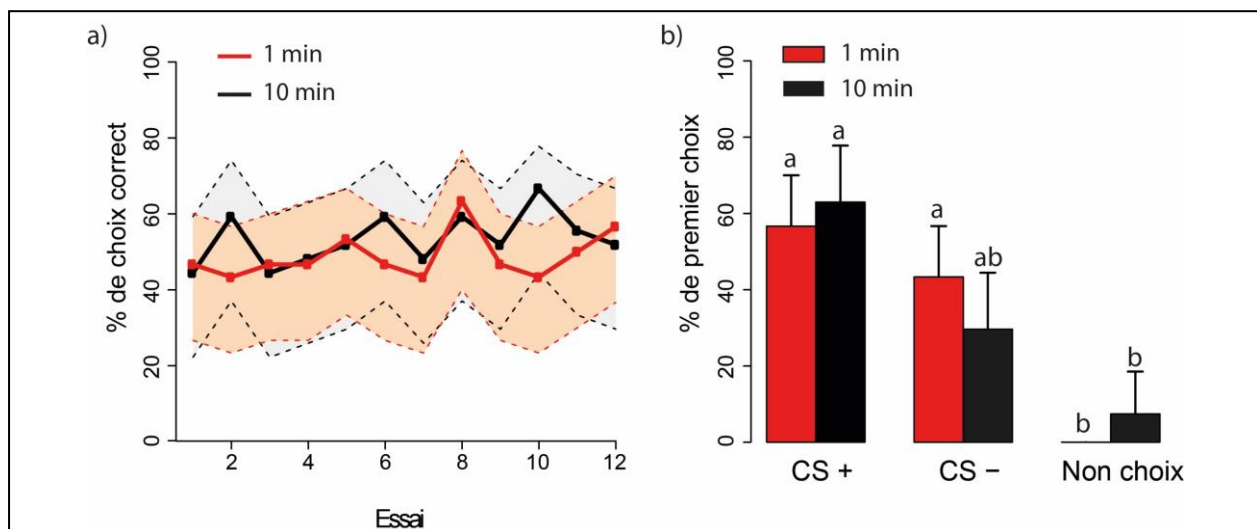
permettre d'indiquer une source de nourriture. Par exemple, une butineuse qui trouve une source de nectar particulièrement abondante, va pouvoir informer ses congénères de l'emplacement de cette source grâce à la danse (**Figure 3**). Les informations de distance et de direction vont être exprimées grâce à des vibrations de l'abdomen et des rotations sur un axe précis, qui vont être perçues et traduites par les autres abeilles par contact direct avec l'individu. Pendant cette danse, l'odeur transportée par la butineuse va aussi être analysée par les congénères et permettre de favoriser le recrutement de nouvelles butineuses (Farina et al., 2005). Hors de la ruche, les contacts sociaux ont aussi leur importance. On sait par exemple que chez le bourdon, certaines butineuses peuvent apprendre simplement en observant leurs congénères (Alem et al., 2016; Dawson et al., 2013). Les contacts sociaux directs avec leurs congénères tels que le toucher ou l'observation semblent avoir une importance cruciale dans les comportements de butinage, et donc indirectement d'apprentissage. Ces interactions semblent primordiales au recrutement d'autres butineuses et donc à transmettre à de nouvelles abeilles la motivation à s'impliquer dans une nouvelle tâche. Il pourrait alors sembler logique que cette interaction n'est pas unilatérale, et qu'elle permette aussi de maintenir la motivation de la butineuse revenant à la ruche. De ce fait, un individu en RV, n'ayant pas accès à ces contacts sociaux pourrait voir sa motivation décroître, ce qui se répercute sur l'apprentissage.



Ces contacts sociaux ne se résument pas seulement à des contacts directs entre les individus. En effet, comme pour nombre d'espèces sociales, la vie d'une colonie est régie par la présence de nombreuses phéromones dont la valence varie selon le contexte. Une phéromone est une substance chimique relâchée dans l'environnement par un individu et qui va déclencher des réponses stéréotypées et/ou des processus physiologiques chez des congénères (Karlson and Lüscher, 1959). Une abeille en danger pourra par exemple relâcher la phéromone d'alarme afin de recruter d'autres congénères et défendre la colonie (Slessor et al., 2005), ou encore relâcher la phéromone de Nasonov afin de guider ses congénères pendant un essaimage (Slessor et al., 2005). Depuis quelques années, le rôle des phéromones a aussi été corrélé à des modulations chez l'abeille tant en termes de performances d'apprentissage (Urlacher, 2010; Vergoz et al., 2007), qu'en termes de sensibilité au renforcement (Baracchi et al., 2017; Pankiw and Page, 2003; Rossi et al., 2018). Etant donné que la sensibilité au renforcement a été démontrée comme étant corrélée à l'apprentissage, c'est-à-dire que les individus les plus sensibles sont ceux apprenant le mieux (Roussel et al., 2009; Scheiner et al., 2001), il est possible que la modulation phéromonale des deux soit également concordante. Par exemple, une abeille stimulée par une phéromone positive telle que le géraniol va voir sa sensibilité au sucre augmentée (Baracchi et al., 2017), ce qui pourrait faciliter l'apprentissage. Notamment, les auteurs suggèrent que les phéromones modulent la motivation de l'individu à s'engager dans une tâche appétitive ou défensive (Nouvian et al., 2015; Rossi et al., 2018). D'autres phéromones vont fournir des informations sur la santé de la colonie, telle que la phéromone de reine, ou la phéromone de couvain (Slessor et al., 2005). La phéromone de couvain va permettre la reconnaissance des larves par les ouvrières et surtout favoriser le recrutement de butineuses de pollen (Slessor et al., 2005). Ces différents résultats suggèrent que les phéromones vont agir de manière conséquente sur la motivation d'un individu à butiner ou sur sa sensibilité au renforcement qui lui est proposé. Une abeille placée dans la VR, n'aura ainsi pas accès à ces phéromones et ne pourra pas maintenir son état de motivation tout au long de l'expérience.

Un aller-retour entre la ruche et le labyrinthe lors d'une expérience en libre vol dure en moyenne  $5 \pm 2$  min, constituant une des différences majeures avec les expériences en RV. En effet, dans les différents chapitres, le temps entre les essais (ITI) utilisé était 1 min. Cet ITI d'1 min a été choisi pour la simple raison qu'il permet de réaliser une expérience en moins d'une heure, période après laquelle une abeille voit sa motricité décroître dans le dispositif, certainement par faute de motivation. L'impact de ce paramètre sur les performances d'apprentissage olfactif a déjà été

largement étudié. Ainsi, il a été démontré que, chez l'abeille, plus la longueur de l'ITI était importante et plus les performances au moment du test de mémoire étaient bonnes (Giurfa and Sandoz, 2012; Menzel et al., 2001). De plus, cette constatation est aussi valable dans le cadre d'apprentissage non élémentaire tel que le « Patterning négatif » olfactif qui nécessite un ITI de 8 min afin d'obtenir de bonnes performances des abeilles (Deisig et al., 2007). Ces résultats laissent à penser qu'un ITI court ne pourrait pas permettre la consolidation de l'information au cours de l'apprentissage et que l'utilisation d'un ITI plus long pourrait résoudre cette différence de performance entre le labyrinthe et la RV. Pour vérifier cette hypothèse, des abeilles ont été conditionnées dans un protocole de discrimination de couleurs dans le labyrinthe contrôlé du chapitre II. Un groupe était conditionné avec un ITI d'1min et un autre groupe avec un ITI de 10 min (**Figure 4**, Données préliminaires). Les performances des abeilles quel que soit l'ITI utilisé ne sont pas différentes. Ces résultats suggèrent donc que l'ITI n'a pas d'impact sur les performances de discrimination de couleurs.



**Figure 4. Performances pendant un apprentissage discriminatif de couleurs pour un ITI de 1 min et de 10min.**

(a) Pourcentage d'abeilles ( $\pm$  intervalle de confiance) choisissant le CS+ pendant les 12 essais de conditionnement espacés de 1 min (rouge) ou 10 min (noir). Il n'y a aucune différence significative entre les courbes d'acquisition des deux groupes (GLMM, Groupe\*CS\*Essai :  $\chi^2=20.38$ , ddl=22,  $p=0.56$ ). (b) Pourcentage d'abeilles ( $\pm$  intervalle de confiance) choisissant en premier le CS+, le CS- ou n'effectuant pas de choix pour le groupe 1 min (rouge) et 10 min (noir). Aucune différence significative n'a été trouvée entre les deux groupes (GLMM, Groupe\*CS\*Essai :  $\chi^2=1.21$ , ddl=1,  $p=0.27$ ).

La totalité des expériences utilisées en RV ont été réalisées dans le noir. L'utilisation de la vidéoprojection nécessite une complète obscurité pour ne pas altérer la qualité des images. De plus, les abeilles sont naturellement attirées par la lumière (Menzel and Greggers, 1985), il est donc primordial de supprimer toute source de lumière autre que le vidéoprojecteur pour éviter un manque d'attention. Néanmoins, lors des expériences en libre vol réalisées dans le chapitre I, seul le labyrinthe était placé dans l'obscurité, mais les abeilles au moment de leur retour à la ruche étaient exposées à la lumière du soleil. On sait que l'exposition à la lumière peut entraîner des modulations tant au niveau des réseaux synaptiques (Scholl et al., 2014), qu'au niveau de l'expression de certains gènes (Sommerlandt et al., 2017). Les réseaux synaptiques sont connus pour jouer un rôle crucial dans l'apprentissage et la mémoire (Bliss and Collingridge, 1993). Si la lumière peut avoir un effet sur ces réseaux, on peut imaginer qu'elle peut aussi avoir un effet sur la consolidation de l'information au cours de l'apprentissage. Une abeille placée dans une obscurité totale, seulement stimulée par la lumière artificielle de la RV ne subirait ainsi peut-être pas ces modifications synaptiques. Ceci se traduirait par une incapacité à consolider l'information et ainsi des performances moindres comparées à celles obtenues en libre vol.

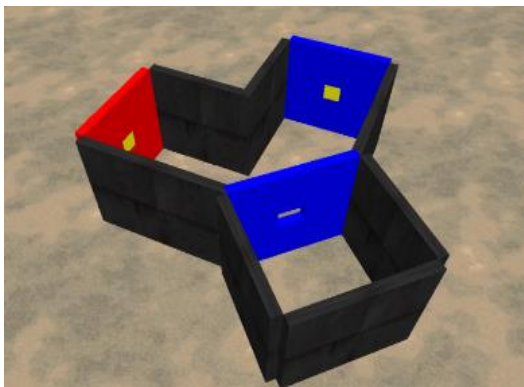
Pour finir, les méthodes de prélèvement des abeilles sont bien différentes pour la RV et pour le libre vol. Pour les expériences de RV, les abeilles sont prélevées directement sur un nourrisseur juste avant qu'elles se nourrissent. Elles sont ensuite attachées puis testées après un temps de familiarisation à l'attache. En libre vol, les abeilles sont entraînées petit à petit à venir jusqu'à l'intérieur du labyrinthe en les attirant avec un petit nourrisseur. Une fois que l'abeille a réalisé quelques allers-retours entre la ruche et l'intérieur du labyrinthe, l'expérience commence. Ceci implique, qu'en libre vol, un tri indirect est réalisé. En effet, l'expérience ne va être réalisée qu'avec des abeilles qui ont démontré une motivation importante à venir à l'intérieur du labyrinthe, ce qui constitue déjà une première forme d'apprentissage. Cette étape n'existant pas pour la RV, il est possible que le seul fait de tester des abeilles « tout-venant » pourrait biaiser les performances globales du groupe.

**Au regard de tous ces éléments, il est évident qu'il existe un nombre impressionnant de paramètres qui pourraient agir sur les performances des abeilles testées en RV. Néanmoins, la mise au point d'un labyrinthe contrôlé (chapitre II) dans lequel les performances sont**

similaires à la RV, va permettre de tester l'influence de chaque paramètre. Dans cette expérience (actuellement en cours), différents groupes d'abeilles sont testés sur un protocole de discrimination de couleurs dans le labyrinthe contrôlé. Chaque groupe subit le même conditionnement mais varie au niveau des paramètres présentés dans cette partie. Les performances seront ainsi comparées afin de valider ou non l'impact de chaque paramètre.

#### d) La réalité virtuelle : vers la réplique d'autres paradigmes

La mise en place de notre dispositif a permis de répliquer des protocoles d'apprentissage simple (discrimination de couleurs), mais aussi plus complexe (Patterning négatif). Ces premiers résultats sont plus qu'encourageants et vont permettre des premières expériences d'électrophysiologie par exemple, afin de caractériser comment le cerveau code l'information au cours de ces apprentissages. En revanche, les environnements utilisés sont relativement simples et ne permettent actuellement pas de se rapprocher exactement de l'expérience visuelle qu'une abeille pourrait avoir



**Figure 5: Labyrinthe en Y virtuel**

Labyrinthe virtuel général grâce au logiciel Simcog développé par André Cyr.

@AndréCyr

lors d'un vol de butinage ou d'une expérience en libre vol. La prochaine étape est donc la mise en place d'un environnement virtuel plus complexe. Par exemple, on pourrait imaginer l'utilisation d'un labyrinthe en Y en 3D (**Figure 5**), qui permettrait d'avoir le mouvement en profondeur discuté plus haut, et ainsi offrir à l'abeille un flux optique plus complet.

Ce type d'environnement permettrait d'explorer de nombreux paradigmes qui ont permis de mettre en évidence les capacités cognitives incroyables des abeilles mises en avant lors de mon introduction. En effet, il serait possible de mettre en place des expériences en RV pour tester la similarité (Giurfa et al., 2001) ou la catégorisation (Benard et al., 2006) par exemple. Récemment, il a été montré que les abeilles étaient capables d'apprendre le concept de zéro (Howard et al.,

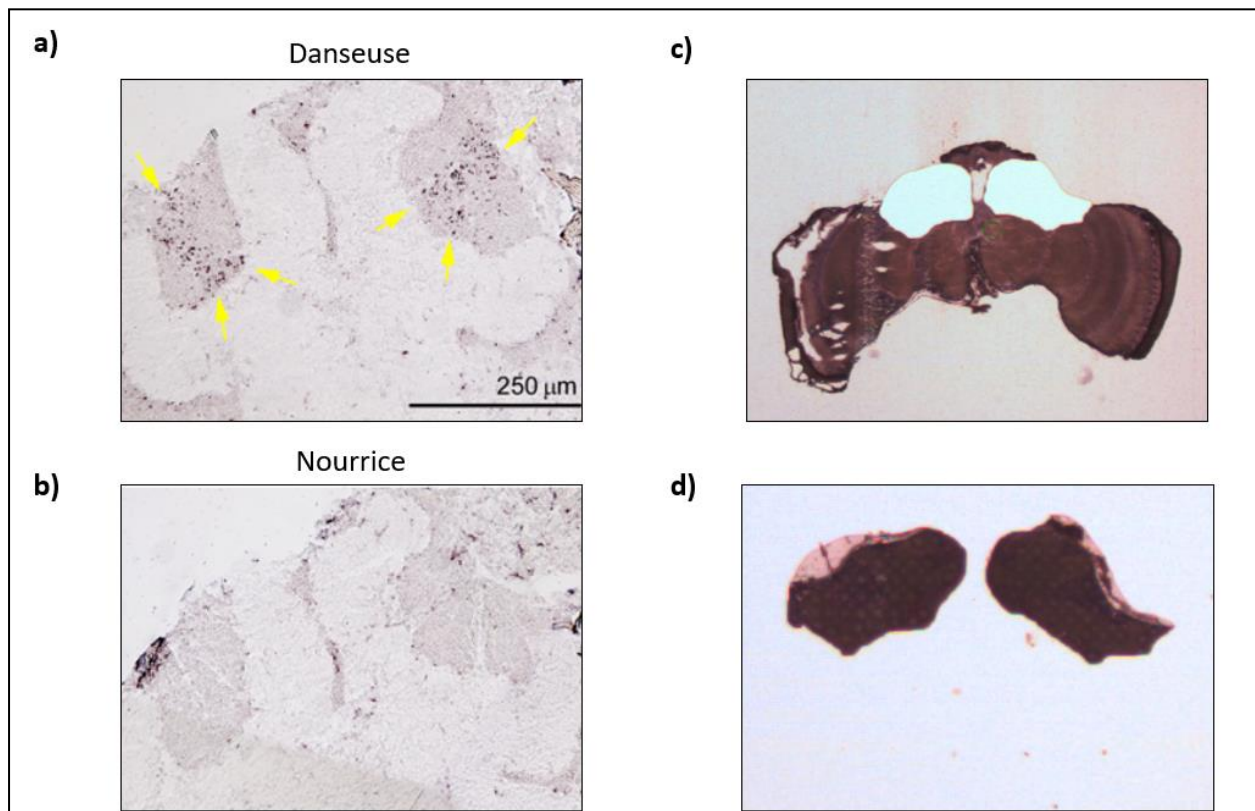
2018). Ceci peut paraître simple, mais c'est pourtant impossible pour un jeune enfant de 3-4 ans. Se pose alors la question de comment les abeilles avec leur cerveau miniature peuvent réussir à résoudre ce genre de problème. Là encore, les expériences ont été réalisées en libre vol, et notre dispositif offrira la possibilité de les répéter sur des individus attachés afin d'explorer les mécanismes cérébraux sous-jacents.

## II. Les bases neurobiologiques de l'apprentissage et de la mémoire visuelle

L'enjeu majeur de la mise en place d'un dispositif de réalité virtuelle est la possibilité d'explorer les mécanismes cérébraux sous-jacents. Au cours de ma thèse, j'ai pu réaliser une première étude préliminaire de l'activation neurale des différentes aires cérébrales au cours d'un test de mémoire de couleur.

Au cours du chapitre IV, l'expérience d'apprentissage de discrimination de couleur en RV a été combinée à une analyse rétrospective de l'expression de gènes précoces immédiats (GPIs) dans différentes zones du cerveau. *Kakusei* et *egr* sont des GPIs considérés comme marqueurs de l'activité neurale (Kiya et al., 2012; Ugajin et al., 2013). Ainsi, leur quantification peut-être indirectement corrélée à une activation neuronale. Plusieurs expériences dans le passé ont permis de montrer qu'une tâche d'apprentissage telle que « le retour à la ruche » entraîne une surrégulation de l'expression de ces gènes (Kiya et al., 2007; Ugajin et al., 2013, 2017). De plus, chez les vertébrés, il est courant d'utiliser la quantification de l'expression des GPIs afin d'établir des cartes d'activation des zones cérébrales (Guzowski et al., 2005; Terleph and Tremere, 2006). Basé sur ces principes, nous avons donc quantifié l'expression de ces deux GPIs dans les lobes optiques (LO), les lobe antennaires (LA), les corps pédonculés (CP), et le reste du cerveau (RC) après un test de mémoire de couleurs dans la RV. Nos résultats n'ont pas permis de mettre en évidence une surrégulation des gènes dans ces différentes aires, suggérant ainsi qu'aucune d'entre elles n'a présenté une activité neuronale plus importante que les autres au cours de la rétention de mémoire. Nos analyses ont été réalisées sur des structures entières, et donc fournissent une précision insuffisante pour pouvoir discuter plus en finesse ces résultats. De plus, il est important de signaler la limite de notre technique. L'expression de ces GPIs peut-être liée à l'activation de n'importe quel type de neurones. Il suffirait donc que ce type d'apprentissage n'implique pas l'activation d'un grand nombre de neurones pour que cette activation soit couverte par le bruit des neurones qui

s'activent pour le traitement sensoriel du stimulus. Il serait donc profitable d'augmenter la finesse de nos analyses en réalisant une hybridation *in situ* sur tranche (**Figure 6a, b**) ou des quantifications des GPIs sur des échantillons obtenus à partir de microdissection laser de tranche de cerveau (**Figure 6c, d**). L'hybridation *in situ* a par exemple permis par marquage des ARNs des GPIs de mettre en évidence une différence d'activation entre une abeille effectuant la danse frétilante et une nourrice en comparant leur profil d'activation (**Figure 6a, b**). En effet, une activation des cellules de Kenyon moyennes (mCK) est observée chez les danseuses et pas chez les nourrices



**Figure 6. Techniques de quantification sur coupe de cerveau.**

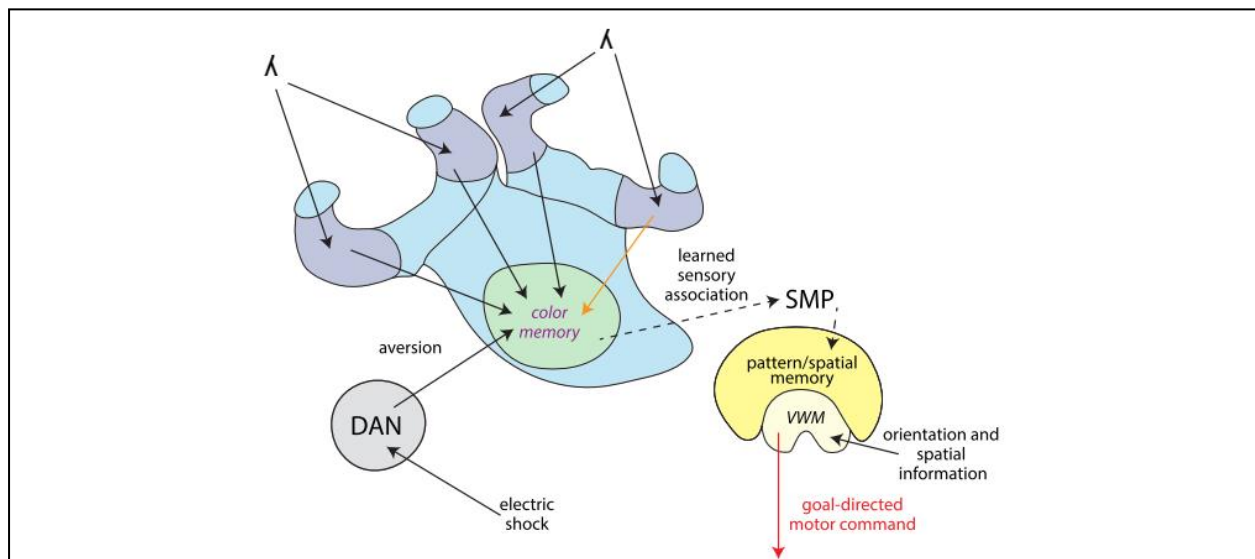
Hybridation *in situ* de *kakusei* sur des coupes de cerveau d'abeille qui effectuait une danse (**a**) et d'une nourrice (**b**). Dans les deux cas, l'image est focalisée sur les CPs, et plus particulièrement sur la zone de regroupement des cellules de Kenyon. Afin d'être plus précis, il est possible de réaliser une microdissection laser qui va permettre de sélectionner une zone précise des corps pédonculés. Seule l'aire des corps cellulaires a été disséquée dans (**c**), puis placée dans une autre coupelle (**d**) en attente d'une RT-qPCR.

(a) et (b) : Kiya et al., 2007 ; (c) et (d) : @ Manon Marque

(Kiya et al., 2007). Afin de compléter cette analyse descriptive, il est aussi possible d'effectuer des

quantifications des GPIs dans des aires plus restreintes grâce à la microdissection laser (**Figure 6c, d**). Cette figure montre l'extraction de l'air contenant les corps cellulaires des cellules de Kenyon. Il est aussi possible d'extraire uniquement les mCKs, et ainsi de réaliser une quantification sur des populations de neurones plus particulières.

Bien que nos résultats préliminaires soient négatifs, ils pourraient traduire un processus impliquant une synergie entre les différentes aires et notamment entre le CPs et le complexe



**Figure 7. Modèle de flux d'information pour l'apprentissage différentiel de couleur**

Les informations lumineuses ( $\lambda$ ) entrent dans la région du collier (violet) par les neuropiles optiques. Cette information visuelle va passer du collier au lobe vertical (vert) par les cellules de Kenyon. Les informations du choc électrique passent par la corde nerveuse ventrale grâce à des neurones dopaminergique (DAN) qui modulent les sorties du/des ? CP. Toutes ces informations vont être mises en relation au niveau du lobe vertical permettant ainsi la formation de la mémoire. Ces informations vont ensuite passer au complexe central par le protocérébrum médial supérieur (SMP). Le complexe central va recevoir les informations spatiales et d'orientation en utilisant la mémoire de travail visuelle (VWM). Celui-ci va alors mettre en relation les informations visuelles des CPs et les informations spatiales pour lancer une réponse motrice. Plath et al., 2017.

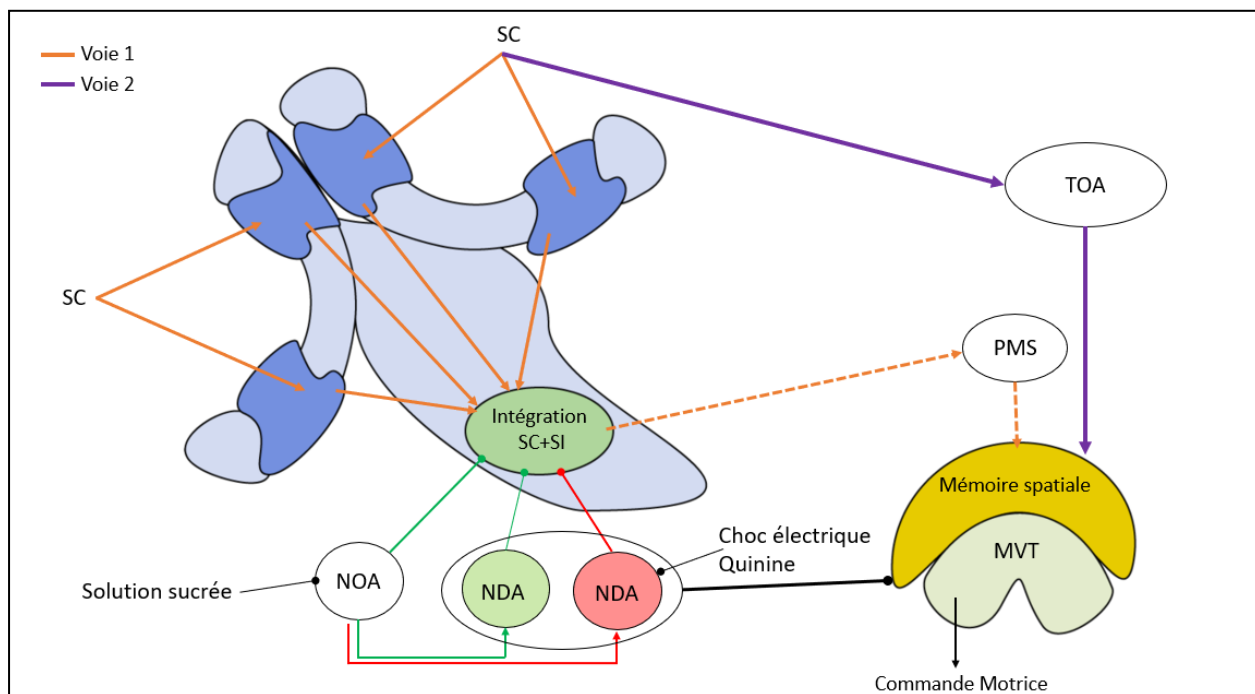
centrale (CC). C'est en tout cas le modèle proposé par Plath et al. (2018), dans lequel l'apprentissage aversif de couleur serait traité d'abord par les CPs qui communiqueraient ensuite l'information au CC qui enclenche un signal moteur (**Figure 7**). Malgré la cohérence anatomique de leur modèle, leurs résultats sont discutables du fait d'un effet similaire de l'injection de

l'anesthésique ou de véhicule dans le lobe vertical des CPs. A la lecture de leurs résultats et au regard de la différence entre anesthésique et véhicule, il semblerait que seule l'injection dans le CC a réellement eu un effet sur l'apprentissage et la mémoire de couleur. Ce modèle n'implique qu'une connexion hypothétique entre le CP et le CC par l'intermédiaire du protocerebrum medial et s'affranchit totalement de l'implication du tubercule optique antérieur (TOA). En effet cette structure, par l'intermédiaire du protocerebrum latéral va communiquer avec le CC (Mota et al., 2016). De plus, le TOA est impliqué dans les processus chromatiques (Mota et al., 2013; Paulk et al., 2008, 2009). Ces derniers éléments pourraient permettre de proposer un modèle théorique du flux d'information visuel impliquant le CP, le TOA, le protocérébrum latéral et le CC lors de l'apprentissage de couleur dans lequel le CP permettrait de traiter l'information visuel et de la lier au renforcement mais pas de stocker ces informations (**Figure 8**). Ce modèle serait en accord avec l'étude de Sommerlandt et al. (2016) dans laquelle suite à un apprentissage de couleur, aucune modulation au niveau des réseaux synaptiques n'a été observée, traduit par une constance du nombre et de la densité des microglomérules dans le collier des CPs. Ces résultats vont à l'encontre de ceux obtenus pour un apprentissage discriminatif d'odeur qui au contraire induit une modulation des microglomérules dans la lèvre des CP et donc des réseaux synaptiques (Hourcade et al., 2010). De plus, plusieurs études chez la drosophile on aussi montré que pour des apprentissages visuel simples les CPs étaient dispensables (Ofstad et al., 2011; Wolf et al., 1998). Ces différentes études anatomiques et comportementales permettent de proposer deux voies par lesquelles l'information visuelle serait traitée au cours d'un apprentissage de couleur (**Figure 8**).

Dans la première, la stimulation colorée (stimulus conditionnel : SC) stimule le lobe optique qui envoie des projections jusqu'au collier des CPs. Ces connections entre lobe optique et CPs ont été largement documentés par Paulk et Gronenberg (2008). Les cellules de Kenyon des CPs vont alors créer des connections synaptiques avec les terminaisons des neurones de projections au sein de capsules synaptique, les microglomérules (Hourcade et al., 2010), et projeter dans le lobe verticale du CPs au sein duquel il est possible que des connexions soient formées entre les terminaisons des cellules de Kenyon et les afférences des neurones dopaminergiques et octopaminergiques. En effet, deux études récentes ont permis de mettre en évidence le rôle de ces deux amines biogènes dans l'apprentissage de discrimination de couleur (Mancini et al., 2018; Rodrigues Vieira et al., 2018). Leur injection entraîne une amélioration des performances tant au niveau de l'apprentissage que de la mémoire des couleurs, alors que l'injection de leurs



antagonistes mène à un effet inverse. Ces expériences n'impliquaient pas l'utilisation de renforcement aversif. La dopamine, pourtant considérée comme synonyme de récompense et de motivation chez les mammifères (Wise, 2004), était jusque-là associée au renforcement négatif chez l'abeille (Mizunami et al., 2009; Schwaerzel et al., 2003). Il semblerait que depuis quelques années le rôle de cet amine biogène soit remis en question. Ainsi, chez la drosophile, il a été démontré que la dopamine pouvait moduler à la fois le traitement des renforcements positifs et négatifs et ce par l'intermédiaire du circuit octopaminergique (Schwaerzel et al., 2003). De plus, le circuit dopaminergique est connu pour projeter au niveau du CP, ainsi que du CC (Tedjakumala



**Figure 8. Modèle théorique du flux d'informations au cours d'un apprentissage de couleur**

Dans la voie 1 (voie orange), le stimulus conditionnel (SC) est traité par les lobes optiques, puis par les colliers des corps pédonculés (bleu foncé). L'information va ensuite dans le lobe vertical afin d'être intégrée avec les informations liées au stimulus inconditionnel (SI) et médiées par les neurones octopaminergique (NOA) ou dopaminergique (NDA). Les flèches vertes correspondent au circuit des renforcements positifs, les rouges aux circuits des renforcement négatifs. Enfin, cette information intégrée va par l'intermédiaire du protocerebrum medial supérieur (PMS) être transmise au complexe central (jaune), puis intégrée aux informations spatiales et aux informations de mémoire visuelle de travail (MVT) ce qui va permettre l'initiation d'une commande motrice. Dans la voie 2 (violette), l'information du SC est aussi traitée par les lobes optiques, puis envoyée au CC par l'intermédiaire du tubercule optique antérieur (TOA).

et al., 2017), Le neurone VUMx1 qui projette vers les calyces des CPs (Hammer, 1993) qui quant à lui a un rôle majeur dans le traitement des renforcement positifs est un neurone octopaminergique. Il n'existe cependant pas à notre connaissance de neurones octopaminergique projetant vers le CC. En s'appuyant sur ces nouveaux résultats comportementaux (Mancini et al., 2018; Rodrigues Vieira et al., 2018) et sur les avancées anatomiques mises en évidence chez la drosophile qui est proche de l'abeille pour l'organisation du cerveau, un rôle plus important a été donné au amines biogènes dans ce nouveau modèle (**Figure 8**). Ainsi, le circuit octopaminergique permettrait la médiation des renforcements positifs jusqu'à intégration au niveau du CP, alors que le circuit dopaminergique lui permettrait la médiation des renforcements négatifs directement vers le CP et le CC, mais aussi des renforcements positifs par l'intermédiaire du circuit octopaminergique. Une fois l'intégration de l'information du SC et du stimulus inconditionnel (renforcement, SI) effectuée, un signal partirait du CP vers le CC par l'intermédiaire du protocerebrum medial supérieur (PMS) entraînant ainsi l'envoi de la commande motrice par le CC qui se traduirait par le comportement moteur de l'abeille vers le stimulus.

La seconde voie, beaucoup plus courte est basée sur les résultats de Mota et al. (2016) qui ont permis l'observation d'une connexion entre le lobe optique, et le CC par l'intermédiaire du tubercule optique antérieur. Il semble que cette voie permet un traitement chromatique (Mota et al., 2013; Paulk et al., 2008) et donc qu'elle est probablement impliquée dans le traitement de l'information lors d'un apprentissage de couleur. De plus, le CC chez les insectes a été identifié comme ayant un rôle majeur dans le codage de la lumière polarisée, la reconnaissance de motif, l'orientation spatiale, et les contrôles moteurs (Liu et al., 2006; Strauss, 2002; Vitzthum et al., 2002; Wang et al., 2008). Les afférences du TOA vers le central complexe suggèrent indirectement que cette voie transmet les informations visuelles spatiales au CC. Comme pour la voie 1, le SC va exciter les neurones des photorécepteurs qui vont projeter dans le TOA, au sein duquel un cluster de microglomérules va se former contenant les connexions synaptiques entre les terminaisons des neurones des photorécepteurs et les dendrites d'autres neurones qui vont projeter jusqu'au CC (Mota et al., 2016).

A ce jour, aucun résultat n'a permis de concrètement identifier les voies impliquées dans l'apprentissage visuel qu'il soit appétitif, aversif ou différentiel. Il est important de garder à l'esprit que le modèle proposé est purement théorique et basé essentiellement sur des observations

anatomiques. Néanmoins, il constitue une bonne base de réflexion pour la suite. En effet, l'essence même de mon projet a été de mettre en place un dispositif permettant d'explorer plus facilement les activations dans le cerveau au cours d'un apprentissage visuel. Ainsi, il sera possible en réalisant des injections, ou de l'électrophysiologie de déterminer si une voie est prédominante sur l'autre, ou si elles sont complémentaires et de valider ou non ce modèle. Par ailleurs, il sera aisé de réaliser des injections de dopamine ou d'octopamine dans certaines aires cérébrales afin là encore d'éprouver ce modèle.

**La réalité virtuelle permet de faciliter l'exploration du cerveau contrairement aux observations de comportement dans la nature. Il est toutefois important de garder à l'esprit que ce qui est observé en réalité virtuelle est peut-être loin de ce que l'on pourrait observer dans des conditions naturelles. Une étude chez le rongeur, a par ailleurs permis de mettre en évidence les différences entre l'activation des cellules de lieu observée en réalité virtuelle et dans une arène bien réelle (Ravassard et al., 2013). Des cellules de lieu étaient observées dans les deux cas, mais moins dans la réalité virtuelle. Le monde virtuel est donc perçu mais avec une définition bien moindre comparé au monde réel. On est alors en droit de penser que ce type de différences pourrait être observé chez toutes les espèces. Le tout est donc maintenant, d'analyser les résultats avec prudence.**

## Conclusion Générale

Au cours de cette thèse nous avons montré :

1. Les abeilles sont capables d'apprendre à discriminer des stimuli visuels virtuels que ce soit en libre vol ou placées dans un système de réalité virtuelle (**Chapitre I, II**).
2. Les abeilles sont capables de résoudre un apprentissage non élémentaire visuel tel que le « patterning négatif » en réalité virtuelle (**Chapitre III**).
3. En réalité virtuelle, la luminosité est un paramètre déterminant au cours de l'apprentissage. Ces résultats ouvrent des portes sur la mise en place de nouveau modèle de distance chromatique spécifique à ce type de système (**Chapitre I, II, III**).
4. Les abeilles adaptent leur stratégie visuelle en fonction des possibilités de vision active offertes. Ces stratégies ne se répercutent pas sur leurs performances d'apprentissage, mais sont limitantes lors d'un transfert entre deux contextes de vision active différents (**Chapitre II**).
5. La quantification de gènes précoces immédiats permet une quantification indirecte de l'activité neurale au cours d'une rétention de mémoire des couleurs. Cette technique n'a pas permis la mise en évidence d'un motif d'activation particulier dans le cerveau (**Chapitre IV**).

Cette thèse fournit de nombreux résultats et l'élaboration d'un système de réalité virtuelle qui va permettre l'étude des apprentissages visuels élémentaires et non-élémentaires sur des abeilles attachées et ainsi ouvre des portes à l'exploration plus poussée des mécanismes cérébraux sous-jacents.

Ces résultats ont permis de mettre en évidence toute l'importance de fournir aux individus un environnement virtuel le plus riche possible. Il est donc essentiel pour la suite d'améliorer encore le système afin d'obtenir un environnement en 3D dans lequel les abeilles pourront pleinement exprimer tout leur potentiel visuel. La mise en place de ce dispositif ouvre aussi tout un nouveau pan sur la sensorialité chez les abeilles et offre la possibilité d'étudier comment un

monde virtuel peut-être perçu par un insecte. Deux paradigmes ont été proposés ici, l'un d'apprentissage élémentaire, et l'autre non élémentaire. Ces deux protocoles solides fournissent une base complète pour avancer vers la compréhension des bases neurales sous-jacentes. De ce fait, la prochaine étape est logiquement la réplication de ces protocoles combinés à des méthodes permettant l'exploration du cerveau, comme l'électrophysiologie, la neuropharmacologie, ou encore la génétique comme lors de mon dernier chapitre. Cette dernière méthode a montré ses limites, mais est améliorable afin de gagner en précision. La réalité virtuelle, et plus particulièrement le dispositif développé au cours de cette thèse, présente des avantages et des limites qu'il est important de résumer.

### **Avantages**

- Contrôle complet de l'expérience visuelle de l'abeille
- Contrôle complet de l'expérience : Temps du stimulus, Temps de récompense, Temps entre les essais
- Gain de temps : il n'est pas nécessaire d'entraîner l'abeille à venir
- Possibilité d'accès au cerveau pendant l'expérience
- Possibilité de réaliser une expérience sur plusieurs jours avec isolement facile de l'abeille entre chaque journée afin d'éviter de la perdre et de contrôler son expérience.

### **Limites**

- La phototaxie
- Le système RVB
- Le manque d'interaction avec la ruche entre les essais
  - Manque de motivation
  - Les abeilles ne peuvent pas vider le nectar récolté

## Références

- Alem, S., Perry, C. J., Zhu, X., Loukola, O. J., Ingraham, T., Søvik, E., et al. (2016). Associative Mechanisms Allow for Social Learning and Cultural Transmission of String Pulling in an Insect. *PLoS Biol.* 14, 1–28. doi:10.1371/journal.pbio.1002564.
- Backhaus, W. (1991). Color opponent coding in the visual system of the honeybee. *Vision Res.* 31, 1381–1397. doi:10.1016/0042-6989(91)90059-E.
- Baracchi, D., Devaud, J. M., D’Ettorre, P., and Giurfa, M. (2017). Pheromones modulate reward responsiveness and non-associative learning in honey bees. *Sci. Rep.* 7, 1–9. doi:10.1038/s41598-017-10113-7.
- Benard, J., Stach, S., and Giurfa, M. (2006). Categorization of visual stimuli in the honeybee *Apis mellifera*. *Anim. Cogn.* 9, 257–270. doi:10.1007/s10071-006-0032-9.
- Bliss, T. V., and Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361, 31–39. doi:10.1038/361031a0.
- Borst, A., and Egelhaaf, M. (1989). Principles of visual motion detection. *Trends Neurosci.* 12, 297–306. doi:10.1016/0166-2236(89)90010-6.
- Buatois, A., Pichot, C., Schultheiss, P., Sandoz, J.-C., Lazzari, C. R., Chittka, L., et al. (2017). Associative visual learning by tethered bees in a controlled visual environment. *Sci. Rep.* 7, 12903. doi:10.1038/s41598-017-12631-w.
- Chittka, L. (1992). The colour hexagon: A chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi:10.1007/BF00199331.
- Colomb, J., Reiter, L., Blaszkiewicz, J., Wessnitzer, J., and Brembs, B. (2012). Open source tracking and analysis of adult *Drosophila* locomotion in Buridan’s paradigm with and without visual targets. *PLoS One* 7, 1–12. doi:10.1371/journal.pone.0042247.
- Dawson, E. H., Avarguès-Weber, A., Chittka, L., and Leadbeater, E. (2013). Learning by observation emerges from simple associations in an insect model. *Curr. Biol.* 23, 727–730. doi:10.1016/j.cub.2013.03.035.

- de Brito Sanchez, M. G., Serre, M., Avargues-Weber, A., Dyer, A. G., and Giurfa, M. (2015). Learning context modulates aversive taste strength in honey bees. *J. Exp. Biol.* 218, 949–959. doi:10.1242/jeb.117333.
- Deisig, N., Sandoz, J. C., Giurfa, M., and Lachnit, H. (2007). The trial-spacing effect in olfactory patterning discriminations in honeybees. *Behav. Brain Res.* 176, 314–322. doi:10.1016/j.bbr.2006.10.019.
- Egelhaaf, M., Boeddeker, N., Kern, R., Kurtz, R., and Lindemann, J. P. (2012). Spatial vision in insects is facilitated by shaping the dynamics of visual input through behavioral action. *Front. Neural Circuits* 6, 1–23. doi:10.3389/fncir.2012.00108.
- Farina, W. M., Grüter, C., and Díaz, P. C. (2005). Social learning of floral odours inside the honeybee hive. *Proc. Biol. Sci.* 272, 1923–1928. doi:10.1098/rspb.2005.3172.
- Giurfa, M. (2004). Conditioning procedure and color discrimination in the honeybee *Apis mellifera*. *Naturwissenschaften* 91, 228–231. doi:10.1007/s00114-004-0530-z.
- Giurfa, M., Nunez, J., Chittka, L., and Menzel, R. (1995). Color Preferences of Flower-Naive Honeybees. *J. Comp. Physiol. a-Sensory Neural Behav. Physiol.* 177, 247–259. doi:10.1007/BF00192415.
- Giurfa, M., and Sandoz, J.-C. (2012). Invertebrate learning and memory: Fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* 19, 54–66. doi:10.1101/lm.024711.111.
- Giurfa, M., Vorobyev, M., Brandt, R., Posner, B., and Menzel, R. (1997). Discrimination of coloured stimuli by honeybees: Alternative use of achromatic and chromatic signals. *J. Comp. Physiol. - A Sensory, Neural, Behav. Physiol.* 180, 235–243. doi:10.1007/s003590050044.
- Giurfa, M., Vorobyev, M., Kevan, P., and Menzel, R. (1996). Detection of coloured stimuli by honeybees: minimum visual angles and receptor specific contrasts. *J. Comp. Physiol. A* 178. doi:10.1007/BF00227381.
- Giurfa, M., Zhang, S., Jenett, A., Menzel, R., and Srinivasan, M. (2001). The concepts of “sameness” and ‘difference’ in an insect. *Nature* 410, 2–5. Available at: <http://www.nature.com/nature/journal/v410/n6831/abs/410930a0.html>.

- Guzowski, J. F., Timlin, J. A., Roysam, B., McNaughton, B. L., Worley, P. F., and Barnes, C. A. (2005). Mapping behaviorally relevant neural circuits with immediate-early gene expression. *Curr. Opin. Neurobiol.* 15, 599–606. doi:10.1016/j.conb.2005.08.018.
- Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* 366, 59–63.
- Hempel de Ibarra, N., and Giurfa, M. (2003). Discrimination of closed coloured shapes by honeybees requires only contrast to the long wavelength receptor type. *Anim. Behav.* 66, 903–910. doi:10.1006/anbe.2003.2269.
- Hourcade, B., Muenz, T. S., Sandoz, J.-C., Rössler, W., and Devaud, J.-M. (2010). Long-term memory leads to synaptic reorganization in the mushroom bodies: a memory trace in the insect brain? *J. Neurosci.* 30, 6461–6465. doi:10.1523/JNEUROSCI.0841-10.2010.
- Howard, S. R., Avarguès-Weber, A., Garcia, J. E., Greentree, A. D., and Dyer, A. G. (2018). Numerical ordering of zero in honey bees. 360, 1124–1126. doi:10.1126/science.aar4975.
- Kaiser, W., Seidl, R., and Vollmar, J. (1977). The participation of all three colour receptors in the phototactic behaviour of fixed walking honeybees. *J. Comp. Physiol. ■ A* 122, 27–44. doi:10.1007/BF00611246.
- Karlson, P., and Lüscher, M. (1959). “Pheromones”: A new term for a class of biologically active substances. *Nature* 183, 55–56. doi:10.1038/183055a0.
- Kirkerud, N. H., Schlegel, U., and Giovanni Galizia, C. (2017). Aversive Learning of Colored Lights in Walking Honeybees. *Front. Behav. Neurosci.* 11, 1–17. doi:10.3389/fnbeh.2017.00094.
- Kiya, T., Kunieda, T., and Kubo, T. (2007). Increased neural activity of a mushroom body neuron subtype in the brains of forager honeybees. *PLoS One* 2. doi:10.1371/journal.pone.0000371.
- Kiya, T., Ugajin, A., Kunieda, T., and Kubo, T. (2012). Identification of kakusei, a nuclear non-coding RNA, as an immediate early gene from the honeybee, and its application for neuroethological study. *Int. J. Mol. Sci.* 13, 15496–15509. doi:10.3390/ijms131215496.
- Liu, G., Seiler, H., Wen, A., Zars, T., Ito, K., Wolf, R., et al. (2006). Distinct memory traces for



- two visual features in the *Drosophila* brain. *Nature* 439, 551–556. doi:10.1038/nature04381.
- Mancini, N., Giurfa, M., Sandoz, J. C., and Avarguès-Weber, A. (2018). Aminergic neuromodulation of associative visual learning in harnessed honey bees. *Neurobiol. Learn. Mem.*, 0–1. doi:10.1016/j.nlm.2018.05.014.
- Menzel, R., and Blakers, M. (1976). Colour receptors in the bee eye - Morphology and spectral sensitivity. *J. Comp. Physiol.* ■ A 108, 11–13. doi:10.1007/BF00625437.
- Menzel, R., and Greggers, U. (1985). Natural phototaxis and its relationship to colour vision in honeybees. *J. Comp. Physiol. A* 157, 311–321. doi:10.1007/BF00618121.
- Menzel, R., Manz, G., Menzel, R., and Greggers, U. (2001). Massed and spaced learning in honeybees: The role of CS, US, the intertrial interval, and the test interval. *Learn. Mem.* 8, 198–208. doi:10.1101/lm.40001.
- Mizunami, M., Unoki, S., Mori, Y., Hirashima, D., Hatano, A., and Matsumoto, Y. (2009). Roles of octopaminergic and dopaminergic neurons in appetitive and aversive memory recall in an insect. *BMC Biol.* 7, 1–16. doi:10.1186/1741-7007-7-46.
- Mota, T., Gronenberg, W., Giurfa, M., and Sandoz, J.-C. (2013). Chromatic Processing in the Anterior Optic Tubercle of the Honey Bee Brain. *J. Neurosci.* 33, 4–16. doi:10.1523/JNEUROSCI.1412-12.2013.
- Mota, T., Kreissl, S., Carrasco Durán, A., Lefer, D., Galizia, G., and Giurfa, M. (2016). Synaptic Organization of Microglomerular Clusters in the Lateral and Medial Bulbs of the Honeybee Brain. *Front. Neuroanat.* 10. doi:10.3389/fnana.2016.00103.
- Nouvian, M., Hotier, L., Claudianos, C., Giurfa, M., and Reinhard, J. (2015). Appetitive floral odours prevent aggression in honeybees. *Nat. Commun.* 6, 1–10. doi:10.1038/ncomms10247.
- Núñez, J. A. (1966). Quantitative beziehungen zwischen den eigenschaften von futterquellen und dem verhalten von sammelbienen. 53, 142–164.
- Ofstad, T. A., Zuker, C. S., and Reiser, M. B. (2011). Visual place learning in *Drosophila melanogaster*. *Nature* 474, 204–7. doi:10.1038/nature10131.
- Pankiw, T., and Page, R. E. (2003). Effect of pheromones, hormones, and handling on sucrose

- response thresholds of honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol.* 189, 675–684. doi:10.1007/s00359-003-0442-y.
- Paulk, A. C., Dacks, A. M., Phillips-Portillo, J., Fellous, J.-M., and Gronenberg, W. (2009). Visual processing in the central bee brain. *J. Neurosci.* 29, 9987–99. doi:10.1523/JNEUROSCI.1325-09.2009.
- Paulk, A. C., and Gronenberg, W. (2008). Higher order visual input to the mushroom bodies in the bee, *Bombus impatiens*. *Arthropod Struct. Dev.* 37, 443–458. doi:10.1016/j.asd.2008.03.002.
- Paulk, A. C., Kirszenblat, L., Zhou, Y., and van Swinderen, B. (2015). Closed-Loop Behavioral Control Increases Coherence in the Fly Brain. *J. Neurosci.* 35, 10304–10315. doi:10.1523/JNEUROSCI.0691-15.2015.
- Paulk, A. C., Phillips-Portillo, J., Dacks, A. M., Fellous, J.-M., and Gronenberg, W. (2008). The processing of color, motion, and stimulus timing are anatomically segregated in the bumblebee brain. *J. Neurosci.* 28, 6319–6332. doi:10.1523/JNEUROSCI.1196-08.2008.
- Paulk, A. C., Stacey, J. A., Pearson, T. W. J., Taylor, G. J., Moore, R. J. D., Srinivasan, M. V., et al. (2014). Selective attention in the honeybee optic lobes precedes behavioral choices. *Proc. Natl. Acad. Sci.* 111, 5006–5011. doi:10.1073/pnas.1323297111.
- Ravassard, P., Kees, A., Willers, B., Ho, D., Aharoni, D., Cushman, J., et al. (2013). Multisensory control of hippocampal spatiotemporal selectivity. *Science (80-. )*. 340, 1342–1346. doi:10.1126/science.1232655.
- Rodrigues Vieira, A., Salles, N., Borges, M., and Mota, T. (2018). Visual discrimination transfer and modulation by biogenic amines in honeybees. *J. Exp. Biol.* doi:10.1242/jeb.178830.
- Rossi, N., d’Ettorre, P., and Giurfa, M. (2018). Pheromones modulate responsiveness to a noxious stimulus in honey bees. *J. Exp. Biol.*, jeb.172270. doi:10.1242/jeb.172270.
- Roussel, E., Carcaud, J., Sandoz, J. C., and Giurfa, M. (2009). Reappraising social insect behavior through aversive responsiveness and learning. *PLoS One* 4. doi:10.1371/journal.pone.0004197.
- Rusch, C., Roth, E., Vinauger, C., and Riffell, J. A. (2017). Honeybees in a virtual reality

- environment learn unique combinations of colour and shape. *J. Exp. Biol.*, jeb.164731. doi:10.1242/jeb.164731.
- Scheiner, R., Page, R. E., and Erber, J. (2001). Responsiveness to sucrose affects tactile and olfactory learning in preforaging honey bees of two genetic strains. *Behav. Brain Res.* 120, 67–73. doi:10.1016/S0166-4328(00)00359-4.
- Scholl, C., Wang, Y., Krischke, M., Mueller, M. J., Amdam, G. V., and Rössler, W. (2014). Light exposure leads to reorganization of microglomeruli in the mushroom bodies and influences juvenile hormone levels in the honeybee. *Dev. Neurobiol.* 74, 1141–1153. doi:10.1002/dneu.22195.
- Schubert, M., Lachnit, H., Francucci, S., and Giurfa, M. (2002). Nonelemental visual learning in honeybees. *Anim. Behav.* 64, 175–184. doi:10.1006/anbe.2002.3055.
- Schultheiss, P., Buatois, A., Avarguès-Weber, A., and Giurfa, M. (2017). Using virtual reality to study visual performances of honeybees. *Curr. Opin. Insect Sci.* 24, 43–50. doi:10.1016/j.cois.2017.08.003.
- Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* 23, 10495–502. doi:Copyright © 2012 by the Society for Neuroscience.
- Slessor, K. N., Winston, M. L., and Le Conte, Y. (2005). Pheromone communication in the honeybee (*Apis mellifera* L.). *J. Chem. Ecol.* 31, 2731–2745. doi:10.1007/s10886-005-7623-9.
- Sommerlandt, F. M. J., Rössler, W., and Spaethe, J. (2017). Impact of light and alarm pheromone on immediate early gene expression in the European honeybee, *Apis mellifera*. *Entomol. Sci.* 20, 122–126. doi:10.1111/ens.12234.
- Sommerlandt, F. M. J., Spaethe, J., Rössler, W., and Dyer, A. G. (2016). Does fine color discrimination learning in free-flying honeybees change mushroom-body calyx neuroarchitecture? *PLoS One* 11, 1–17. doi:10.1371/journal.pone.0164386.
- Srinivasan, M. V., and Lehrer, M. (1984). Temporal acuity of honeybee vision: behavioural studies

- using flickering stimuli. *Physiol. Entomol.* 9, 447–457. doi:10.1111/j.1365-3032.1984.tb00787.x.
- Srinivasan, M. V., and Zhang, S. (2004). Visual Motor Computations in Insects. *Annu. Rev. Neurosci.* 27, 679–696. doi:10.1146/annurev.neuro.27.070203.144343.
- Srinivasan, M. V., Zhang, S. W., Berry, J., Cheng, K., and Zhu, H. (1999). Honeybee navigation: Linear perception of short distances travelled. *J. Comp. Physiol. - A Sensory, Neural, Behav. Physiol.* 185, 239–245. doi:10.1007/s003590050383.
- Strauss, R. (2002). The central complex and the genetic dissection of locomotor behaviour. *Curr. Opin. Neurobiol.* 12, 633–638. doi:10.1016/S0959-4388(02)00385-9.
- Taylor, G. J., Luu, T., Ball, D., and Srinivasan, M. V. (2013). Vision and air flow combine to streamline flying honeybees. *Sci. Rep.* 3, 2614. doi:10.1038/srep02614.
- Tedjakumala, S. R., Rouquette, J., Boizeau, M.-L., Mesce, K. A., Hotier, L., Massou, I., et al. (2017). A Tyrosine-Hydroxylase Characterization of Dopaminergic Neurons in the Honey Bee Brain. *Front. Syst. Neurosci.* 11. doi:10.3389/fnsys.2017.00047.
- Terleph, T. A., and Tremere, L. A. (2006). “The Use of Immediate Early Genes as Mapping Tools for Neuronal Activation: Concepts and Methods,” in *Immediate Early Genes in Sensory Processing, Cognitive Performance and Neurological Disorders*, eds. R. Pinaud and L. A. Tremere (Boston, MA: Springer US), 1–10. doi:10.1007/978-0-387-33604-6\_1.
- Ugajin, A., Kunieda, T., and Kubo, T. (2013). Identification and characterization of an Egr ortholog as a neural immediate early gene in the European honeybee (*Apis mellifera* L.). *FEBS Lett.* 587, 3224–3230. doi:10.1016/j.febslet.2013.08.014.
- Ugajin, A., Uchiyama, H., Miyata, T., Sasaki, T., Yajima, S., and Ono, M. (2017). Identification and initial characterization of novel neural immediate early genes possibly differentially contributing to foraging-related learning and memory processes in the honeybee. *Insect Mol. Biol.* 27, 154–165. doi:10.1111/imb.12355.
- Urlacher (2010). An alarm pheromone modulates appetitive olfactory learning in the honeybee (*Apis mellifera*). *Front. Behav. Neurosci.* 4, 1–10. doi:10.3389/fnbeh.2010.00157.

- Vergoz, V., Roussel, E., Sandoz, J. C., and Giurfa, M. (2007). Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *PLoS One* 2. doi:10.1371/journal.pone.0000288.
- Vitzthum, H., Müller, M., and Homberg, U. (2002). Neurons of the central complex of the locust *Schistocerca gregaria* are sensitive to polarized light. *J. Neurosci.* 22, 1114–1125. doi:10.1016/J.CUB.2010.02.055.
- von Frisch, K. (1967). *The Dancing language and orientation of bees*. Cambridge, Massachusetts: The Belknap press of Harvard University Press Available at: [http://books.google.co.in/books/about/The\\_Dancing\\_Bees.html?id=xt4OAAAAQAAJ&pgis=1](http://books.google.co.in/books/about/The_Dancing_Bees.html?id=xt4OAAAAQAAJ&pgis=1).
- Wakakuwa, M., Kurasawa, M., Giurfa, M., and Arikawa, K. (2005). Spectral heterogeneity of honeybee ommatidia. *Naturwissenschaften* 92, 464–467. doi:10.1007/s00114-005-0018-5.
- Wang, Z., Pan, Y., Li, W., Wang, Z., Pan, Y., Li, W., et al. (2008). complex of *Drosophila* Visual pattern memory requires foraging function in the central complex of *Drosophila*. 133–142. doi:10.1101/lm.873008.
- Webb, B. (2004). Neural mechanisms for prediction: Do insects have forward models? *Trends Neurosci.* 27, 278–282. doi:10.1016/j.tins.2004.03.004.
- Winston, M. (1991). *The Biology of the Honey Bee*. Harvard Un. Cambridge, Massachusetts.
- Wise, R. A. (2004). Dopamine, learning and motivation. *Nat. Rev. Neurosci.* 5, 483–494. doi:10.1038/nrn1406.
- Wolf, R., Wittig, T., Liu, L., Wustmann, G., Eyding, D., and Heisenberg, M. (1998). *Drosophila* Mushroom Bodies Are Dispensable for Visual, Tactile, and Motor Learning. *Learn. Mem.* 5, 166–178. doi:10.1101/lm.5.1.166.
- Zeil, J. (1993). Orientation flights of solitary wasps (*Cerceris*; Sphecidae; Hymenoptera).~I. Description of flights. *J. Comp. Physiol. A* 172, 189–205.
- Zeil, J. (1997). The control of optic flow during learning flights. *J. Comp. Physiol. - A Sensory, Neural, Behav. Physiol.* 180, 25–37. doi:10.1007/s003590050024.

# Annexe



# Using virtual reality to study visual performances of honeybees

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and Martin Giurfa



Virtual reality (VR) offers an appealing experimental framework for studying visual performances of insects under highly controlled conditions. In the case of the honeybee *Apis mellifera*, this possibility may fill the gap between behavioural analyses in free-flight and cellular analyses in the laboratory. Using automated, computer-controlled systems, it is possible to generate virtual stimuli or even entire environments that can be modified to test hypotheses on bee visual behaviour. The bee itself can remain tethered in place, making it possible to record neural activity while the bees is performing behavioural tasks. Recent studies have examined visual navigation and attentional processes in VR on flying or walking tethered bees, but experimental paradigms for examining visual learning and memory are only just emerging. Behavioural performances of bees under current experimental conditions are often lower in VR than in natural environments, but further improvements on current experimental protocols seem possible. Here we discuss current developments and conclude that it is essential to tailor the specifications of the VR simulation to the visual processing of honeybees to improve the success of this research endeavour.

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## Introduction

Honeybees (*Apis mellifera*) constitute a privileged model system for the study of perception, learning and memory [1–5]. Despite their relatively small brain size, their perceptual and learning abilities are impressive. Foraging bees are able to navigate in complex environments, in

which they can locate and repeatedly visit profitable food sources such as flowers [6]. Fundamental to these performances is their ability to associate certain environmental cues with food reward, namely the nectar or pollen found in flowers. In freely-flying bees, visually-driven performances of varying degrees of plasticity have been studied in controlled and carefully designed experiments in which a variety of sensory cues has been paired with a reward of sucrose solution [5]. In such free-flight conditions, it was possible to glean insights into sensory processing mechanisms. For example, honeybees, which possess trichromatic colour-vision [7], are colour-blind for visual tasks that involve edge-detection or motion sensing, as they rely on the exclusive sensory input to long-wave photoreceptors for these tasks [8]. Further, bees use the apparent image speed across the retina to perceive their distance from a visual cue [9]. However, in the visual domain, detailed investigations into the neural correlates of such interesting behaviours and processes have been stalled until quite recently, largely due to the absence of an experimental procedure in which bees are immobilised but perform sufficiently well on visual tasks. The same problem exists for the study of visual learning: while freely-flying bees learn to efficiently solve simple as well as complex discrimination problems [5], the neural underpinnings of these performances have remained elusive. Immobilisation of the bee remains essential for the application of most current invasive methods for recording neural activity, even in today's age of technological miniaturisation.

A first attempt to study visual associative learning in immobilised bees made use of the proboscis extension response (PER), a reflexive, appetitive behaviour exhibited by hungry bees when sucrose solution and other sweet tastants contact their antennae, tarsi and/or mouth-pieces [10,11]. Makoto Kuwabara was the first to report visual conditioning of PER using chromatic lights paired with sucrose solution delivered to the tarsi [12]. Yet, since then, and despite repeated attempts, learning rates and discrimination capabilities as revealed by this protocol remain low and far from those of freely-flying bees (see review in [13]).

Novel attempts were therefore developed and among them, virtual reality (VR, see [Box 1](#)) offers an appealing experimental framework to overcome these limitations. It refers to a simulated environment, perceived and updated by the actions of an animal immersed in the simulation



**Box 1 Virtual reality for insects — a potted history.**

An early predecessor of a VR system for walking insects was published more than 60 years ago by Bernhard Hassenstein to characterise for the first time optomotor responses in insects [46]. In this setup, a tethered beetle held onto a very lightweight 'Y-maze globe' made of thin straws. This ball turned below the beetle as the beetle 'walked' along a blade of straw, thus repeatedly confronting the beetle with Y-maze choices of diverging straws. The tethered beetle could then be exposed to highly controlled, moving visual stimuli, simultaneously recording its directional choices on the globe [47]. Following designs, so-called locomotion compensators, then allowed for less constrained movements of the insect on two-dimensional surfaces (flies: [48], silkworms: [49]). These early setups were technically demanding, and involved insects walking unrestrained (untethered) on top of a rolling ball. The movement of the insect on the ball is constantly monitored and recorded, and servomotors at the side or bottom of the ball compensate for this locomotion by moving the ball in opposite directions. The insect therefore always remains at the apex of the ball, where it is presented with controlled olfactory or visual stimuli. Locomotion compensators have been very successful for behavioural analyses [50]. For simultaneous neuronal recordings, the insect itself is immobilised by a tether holding it in place on top of a lightweight trackball, which is suspended freely on an airflow. The walking movements of the insect are thus directly translated into ball movements, which can be recorded with precision and also used to directly manipulate the presented cues in real-time (closed-loop). While walking VR setups have been used for investigating olfactory cues (e.g. in bees: [20,51,52]) or acoustic cues (e.g. in crickets: [50]), they are particularly useful for the presentation of visual cues. Screens consisting of LED bulb arrays are commonly employed (e.g. [21\*\*,53,54]), but projection-based designs have also been developed more recently (see [39\*] for an example in spiders). Trackball setups can be profitably used in natural visual surroundings too [55\*].

However, for many insects the main mode of locomotion is flight. While VR systems for behavioural studies on free-flying insects have been developed [56,57], apparatuses for tethered flying insects have been in much wider use, especially when the neurobiology is of interest. Early systems established the use of torque metres for monitoring and measuring the forces acting on the tether by the flying insect [58,59], information that can be fed back for closed-loop control of the stimuli. Such VR systems allow for a combination or a full separation of classic and operant conditioning protocols [60], and enable the implementation of a versatile range of relationships between environmental cues and behavioural decisions [61]. As a consequence, they are still in wide use today (e.g. [62,63]). The insect's flight behaviour has also been filmed for detailed quantification [64].

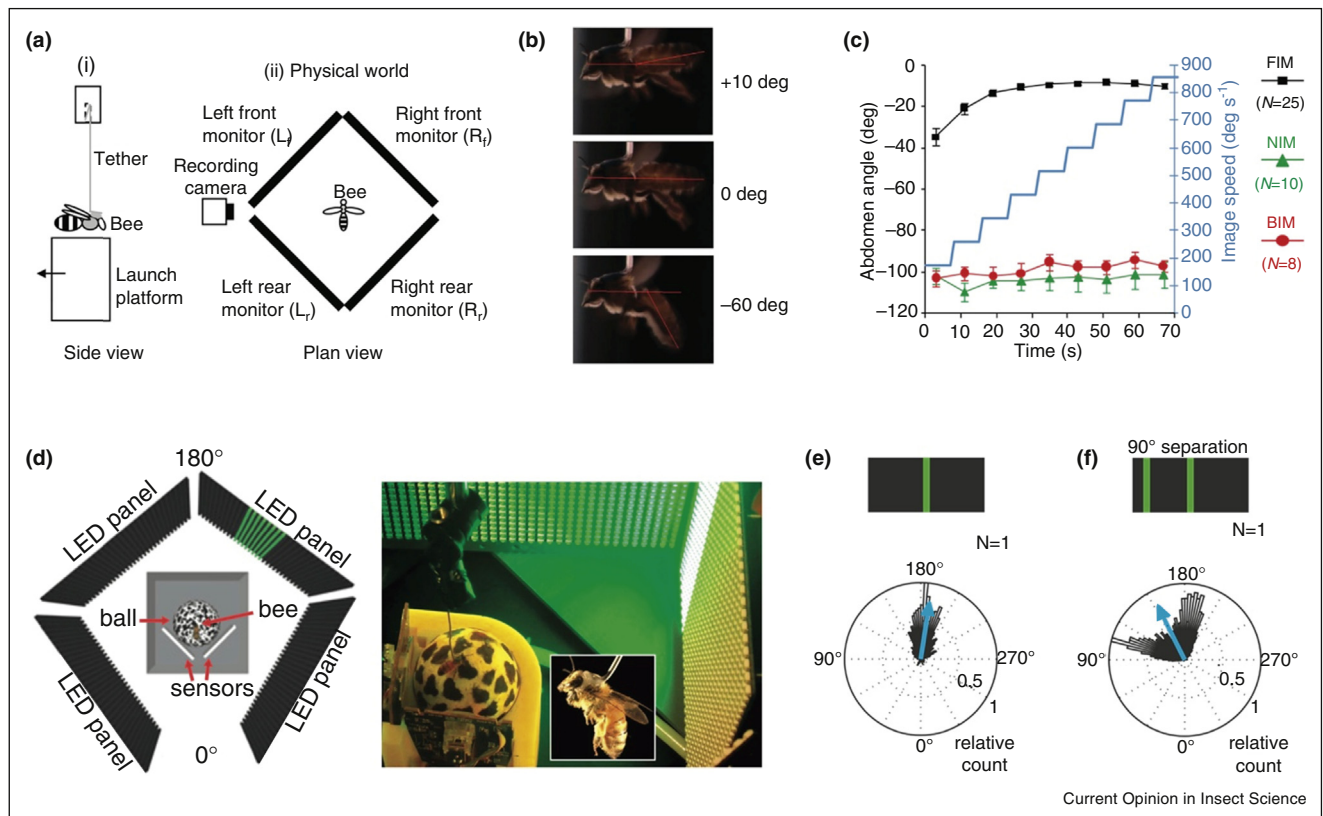
[14]. Using automated, computer-controlled systems, it is possible to generate abstract or realistic virtual stimuli and even entire landscape displays that can be modified to test specific hypotheses on visual behaviour. This approach offers, therefore, a valuable compromise between a controlled experimental environment and an ecologically rich surrounding in which an individual animal can be studied [15]. Here we will focus on discussing some recent attempts to study honeybee visual behaviour in virtual reality environments. This may help to overcome the limitations of using only free-flying bees to study visually-driven behaviour and may stimulate further efforts in this direction.

**Bees in virtual reality: studying navigation and attentional processes**

A first important breakthrough was achieved by Luu *et al.* [16], who placed a tethered bee in the middle of a setup of four LCD monitors that displayed a moving panorama (Figure 1a). The goal was to study how passive image motion affects the behaviour of a flying bee *en route* to the goal. The authors were able to make the tethered bees fly in these experimental conditions and noticed that, upon such suspended flight, bees slightly raise their abdomen, a response that is interpreted as a 'streamlining response', presumably to reduce aerodynamic drag. This response is elicited by pure visual exposure ([16]; Figure 1b,c) and is strongest when the image motion is in the direction that would be experienced during forward flight and when it covers the full visual panorama of the bee. It shows highest sensitivity in the lateral rather than in the frontal and rear fields, and is also modulated by air-flow cues simulating head-wind [17].

An alternative to bees flying stationary is the study of tethered bees walking on top of a light-weight trackball suspended on an airflow. Ball movements can be tracked accurately by appropriate optical mouse sensors [18] or a video camera [19]. This kind of device, usually termed locomotion compensator, running sphere or treadmill, has been used since more than four decades to study different aspects of insect behaviour, in particular stereotyped responses to environmental stimuli [20]. Yet, the coupling with a visual environment that is directly updated by the movements of the insect walking stationary (closed-loop) represents a novelty. Paulk *et al.* [21\*\*] used a variant of such a closed-loop VR setup for studying attention-like processes in tethered walking honeybees. Bees walking stationary in the middle of an LED arena (Figure 1d) were presented with one (Figure 1e) or two competing green vertical bars separated by 90° (Figure 1f) and flickering at different frequencies. The authors were able to combine the recording of behavioural fixation of these stimuli, reflecting attention, with an electrophysiological analysis of neural activity in different parts of the bee brain, inspired by earlier work on *Drosophila* [22]. In this way, neural responses to a specific stimulus could be 'frequency tagged' and thus traced in measurements of local field potentials, showing that attention-like processes occurred in the optic lobes before the bee displayed a behavioural choice. The use of closed-loop instead of open-loop controlled visual stimuli seems to be an important parameter, as it increases the temporal coordination of neural activity in the insect brain [23\*]. In a follow-up study, van de Poll *et al.* [24\*\*] focussed on a detailed exploration of choice behaviour of honeybees. Tethered bees were again placed on a trackball and surrounded by a hexagonal LED arena. The LED screens presented two or more vertical green bars that differed in their visual flicker frequency. The bee had closed-loop control over the stimulus movements,

Figure 1



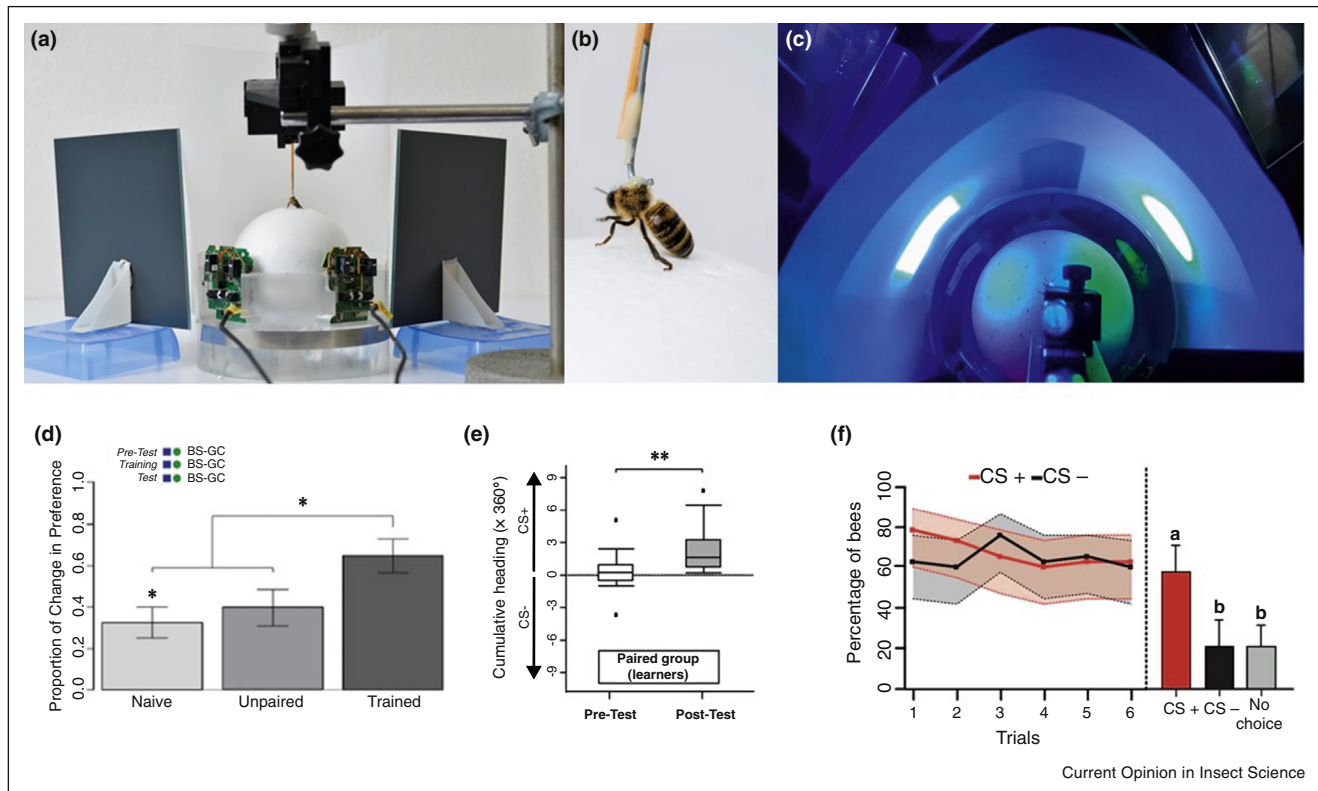
Two experiments on stereotyped visual responses in bees in VR conditions. In the first experiment (a–c), reproduced/adapted with permission from *Journal of Experimental Biology* [16] visual orientation *en route* to the goal was studied and the tethered bee flew stationary. In the second experiment (d–f; reproduced with permission from *Proceedings of the National Academy of Sciences of the USA* [21\*\*]; photo in (d) reprinted from [19], with permission from Elsevier), visual attention towards moving bars was studied and the tethered bee walked on a ball. **(a)** A flying bee was tethered by a thin metallic rod and placed in the centre of an arena consisting of four LCD monitors (termed ‘physical world’). A recording camera filmed a side view of the bee. From the perspective of the bee, the projected visual cues on the monitors appeared to form an infinite tunnel, whose walls were lined with vertical red and white sinusoidal gratings. **(b)** A simulated displacement was achieved by horizontally moving the vertical gratings in the virtual tunnel wall; the bees responded by raising or lowering their abdomens, the so-called streamlining response. **(c)** Streamlining response of bees during forward image motion (FIM, black line), no image motion (NIM, green line) and backward image motion (BIM, red line). The blue line shows the stepwise increasing image speed. As speed was increased during forward image motion, the bees progressively raised their abdomens, and then kept it in this elevated position during high speeds. **(d)** The tethered walking bees were placed on a lightweight trackball at the centre of four LED panels, which displayed vertical green bars. The bee had closed-loop control over the lateral movements of the bars. **(e)** When only one bar was displayed, the bee fixated it in a narrow ‘fixation window’ in front of her. As shown in the circular histogram, the bee had a strong tendency to keep the green bar in front of her during the experiment (blue arrow shows mean vector). **(f)** When two green bars were displayed simultaneously, the bee switched between keeping one or the other bar in its ‘fixation window’, in a behaviour reminiscent of attentional split. The circular histogram shows this alternative fixation of both bars over the course of the experiment (blue arrow shows mean vector).

and could spontaneously choose among the presented stimuli through fixation. The authors were able to show that honeybees payed more attention to fixated bars flickering at 20–25 Hz, while they avoided higher or lower frequencies (50–100 Hz and 2–4 Hz, respectively).

The VR setups used across these studies are constantly evolving, and each study so far has used slightly different parameters and materials (see also Box 1). Most importantly, the techniques used for visual stimulus presentation have changed from LCD to LED screens, as LCD screens do not allow for easy control over parameters such

as colour, brightness, light polarisation or flicker frequency [25]. LED bulbs allow precise control, and arrays of bulbs can be adjusted to match the visual resolution of bee eyes [26]. So far, these arrays have only been used to display monochromatic light stimuli. A further parameter that changes between studies is the use of open-loop or closed-loop control over stimulus movements. While early studies might have suffered from the technical difficulty of producing closed-loop control in real-time, current software readily allows for its implementation. Thus, the choice of open-loop or closed-loop today ultimately depends on the research question. In any case, the

Figure 2



Visual learning of honeybees on a treadmill. Panel (d) is reproduced/adapted with permission from *Journal of Experimental Biology*, all others are reproduced from *Scientific Reports*. **(a)** A tethered bee placed on a lightweight polystyrene trackball floating on a constant airflow. Two opti-mouse sensors were placed on the ball support, at 90° of each other to record the ball movements. The setup translates the movements of the walking bee into rotations of the ball (from [28]; © Cyril Frésillon/CNRS). **(b)** A bee tethered by the thorax by means of a vertical attachment made of a wooden toothpick and a metal piece glued to the thorax. The toothpick was held by a micromanipulator, which allowed adjusting the position of the bee on the treadmill (from [28]; © Cyril Frésillon/CNRS). **(c)** Setup viewed from above during a test situation (from [28]); two different visual stimuli, a blue square and a green disc, were projected on a semi-circular, semi-transparent screen in front of the bee. During training, one of them was paired with a sucrose reward, and the other with a quinine punishment. **(d)** Test performance under closed-loop conditions expressed in terms of 'proportion of change in preference', which compares preference after and before training, when the bees were presented with the two stimuli to be discriminated simultaneously (from [27]). Trained bees change their preference compared to naïve bees, thus showing that they learned the discrimination. Unpaired bees, which had no explicit pairing of the visual stimuli with reinforcement, did not learn the discrimination and thus did not change their preference. BS: blue square, GC: green circle. **(e)** Test performance expressed in terms of cumulative heading at the treadmill in open-loop conditions (from [28]). Values correspond to multiples of a 360° rotation and reflect the tendency of a bee to walk towards one of the two visual stimuli presented simultaneously, the CS+ (rewarded during training) or the CS- (punished during training). Performance of the paired group, which experienced the visual stimuli and the reinforcements explicitly paired, changed from a pre-test assessing naïve preference to a post-test following conditioning. After training, these bees walked more towards the stimulus previously rewarded (CS+), thus showing that they learned the discrimination. Other control groups (not shown) did not exhibit this change in performance. **(f)** Training with a single illuminated stimulus hides learning at the treadmill (from [28]). *Left*: the red curve shows the choice of the CS+ and the black curve the choice of the CS- during training of a paired group (percentage of bees choosing a given stimulus). The 95% confidence interval is shown (dashed lines and shading; in pink for the CS+ and in grey for the CS-). The bees do not show any evidence of discrimination during training. *Right*: post training performance of the bees in a test situation presenting the CS+ and the CS- simultaneously without reinforcement. The test shows that despite the apparent lack of learning during training, bees had indeed learned the discrimination. The proportion of bees choosing the CS+ (red bar) was significantly higher than the proportions of bees choosing the CS- (black bar) or not choosing any stimulus.

studies described above, while still limited in number, show clearly how successful visual VR setups can be when behavioural or neural responses of bees to stimulus presentations are of interest. The responses described above are stereotyped and thus easy to elicit in simplified VR environments. Can visual learning also be studied under such conditions?

### Bees in virtual reality: studying associative learning and memory

Immobilised bees readily perform stereotyped responses, but do not perform well on visual learning and memory tasks (see 'Introduction' section). Could these performances be improved by providing bees with some freedom of movement?

Promising results were obtained in two studies in which tethered bees had to learn a visual discrimination while walking stationary on a trackball [27,28]. In both cases, a visual projection system displayed visual stimuli on a semi-cylindrical screen placed in front of the tethered walking bee (Figure 2a–c). After a pre-test assessing naïve preference for the two visual stimuli to be discriminated, bees were trained by pairing one of them with appetitive sucrose solution and the other with aversive quinine solution. Training was performed under open-loop conditions, presenting one stimulus at a time. Thereafter, bees were tested with the two visual stimuli displayed simultaneously and without reinforcement (Figure 2c), to determine whether learning induced a change in the original preference. In one work, this test was performed under closed-loop conditions [27] while in the other, the open-loop condition was maintained [28]. Both studies showed that bees learned to discriminate the trained stimuli and modified their original preferences because of the reinforcement received during training (Figure 2d, e). As stimuli varied in shape and colour (e.g. a green disc versus a blue square), one study addressed the question of the dominance of these parameters when set in competition; it showed that visual stimuli are learned in a non-additive manner with the interaction of specific colour and shape combinations being critical for learned responses [27]. The other study analysed acquisition during training and showed that presentation of a single stimulus during training hides learning, as bees tend to fixate and/or respond in a positive phototactic manner to any single illuminated stimulus displayed in front of them [28], even if in subsequent tests with two stimuli learning was evident (Figure 2f). Moreover, it showed that different negative reinforcements associated with the stimulus to be avoided had a different impact on discrimination learning, with quinine solution and distilled water being more efficient in improving discrimination [28].

### Benefits and caveats of using virtual reality to study learning and memory in bees

The most important benefit of VR setups is the opportunity to combine controlled behavioural analyses with invasive analyses of underlying neural performances [21<sup>••</sup>], which are not feasible in freely-flying or moving insects (but see [29]). Recent advances in neurophysiological methods, such as stable extracellular long-term recordings [30–33], have established the honeybee as an ideal organism for the study of learning-related plasticity. Paulk *et al.* [21<sup>••</sup>] have shown that electrophysiological recordings on behaving honeybees in VR setups are feasible. VR can now offer the opportunity to characterise neural activity in a behaving bee during learning experiments, a goal that has remained elusive until now.

In VR, parameters of interest can be manipulated singly, systematically, in specific combinations, or even have properties that are impossible in the natural world. The

complexity of possible stimuli can vary greatly, ranging from very simple open-loop presentations to naturalistic, immersive, multimodal scenarios in closed-loop. The experimenter can also have complete control over the timing of stimuli, and over the bees, including their entire sensory exposure over the course of the experiment, which is impossible under free-flight conditions. As tethered bees can be kept for long periods if they are fed regularly and controlled for their motivation, this opens up the possibility of studying the neurobiological processes of long-term memory formation. For example, such tight control over visual experience is crucial for the characterisation of synapse reorganisation during learning and memory formation, which has failed in experiments using free-flying animals due to numerous experimental caveats [34,35].

Of course, despite these benefits, VR systems present some deficits. Indeed, they aim at simulating environmental stimuli or parts thereof, to trigger behavioural and/or neurological responses in the animal studied. The exact design of the stimuli is therefore to some degree hypothesis-driven and will not include all potential cues available in real-world environments. It is important to ensure that the appropriate responses are recorded, that responses are naturalistic, and that they do not include artefacts. For example, presenting a single coloured light stimulus at a time during a discrimination-learning task posed a problem, as bees always oriented towards the single stimulus shown in any training trial (Figure 2f, [28]). This was due to a phototactic reaction (the stimulus was the brightest area as the setup was placed in dark surroundings) or to a tendency to fixate the object on the screen, a well-known behaviour in *Drosophila* that has been used to analyse walking ability in ‘Buridan’s paradigm’ [36]. As a consequence, no clear acquisition could be seen, even if the post-acquisition tests revealed a significant learning effect. Introducing closed-loop control and thus the possibility of displaying both rewarded and punished stimuli during each training trial will allow accessing acquisition performances in a clearer way. Restricting the freedom of movement may also introduce some problems, as learning success is reduced under such conditions compared to performances of freely-flying bees (i.e. 60% versus the typical 90 or 100% success rates of freely-flying bees trained to discriminate visual stimuli in Y-mazes [28]). This may be because under tethered conditions, bees are lacking some essential proprioceptive input (such as antennal deflections during flight) for complete multisensory integration. Missing mechanosensory input is known to influence responses of *Drosophila* to visual stimuli [37], and is believed to be a cause of ‘cybersickness’ in human applications of VR [38]. In some bees, tethering may also induce a decrease or a switch from the appetitive motivation necessary for visual training to an escape motivation, which will interfere with learning. Also, when keeping bees tethered for longer periods, a proper control of

appetitive motivation is necessary as well as regular checks of normal motor (walking) behaviour to avoid fatigue effects.

To work around some of these caveats, two solutions could be useful. The first would be to use a very detailed VR that provides as much information as possible: for example, a three-dimensional virtual Y-maze that includes polarised light information and optical flow of the walls. Such a virtual world would be close to the visual setting in a free-flight experiment, without making any assumptions about the importance of specific cues. A second solution would be to make use of transfer experiments (as done in [39]). Bees would be trained on a real Y-maze and finally tested in a VR system. This will ensure that bees learn the appropriate stimuli, but will also allow the study of neural correlates of memory retention.

Finally, one also needs to account for the specific properties of the bee visual system when designing VR setups [25,40]. Bees have compound eyes with photoreceptor sensitivities peaking in the green, blue and UV regions of the spectrum [7]. Common technology for creating visual stimuli is, however, designed for human vision, in which, for example, yellow will be a blend of green and red. As bees cannot perceive red light, the colour perception will be very different [41]. Attempts to produce images taking into account the properties of the bee visual system have been performed [42]. Adapting these attempts to the visual display of images in VR setups is necessary and possible [43]. In addition, bee vision has high temporal resolution (almost 200 Hz [44]), which should therefore be the minimum frequency of any VR display system.

## Conclusion

Science and technology lead a parallel race, triggering a co-evolution of the two. Virtual reality is already widely applied in several industries such as video game development or the sport industry, and can be of great use for the study of human and animal behaviour. While off-the-shelf VR systems are designed for the direct application in humans (e.g. [45]), the use of VR in animals and especially in bees demands some additional considerations. Indeed, two aspects require particular attention in order to approximate natural conditions. Firstly, the animal needs to be kept in one position without restraining its movements, for which both the flight simulator and the locomotion compensator seem to be appropriate devices. Secondly, and more importantly, the virtual environment has to be created in a manner adapted to the sensory system of the study animal, best provided by either an LED screen or a high frequency videoprojector. The former prevents, in principle, the possibility of displaying complex landscapes and images with a finer spatial resolution due to the physical separation of the light-producing LEDs. The latter offers a more promising perspective for visual experiments on tethered bees. These advances will facilitate the

manipulation of bees in VR setups and accelerate the study of visual processing and learning in the honeybee. We expect that the advent and development of this technology will finally enable access to the neural underpinnings of the numerous visually driven performances of bees that have fascinated researchers for decades.

## Conflicts of interest

None declared.

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## References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Menzel R: **Memory dynamics in the honeybee.** *J Comp Physiol A* 1999, **185**:323-340 <http://dx.doi.org/10.1007/s003590050392>.
2. Menzel R: **Searching for the memory trace in a mini-brain, the honeybee.** *Learn Mem* 2001, **8**:53-62 <http://dx.doi.org/10.1101/lm.38801>.
3. Giurfa M: **Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well.** *J Comp Physiol A* 2007, **193**:801-824 <http://dx.doi.org/10.1007/s00359-007-0235-9>.
4. Srinivasan MV: **Honey bees as a model for vision, perception, and cognition.** *Annu Rev Entomol* 2010, **55**:267-284 <http://dx.doi.org/10.1146/annurev.ento.010908.164537>.
5. Avarguès-Weber A, Deisig N, Giurfa M: **Visual cognition in social insects.** *Annu Rev Entomol* 2011, **56**:423-443 <http://dx.doi.org/10.1146/annurev-ento-120709-144855>.
6. Menzel R: **Learning in honey bees in an ecological and behavioral context.** In *Experimental Behavioral Ecology and Sociobiology*. Edited by Hölldobler B, Lindauer M. Gustav Fischer Verlag; 1985:55-74.
7. Menzel R, Backhaus W: **Color vision in insects.** In *Vision and Visual Dysfunction*. Edited by Gouras P. Macmillan; 1991:262-288.
8. Lehrer M: **Spatial vision in the honeybee: the use of different cues in different tasks.** *Vis Res* 1994, **34**:2263-2285 [http://dx.doi.org/10.1016/0042-6989\(94\)90282-8](http://dx.doi.org/10.1016/0042-6989(94)90282-8).
9. Srinivasan MV, Lehrer M, Kirchner WH, Zhang SW: **Range perception through apparent image speed in freely flying honeybees.** *Vis Neurosci* 1991, **6**:519-535 <http://dx.doi.org/10.1017/S095252380000136X>.
10. Minnich DE: **The chemical senses of insects.** *Q R Biol* 1929, **4**:100-112 <http://dx.doi.org/10.1086/394325>.
11. Minnich DE: **The contact chemoreceptors of the honey bee, *Apis mellifera* Linn.** *J Exp Zool* 1932, **61**:375-393 <http://dx.doi.org/10.1002/jez.1400610303>.
12. Kuwabara M: **Bildung des bedingten Reflexes von Pavlows Typus bei der Honigbiene, *Apis mellifica*.** *J Fac Sci Hokkaido Univ* 1957, **13**:458-464.
13. Avarguès-Weber A, Mota T: **Advances and limitations of visual conditioning protocols in harnessed bees.** *J Physiol (Paris)* 2017, **110**:107-118 <http://dx.doi.org/10.1016/j.jphysparis.2016.12.006>.
14. Dombeck DA, Reiser MB: **Real neuroscience in virtual worlds.** *Curr Opin Neurobiol* 2012, **22**:3-10 <http://dx.doi.org/10.1016/j.conb.2011.10.015>.

15. Bohil CJ, Alicea B, Biocca FA: **Virtual reality in neuroscience research and therapy.** *Nat Rev Neurosci* 2011, **12**:752-762 <http://dx.doi.org/10.1038/nrn3122>.
16. Luu T, Cheung A, Ball D, Srinivasan MV: **Honeybee flight: a novel 'streamlining' response.** *J Exp Biol* 2011, **214**:2215-2225 <http://dx.doi.org/10.1242/jeb.050310>.
17. Taylor GJ, Luu T, Ball D, Srinivasan MV: **Vision and air flow combine to streamline flying honeybees.** *Sci Rep* 2013, **3**:2614 <http://dx.doi.org/10.1038/srep02614>.
18. Taylor GJ, Paulk AC, Pearson TW, Moore RJ, Stacey JA, Ball D, van Swinderen B, Srinivasan MV: **Insects modify their behaviour depending on the feedback sensor used when walking on a trackball in virtual reality.** *J Exp Biol* 2015, **218**:3118-3127 <http://dx.doi.org/10.1242/jeb.125617>.
19. Moore RJ, Taylor GJ, Paulk AC, Pearson T, van Swinderen B, Srinivasan MV: **FicTrac: a visual method for tracking spherical motion and generating fictive animal paths.** *J Neurosci Methods* 2014, **225**:106-119 <http://dx.doi.org/10.1016/j.jneumeth.2014.01.010>.
20. Kramer E: **The orientation of walking honeybees in odour fields with small concentration gradients.** *Physiol Entomol* 1976, **1**:27 <http://dx.doi.org/10.1111/j.1365-3032.1976.tb00883.x>.
21. Paulk AC, Stacey JA, Pearson TW, Taylor GJ, Moore RJ, Srinivasan MV, van Swinderen B: **Selective attention in the honeybee optic lobes precedes behavioral choices.** *Proc Natl Acad Sci U S A* 2014, **111**:5006-5011 <http://dx.doi.org/10.1073/pnas.1323297111>.
- This study was the first to record neural activity in the brain of a walking bee performing a visual task in VR.
22. van Swinderen B: **Competing visual flicker reveals attention-like rivalry in the fly brain.** *Front Integr Neurosci* 2012, **6**:96 <http://dx.doi.org/10.3389/fnint.2012.00096>.
23. Paulk AC, Kirszenblat L, Zhou Y, van Swinderen B: **Closed-loop behavioral control increases coherence in the fly brain.** *J Neurosci* 2015, **35**:10304-10315 <http://dx.doi.org/10.1523/JNEUROSCI.0691-15.2015>.
- Shows that closed-loop control of VR stimulus movements can improve the coherence of neural activity.
24. van de Poll MN, Zajackowski EL, Taylor GJ, Srinivasan MV, van Swinderen B: **Using an abstract geometry in virtual reality to explore choice behaviour: visual flicker preferences in honeybees.** *J Exp Biol* 2015, **218**:3448-3460 <http://dx.doi.org/10.1242/jeb.125138>.
- A good example of how to use VR for designing experimental paradigms that are impossible in real-world settings.
25. D'Eath RB: **Can video images imitate real stimuli in animal behaviour experiments?** *Biol Rev* 1998, **73**:267-292 <http://dx.doi.org/10.1111/j.1469-185X.1998.tb00031.x>.
26. Reiser MB, Dickinson MH: **A modular display system for insect behavioral neuroscience.** *J Neurosci Methods* 2008, **167**:127-139 <http://dx.doi.org/10.1016/j.jneumeth.2007.07.019>.
27. Rusch C, Roth E, Vinauger C, Riffell JA: **Honeybees in a virtual reality environment learn unique combinations of colour and shape.** *J Exp Biol* 2017 <http://dx.doi.org/10.1242/jeb.164731>. (in press).
28. Buatois A, Pichot C, Schultheiss P, Sandoz J-C, Lazzari CR, Chittka L, Avargués-Weber A, Giurfa M: **Associative visual learning by tethered bees in a controlled visual environment.** *Sci Rep* 2017. (in press).
29. Grover D, Katsuki T, Greenspan RJ: **Flyception: imaging brain activity in freely walking fruit flies.** *Nat Methods* 2016, **13**:569-572 <http://dx.doi.org/10.1038/nmeth.3866>.
30. Okada R, Rybak J, Manz G, Menzel R: **Learning-related plasticity in PE1 and other mushroom body-extrinsic neurons in the honeybee brain.** *J Neurosci* 2007, **27**:11736-11747 <http://dx.doi.org/10.1523/JNEUROSCI.2216-07.2007>.
31. Hussaini SA, Menzel R: **Mushroom body extrinsic neurons in the honeybee brain encode cues and contexts differently.** *J Neurosci* 2013, **33**:7154-7164 <http://dx.doi.org/10.1523/JNEUROSCI.1331-12.2013>.
32. Strube-Bloss MF, Nawrot MP, Menzel R: **Mushroom body output neurons encode odor-reward associations.** *J Neurosci* 2011, **31**:3129-3140 <http://dx.doi.org/10.1523/JNEUROSCI.2583-10.2011>.
33. Strube-Bloss MF, Nawrot MP, Menzel R: **Neural correlates of side-specific odour memory in mushroom body output neurons.** *Proc R Soc B: Biol Sci* 2016, **283**:20161270 <http://dx.doi.org/10.1098/rspb.2016.1270>.
34. Sommerlandt FM, Spaethe J, Rössler W, Dyer AG: **Does fine color discrimination learning in free-flying honeybees change mushroom-body calyx neuroarchitecture?** *PLOS ONE* 2016, **11**:e0164386 <http://dx.doi.org/10.1371/journal.pone.0164386>.
35. van Nest BN, Wagner AE, Marrs GS, Fahrbach SE: **Volume and density of microglomeruli in the honey bee mushroom bodies do not predict performance on a foraging task.** *Dev Neurobiol* 2017 <http://dx.doi.org/10.1002/dneu.22492>.
36. Strauss R, Hanesch U, Kinkelin M, Wolf R, Heisenberg M: **No-bridge of *Drosophila melanogaster*: portrait of a structural brain mutant of the central complex.** *J Neurogenetics* 1992, **8**:125-155 <http://dx.doi.org/10.3109/01677069209083444>.
37. Mureli S, Thanigaivelan I, Schaffer ML, Fox JL: **Cross-modal influence of mechanosensory input on gaze responses to visual motion in *Drosophila*.** *J Exp Biol* 2017, **220**:2218-2227 <http://dx.doi.org/10.1242/jeb.146282>.
38. Rebenitsch L, Owen C: **Review on cybersickness in applications and visual displays.** *Virtual Real* 2016, **20**:101-125 <http://dx.doi.org/10.1007/s10055-016-0285-9>.
39. Peckmezian T, Taylor PW: **A virtual reality paradigm for the study of visually mediated behaviour and cognition in spiders.** *Anim Behav* 2015, **107**:87-95 <http://dx.doi.org/10.1016/j.anbehav.2015.06.018>.
- An innovative study that uses projected images in a VR system for arthropods.
40. Fleishman LJ, Endler JA: **Some comments on visual perception and the use of video playback in animal behavior studies.** *Acta Ethol* 2000, **3**:15-27 <http://dx.doi.org/10.1007/s102110000025>.
41. Fleishman LJ, McClintock WJ, D'Eath RB, Brainard DH, Endler JA: **Colour perception and the use of video playback experiments in animal behaviour.** *Anim Behav* 1998, **56**:1035-1040 <http://dx.doi.org/10.1006/anbe.1998.0894>.
42. Vorobyev M, Gumbert A, Kunze J, Giurfa M, Menzel R: **Flowers through insect eyes.** *Isr J Plant Sci* 1997, **45**:93-101 <http://dx.doi.org/10.1080/07929978.1997.10676676>.
43. Tedore C, Johnsen S: **Using RGB displays to portray color realistic imagery to animal eyes.** *Curr Zool* 2017, **63**:27-34 <http://dx.doi.org/10.1093/cz/zow076>.
44. Srinivasan MV, Lehrer M: **Temporal acuity of honeybee vision: behavioural studies using moving stimuli.** *J Comp Physiol A* 1984, **155**:297-312 <http://dx.doi.org/10.1007/BF00610583>.
45. Foerster RM, Poth CH, Behler C, Botsch M, Schneider WX: **Using the virtual reality device Oculus Rift for neuropsychological assessment of visual processing capabilities.** *Sci Rep* 2016, **6**:37016 <http://dx.doi.org/10.1038/srep37016>.
46. Hassenstein B: **Wandernde geometrische Interferenzfiguren im Insektenauge.** *Naturwissenschaften* 1950, **2**:45-46 <http://dx.doi.org/10.1007/BF00645369>.
47. Hassenstein B: **Ommatidienraster und afferente Bewegungsintegration. (Versuche an dem Rüsselkäfer *Chlorophanus viridis*).** *Z Vgl Physiol* 1951, **33**:301-326 <http://dx.doi.org/10.1007/BF00339334>.
48. Goetz KG, Gambke C: **Zum Bewegungssehen des Mehlkäfers *Tenebrio molitor*.** *Kybernetik* 1968, **4**:225-229 <http://dx.doi.org/10.1007/BF00272520>.
49. Kramer E: **Orientation of the male silkworm to the sex attractant bombycol.** In *Olfaction and Taste V.* Edited by Denton D, Coghlan JD. Academic Press; 1975:329-335.
50. Gerhardt HC, Huber F: *Acoustic Communication in Insects and Anurans: Common Problems and Diverse Solutions.* Chicago: University of Chicago Press; 2002.

51. Brandstaetter AS, Bastin F, Sandoz JC: **Honeybee drones are attracted by groups of conspecifics in a walking simulator.** *J Exp Biol* 2014, **217**:1278-1285 <http://dx.doi.org/10.1242/jeb.094292>.
52. Yamashita T, Haupt SS, Ikeno H, Ai H: **Walking patterns induced by learned odors in the honeybee, *Apis mellifera* L.** *J Exp Biol* 2016, **219**:12-16 <http://dx.doi.org/10.1242/jeb.123356>.
53. Seelig JD, Chiappe ME, Lott GK, Dutta A, Osborne JE, Reiser MB, Jayaraman V: **Two-photon calcium imaging from head-fixed *Drosophila* during optomotor walking behavior.** *Nat Methods* 2010, **7**:535-540 <http://dx.doi.org/10.1038/nmeth.1468>.
54. Seelig JD, Jayaraman V: **Neural dynamics for landmark orientation and angular path integration.** *Nature* 2015, **521**:186-191 <http://dx.doi.org/10.1038/nature14446>.
55. Dahmen H, Wahl VL, Pfeffer SE, Mallot HA, Wittlinger M:  
 • **Naturalistic path integration of *Cataglyphis* desert ants on an air-cushioned lightweight spherical treadmill.** *J Exp Biol* 2017, **220**:634-644 <http://dx.doi.org/10.1242/jeb.148213>.  
 Describes a novel treadmill design that records insect movements with high precision.
56. Fry SN, Rohrseitz N, Straw AD, Dickinson MH: **TrackFly: virtual reality for a behavioral system analysis in free-flying fruit flies.** *J Neurosci Methods* 2008, **171**:110-117 <http://dx.doi.org/10.1016/j.jneumeth.2008.02.016>.
57. Straw AD, Lee S, Dickinson MH: **Visual control of altitude in flying *Drosophila*.** *Curr Biol* 2010, **20**:1550-1556 <http://dx.doi.org/10.1016/j.cub.2010.07.025>.
58. Goetz KG: **Optomotorische Untersuchung des visuellen Systems einiger Augenmutanten der Fruchtfliege *Drosophila*.** *Kybernetik* 1964, **2**:77-92 <http://dx.doi.org/10.1007/BF00288561>.
59. Heisenberg M, Wolf R: *Vision in Drosophila: Genetics of Microbehavior.* Berlin: Springer; 1984.
60. Brembs B, Heisenberg M: **The operant and the classical in conditioned orientation of *Drosophila melanogaster* at the flight simulator.** *Learn Mem* 2000, **7**:104-115 <http://dx.doi.org/10.1101/lm.7.2.104>.
61. Brembs B, Wiener J: **Context and occasion setting in *Drosophila* visual learning.** *Learn Mem* 2006, **13**:618-628 <http://dx.doi.org/10.1101/lm.318606>.
62. Maimon G, Straw AD, Dickinson MH: **Active flight increases the gain of visual motion processing in *Drosophila*.** *Nat Neurosci* 2010, **13**:393-399 <http://dx.doi.org/10.1038/nn.2492>.
63. Brembs B: **Spontaneous decisions and operant conditioning in fruit flies.** *Behav Process* 2011, **87**:157-164 <http://dx.doi.org/10.1016/j.beproc.2011.02.005>.
64. Fry SN, Sayaman R, Dickinson MH: **The aerodynamics of hovering flight in *Drosophila*.** *J Exp Biol* 2005, **208**:2303-2318 <http://dx.doi.org/10.1242/jeb.01612>.

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## Résumé

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Les abeilles en libre vol montrent des capacités cognitives visuelles remarquables mais les bases neurales sous-jacentes ne peuvent pas être étudiées chez des insectes en vol. À l'inverse, le cerveau des abeilles immobilisées est accessible mais ne permet pas d'explorer l'apprentissage visuel. Pour dépasser cette limite, notre objectif a été d'établir un dispositif de réalité virtuelle pour pouvoir tester l'apprentissage visuel sur des abeilles attachées.

Dans un premier temps, les abeilles ont été testées sur leur capacité à discriminer des couleurs en s'appuyant sur les renforcements positifs ou négatifs qui leur étaient associés. Ces expériences ont permis de mettre en évidence le rôle de la vision active dans la réalité virtuelle et l'importance de la phototaxie dans ce type de système.

Grâce à ce dispositif, un apprentissage non élémentaire en réalité virtuelle, le patterning négatif, a été mis en place. Ainsi il a été montré que les abeilles étaient capables de résoudre cette tâche en réalité virtuelle malgré sa complexité.

Enfin, en s'appuyant sur le protocole de discrimination de couleurs, l'activation du cerveau a été étudiée au cours d'un test de mémoire des couleurs en analysant l'expression de gènes considérés comme des marqueurs de l'activité neurale.

Les résultats de cette thèse, fournissant deux protocoles de réalité virtuelle solides pour étudier les apprentissages visuels élémentaires et non élémentaires, constituent une avancée considérable vers la possibilité de comprendre les bases neurales sous-jacentes à ces apprentissages chez l'abeille qu'ils soient simples ou complexes.

**Mots clefs** : Abeille, Réalité virtuelle, Apprentissage visuel, Discrimination de couleur, Patterning négatif, Gènes précoces immédiats



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## Abstract

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### **Behavioral and Neurobiological studies of visual learning in honey bees in virtual reality**

Free flying bees are known for their impressive visual cognition abilities, but the neural bases underlying those are poorly studied because of the difficulty to explore the brain in a flying insect. Conversely, it is possible to have access to the brain with tethered bees but, until now, no studies explored visual learning. To bypass this limitation, our aim was to establish a virtual reality device to test visual learning in tethered bees.

First, bees were tested for their abilities to learn to discriminate colors according to the reinforcement associated to each of them. These experiments allowed to highlight the role of the active vision in virtual reality and the importance of phototaxis in this kind of device.

Driven by these good results, we explored the non-elementary visual learning in bees and, more specifically, the negative patterning. The results suggest that bees were able to resolve this task in virtual reality despite its complexity.

Finally, using the protocol of color discrimination, the brain activation has been explored during a color memory recall thanks to a quantification of immediate early genes considered as neural markers.

This thesis provides two solid virtual reality protocols to study elementary and non-elementary visual learning in honey bees. This constitutes a huge advance and will allow to go further towards the understanding of the neural bases of simple and complex visual learning.

**Keywords :** Honey bees, Virtual reality, Visual learning, Color discrimination, Negative patterning, Immediate early genes