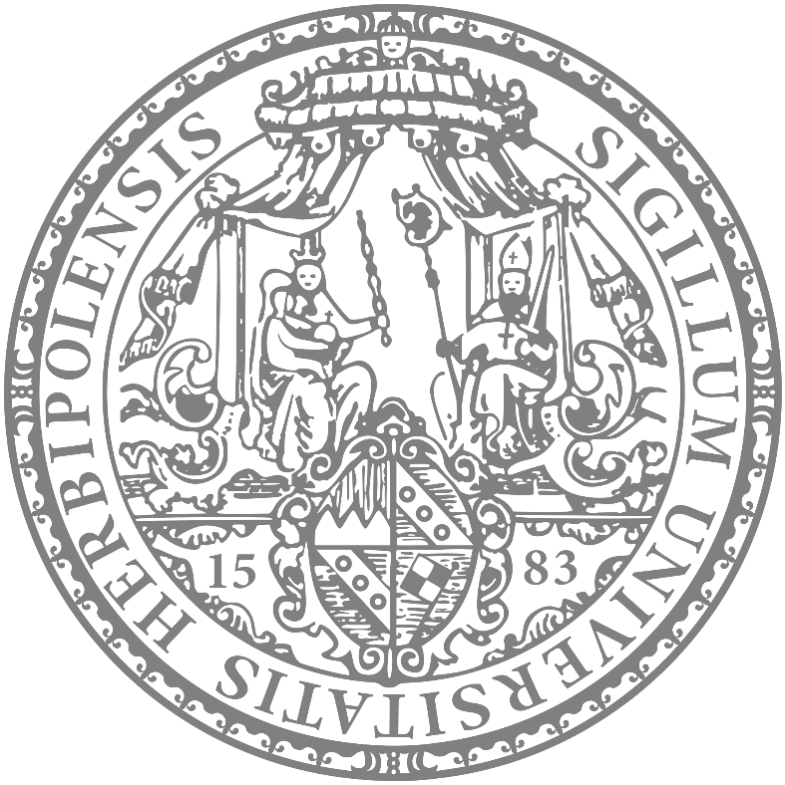
Are some bees smarter than others?

An examination of consistent individual differences in the cognitive abilities of honey bees

Sind manche Bienen schlauer als andere?

Eine Untersuchung von konsistenten individuellen Unterschieden in den kognitiven Fähigkeiten von Honigbienen



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“The bee’s life is like a magic well: the more you draw from it, the more it fills with water“

- Karl von Frisch -

(Bees: Their Vision, Chemical Senses and Language, 1950)

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List of abbreviations

1st RL	first phase of reversal learning
2nd RL	second phase of reversal learning
AL	antennal lobe
CR	conditioned response
CS	conditioned stimulus
CS-	unrewarded conditioned response
CS+	rewarded conditioned stimulus
DNA	deoxyribonucleic acid
g factor	general factor
GABA	gamma-aminobutyric acid
GLMM	generalized linear mixed model
GRS	gustatory response score
ID	subject identity
KC	Kenyon cell
L	long-range wavelength
LED	light-emitting diode
LH	lateral horn
LN	local interneuron
M	mid-range wavelength
MB	mushroom body
MBON	mushroom body output neuron
NP	negative patterning
ORN	olfactory receptor neuron
PER	proboscis extension response

PN	projection neuron
RFID	radio frequency identification
S	short-range wavelength
SEM	standard error of the mean
SER	sting extension response
UR	unconditioned response
US	unconditioned stimulus
Λmax	maximal light absorption

Summary

Cognition refers to the ability to of animals to acquire, process, store and use vital information from the environment. Cognitive processes are necessary to predict the future and reduce the uncertainty of the ever-changing environment. Classically, research on animal cognition focuses on decisive cognitive tests to determine the capacity of a species by the testing the ability of a few individuals. This approach views variability between these tested key individuals as unwanted noise and is thus often neglected. However, inter-individual variability provides important insights to behavioral plasticity, cognitive specialization and brain modularity. Honey bees *Apis mellifera* are a robust and traditional model for the study of learning, memory and cognition due to their impressive capabilities and rich behavioral repertoire. In this thesis I have applied a novel view on the learning abilities of honey bees by looking explicitly at individual differences in a variety of learning tasks. Are some individual bees consistently smarter than some of her sisters? If so, will a smart individual always perform good independent of the time, the context and the cognitive requirements or do bees show distinct isolated 'cognitive modules'?

My thesis presents the first comprehensive investigation of consistent individual differences in the cognitive abilities of honey bees. To speak of an individual as behaving consistently, a crucial step is to test the individual multiple times to examine the repeatability of a behavior. I show that free-flying bees remain consistent in a visual discrimination task for three consecutive days. Successively, I explored individual consistency in cognitive proficiency across tasks involving different sensory modalities, contexts and cognitive requirements. I found that free-flying bees show a cognitive specialization between visual and olfactory learning but remained consistent across a simple discrimination task and a complex concept learning task. I wished to further explore individual consistency with respect to tasks of different cognitive complexity, a question that has never been tackled before in an insect. I thus performed a series of four experiments using either visual or olfactory stimuli and a different training context (free-flying and restrained) and tested bees in a discrimination task, reversal learning and negative patterning. Intriguingly, across all these experiments I evidenced the same results: The bees' performances were consistent across the discrimination task and reversal learning and negative patterning respectively. No association was evidenced between reversal learning and negative patterning. After establishing the existence of consistent individual differences in the cognitive proficiency of honey bees I wished to determine factors which could

underlie these differences. Since genetic components are known to underlie inter-individual variability in learning abilities, I studied the effects of genetics on consistency in cognitive proficiency by contrasting bees originating from either from a hive with a single patriline (low genetic diversity) or with multiple patrilines (high genetic diversity). These two groups of bees showed differences in the patterns of individually correlated performances, indicating a genetic component accounts for consistent cognitive individuality. Another major factor underlying variability in learning performances is the individual responsiveness to sucrose solution and to visual stimuli, as evidenced by many studies on restrained bees showing a positive correlation between responsiveness to task relevant stimuli and learning performances. I thus tested whether these relationships between sucrose/visual responsiveness and learning performances are applicable for free-flying bees. Free-flying bees were again subjected to reversal learning and negative patterning and subsequently tested in the laboratory for their responsiveness to sucrose and to light. There was no evidence of a positive relationship between sucrose/visual responsiveness and neither performances of free-flying bees in an elemental discrimination, reversal learning and negative patterning. These findings indicate that relationships established between responsiveness to task relevant stimuli and learning proficiency established in the laboratory with restrained bees might not hold true for a completely different behavioral context i.e. for free-flying bees in their natural environment.

These results show that the honey bee is an excellent insect model to study consistency in cognitive proficiency and to identify the underlying factors. I mainly discuss the results with respect to the question of brain modularity in insects and the adaptive significance of individuality in cognitive abilities for honey bee colonies. I also provide a proposition of research questions which tie in this theme of consistent cognitive proficiency and could provide fruitful areas for future research.

Zusammenfassung

Unter Kognition versteht man die Fähigkeit von Tieren, essenzielle Informationen aus der Umwelt zu erfassen, zu verarbeiten, zu speichern und zu nutzen. Kognitive Prozesse sind notwendig, um die Zukunft vorherzusagen und die Unvorhersehbarkeit der sich ständig verändernden Umwelt zu verringern. Die Forschung der Kognition von Tieren konzentriert sich klassischerweise auf entscheidende kognitive Tests, um die Fähigkeit einer Spezies anhand der Leistungen einiger weniger Individuen zu bestimmen. Bei diesem Ansatz wird die Variabilität zwischen Individuen als unerwünschtes Rauschen betrachtet und daher vernachlässigt. Die interindividuelle Variabilität liefert jedoch wichtige Erkenntnisse über die Plastizität des Verhaltens, die kognitive Spezialisierung und die Modularität des Gehirns. Die Honigbiene *Apis mellifera* ist aufgrund ihrer eindrucksvollen Fähigkeiten und ihres reichen Verhaltensrepertoires ein robuster und traditioneller Modellorganismus für die Untersuchung von Lernen, Gedächtnis und Kognition. In dieser Arbeit habe ich das Lernverhalten von Honigbienen in einem neuen Blickwinkel betrachtet, indem ich explizit die individuellen Unterschiede bei diversen Lernaufgaben untersucht habe. Zeigen manche Bienen durchweg eine erhöhte Lernleistung im Vergleich zu ihren Schwestern? Wenn ja, erbringt ein Individuum unabhängig von der Zeit, dem Kontext und den kognitiven Anforderungen der Lernaufgaben immer gute Leistungen, oder zeigen Bienen ausgeprägte unabhängige "kognitive Module"?

Die vorliegende Doktorarbeit stellt die erste umfassende Untersuchung konsistenter individueller Unterschiede in den kognitiven Fähigkeiten von Honigbienen dar. Um von einem konsistenten Verhalten sprechen zu können, ist es entscheidend das Individuum mehrfach zu testen, um die Wiederholbarkeit eines Verhaltens zu untersuchen. Ich konnte zeigen, dass frei fliegende Bienen bei einer visuellen Unterscheidungsaufgabe an drei aufeinanderfolgenden Tagen eine konsistente Lernleistung zeigen. Im Anschluss untersuchte ich die individuelle Konsistenz der kognitiven Fähigkeiten bei Lernaufgaben mit unterschiedlichen sensorischen Modalitäten, Kontexten und kognitiven Anforderungen. Frei fliegende Bienen zeigten eine kognitive Spezialisierung zwischen visuellem und olfaktorischem Lernen, während sie bei einer einfachen Unterscheidungsaufgabe und einer komplexen Konzeptlernaufgabe konsistent im Lernverhalten blieben. Anschließend wollte ich die individuelle Konsistenz im Lernverhalten bei Aufgaben unterschiedlicher kognitiver Komplexität weiter erforschen, eine Frage, die bisher noch nie bei einem Insekt behandelt wurde. Ich führte dazu eine Reihe von vier Experimenten durch, bei denen entweder visuelle oder olfaktorische Stimuli und ein

unterschiedlicher Trainingskontext (frei fliegend oder eingespannt) verwendet wurden. Die Bienen wurden in einer Unterscheidungsaufgabe, einer Umlernaufgabe und in Negative Patterning getestet. Erstaunlicherweise wurden bei diesen Experimenten die gleichen Ergebnisse festgestellt: Die Lernleitung der Bienen in der Unterscheidungsaufgabe zeigte eine positive Korrelation mit der Lernleistung im Umlernen und Negative Patterning. Zwischen dem Umkehrlernen und Negative Patterning konnte jedoch kein Zusammenhang festgestellt werden.

Nachdem ich festgestellt hatte, dass es konsistente individuelle Unterschiede in den kognitiven Fähigkeiten von Bienen gibt, wollte ich die Faktoren ermitteln, die diesen Unterschieden zugrunde liegen könnten. Es war bereits bekannt, dass genetische Komponenten der interindividuellen Variabilität im Lernverhalten zugrunde liegen. Deshalb untersuchte ich den Einfluss von genetischer Vielfalt auf die Beständigkeit von kognitiven Fähigkeiten, indem ich Bienen gegenüberstellte, die entweder aus einem Bienenstock mit einer einzigen Patriline (geringe genetische Vielfalt) oder mit mehreren Patrilinen (hohe genetische Vielfalt) stammten. Diese beiden Gruppen von Bienen wiesen Unterschiede in den Mustern der individuellen korrelierten Lernleistungen auf, was darauf hindeutet, dass eine genetische Komponente für kognitive Individualität verantwortlich ist. Ein weiterer wichtiger Faktor, welcher der Variabilität im Lernverhalten zugrunde liegt, ist die individuelle Reaktionsfähigkeit auf Saccharose Lösungen und auf visuelle Stimuli. Dies wurde durch viele Studien an eingespannten Bienen gezeigt, die eine positive Korrelation zwischen der Reaktionsfähigkeit auf aufgabenrelevante Reize und den Lernfähigkeiten feststellten. Ich habe daher untersucht, ob diese Beziehungen zwischen der Reaktionsfähigkeit auf Saccharose und visuellen Stimuli und den Lernleistungen auch für frei fliegende Bienen zutreffen. Die individuellen Lernleistungen im Umlernen und Negative patterning von frei fliegenden Bienen wurden erneut ermittelt und anschließend wurde im Labor die Reaktionsfähigkeit auf Saccharose und Licht getestet. Es gab keine Hinweise auf eine positive Korrelation zwischen der Reaktionsfähigkeit auf Saccharose und Licht und den Lernleistungen von frei fliegenden Bienen. Diese Ergebnisse deuten darauf hin, dass Beziehungen zwischen der Reaktionsfähigkeit auf aufgabenrelevante Stimuli und der Lernleistung, die im Labor mit eingespannten Bienen festgestellt wurden, möglicherweise nicht für einen anderen Verhaltenskontext gelten, d. h. für frei fliegende Bienen in ihrer natürlichen Umgebung.

Diese Ergebnisse zeigen, dass die Honigbiene ein hervorragendes Insektenmodell ist, um die Konsistenz kognitiver Fähigkeiten zu untersuchen und die zugrunde liegenden Faktoren zu ermitteln. Ich diskutiere die Ergebnisse vor allem im Hinblick auf die Frage der Modularität des Gehirns bei Insekten und die adaptive Bedeutung von individuellen konsistenten kognitiven

Fähigkeiten für Honigbienenvölker. Ich schlage auch Forschungsfragen vor, die mit individuellen konsistenten kognitiven Fähigkeiten zusammenhängen und wertvolle Bereiche für künftige Forschungen darstellen könnten.

1. General Introduction

1.1 The origins of studying consistent individual cognitive proficiency

The basis of psychometrics involves the finding that humans show consistent individual differences in a variety of cognitive capabilities. Cognition can be defined as the ability to acquire, process, store and use critical environmental information (Shettleworth 2009). Charles Spearman conducted pioneering studies where he provided first evidence that the performances of individual people in a battery of tasks testing different cognitive abilities were positively correlated, to which he referred to as 'general intelligence' (Spearman 1904). Since then, general intelligence has been studied extensively and is now described as one of the most heritable and reliable traits in humans (Plomin 1999; Duncan et al. 2000; Plomin and Spinath 2002; Gläscher et al. 2010).

The topic of consistent individual differences in the cognitive abilities of non-human animals has long been neglected. This can probably be explained by the traditional view of behavioral biologists that inter-individual variability in behavior represents noise around the optimal behavioral mean (Thomson and Chittka 2001; Bergmüller and Taborsky 2010). Traditionally, the study of cognitive abilities in animals involves testing a few subjects individually and then these individual performances are pooled to an average group performance which is then used as a representative measure of proficiency for the whole species. This approach, however, does not allow to draw any conclusions on if and how the subjects within the sample exhibit inter-individual variability (Gallistel et al. 2004; Pamir et al. 2011). However, in the past two decades the topic of 'animal personality', i.e. individual differences in behavior that are consistent across time and contexts, has gained increasing interest and has shifted the focus from the average group performances in behavior to those of individuals (Gosling 2001; Sih et al. 2004; Réale et al. 2007, 2010; Dall et al. 2012; Jandt et al. 2014; Jeanson and Weidenmüller 2014). Consistent individual differences in cognitive proficiency have been demonstrated in a wide range of vertebrate species (Matzel et al. 2003; Herrmann and Call 2012; Guenther and Brust 2017; Boogert et al. 2018; Cauchoix et al. 2018). They are studied by themselves to identify their causes and consequences to gain a better understanding of the evolution, structure and mechanisms underlying cognitive abilities (Galsworthy et al. 2005; Kolata et al. 2005; Healy et al. 2009; Healy 2012; Thornton and Lukas

2012; Thornton et al. 2014). Indeed, natural selection acts on variability and thus an essential premise to comprehend the evolution of cognition and the underlying neurobiological structures is to study individual variability in cognitive traits (Endler 1986).

Contrarily, these questions have been mostly neglected in invertebrate research, which can be mainly attributed to the wide-spread assumption that insects are 'mindless machines' (*sensu* Gould and Gould 1982) i.e. that their behavior is rather stereotypical and inflexible expressing only restricted experience-dependent plasticity (Pinker 2010). Several rationales which I will discuss in the following sections below, justify the study of consistent individual differences in cognitive proficiency in the honey bee (*Apis mellifera*) among which the versatile cognitive capabilities and the rich behavioral repertoire are major arguments.

1.2 The honey bee as model for learning, memory, and cognition

The honey bee is a eusocial insect that lives in integrated societies with several thousand individuals in a single colony (Winston 1987). Within a colony, different tasks are being performed simultaneously by specialized individuals referred to as division of labor (Oster and Wilson 1978; Wilson 1985). The division of labor in honey bees is mainly regulated by age polyethism as a task an individual performs is highly influenced by its age (Seeley 1982): younger bees (~ 1-21 days of age) are involved in inside-hive tasks related to brood care, cleaning, and food processing while the oldest bees (> 21 days of age) are be involved in searching for different food sources outside of the nest. These forager bees navigate in the complex environment search for profitable food sources. Different groups of foragers exist which preferentially collect nectar, pollen or water. They can fly up to several kilometers to collect pollen or nectar from flowering plants (von Frisch 1965). To navigate through the environment they use landmarks, polarized light patterns and a sun compass (von Frisch 1950; Lindauer 1960; von Frisch et al. 1960; Cartwright and Collett 1983; Kraft et al. 2011).

Flowering plants provide attractive signals such as colors and odorants to attract animals. Different angiosperm species compete for these pollinators, which serve as pollen vectors (Darwin 1876; Kevan 1978; Waser 1983; Chittka and Menzel 1992). When searching for food sources, foragers may first rely on innate search images by expressing inborn preferences to features of flowers such as certain colors, shapes and odors (Koltermann 1973; Lehrer et al. 1995; Giurfa et al. 1995a).

However, learning and memorizing the positions and physical characteristics of a floral patch and the nest are highly important for successful foraging to differentiate between profitable and unprofitable food sources (Menzel and Müller 1996) or to communicate the location and quality of food sources to their nestmates (von Frisch 1965). The capability to establish a predictive relationship between a stimulus (i.e. a specific flower) and a reinforcement (such as pollen or nectar reward provided by the flower) is referred to as associative learning. The importance of associative learning for foragers becomes even more apparent when we contemplate a foraging behavior called flower constancy. Flower constancy describes the phenomenon that an individual temporarily specializes in collecting resources only from one angiosperm species as long as it remains profitable (Waser 1986; Chittka et al. 1999). Thereby, critical premises are adequate sensory perception and processing faculties allowing to discriminate between different flowering plant species (Chittka and Menzel 1992; Chittka and Thomson 2001).

1.2.1 The visual system of honey bees

Honey bees see the world through two apposition compound eyes each consisting of approximately 5000 optically isolated ommatidia (Jander and Jander 2002). Each ommatidium samples a small fraction of the image perceived by the compound eye (Varela and Wiitanen 1970). Due to the structure of the compound eyes, movements are detected with a high temporal resolution (Srinivasan et al. 1999) but the visual acuity is poor compared to animals with single aperture eyes like humans (Land 1997). Honey bees have trichromatic color vision with three spectral photoreceptor types depending on the spectral sensitivity of the photopigments (Autrum and von Zwehl 1964; Menzel and Blakers 1976; Peitsch et al. 1992): The long-range wavelength (L) photoreceptor has a maximal light absorption (λ_{max}) at ~ 530 nm (green), the mid-range wavelength (M) photoreceptor with λ_{max} ~ 460 nm (blue) and the short-range wavelength (S) photoreceptor with λ_{max} ~ 340 nm (ultraviolet). The responses of these photoreceptors are processed by two neural subsystems (Backhaus 1991; Vorobyev and Brandt 1997). One subsystem, the achromatic channel, receives its input from a single photoreceptor. The L photoreceptor perceives the ‘green contrast’, i.e. the difference in the excitations of a stimulus and its background (Srinivasan and Lehrer 1984). Green contrast is involved in motion perception (Kaiser and Liske 1974) and edge detection (Lehrer et al. 1990). This subsystem is achromatic as chromatic information can only be processed by receiving input from two or more photoreceptors. The second subsystem, the chromatic channel, is mediated by color

opponent processing between all three photoreceptors (Backhaus 1991; Vorobyev and Brandt 1997). Color opponent processing means that neurons exist which show excitatory and/or inhibitory interactions between two or more photoreceptor types in a combination-sensitive manner thus providing chromatic contrast (Ribi 1975; Menzel and Blakers 1976; Backhaus 1991; Yang et al. 2004; Paulk et al. 2009). Interestingly, both the chromatic and achromatic channels are used by bees to detect and discriminate colored stimuli (Giurfa et al. 1996b, 1997). When the colored stimuli subtended large visual angles to the bees' eyes (> 15°), only information of the chromatic channel was sufficient to explain the bees' choice behavior. At small visual angles (5°-15°), close to the minimum detection angle of the visual system of 5° (Lehrer and Bischof 1995; Giurfa et al. 1996b) only the green contrast is accessible to detect and discriminate colored stimuli (Giurfa et al. 1996b, 1997).

Besides color, bees can learn a variety of different shapes and patterns (Srinivasan 1994; Giurfa and Lehrer 2001; Hempel de Ibarra et al. 2001, 2002). The visual information from the photoreceptors is processed through three successive neuropils, the lamina, medulla and lobula. The axons of the L photoreceptor terminate in the lamina and synapse with monopolar cells (Menzel 1974). Monopolar cells are involved in the spectral, temporal and spatial coding of visual information received by the photoreceptors for further processing in the brain (de Souza et al. 1992). The axons of the S photoreceptor, M photoreceptor and monopolar cells further project into the medulla, a layered structure organized in a retinotopic fashion (Ribi 1975; Straußfeld 1976; Ribi and Scheel 1981). Most medulla neurons project to the third visual neuropil, the lobula (Ribi 1981; Ribi and Scheel 1981). The lobula has a motion-sensitive (mostly achromatic) region in the outer layers and a color-sensitive region located in the inner layers (Ribi and Scheel 1981; Paulk et al. 2008). The color-sensitive neurons of the lobula project predominantly to the anterior lateral protocerebrum (Ehmer and Gronenberg 2002; Paulk and Gronenberg 2008) including the mushroom bodies (MBs) which are associated with learning and memory (Heisenberg 1998, 2003).

1.2.2 The olfactory system of honey bees

Honey bees also have a sophisticated olfactory system as odorants play a major role in the life of the bees inside and outside the hive (Menzel 1983; Menzel et al. 1993; Chittka and Raine 2006; Paoli and Galizia 2021). Olfactory cues seem to be more readily learned compared to visual cues provided by flowers (Menzel 1985; Menzel et al. 1993). Recent evidence based on

computational models indeed suggests that odors are the most common sensory cue encountered by pollinators during foraging (Sprayberry 2018). Honey bees perceive odorants via approximately 60.000 olfactory receptor neurons (ORNs) located in sensillae on the two antennae (Esslen and Kaissling 1976). Single-sensillum recordings showed that they exhibit responses to a wide range of odorants (Getz and Akers 1993; Akers and Getz 1993). The ORNs convey the broadly tuned olfactory information to the primary olfactory neuropil, the antennal lobe (AL) (Suzuki 1975; Mobbs 1982; Abel et al. 2001). More specifically, the axons of the ORNs extend to 165 glomeruli, the functional units of the AL (Anton and Homberg 1999). Each odorant causes a distinct spatio-temporal pattern of activated glomeruli (Galizia et al. 1997, 1998; Joerges et al. 1997). Within the glomeruli, the ORNs synapse with local interneurons (LNs) which interconnect the glomeruli and outgoing projection neurons (PNs) which project to higher-order brain structures (Gascuel and Masson 1991; Kirschner et al. 2006; Rybak 2012). Within the MB, the Kenyon cells (KCs) form cup-shaped regions called calyces subdivided into the lip, the collar and the basal ring (Mobbs 1982; Strausfeld 2002). The lip region receives olfactory input, the collar receives visual input and the basal ring receives both (Mobbs 1982; Gronenberg 2001; Strausfeld 2002). PNs synapse with KCs in the lip region forming multisynaptic microcircuits called microglomeruli (Ganeshina and Menzel 2001). The axons of the KCs project into the central brain and form the pedunculus as well as the α-lobe and β-lobe (Mobbs 1982; Strausfeld 2002). Gamma-aminobutyric acid (GABA)ergic feedback neurons in the lobes project back into the calyces, providing a negative feedback loop between MB output and input areas (Bicker et al. 1985). The dendrites of mushroom body output neurons (MBONs) branch in the MB lobes and the lateral protocerebrum and receive input from different sensory modalities (Rybak and Menzel 1993). They convey the olfactory information from the MB lobes to other subcompartments of the protocerebrum such as the lateral horn (LH) and relay experience-dependent information about the olfactory input (Rybak and Menzel 1993; Okada et al. 2007; Galizia 2014).

1.3 Associative learning in honey bees

Associative learning can be defined as the process by which animals acquire information about predictive relationships between co-occurring events or stimuli in the environment (Christian 2010). It can be divided into two principal categories: Classical conditioning (Pavlov 1927) and operant conditioning (Skinner 1938). In classical conditioning (see section 1.3.1) an animal learns to associate an originally neutral stimulus (conditioned stimulus, CS) with a stimulus that is biologically relevant to the animal (unconditioned stimulus, US). In operant conditioning (see section 1.3.2) an animal learns to associate its own behavior with a reinforcement. With his experiments in rats, Skinner (1938) could show that a positive reinforcement such as food following the to-be conditioned behavior strengthens the conditioned behavior while a punishment such as an electric shock suppresses it. Both forms of associative learning admit different levels of complexity based on the establishment of unambiguous (elemental learning, see section 1.3.3) or ambiguous links (non-elemental learning, see section 1.3.4) between stimuli.

1.3.1 Classical conditioning protocols

In honey bees, the conditioning of the proboscis extension response (PER), developed by Takeda (1961) is now a well-established and commonly used protocol for classical conditioning (Matsumoto et al. 2012; Giurfa and Sandoz 2012; Scheiner et al. 2013). The PER is an innate feeding behavior of hungry bees. Once the antennae, mouthparts or tarsi are stimulated with a sucrose solution that exceeds the response threshold of a bee, its proboscis extends (Frings 1944; Frings and Frings 1949).

In the classic protocol a neutral odorant which does not elicit an innate response serves as CS and is presented by the experimenter in close temporal association with a sucrose solution (US) that evokes an innate response, the PER (unconditioned response, UR) (Fig. 1A). If this association is learned, the conditioned response is shown (CR, i.e. the PER) when the odorant is presented in absence of the US during a non-reinforced test (Takeda 1961) (Fig. 1B). The procedure of Takeda corresponds to absolute conditioning and was later further developed by Bitterman (1983) to fit a differential conditioning scheme by pairing one CS with a US (CS+) and another CS that is not rewarded (CS-). Bees usually perform exceptionally well in differential olfactory PER conditioning and are able to learn numerous different CS-US associations (Matsumoto et al. 2012; Giurfa and Sandoz 2012 for review). More recently,

despite many initial methodological issues (Kuwabara 1957; Hori et al. 2006, 2007; Niggebrügge et al. 2009), successful PER conditioning has been demonstrated for elemental visual learning (Dobrin and Fahrbach 2012; Mancini et al. 2018).

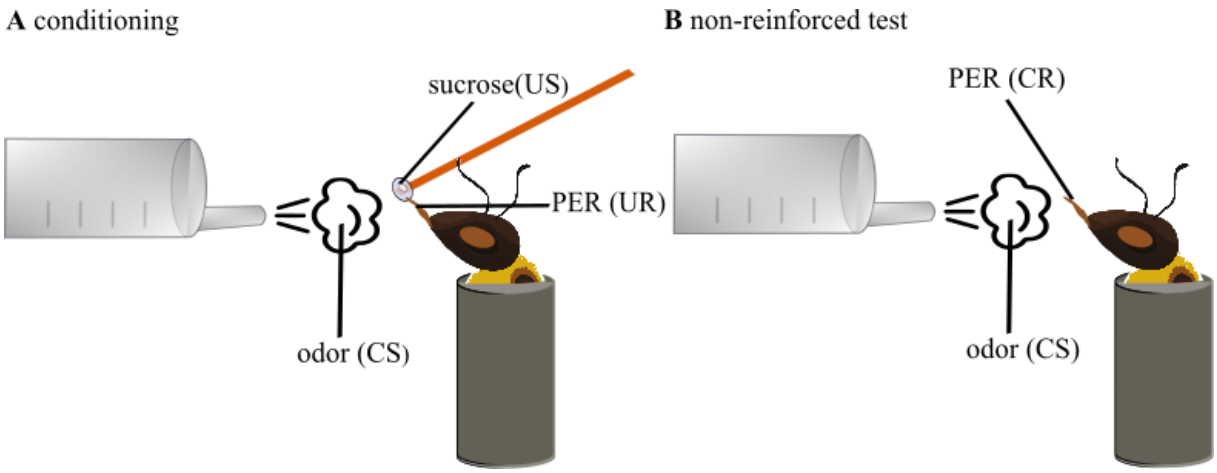

Fig. 1 Schematic overview of the classical PER conditioning protocol for honey bees. A) During conditioning a neutral odor which does not elicit an innate response is used as CS and is presented in close temporal association with a sucrose solution (US) that elicits an innate response, the PER (UR). **B)** In the non-reinforced test the CS is not presented in association with a US. If a bee has learned the association during conditioning, it will show a PER in the absence of reinforcement in the test.

1.3.2 Operant conditioning protocols

In honey bees, the experimental procedure for operant conditioning was developed by von Frisch (1914). In his pioneering experiments von Frisch trained individually marked free-flying honey bees to collect a sugar reward from a blue-colored cardboard via absolute conditioning (Fig. 2A). The basis for such conditioning is the flower constancy behavior of bees as they will usually return reliably to the experimental set-up as long as a positive reinforcement is provided. After several trials the positive reinforcement was removed from the blue cardboard and several novel stimuli were added to the experimental set-up to conduct a non-reinforced test (Fig. 2B). Nevertheless, the bees preferentially landed on the blue cardboard stimulus irrespective of the location. With this technique von Frisch also conducted differential conditioning with free-flying bees. Importantly, associations established in this free-flying context can be classical, operant or a mixture of both. For example, bees could form an operant association between their behavior (landing on a visual stimulus) and the reinforcement or they could form a classical association between the visual stimulus itself and the reinforcement or they could form both associations. However, as the behavior of the bees is crucial in order to receive the

reinforcement, the free-flying context can predominantly be viewed as being operant (Avarguès-Weber et al. 2011a).

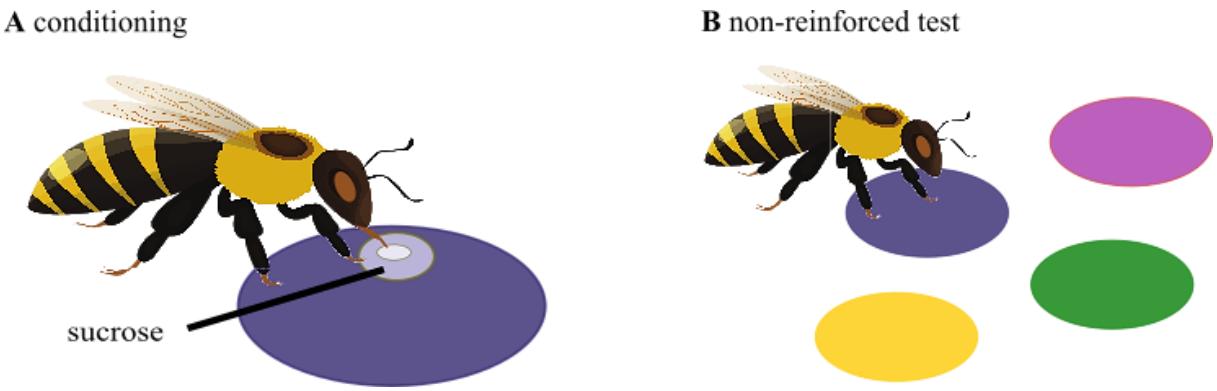

Fig. 2 Schematic overview of an operant conditioning protocol for honey bees. A) During conditioning one visual stimulus (here the blue cardboard) is associated with a sucrose reward. A free-flying bee lands on the cardboard in order to collect the sucrose reward. **B)** In the non-reinforced test no reward is provided and the blue cardboard is presented together with novel stimuli. If a bee has learned the association, it will prefer the blue cardboard over the alternatives presented.

1.3.3 Comparison between classical and operant conditioning protocols

Classical PER conditioning follows strict standardized protocols by exact timing of CS and US presentation, the inter-trial interval, and the duration of a trial in a laboratory environment with constant external conditions (e.g. temperature, humidity, illumination) so that all animals are treated equally (Matsumoto et al. 2012; Giurfa and Sandoz 2012 for review). Several individuals (usually up to 10) are trained in parallel which allows fast data acquisition and consequently high sample sizes. However, the animals are harnessed and 'forced' to participate in the experiment ignoring important aspects of learning such as motivation and these unnatural conditions complicate the interpretation of the results in a natural context. The conditioning of bees in a free-flying context on the other side represents a far more natural scenario for the bees with respect to their foraging behavior. The bees participate in the experiments voluntarily as they are free to leave the experimental set-up and unload their crop load when returning to the colony. Consequently, most of the tested individuals show a high appetitive motivation and attention to the stimuli throughout conditioning. In PER conditioning, however, US strength and consequently the attention to the CS may vary because the bees cannot unload their crop content and thus they become increasingly satiated which may alter their evaluation of the US (Rescorla and Wagner 1972). Furthermore, the harnessing procedure is likely to be associated with increased stress levels (De Brito Sanchez et al. 2015).

Some highly cognitive demanding learning tasks (further explanation in section 1.3.5) such as concept learning among others have so far only been evidenced in free-flying conditions (Giurfa et al. 2001; Avarguès-Weber et al. 2012, 2020; Howard et al. 2018). Additionally, in free-flying experiments researchers obtain a fine-grain measure of an individuals' learning performance as a proportion of correct choices is usually calculated from the number of correct choices made during the several trials of the non-reinforced test following conditioning phases. In PER conditioning, on the other side, the quantification of an individuals' learning performance in the non-reinforced test is limited to the classification as 'learner' i.e. responding to the correct but not the incorrect stimuli, 'generalist' i.e. responding to all stimuli and non-learner by responding to none of the stimuli. Free-flying experiments have nevertheless not to be neglected disadvantages such as variable external (humidity, temperature, illumination etc.) and experimental (duration of the inter-trial interval, duration of conditioning etc.) conditions as well as an immense time expenditure as only one individual can be conditioned at a time and depending on the complexity of the task, conditioning one bee can take up to several hours.

1.3.4 Elemental associative learning

In elemental associative learning a linear and unambiguous link is established between two specific co-occurring events (Rescorla 1972, 1973; Rudy and Sutherland 1992; Giurfa 2007). Unambiguous in this scenario means that the learned association for example between a sugar reward and a pale blue stimulus is only valid for exactly this specific color shade and not an alternative blue shade. Elemental learning can be assessed via absolute and differential conditioning. In absolute conditioning a stimulus is unambiguously associated with a reinforcement (A+) while in differential conditioning one stimulus is associated with a reinforcement (A+) while a second stimulus is not reinforced (B-) (see Fig. 3). Both learning tasks test discrimination ability. However, when the stimuli are perceptually similar bees can only discriminate between them when subjected to differential conditioning as opposed to absolute conditioning (Giurfa 2004). Furthermore, the use of a bitter quinine solution as punishment instead of no reinforcement of B- significantly improves the performances of bees in a differential conditioning task in free-flying conditions (Avarguès-Weber et al. 2010a). Elemental learning in honey bees has been demonstrated extensively both in a classical and an classical-operant context with the use of visual, olfactory and tactile stimuli (von Frisch 1914; Bitterman et al. 1983; Giurfa et al. 1997; Laska et al. 1999; Hempel de Ibarra et al. 2002; Giurfa 2004, 2007; Guerrieri et al. 2005; Scheiner et al. 2005; Scheiner and Amdam 2009;

Niggebrügge et al. 2009; Matsumoto et al. 2012; Giurfa and Sandoz 2012; Mancini et al. 2018). The MBs, which have been frequently been associated with learning and memory proficiency (Menzel et al. 1974; Erber et al. 1980; Menzel 1999, 2001), seem to be dispensable for the success during conditioning in elemental learning tasks as evidenced for elemental olfactory PER conditioning (Malun et al. 2002; Devaud et al. 2007) and elemental tactile learning in an operant context (Scheiner et al. 2001c). However, the formation, consolidation and retrieval of memory especially long-term memory, requires MB function (Menzel et al. 1974; Erber et al. 1980; Komischke et al. 2005).

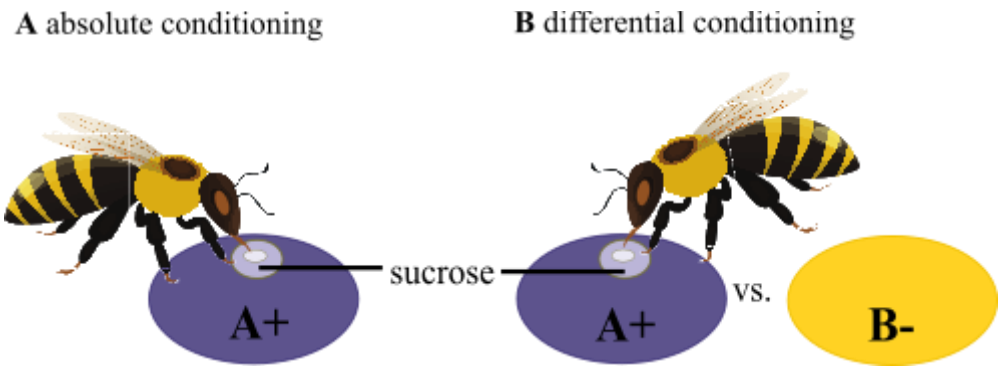

Fig. 3 Schematic overview of A) absolute conditioning and B) differential conditioning. A) In absolute conditioning one stimulus is unambiguously associated with a sucrose reward (A+). **B)** In differential conditioning one stimulus is associated with a sucrose reward (A+) and a second stimulus is not rewarded (B-).

1.3.5 Non-elemental associative learning

Besides the linear and unambiguous associations I explained above, honey bees are capable to solve complex problems where non-linear associations are established due to transient or permanent stimulus ambiguity, referred to as non-elemental learning (Giurfa 2003, 2007). In the following section I will concentrate on the description of non-elemental learning paradigms I used for my experiments: reversal learning, negative patterning, and concept learning.

1.3.5.1 Reversal learning

The reversal learning task (Pavlov 1927) consists of two distinct phases that differ in terms of their cognitive requirements. The first phase of reversal learning (1st RL) is a differential conditioning in which one stimulus (A+) is associated with a reward and a second stimulus is not (B-) (see Fig. 4A). In the second phase (2nd RL) the reward contingencies of the previous

phase (A+B-) are reversed, so that A becomes unrewarding and B becomes rewarding (A-B+) (see Fig. 4B). Strictly speaking, both phases follow a differential conditioning protocol and thus when viewed in isolation, test elemental learning. However, the switch in reward contingencies induces a transient ambiguity of the stimuli A and B, a characteristic of non-elemental learning (Giurfa 2003, 2007). Importantly, reversal learning ability can only be evaluated in animals that have successfully learned the initial A+B- association in the 1st phase (Mota and Giurfa 2010). Two opposing mechanisms are required to learn reversed reward contingencies: new excitatory learning of B+ and new inhibitory learning of A-.

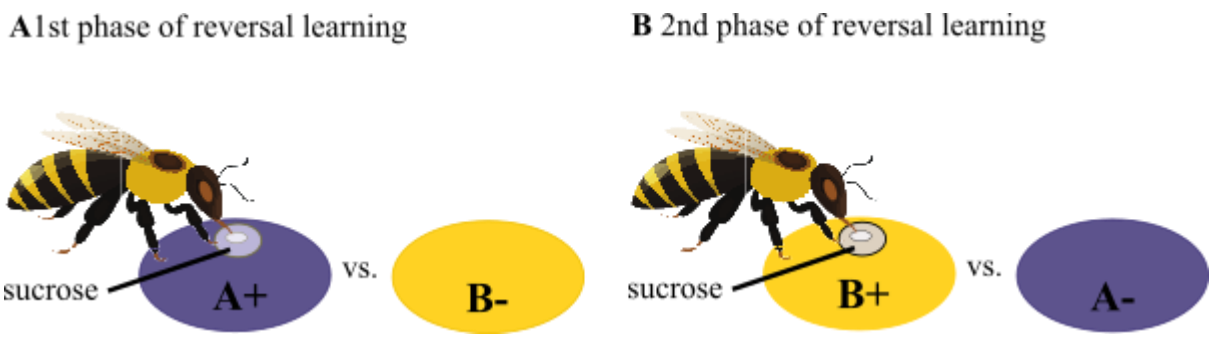

Fig. 4 Schematic overview of the A) 1st and B) 2nd phase of reversal learning. A) In the first phase of reversal learning one stimulus is associated with a sucrose reward (A+) and a second is not rewarded (B+). **B)** In the second phase of reversal learning the previously rewarded stimulus becomes unrewarding (A-) and the previously unrewarded stimulus becomes rewarding.

Reversal learning is frequently used by scientists to assess thee cognitive flexibility of humans and non-human animals (Izquierdo et al. 2017). Furthermore, it offers the great advantage of testing elemental and non-elemental learning within a single task. The capacity to solve reversal learning has been repeatedly demonstrated in honey bees in restrained and free-flying contexts (Chandra et al. 2000; Ferguson et al. 2001; Komischke et al. 2002; Hadar and Menzel 2010; Mota and Giurfa 2010; Dyer et al. 2014; Cabirol et al. 2017). Interestingly, individual honey bees seem to apply different strategies for reversal learning in both free-flying and restrained conditions (harnessed: Mota and Giurfa 2010; free-flying: Dyer et al. 2014). Some individuals were quickly able to switch their responses after reversing of the reward contingencies in the 2nd phase. Other bees learned the initial discrimination but were unable to perform the reversal and a third group of bees that did not learn the initial discrimination in the first phase and thus failed in both phases (Mota and Giurfa 2010; Dyer et al. 2014). Olfactory reversal learning is impaired when MB function is blocked via procaine injection while it did not affect the success in the initial elemental discrimination (Devaud et al. 2007). Thus, it seems

that the MBs are required for non-elemental tasks while being dispensable for elemental learning involving olfactory stimuli. Furthermore, feedback neurons which provide ionotropic GABAergic signaling from the MB lobes to the calyces are required for successful reversal learning (Boitard et al. 2015).

1.3.5.2 Negative patterning

In negative patterning (Whitlow and Wagner 1972) two single stimuli are associated with a reward when presented in isolation (C+ and D+) but are not reinforced when presented together as a compound stimulus (CD-). The subject needs to learn to treat the compound differently than the sum of its components by responding to C and D but not to CD (Fig. 5). For performing a negative patterning task, it is of major importance not to negatively reinforce the compound CD as such an experimental design would confound negative (C+ and D+ vs. CD-) and positive patterning problems (C- and D- vs. CD+) (Deisig et al. 2001). Elemental learning theory cannot account for success in negative patterning. The associative strength of CD would be the linear sum of C and D and thus the subject would respond twice as much to the compound compared to the single elements (Whitlow and Wagner 1972). Two configural theories could account for the processing of compound stimuli allowing to solve this discrimination. The unique-cue theory postulates that a compound is processed as linear sum of its two components including a cue that provides a unique signature to the combination of these components (Rescorla 1972, 1973). Pearce configural theory postulates that a compound is processed by combining the components into a single association without linear summation of their associative strengths (Pearce 1987, 1994).

Fig. 5 Schematic overview of the conditioning procedure of negative patterning. Two single stimuli are rewarded when presented in isolation (C+ and D+), while their mixture is not reinforced (CD-).

Honey bees were repeatedly shown to be capable of solving negative patterning discriminations both in free-flying conditions with visual stimuli (Schubert et al. 2002) and in restrained conditions with olfactory stimuli (Deisig et al. 2001, 2002, 2003). Thereby, odor compound processing seems to support the unique-cue theory in bees (Deisig et al. 2003). In the AL, odor mixture processing follows an elementary strategy as glomerular activity patterns of odor mixtures correspond predominantly to the sum of activated glomeruli patterns evoked by the single odorants (Deisig et al. 2006). However, at the level of PNs, which convey the olfactory information from the AL to higher-order brain centers such as the MBs, the responses are not as linear suggesting that inhibitory processing decreases this linearity at higher-order processing stages (Deisig et al. 2010). Indeed, bees injected with procaine to block the neuronal activity of the MB vertical lobes were unable to solve negative patterning, indicating that inhibitory GABAergic feedback neurons from the MB lobes to the MB calyces are necessary for the success in olfactory negative patterning (Devaud et al. 2015).

1.3.5.3 Concept learning

The theory of concept learning originates from human psychology studies and refers to the ability to discriminate and group objects into distinct categories based on shared features (Keller and Schoenfeld 1950). The formation of concepts enables the transfer of previously learned rules to novel and unknown objects or contexts. Such categories can be based on shared physical properties referred to as perceptual concept learning (Zentall et al. 2002, 2008). A perceptual category is for example “cat”, so even if different cat species such as the tiger, the leopard and the jaguar differ in size and coloration they share similar physical attributes such as a tail, claws, whiskers and fur. Imagine a person which once had a dangerous encounter with a tiger. When this person sees a leopard for the first time, he will most likely avoid this animal due to his previous experience with the tiger. Such a categorical classification promotes learning efficiency by simplifying the complex environment as not every new object or context encountered needs to be learned as specific entity and allows behavioral adaptation to unknown objects and/or situations (Murphy 2002; Zentall et al. 2002, 2008). However, concept learning can also occur independently of shared physical characteristics among objects referred to as relational concept learning (Zentall et al. 2002, 2008). Relational concept learning involves the relationships between objects which must be processed independently of their physical properties (Murphy 2002; Doumas et al. 2008). Typical examples for relational concepts are ‘same/different’, ‘above/below’, or ‘larger/smaller’ (Zentall et al. 2002, 2008). The formation

of relational concepts is thus dependent on abstraction faculties which require high-level cognitive capacities (Yee 2019) not necessarily expected in insects (Avarguès-Weber and Giurfa 2013; Giurfa 2021).

Honey bees can categorize objects based on shared physical features (Giurfa et al. 1996a; Zhang et al. 2004; Benard et al. 2006; Avarguès-Weber et al. 2010b) and were shown to form the relational concepts ‘sameness/difference’ (Giurfa et al. 2001) and ‘above/below’ (Avarguès-Weber et al. 2011b). Intriguingly, bees can even form and discriminate between the two concepts ‘above/below’ and ‘left/right’ simultaneously (Avarguès-Weber et al. 2012) (Fig. 6). So far, the study of concept learning in honey bees has only been performed under free-flying conditions with the use of visual stimuli.

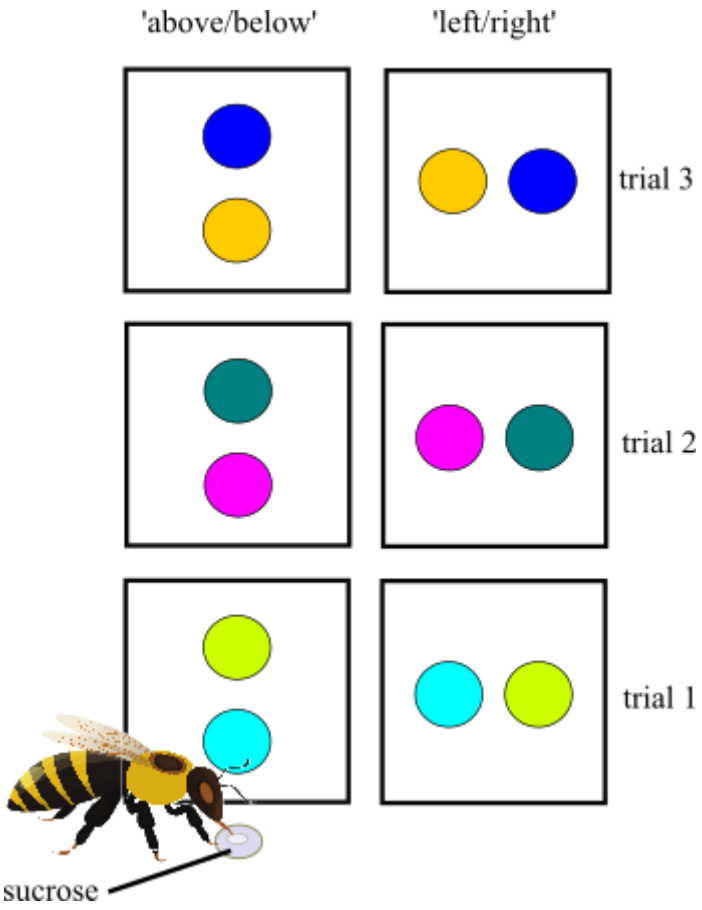

Fig. 6 Schematic overview of the conditioning procedure of a relational concept learning task. In the relational concept learning task described by Avarguès-Weber et al. (2012) the bees had to learn that one relational concept (here ‘above/below’ i.e. two stimuli that are always arranged above and below each other) is associated with a sucrose reward while the second relational concept (here ‘left/right’) was not associated with a sucrose reward.

In summary, honey bees possess extraordinary and sophisticated cognitive abilities which were often not thought to be possible with a small brain containing only around 960.000 neurons. They can be reliably conditioned under free-flying conditions to study their capabilities in an ecologically relevant context. They can also be easily conditioned in the laboratory under restrained conditions which allows to precisely control experimental factors and to couple learning experiments with non-invasive pharmacological and neurobiological techniques to study the neurobiological mechanisms underlying cognitive capabilities.

1.4 Inter-individual variability in the learning proficiency of honey bees

In honey bees, natural variation in behaviors are ubiquitous and exist at multiple levels: between colonies, between castes and individuals within a colony (Jandt et al. 2014; Jeanson and Weidenmüller 2014; Jandt and Gordon 2016; Jeanson 2019). They are highly suited to study consistent individual differences in cognitive proficiency because inter-individual variability in a variety of behavioral and physiological traits are the basis underlying their division of labor (Oster and Wilson 1978; Wilson 1985). The response threshold model is a theoretical framework highlighting the importance of inter-individual variability for effective task allocation (Beshers and Fewell 2001 for review). The main assumption is that individuals within a colony exhibit intrinsic sensory differences (response thresholds) to task-relevant stimuli (Winston and Katz 1982; Wilson 1985; Robinson 1992). An individual will start to engage in a certain task once the intensity of the task-associated stimulus extends the individuals' response threshold to that stimulus (Robinson and Page 1989b; Robinson 1992; Bonabeau et al. 1996). Consequently, individuals with low thresholds will engage in the task at lower intensities of the task-associated stimulus and individuals with higher thresholds require higher stimulus intensities for their engagement. Variability in response thresholds thus creates a task allocation which admits both task specialization and flexibility. Usually only a small proportion of workers within a colony exhibit very low thresholds for a stimulus associated with a certain task. These individuals perform this task at low stimulus intensities, leading to behavioral specialization (Robinson and Page 1989b). For example, some individuals have low thresholds to the alarm pheromone and thus are likely to engage readily in colony defense. However, in case of a high treat such as a robbing event, more alarm pheromone is released resulting in the recruitment of bees with higher thresholds. Then, because a high number of individuals join in colony defense, the treat level should decrease also leading to a decrease in alarm pheromone

intensity. This example nicely illustrates that a negative feedback loop exists in task allocation which allows behavioral flexibility of workers according to the needs of the colony (Page and Mitchell 1998).

Importantly, response thresholds of individuals are plastic and change with age, experience and in response to environmental factors (Pankiw and Page 1999; Pankiw et al. 2001; Scheiner et al. 2003a). A major factor contributing to behavioral variability is genetic diversity due to extreme polyandry of honey bee queens (matings with 17 males on average) (Tarpy et al. 2012) resulting in different patrilines co-existing within a colony. Patrilines differ from each other in how sensitive they are to task-related stimuli (Frumhoff and Baker 1988; Robinson and Page 1988; Pankiw and Page 1999; Kryger et al. 2000; Scheiner et al. 2004; Scheiner and Arnold 2010; Junca et al. 2014)

Inter-individual variability in learning performances have long been recognized in honey bees and have been linked to genetic diversity, the division of labor and associated differences response thresholds, experience and neurobiological parameters (Free 1958; Menzel 1969; Pham-Delègue et al. 1990; Smith et al. 1991; Bhagavan et al. 1994; Ray and Ferneyhough 1997, 1999; Scheiner et al. 1999; Ben-Shahar et al. 2000; Ferguson et al. 2001; Laloi et al. 2001; Laloi and Pham-Delegue 2010). Importantly, these factors are often connected as the division of labor is closely linked to the age, response thresholds and physiological parameters such as their brain structure of individuals (Calderone and Page 1988; Frumhoff and Baker 1988; Calderone and Page 1991; Page and Robinson 1991; Withers et al. 1993; Durst et al. 1994; Fahrbach and Robinson 1995; Page et al. 2006; Reim and Scheiner 2014).

Many studies have examined inter-individual variability in learning proficiency in the light of genetic determinants. For example, the learning phenotype shown by drones predicted the performances of their female worker offspring in appetitive PER conditioning (Benatar et al. 1995). Heritable components accounting for variability in learning performances have also been evidenced for appetitive reversal learning (Ferguson et al. 2001) and aversive conditioning of the sting extension response (SER) (Junca et al. 2014). Bhagavan and colleagues (1994) examined simultaneously the effects of age, caste and genotype on variability in appetitive olfactory learning and found that only the bees' genotype affected their performances. More recently Laloi & Pham-Delegue (2010) contrasted the inter-patriline (between different patrilines within a hive) and intra-patriline (between individuals within a patriline) variability of appetitive learning performances. Intriguingly, a high proportion of the variability in learning performances was explained by differences among workers from the same patriline (88.4 %)

and only a minor proportion of variability was attributed to differences of individuals between different patrilines (11.2 %). These results indicate that other factors besides genetic determinants also contribute substaintially to inter-individual variability in learning proficiency.

Some studies have found a significant relationship between the age of individuals and their learning performances. Pham-Delègue et al. (1990) found that middle-aged bees (12-18 days of age) performed better compared to younger and older bees in an olfactory PER discrimination task. The result that younger bees performed worse in an olfactory PER conditioning task compared to older bees seems to be confirmed by other studies (Ray and Ferneyhough 1997; Laloi et al. 2001). Due to the structure of the age- related division of labor in natural colonies, it is however impossible to examine the effects of age and social role on learning independently. Manipulations of the hive structure can be used to artificially induce foraging behavior in nurse bees (precocious foragers). These precocious foragers are consequently younger than normal foragers. This technique was used by Ray and Ferneyhough (1999) to disentangle the effects of age and caste on learning performances. They found that when the hive structure is not manipulated, forager bees showed higher learning capabilities than nurse and guard bees. However, when nurses turned into precocious foragers their learning performances were comparable to those of normal-aged foragers. Their results indicate that not the chronological age *per se* but rather that the transition from in-hive duties to foraging leads to increased cognitive capabilities (Ray and Ferneyhough 1999). Indeed, this transition is associated with neuro-anatomical and physiological changes thought to facilitate learning in complex environments (Free 1958; Robinson 1987; Withers et al. 1993; Farris et al. 2001; Schulz et al. 2002; Ben-Shahar et al. 2003; Groh et al. 2012; Reim and Scheiner 2014).

A major factor that has a decisive impact on associative learning performances is the individual perception and evaluation of the CS and US (Annau and Kamin 1961; Rescorla and Wagner 1972; Scheiner et al. 2005). Indeed, the observed differences in the associative learning performances between nurse and forager bees could be explained by differences in their response thresholds to sucrose (Scheiner et al. 2017). Bees with a higher sucrose responsiveness give a higher value to a certain sucrose concentration compared to bees with lower sucrose responsiveness (Scheiner et al. 2005). Consequently, bees with higher responsiveness respond to lower concentrations of sucrose solution compared to bees with a lower responsiveness. Many studies have demonstrated positive correlations between sucrose responsiveness and different appetitive elemental learning tasks in restrained bees (Scheiner et al. 1999, 2001a, b,

2004, 2005; Scheiner 2004; Scheiner and Arnold 2010). A similar association was found between aversive learning and responsiveness to the aversive US in restrained bees (Junca et al. 2014). Collectively, these results indicate that differences in learning proficiency of bees with different genotypes, age and foraging specialization could all be ultimately attributed to differences in sucrose responsiveness.

However, inter-individual variability in learning performances were still observed even when bees received equal subjective sucrose rewards (Scheiner et al. 2005). This indicates that the individual perception of the CS also accounts for a proportion of inter-individual variability in learning performances (Scheiner et al. 2005). Interestingly, sucrose responsiveness also correlated positively with responsiveness to odors and visual stimuli used as CS in PER conditioning tasks (Scheiner et al. 2004; Erber et al. 2006; Tsuruda and Page 2009). Sucrose responsiveness is not constant and can vary with season (Scheiner et al. 2003a), feeding states (Scheiner et al. 2003b) and individual experience (Scheiner et al. 1999; Pankiw et al. 2001). Indeed, some evidence suggests that early experience and the foraging experience also lead to neuroanatomical changes in the MB and affect elemental and non-elemental learning performances (Cabirol et al. 2017, 2018).

1.5 Consistent individual differences in the learning abilities of honey bees and other insects

Despite the extensive knowledge detailed above about how and why inter-individual variability in cognitive abilities arise between workers belonging to different castes, much less is known about variability between workers within the same castes, such as nectar foragers for example. It remains however understudied if these differences in cognitive proficiency remain consistent within the same individual. Does an individual that performs exceptionally well in a given cognitive task also have increased capabilities for alternative cognitive tasks? In other words, do individual bees show a distinct cognitive profile which underlies its learning performances over time and across different contexts? These questions are highly relevant to further understand how inter-individual variability in diverse behaviors contribute to the division of labor and the ecological success of social insect colonies.

Some evidence indeed suggests the existence of individually distinct cognitive capabilities in different insect species. The learning performances of individual bumble bees were positively correlated across elemental learning tasks involving stimuli of different sensory

modalities (color, shape and odors) (Muller and Chittka 2012). However, such a positive correlation between olfactory and visual learning was not confirmed by other results showing no association (Smith and Raine 2014). Similar results were found in isogenic fruit flies which showed individual consistency in an aversive olfactory learning task over time and that the performances of individual flies remained consistent across learning tasks using different aversive stimuli (bitter taste or electric shock) (Smith et al. 2022). In honey bees, the individual performances of bees were positively associated between latent inhibition and reversal learning abilities (Chandra et al. 2000). Similarly, bee strains selected for either high or low performances in an olfactory PER conditioning task showed positively correlated performances in a visual learning task under free-flying conditions (Brandes and Menzel 1990). However, some evidence also suggests the existence of individual cognitive specialization in honey bees i.e. increased proficiency in one cognitive trait at the expense of proficiency in an alternative trait. Individual trade-offs in performances have been found between appetitive and aversive learning (Junca et al. 2019) and olfactory and landmark learning (Tait et al. 2019). It remains currently unknown if honey bees also show consistent individuality in their cognitive skill over time, across tasks of different cognitive requirements and in different contexts. These points are however necessary to conclude on the existence of distinct individual cognitive profiles (Griffin et al. 2015).

2 Thesis outline

Throughout the previous sections I have highlighted the impressive cognitive capabilities and the rich behavioral repertoire of honey bees that justify their relevance as model for the study of learning, memory and cognition. Consequently, they offer a unique opportunity to study the existence and factors underlying consistent individual differences in cognitive proficiency of foragers. Importantly, the existence of inter-individual variability in cognitive performances is well-established in honey bees, at least between workers of different castes. Less is known about the causes and consequences of inter-individual variability in the cognitive proficiency of individuals of the same caste such as nectar foragers. I have thus employed a novel view on the learning proficiency of nectar foragers by focusing on the individual level, rather than the average-group performance usually of interest in the field of animal cognition. The first two chapters of this thesis examine the existence of consistent individual differences in cognitive proficiency. I performed a series of learning experiments with bees which aimed to test for consistent individual differences in the cognitive abilities of bees over time, across learning tasks of different complexity and across different contexts (either restrained conditions in the laboratory or free-flying conditions in the field) as it has been suggested by Griffin et al. (2015). The remaining two chapters examined potential factors which could underlie these consistent individual differences.

In the **first chapter** I performed a series of three visual learning experiments with free-flying honey bees to examine if individuals perform consistently in learning tasks 1) over time, 2) involving stimuli from different sensory modalities and 3) of different cognitive complexity. In experiment 1 I subjected the same bees to an elemental discrimination task each day with the use of novel visual stimuli for a total duration of three consecutive days. In experiment 2 I subjected the same bees to two elemental discrimination tasks using either visual or olfactory stimuli respectively. In experiment 3, bees were subsequently tested for their performances in an elemental discrimination task and a non-elemental concept learning task. The results showed that individual bees remained consistent in their learning performances over time. This was an important finding as repeatability of a behavior constitutes the cornerstone of individual behavioral consistency (Griffin et al. 2015). Intriguingly, bees were also consistent across tasks of different complexity. However, individual performances in visual and olfactory learning were not correlated, indicating the existence of individual cognitive specialization.

In the **second chapter** I wished to further examine the relationship between individual performances in elemental and different non-elemental learning tasks. Therefore, individual bees were tested in three learning tasks: an elemental discrimination task and the two non-elemental tasks reversal learning and negative patterning. Furthermore, I wished to investigate whether similar patterns of individually correlated performances can be observed in different experimental contexts. I thus performed a series of four experiments where I varied the sensory modality of the stimuli used (colors or odors) and the experimental condition (free-flying in the field or restrained in the laboratory). Intriguingly, the patterns of correlated performances were the same across all four experimental conditions, which lead me to conclude that consistent individual differences in learning proficiency may represent a distinct characteristic of honey bees' cognitive profile.

In the **third chapter** I examined whether the pattern of individually correlated performances evidenced in chapter 2 can be attributed to the genetic diversity that naturally exists within bee colonies. Therefore, I contrasted the learning performances of bees originating either from a polyandrous or a monandrous colony. Additionally, I also assessed whether the age of the individuals had an effect on their learning performances. I demonstrate that a genetic component underlies individual cognitive proficiency while the age of individuals could not predict bees' performances.

In the **fourth chapter** I assessed whether the individual evaluation of the CS and US stimuli i.e. the responsiveness to sucrose and to a colored light can account for consistent differences in learning proficiency under free-flying conditions. Due to the tremendous evidence from restrained bees, we hypothesized that bees with a higher responsiveness to task relevant stimuli would perform better in learning tasks compared to bees with a lower responsiveness. We further hypothesized that the effect of sensory responsiveness on learning performances would decrease with increasing complexity of the task. Contrary to my hypothesis, visual and sucrose responsiveness could not predict neither elemental nor non-elemental learning performances in free-flying conditions.

Finally, in the **general discussion** I will highlight the suitability of honey bees as model for the study of individuality in cognitive proficiency. I will further discuss the results of this thesis with respect to the long-standing question of the modularity of cognition: I will also tackle the question of how individuality in cognition contributes to the division of labor and ecological success of honey bee colonies.

3 Chapter 1: Evidence of cognitive specialization in an insect: proficiency is maintained across elemental and higher-order visual learning but not between sensory modalities in honey bees

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RESEARCH ARTICLE

Evidence of cognitive specialization in an insect: proficiency is maintained across elemental and higher-order visual learning but not between sensory modalities in honey bees

Valerie Finke[1,2], David Baracchi[1,3], Martin Giurfa[1,4], Ricarda Scheiner[2] and Aurore Avarguès-Weber[1,*]

ABSTRACT

Individuals differing in their cognitive abilities and foraging strategies may confer a valuable benefit to their social groups as variability may help them to respond flexibly in scenarios with different resource availability. Individual learning proficiency may either be absolute or vary with the complexity or the nature of the problem considered. Determining whether learning ability correlates between tasks of different complexity or between sensory modalities is of high interest for research on brain modularity and task-dependent specialization of neural circuits. The honeybee *Apis mellifera* constitutes an attractive model to address this question because of its capacity to successfully learn a large range of tasks in various sensory domains. Here, we studied whether the performance of individual bees in a simple visual discrimination task (a discrimination between two visual shapes) is stable over time and correlates with their capacity to solve either a higher-order visual task (a conceptual discrimination based on spatial relationships between objects) or an elemental olfactory task (a discrimination between two odorants). We found that individual learning proficiency within a given task was maintained over time and that some individuals performed consistently better than others within the visual modality, thus showing consistent aptitude across visual tasks of different complexity. By contrast, performance in the elemental visual-learning task did not predict performance in the equivalent elemental olfactory task. Overall, our results suggest the existence of cognitive specialization within the hive, which may contribute to ecological social success.

KEY WORDS: *Apis mellifera*, Cognitive consistency, Honey bee, Insect cognition, Inter-individual variability, Visual cognition

INTRODUCTION

Cognitive skills are often attributed to a species based on the ability of a few representative members to pass or fail key cognitive tests. Yet, depicting cognition at a species level ignores the inter-individual variability that may reveal fundamental properties in terms of behavioural plasticity and, in the case of eusocial animals, specialization within a species. Indeed, inter-individual differences may involve variation in different domains such as motivation to complete the task, choice strategy, personality, or any combination of these factors. However, variable performance between individuals may also be due to intrinsic differences in cognitive abilities, which may occur in multiple dimensions, from differences in gene expression to variability in neural population responses and hormonal levels (Akhund-Zade et al., 2019; Honegger et al., 2020). Shifting the focus from the species to the individual level in the study of cognitive abilities provides a remarkable opportunity to reveal key underpinning mechanisms. Moreover, it also offers novel perspectives to understand the link between fitness and cognition (Raine and Chittka, 2008; Thornton and Lukas, 2012; Thornton et al., 2014; Evans et al., 2017; Boogert et al., 2018).

Social insects have attracted wide attention as a result of their remarkable cognitive feats (Giurfa, 2007; Srinivasan, 2010; Avarguès-Weber and Giurfa, 2013; Chittka, 2017). The co-existence of individuals with variable cognitive abilities within a social group may be favoured because of the significant energetic cost of investing in important learning faculties or problem-solving abilities (Mery and Kawecki, 2003; Burns, 2005; Burns and Rodd, 2008; Kawecki, 2010; Burns et al., 2011; Jaumann et al., 2013; Kotrschal et al., 2013). Such a co-existence has been documented, for example, in bumblebees colonies where some individuals consistently make fast but inaccurate foraging decisions while others decide more slowly yet with higher accuracy (Chittka et al., 2003). Colony success indeed benefits from the co-existence of costly but highly skilled foragers and cheaper but less accurate animals, as this heterogeneity may improve exploitation of different food sources and information distribution within the colony (Burns, 2005; Burns and Dyer, 2008; Muller and Chittka, 2008; Chittka et al., 2009). For example, the distinction between scout foragers searching novel resources and recruited bees relying on social information to massively exploit a unique resource as long as it remains profitable might be based on different cognitive abilities (Cook et al., 2019).

Bees are particularly appealing to study inter-individual cognitive variability as forager bees demonstrate diverse learning abilities ranging from elemental associative tasks to higher-order forms of learning such as categorization, numerical tasks or concept formation, among others (Srinivasan, 2010; Avarguès-Weber et al., 2011; Avarguès-Weber and Giurfa, 2013; Giurfa, 2013, 2019). Inter-individual variability has been generally neglected in standard tests of bee learning, which rely on mean group performance. Exceptions to this trend are the identification of individual variability in sucrose responsiveness as an important factor determining individual learning performance in honeybees (Scheiner et al., 1999; 2001, 2005) and the analysis of variability in olfactory learning performance on an individual basis (Pamir et al.,

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3.1 Abstract

Individuals differing in their cognitive abilities and foraging strategies may confer a valuable benefit to their social groups as variability may help them to respond flexibly in scenarios with different resource availability. Individual learning proficiency may either be absolute or vary with the complexity or the nature of the problem considered. Determining whether learning ability correlates between tasks of different complexity or between sensory modalities is of high interest for research on brain modularity and task-dependent specialization of neural circuits. The honeybee Apis mellifera constitutes an attractive model to address this question because of its capacity to successfully learn a large range of tasks in various sensory domains. Here, we studied whether the performance of individual bees in a simple visual discrimination task (a discrimination between two visual shapes) is stable over time and correlates with their capacity to solve either a higher-order visual task (a conceptual discrimination based on spatial relationships between objects) or an elemental olfactory task (a discrimination between two odorants). We found that individual learning proficiency within a given task was maintained over time and that some individuals performed consistently better than others within the visual modality, thus showing consistent aptitude across visual tasks of different complexity. By contrast, performance in the elemental visual-learning task did not predict performance in the equivalent elemental olfactory task. Overall, our results suggest the existence of cognitive specialization within the hive, which may contribute to ecological social success.

3.2 Introduction

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Pioneer studies in bumblebees investigated whether such individual differences are consistent between cognitive tasks. Thus, relative learning performance was compared between

visual and olfactory tasks, with contradictory results between studies concerning the existence of a correlation (Muller and Chittka 2012; Smith and Raine 2014). Bumblebees' ability to solve an elemental discrimination task (A+ versus B−) seems to be correlated with the faculty to then learn reversed reward contingencies in a non- elemental reversal learning phase (A− versus B+) (Raine and Chittka 2012). In contrast, recent studies on honeybees showed no correlation between individual relative performance in odour discrimination tasks when the reinforcement was appetitive or aversive (Junca et al. 2019), or between landmark and olfactory learning (Tait et al. 2019), which suggests some level of cognitive specialization.

Here, we analyzed individual learning performance in a simple visual discrimination task in which bees had to discriminate a rewarded from a non-rewarded visual target. We determined whether learning proficiency was stable over time (3 consecutive days) despite inter-individual differences. After confirming that inter-individual differences were consistent and unaffected by the kind of visual stimulus used in this task, we determined whether performance across visual tasks of different complexity, or across sensory modalities (visual versus olfactory), was correlated. To this end, we trained bees in two consecutive tasks: (i) the same elemental visual discrimination task described above and either (ii) a conceptual visual discrimination based on learning the constant spatial relationships between variable patterns ('choosing the picture presenting an object above/below the other, or to the left/right of the other, independently of the physical properties the objects present') (Avarguès-Weber et al. 2012) or (ii) a simple olfactory discrimination (discrimination between a rewarded and a non-rewarded odorant). While the simple visual and olfactory tasks represent basic forms of learning in which two stimuli have unambiguous outcomes (A+ versus B−), the conceptual task requires transfer to novel unknown stimuli preserving the appropriate spatial relationship, and therefore represents a higher-order learning form (Giurfa 2003; Avarguès-Weber and Giurfa 2013).

3.3 Material and methods

3.3.1 Ethical statement

Our protocols comply with standard welfare practice in our field. The bees were not manipulated and were free to visit our apparatus. The experiment involved bees from an apiary dedicated to research.

3.3.2 General procedure

Free-flying honeybees (*Apis mellifera Linnaeus* 1758) originating from a single hive and trained to forage for nectar on a sucrose gravity feeder were used in all our experiments. Only bees with intact wings were used, as wing damage could account for reduced foraging performance (Higginson and Barnard 2004; Higginson et al. 2011). The age of the bees was not controlled. Bees were recruited from the feeder to the setup by offering them a drop of sucrose solution with a concentration (1.8 mol l^{-1}) higher than that of the feeder (variable depending on foraging motivation). While drinking, the bees were gently placed at the entrance of our Y-maze setup. Once satiated, the bees flew back to their hive. Returning bees on their subsequent foraging trip were colour marked and individually trained in a stepwise fashion to enter the Y-maze to collect a sucrose reward (1.8 mol l^{-1}) placed on the back walls of the maze. This pre-training usually took 1–2 h per bee. The maze consisted of a pre-chamber, a decision chamber and the two arms in which the stimuli to be learned were presented. The pre-chamber was equipped with a sliding door, which allowed the traffic of foraging bees to be controlled. Only one marked bee was allowed at a time in the Y-maze. After entering the pre-chamber, the bee could fly into the decision chamber through a hole (5 cm diameter), leading to the two arms (40×20×20 cm, L×H×W) of the apparatus. The stimuli were presented on a UV-reflecting white background covering the back walls (20×20 cm) of the arms. The back walls were placed 15 cm from the centre of the decision chamber.

During the learning tasks, bees always had to discriminate a stimulus rewarded with 1.8 mol l^{-1} sucrose solution from an alternative stimulus punished with 60 mmol l^{-1} quinine solution (Avarguès-Weber et al. 2010a). A choice was scored when the bee approached one stimulus (<5 cm). If the bee chose the rewarded stimulus, it was allowed to drink the sucrose solution ad libitum until it returned to the hive to deliver the sucrose. An incorrect choice led to the tasting of quinine, which was followed by the possibility of flying to the alternative arm to collect sucrose on the rewarded stimulus. Only the first choice within a foraging bout was recorded. The sides of the rewarded and punished stimuli were exchanged in a pseudo-random sequence (i.e. a stimulus was presented no more than twice consecutively on the same side) throughout all experiments to prevent positional learning. After the last training trial, non-reinforced tests were performed using fresh stimuli. The tests were repeated twice to swap stimulus side and they were spaced by three refreshing reinforced trials in order to maintain appetitive motivation. Each test lasted 45 s, during which the contacts the bees made with the

surface of the stimuli were recorded. This period is typically used in such tests as it allows uncovering of the learning induced by the training; longer periods may result in a switch of choice strategy owing to the extinction conditions. The percentage of correct choices for a given test was calculated for each bee using the number of contacts with the stimuli in both repetitions of the test.

3.3.3 Experiment 1: performance over three consecutive visual elemental discriminations

In this experiment, 18 bees were individually trained over 3 days to learn three consecutive visual discriminations, one per day. A minimum of two, but more often at least three, data points are typically used to show consistency in individual traits (Stamps and Groothuis 2010). We chose to replicate the visual task over 3 consecutive days to determine the stability of individual performance. We did not extend the measurement period beyond 3 days to avoid losing bees as a result of natural death or recruitment to alternative food places. On each day, the bee experienced a 15- trial conditioning (i.e. 15 consecutive visits to the maze) during which it had to learn to discriminate two visual achromatic patterns, one (CS+) being consistently associated with reward (sucrose) and the other (CS−) with punishment (quinine). Training was followed by a test in which the stimuli used for training were presented without reinforcement (Fig. 1A). Training and testing took 1−2h per bee. In the next 2 days, this procedure was repeated using a new set of visual stimuli every day. All the bees that completed the experimental schedule returned voluntarily to the setup every day. They were not maintained captive overnight in the laboratory. The bees returned reliably to the experimental set-up throughout the 3 day period and only one bee trained on day 1 did not come back the next day to complete the training sequence. The stimuli used were 7×7 cm black patterns printed on UV- reflecting white paper. Six different patterns were used, which varied between bees and were presented as counterbalanced pair combinations on each experimental day (Fig. S1). These patterns were originally used in the study of Avarguès-Weber et al. (2012) and could be well resolved by the visual system of honeybees.

3.3.4 Experiment 2: performance in elemental visual and olfactory discriminations

In this experiment, a novel set of bees (n=18) were trained consecutively within a day to solve a visual discrimination task and an olfactory discrimination task. The sequence of visual and olfactory tasks was randomized between bees. The tasks were spaced by approximately 30 min during which the bees could collect sucrose solution outside of the Y-maze. This delay allowed preparation of the setup and stimuli for the next learning task. Both training phases consisted of 15 trials in which the bees had to discriminate between a rewarded stimulus (CS+) associated with sucrose solution and a second stimulus (CS−) associated with a quinine solution (Fig. 2A). Both training phases were followed by a test in which the respective stimuli were presented without reinforcement. The stimuli used in the visual task were the same as those described for experiment 1. The pair combinations were counterbalanced across bees. For the olfactory task, 10 µl of pure odorant (2-octanol and limonene, Sigma-Aldrich) were applied to 7×7 cm squares of filter paper. For each trial, fresh stimuli were used to ensure that the odours could be well perceived throughout training. Between trials, when the bees were absent from the set-up, the Y-maze was ventilated, and the arms of the maze were cleaned with 30% ethanol to remove potential odour residues. In both tasks, reinforcement contingencies were counterbalanced between bees. The whole procedure took between 2 and 3.5 h per bee.

3.3.5 Experiment 3: performance in visual discriminations of different cognitive complexity

An additional group of bees (N=18) were trained within the same day in two successive visual tasks, one elemental discrimination similar to the ones described in experiment 1 and one non-elemental conceptual discrimination based on spatial relational learning (Avarguès-Weber et al. 2012). The sequence of the elemental and non-elemental tasks was randomized between bees. The tasks were spaced by approximately 30 min during which the bees had access to a sucrose solution outside of the training apparatus. The tasks differed in the number of conditioning trials (15 for the elemental task and 30 for the non-elemental task) because of their different complexity. Training lengths were decided to ensure significant learning of the majority of bees. The procedure and stimuli of the elemental task were identical to those used in experiment 1 or in the visual task of experiment 2. In the non-elemental task, the bees were trained to discriminate between two composite images, each consisting of two coloured discs (7 cm diameter), but arranged in two different spatial configurations: above/below (discs

aligned vertically, i.e. one above/below the other) and left/right (discs aligned horizontally, i.e. one to the left/right of the other). The discs differed only in their chromatic properties. They were cut from uncoated HKS paper (K+E Stuttgart, Stuttgart-Feuerbach, Germany; 1N, 3N, 29N, 32N, 48N, 71N; Fig. S1). The reinforcement contingency (above/below+ or left/right+) was counterbalanced between bees. The colour of the discs and their position on the back walls of the Y-maze were pseudo-randomized over trials but keeping their alignment (Fig. 3A). By doing this, we ruled out that bees could use either the absolute spatial locations or the centre of gravity as cue to solve the task (Avarguès-Weber et al. 2012). The spatial relationship between the discs was consequently the only reliable predictor of the reward. The conditioning phase was followed by non-reinforced tests, in which novel achromatic (black) geometric shapes were used to recreate the trained spatial relationships. This allowed us to examine whether bees learned the spatial concept, irrespective of the stimuli properties. We took special care to choose shapes differing as much as possible from the patterns used in the elemental learning task (Fig. 3A). Different shapes were used to this end (Fig. S1). Stimuli were printed on UV-reflecting copy paper and had a size of 7×7 cm. Training and testing took 3–4.5 h per bee.

3.4 Statistical Analysis

Individual bee responses (correct or incorrect) during the acquisition phases were examined using generalized linear mixed models (GLMM) with a binomial error structure and logit-link function, glmer function of R package lme4 (Bates et al. 2015). In the models, the bee's choices (0 or 1) were entered as the dependent variable, while the trial number, the task [Day number (experiment 1), Visual/Olfactory (experiment 2) or Elemental/Non-Elemental (experiment 3)], the stimuli used and the order of the tasks were entered as fixed factors. Subject identity (ID) was entered as a random factor to account for the repeated-measure design. Several models were run by testing interactions between factors and by dropping each factor subsequently to select the significant model with the highest explanatory power (i.e. the lowest AIC value) (see Tables S1–S3).

Performance during the non-reinforced tests was analysed with a GLMM with a binomial error structure and logit-link function, where the proportion of correct choices for each bee was entered as a dependent variable, and the task and the task order were entered as fixed factors when appropriate. The intercept term informed us whether the mean proportion of correct

choices is different from chance level. Correlations were computed using both the Pearson and Spearman correlation coefficients. All statistical analyses were performed with R 3.4.2 (http://www.R-project.org/).

3.5 Results

3.5.1 Experiment 1: performance over three consecutive visual elemental discriminations

Honeybees successfully learned the three elemental visual discriminations between the achromatic visual patterns (Fig. 1B) as they significantly improved their performance during the acquisition phases (GLMM, n=17, Trial: χ^2=5.6, P=0.02; Fig. 1C; Table S1). There was neither a significant effect of the pair of stimuli used (Stimuli: χ^2=15.4, P=0.97; Table S1) nor a significant effect of the training sequence as performance did not improve over the three consecutive visual discriminations (Day: χ^2=0.01, P=0.92; Table S1). Accordingly, performance in the non-reinforced tests (Fig. 1D) was significantly higher than chance on all 3 days with no significant influence of task repetition [n=17; day 1: 76.1±3.3% (mean±s.e.m.), Z=6.17, P<0.001 (GLMM); day 2: 76.0±2.3%, Z=8.79, P<0.001; day 3: 72.0±2.7%, Z=7.85, P<0.001; Day: Z=1.34, P=0.17 (GLMM)].

Individual learning performance (proportion of correct choices) was highly variable between individuals (Fig. 1E), yet it was consistent over the 3 days (day 1 versus day 2: Spearman correlation, r_s=0.62, P=0.009, Pearson correlation, r_p=0.63, P=0.007; day 2 versus day 3: r_s=0.66, P=0.004; r_p=0.78, P<0.001; day 1 versus day 3: r_s=0.70, P=0.002; r_p=0.66, P=0.004; Fig. 1E). This result indicates that despite population variability in learning proficiency, individual proficiency remained stable across days and visual discrimination tasks.

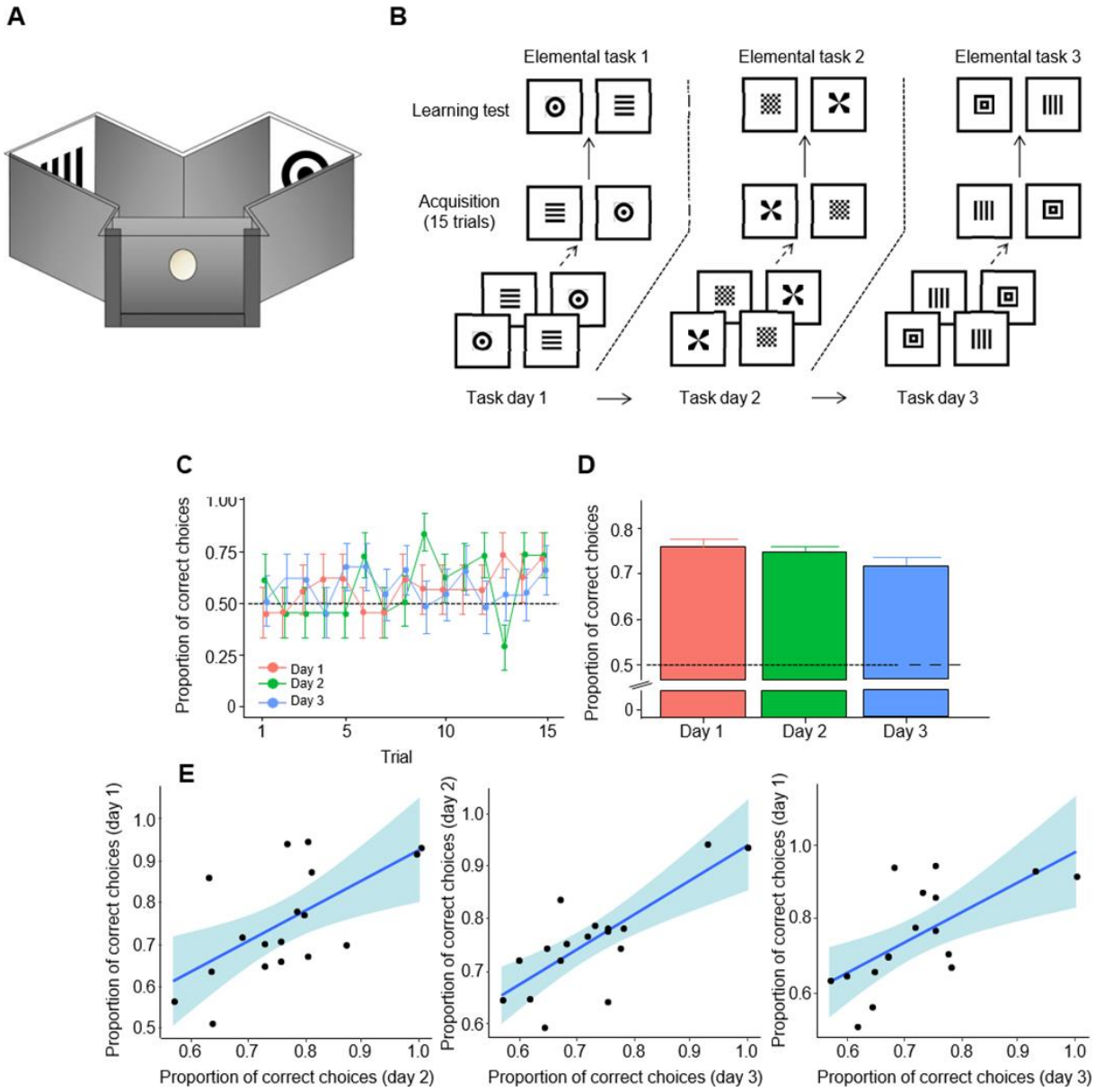

Fig. 1 Experiment 1: comparison of performance in three visual elemental discriminations. (A) Diagram of the Y-maze apparatus used to train bees in this study. **(B)** Schematic representation of one visual elemental learning task that bees were subjected to. The visual pattern that bees had to discriminate varied between bees and between days. **(C)** Acquisition curves expressed as the proportion of correct choices (±s.e.m.) of forager bees confronted with three consecutive elemental visual tasks consisting of 15 trials over 3 days. Bees improved their performance over the course of training (GLMM, n=17, Trial: χ^2=5.6, P=0.02). **(D)** Choice accuracy expressed as the proportion of correct choices (±s.e.m.) of forager bees in the non-reinforced learning tests following each training session. Performance was significantly higher than chance level (GLMM, n=17; day 1: Z=6.17, P<0.001; day 2: Z=8.79, P<0.001; day 3: Z=7.85, P<0.001). **(E)** Correlation between performance (proportion of correct choices) of individual bees in the non-reinforced learning tests of the repetition of elemental visual tasks involving different stimuli. Each dot shows data for one individual bee. The blue line represents the regression line; blue shading indicates the 95% confidence interval. Performance was correlated over the 3 days (Spearman correlation, day 1 versus day 2: r_s=0.62, P=0.009, day 2 versus day 3: r_s=0.66, P=0.004; day 1 versus day 3: r_s=0.70, P=0.002).

3.5.2 Experiment 2: performance in elemental visual and olfactory discriminations

Although no significant improvement of performance could be detected over trials (GLMM, Trial: χ^2=1.7, P=0.19; Fig. 2B; Table S2), the bees (n=18) learned both tasks as shown by their correct stimulus, be it visual (74.9±3.2% of correct choices, GLMM, Z=6.54, P<0.001; Fig. 2C) or olfactory (84.0±2.6%,Z=8.24, P<0.001; Fig. 2C). Bees were generally more accurate in the olfactory task than in the visual task (GLMM, Task: Z=3.58, P<0.001; Fig. 2C). As in the previous experiment, a high variability in learning proficiency was observed between the trained individuals (Fig. 2D). However, this time no correlation between individual performances was found (Olfactory versus Visual: r_s=0.31, P=0.21; r_p=0.26, P=0.29; Fig. 2D), thus showing that individual proficiency is not stable between tasks involving different sensory modalities.

3.5.3 Experiment 3: performance in visual discriminations of different cognitive complexity

Honeybees improved their performance during the acquisition phase in both the elemental and the conceptual task (GLMM, n=18, Trial: χ^2=9.0, P=0.003; Fig. 3B; Table S3). The task sequence did not affect the bees' performance (Order, χ^2=0.05, P=0.82; Fig. S2, Table S3). Overall, the bees' accuracy was higher in the elemental task than in the non-elemental task (Task, χ^2=15.0, P<0.001, Fig. 3C; Table S3), a result that is consistent with the different levels of complexity of these tasks. Performance in the non-reinforced tests was significantly higher than chance in both tasks (Elemental task: 75.4±2.7% of correct choices, GLMM, Z=7.18, P<0.001; non-Elemental task: 68.0±2.9% of correct choices, Z=4.73, P<0.001; Fig. 3C). Yet, it was also affected by the complexity of the task (GLMM, Task: Z=2.36, P=0.02; Fig. 3C) as test performance was better after the elemental conditioning than after the non-elemental conditioning. Individual learning proficiency was variable but it correlated between individuals across the two learning tasks (r_s=0.64, P=0.004; r_p=0.69, P=0.002; Fig. 3D), with some individuals being consistently more error-prone than others in both tests.

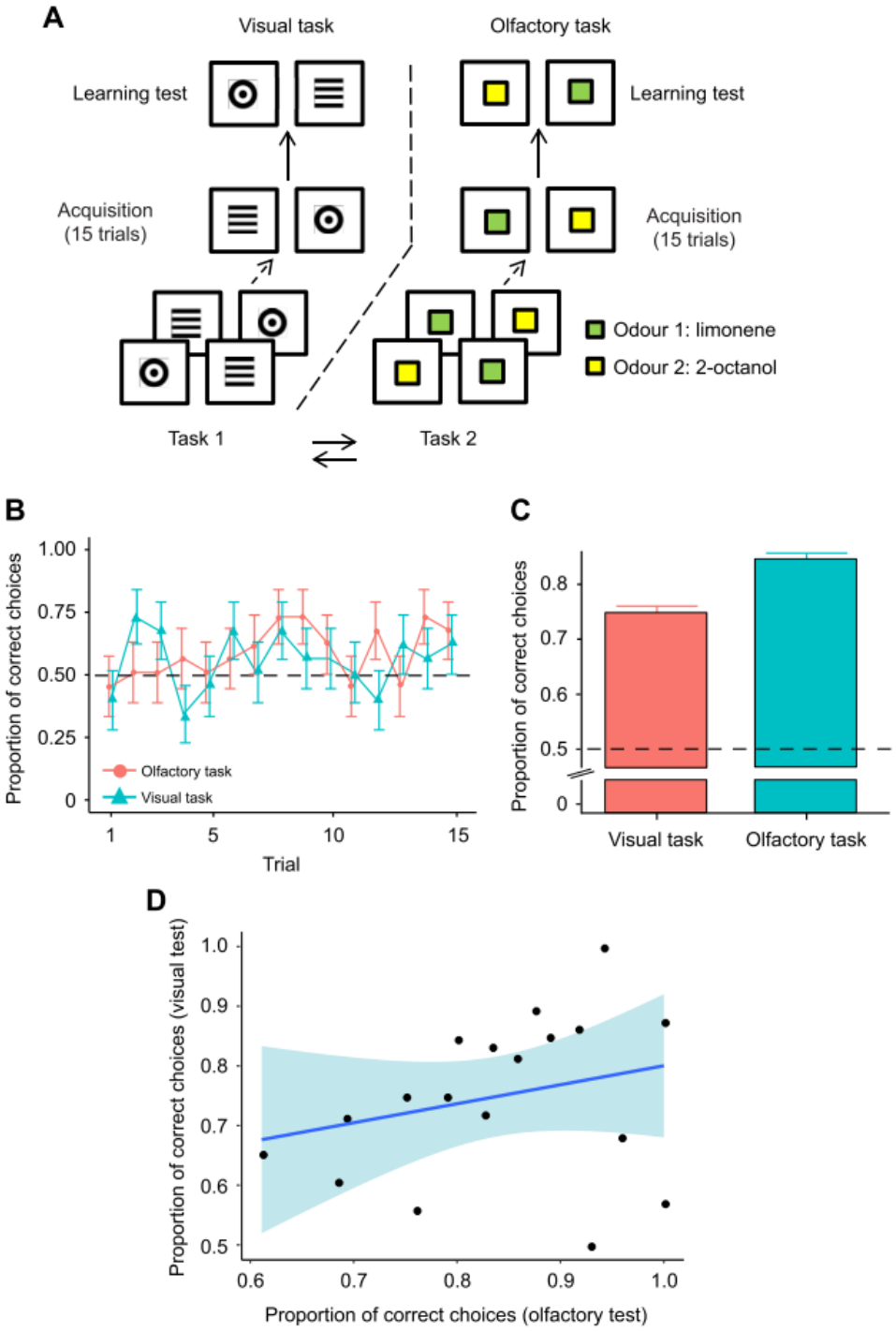

Fig. 2 Experiment 2: comparison of performance in a visual and an olfactory elemental discrimination. (A) Schematic representation of the tasks that bees were subjected to. **(B)** Acquisition curves expressed as the proportion of correct choices (±s.e.m.) of forager bees (n=18) confronted with a visual task and an olfactory task consisting of 15 trials. No significant improvement of performance could be detected over trials (GLMM, Trial: χ^2=1.7, P=0.19). **(C)** Choice accuracy expressed as the proportion of correct choices (±s.e.m.) of forager bees confronted with the visual and the olfactory tasks in the non-reinforced learning test. Bee performance was above chance level for both tasks (GLMM, Vision: Z=6.54, P<0.001; Olfaction: Z=8.24, P<0.001). **(D)** Correlation between the performance (proportion of correct choices) of individual bees in the non-reinforced learning tests. Each dot shows data for one individual bee. The blue line represents the regression line; blue shading indicates the 95% confidence interval. No correlation between individual performances was observed (Spearman correlation, r_s=0.31, P=0.21)

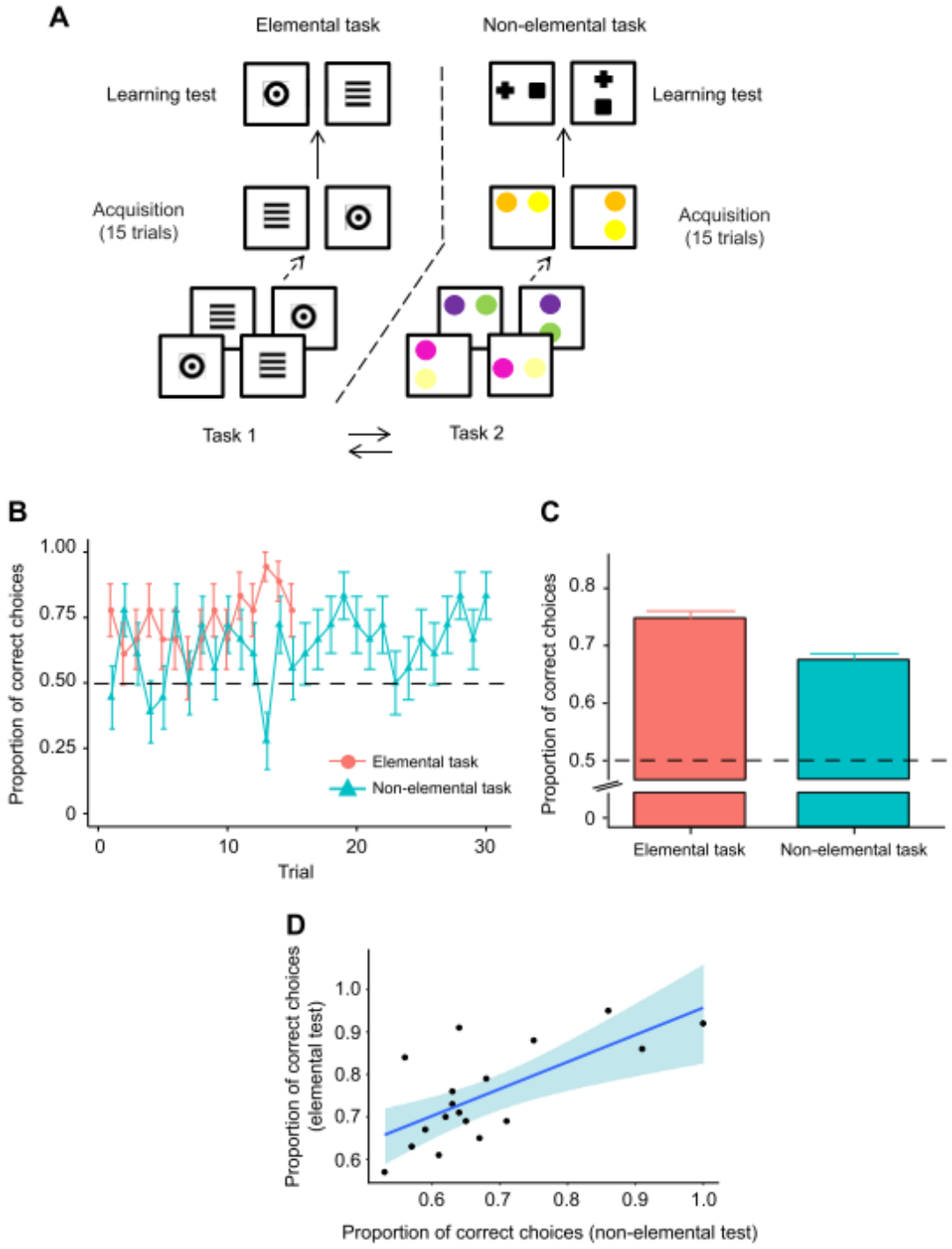

Fig. 3 Experiment 3: comparison of performance in an elemental and a non-elemental visual task. (A) Schematic representation of the visual learning tasks that bees were subjected to. **(B)** Acquisition curves expressed as the proportion of correct choices (±s.e.m.) of forager bees (n=18) confronted with an elemental task consisting of 15 trials and a non- elemental task consisting of 30 trials. There was a significant improvement in performance over trial repetition (GLMM, n=18, Trial: χ^2=9.0, P=0.003). **(C)** Choice accuracy expressed as the proportion of correct choices (±s.e.m.) of forager bees confronted with the elemental and the non-elemental tasks in the non-reinforced learning test. Bee performance was significantly above chance level in both tasks (GLMM, n=18, Elemental: Z=7.18, P<0.001; Non- Elemental: Z=4.73, P<0.001). **(D)** Correlation between the performance (proportion of correct choices) of individual bees in the elemental and non- elemental non-reinforced learning tests (Spearman correlation, r_s=0.64, P=0.004). Each dot shows data for one individual bee. The blue line represents the regression line; blue shading indicates the 95% confidence interval.

3.6 Discussion

Our results highlight the importance of individual variability in cognitive tasks and its relationship with the nature of the task considered. By testing the same bees on consecutive days with tasks that were either similar (experiment 1) or different (experiments 2 and 3), we observed an important inter-individual variability in learning performance as in all cases the proportion of correct choices varied at the population level, with some bees being efficient learners and others, in contrast, poor learners. Importantly, this proficiency was maintained across time when individuals were tested on three similar consecutive visual discriminations (experiment 1). Thus, the variable response observed within a given task seems to be a consequence of individual stable factors rather than being noise resulting from transitory variability in foraging activity, appetitive motivation or stochasticity in choice persistence. In addition, we showed that proficiency is maintained across elemental and higher-order learning tasks within the same sensory modality (experiment 3), even if performance was again highly variable at the population level. This result is important as it shows that bees trained within the visual modality will conserve their success irrespective of the cognitive complexity of the task, a problem that has never previously been addressed in invertebrates. Finally, we showed that consistency in performance was not maintained when bees were trained using tasks involving different modalities (vision and olfaction; experiment 2), thus arguing in favour of within-modality cognitive specialization.

Several factors can account for the inter-individual variability observed. But some of them can be ruled out in our study. In our experiments, only nectar foragers captured at a sucrose feeder and consequently motivated for foraging were used, which discards differences due to division of labour and appetitive motivation. In addition, the temporal sequence did not influence the performance of the bees. We expected that familiarization with the setup and enhanced attention might be promoted by prior training experience, resulting in faster acquisition in subsequent tasks. However, such an improvement was not observed in our conditions.

Consistent inter-individual differences in performance maintained within a visual task or across elemental and higher- order visual tasks could have a genetic basis. *Drosophila* from a population selected over several generations on the basis of their good learning ability in an aversive olfactory task exhibited an equally good performance in a different aversive olfactory task (reinforced by an electric shock rather than a bitter substance), thus highlighting the

importance of genetic selection for learning ability (Mery and Kawecki 2002; Mery et al. 2007). Numerous studies suggest that genetic factors influence cognitive performance in invertebrates (Raine et al. 2006; Orr et al. 2009; Ings et al. 2009; Raine and Chittka 2012; Scheiner et al. 2021). The bees of our study originated from a single hive, a fact that reduces but does not abolish the genetic diversity among the bees tested, as different patrilines typically coexist within a hive as a result of multiple mating of the queen during the nuptial flight. The learning performance of individual worker bees in elemental olfactory tasks can indeed be predicted partially by their patriline (Brandes 1988; Bhagavan et al. 1994; Scheiner and Arnold 2010; Junca et al. 2019). Genetic variability has a strong impact on responsiveness to appetitive and aversive stimuli such as sucrose or thermic shocks, respectively (Scheiner and Arnold 2010; Junca et al. 2019). This variable responsiveness translates into variation of performance observed in associative learning protocols in which such stimuli are used as unconditioned stimuli (Scheiner et al. 2005; Roussel et al. 2009; Scheiner and Arnold 2010). Thus, the variable success of foragers co-opted for our experiments could be due to their belonging to different genetic patrilines within the colony.

Variability in learning performance in our study could also be influenced by prior visual experience gathered on a larger time scale than the duration of our experiment during foraging activities. Both age and sensory experience influence brain structural development of forager bees, which, in turn, can modulate learning performance, although mostly in the form of a cognitive decline with ageing (Withers et al. 1993; Durst et al. 1994; Farris et al. 2001; Münch et al. 2010; Groh et al. 2012; Scholl et al. 2014; Cabirol et al. 2018). The mushroom bodies, the main higher-order structures of the insect brain, show experience-dependent variation in their volume or density of synaptic buttons, following light exposure, age, foraging experience or learning events (Hourcade et al. 2010; Scholl et al. 2014; Cabirol et al. 2017, 2018). Individual variability in mushroom body development may have an impact on cognitive faculties (Li et al. 2017). Therefore, it is likely that the stability of learning proficiency observed across days or tasks of different complexity relies, at least partially, on neurobiological variability resulting from different life experiences.

Learning differences could also emerge from variation in the processing of the stimuli to be learned such as odours or visual cues. This possibility is supported by our study as we found that some bees were relatively better at learning olfactory cues than visual cues, and vice versa, thus reflecting potential variation in perceptual salience between modalities. Honeybees are known to differ in their responsiveness to odours (Scheiner et al. 2004), which could be linked

to inter-individual differences in the activity of olfactory neural circuits. In fruit flies, for instance, stable inter-individual variability was found in an odour-preference assay, which translated into consistent inter-individual differences in Ca^{2+} activity levels in a key structure of the olfactory circuit, the projection neurons of the antennal lobes (Honegger et al. 2020). Similar arguments could apply to visual processing and its underlying visual circuits.

The question of whether learning ability correlates across problems of different complexity or sensory domains is particularly relevant for the analysis of brain modularity and for understanding the contributions of different neural circuits to different forms of learning. In honeybees, different brain structures have been associated with different levels of complexity in olfactory learning. The mushroom bodies are required for non-elemental olfactory discrimination tasks such as the negative patterning problem (A+, B+, AB−) (Devaud et al. 2015) or for reversal learning (A+ B−→A− B+) (Boitard et al. 2015) but are dispensable for elemental olfactory discrimination (A+, B+, CD−) (Devaud et al. 2015). From this perspective, proficiency in non-elemental olfactory learning may not necessarily be correlated with proficiency in elemental olfactory learning, given that these learning forms are mediated by different brain structures. Applying this reasoning to our results in the visual domain suggests that the two learning forms, which are highly correlated, may require similar visual circuits/structures. To date, the brain neuropils involved in different forms of visual learning remain unknown because of the difficulty of reproducing successful visual learning in the laboratory, which would allow coupling with invasive recordings of neural activity (Avarguès-Weber and Mota 2016). However, given the massive visual afferences to the mushroom bodies (Ehmer and Gronenberg 2002; Paulk and Gronenberg 2008) and to the central complex (Pfeiffer and Homberg 2014), participation of these structures is expected. For instance, in *Drosophila*, both mushroom bodies and the central complex are involved in visual learning depending on the specific task and setup used (Liu et al. 1999; Pan et al. 2009; Ofstad et al. 2011; Vogt et al. 2014, 2016).

The fact that we observed a positive correlation of performance across an elemental task and a conceptual task in the visual modality suggests that a similar brain circuitry underlies the two discriminations. An alternative explanation may be that differences in general visual processing ability and attentional processes could have a major influence on performance in both tasks. In humans and rodents, general intelligence has been linked to selective attention and working memory abilities (reviewed in Matzel and Kolata, 2010) which encompassed both the storage of information and the processing and integration of information (Baddeley 2003;

Jarrold and Towse 2006). Increasing evidence indicates that insects are capable of selective attention mediated by several higher-order brain areas (van Swinderen 2011; Nityananda 2016).

Shifting our focus from group to individual performance in cognitive studies could thus contribute to the elucidation of the underlying mechanisms (Thornton and Lukas 2012; Pamir et al. 2014; Boogert et al. 2018; Klein 2018). It also raises fascinating ecological questions such as the possible existence of cognitive specialization between workers from different patrilines in a hive. In honeybees, the best learners might deal with more demanding, complex tasks and concentrate their foraging effort towards the exploration of novel food sources. Others might simply copy the former and use social information when facing difficult tasks, as occurs in bumblebees (Baracchi et al. 2018). This would explain the known differentiation between scouts and recruits (Biesmeijer and De Vries 2001; Beekman et al. 2007). The diversity of foraging strategies within a colony has been shown to increase its fitness (Burns 2005; Burns and Dyer 2008; Jeanson and Weidenmüller 2014; Klein 2018) but, interestingly, the best learners are not necessarily the best foragers, as demonstrated in bumblebees, where bad learners foraged for a longer time frame and collected more resources, potentially as a result of the energetic cost associated with cognition (Evans et al. 2017). Thus, the complex interplay between inter-individual cognitive skill diversity, task allocation and colony fitness remains to be fully elucidated.

Acknowledgements

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.A.-W.; Methodology: A.A.-W, V.F; Validation: V.F., M.G., R.S., A.A.-W.; Formal analysis: D.B., A.A.-W., V.F.; Investigation: V.F.; Resources: A.A.-W.; Writing - original draft: A.A.-W., V.F.; Writing - review & editing: D.B., M.G., R.S.; Visualization: D.B.,

A.A.-W.; Supervision: M.G., R.S., A.A.-W.; Project administration: A.A.-W.; Funding acquisition: A.A.-W.

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Data availability

The datasets supporting this article are available from the Dryad digital repository (Finke et al., 2021): doi:10.5061/dryad.1ns1rn8v5.

3.7 Supplementary material

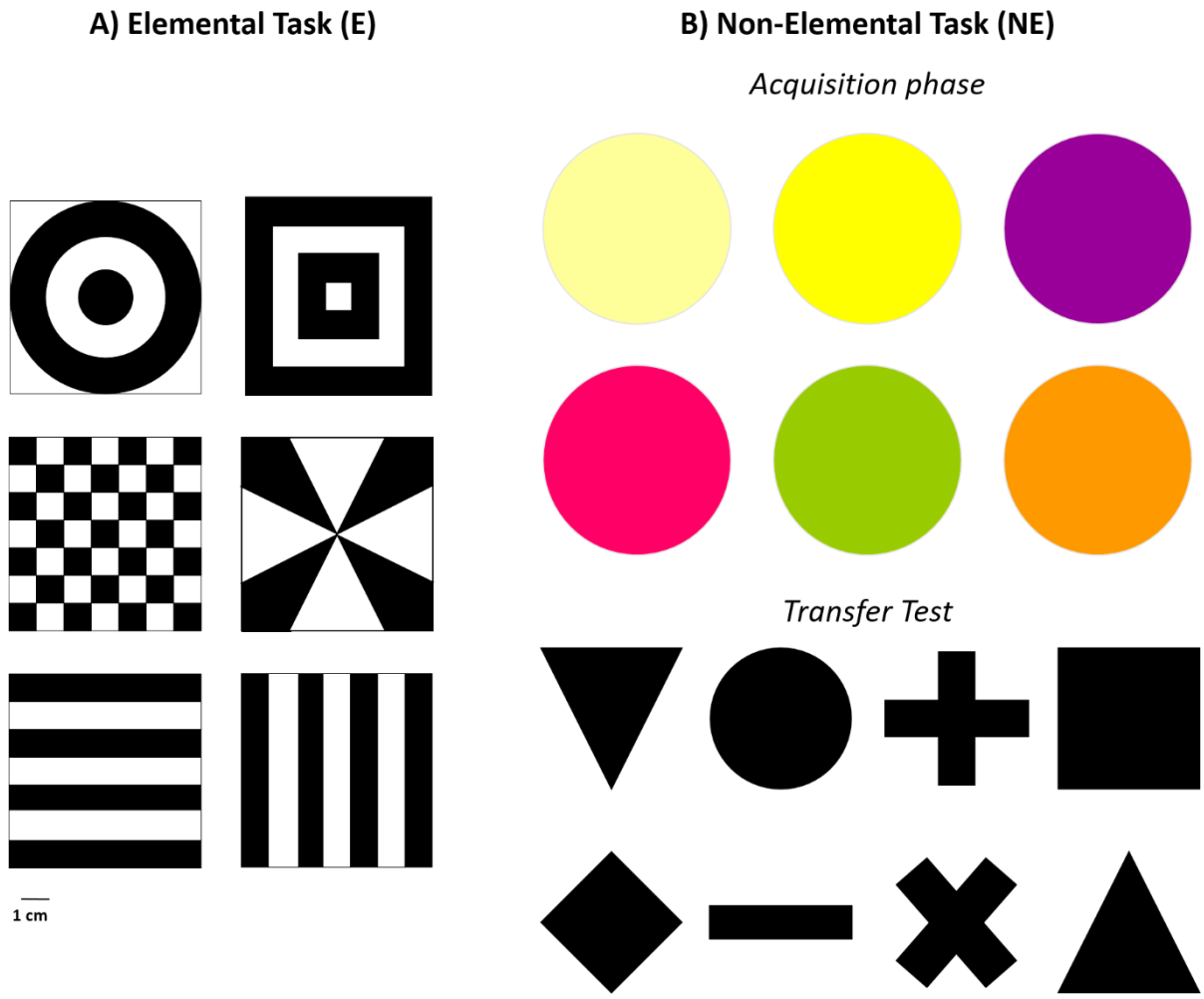

Fig. S1 Stimuli used in our visual learning experiments A) In the Elemental discrimination (Experiments 1, 2 and 3), bees had to differentiate between a pair of stimuli randomly chosen between the six alternatives available: one stimulus was rewarded with sucrose solution (CS+) while the other was punished with quinine solution (CS-). After training, a learning test using the same stimuli that were trained but in the absence of reinforcement, was performed. **B)** In the Non-Elemental conceptual discrimination, all six alternative colour stimuli were used during the acquisition phase for all bees, which had to learn to choose a specific spatial relationship vs. a different spatial relationship (above/left vs. left/right). Each possible pair of stimuli was used to build the spatial relationships that had to be discriminated. Stimuli varied randomly from one trial to the other in order to vary stimuli appearance while keeping constant the spatial relations. After training, a non-reinforced transfer test was conducted with a pair of achromatic stimuli randomly chosen for each individual among the eight alternatives.

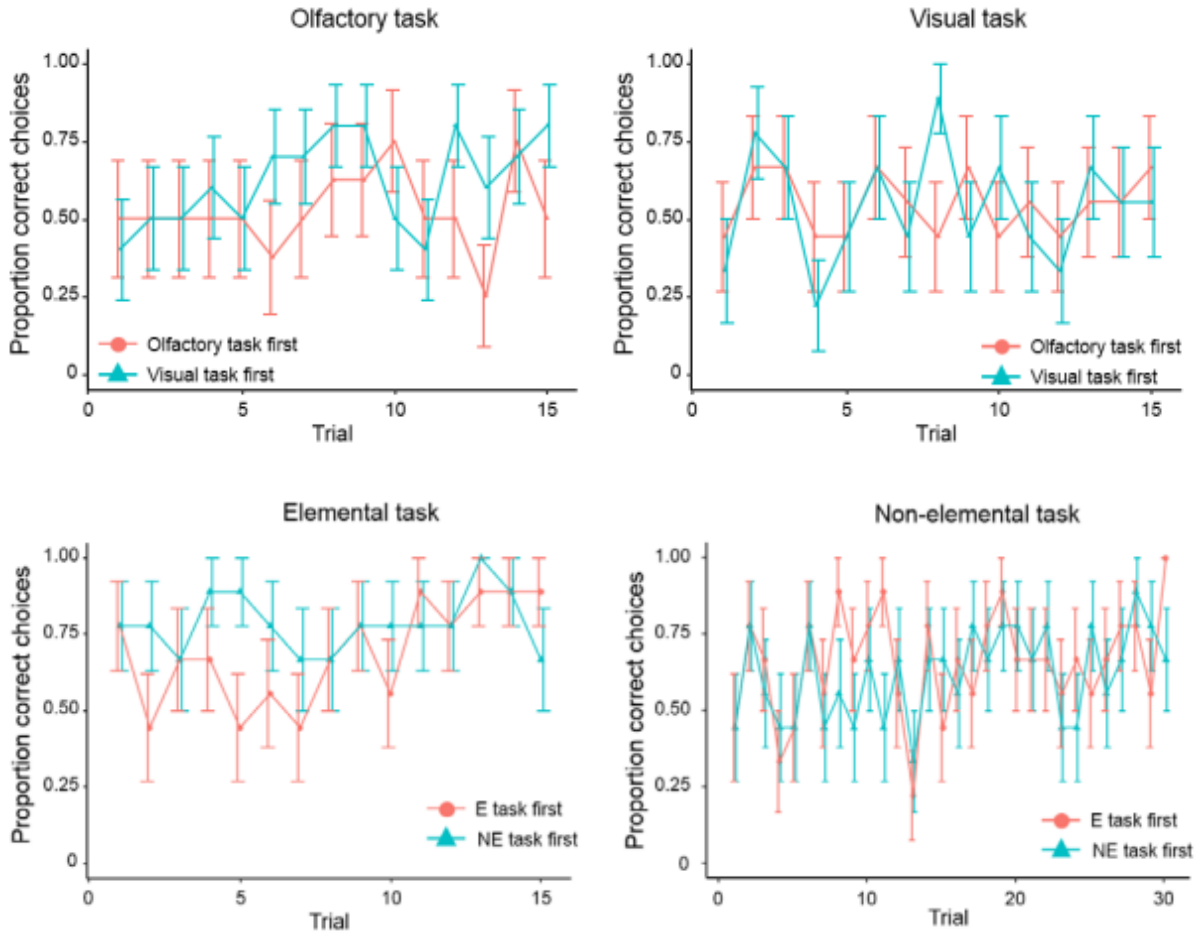

Fig. S2 Representation of the tasks sequence effect. Above Experiment 2: Acquisition curves expressed as the proportion of correct choices ± SEM across trials of forager bees (*n = 18*) confronted to the Olfactory Elemental task (left panel) or the Visual Elemental task (right panel) depending on this task being performed at first or after the other task. Below Experiment 3: Acquisition curves expressed as the proportion of correct choices ± SEM across trials of forager bees (*n = 18*) confronted to the Elemental task (left panel) or the Non-elemental task (right panel) depending on this task being performed at first or after the other task.

Table S1. GLMM Analysis on honeybees' performance in the acquisition phases of the Experiment 1 (repetition of Visual Elemental tasks during 3-days). The model presenting the best fit to the data (lowest AIC) is highlighted in bold. P-values represent the comparison with the model with one more lovel of complexity. Significant p-values mean that removing the corresponding interaction or factor from the model reduces significantly the model fit.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
Model 1: Choice ~ Trial*Day*Stimuli + (1|Bee)	9	1156.4	-487.2		
Model 2: Choice ~ Trial*Day + Stimuli + (1|Bee)	6	1088.2	-511.1	47.8	0.83
Model 3: Choice ~ Trial*Stimuli +Day + (1|Bee)	6	1123.9	-501.9	29.5	0.54
Model 4: Choice ~ Trial + Day + Stimuli + (1|Bee)	5	1088.2	-512.1	20.3	0.85
Model 5: Choice ~ Trial + Day + (1|Bee)	4	1047.6	-519.8	15.4	0.97
Model 6: Choice ~ Trial + (1|ID)	**3**	**1045.6**	**-519.8**	**0.01**	**0.92**
Model 7: Choice ~ Day + (1|ID)	3	1047.6	-522.6	5.6	0.02*

Table S2. GLMM Analysis on honeybees' performance in the acquisition phases of the Experiment 2 (comparison of performance between Visual and an Olfactive Elemental tasks). The model presenting the best fit to the data (lowest AIC) is highlighted in bold. P-values represent the comparison with the model with one more lovel of complexity. Significant p-values mean that removing the corresponding interaction or factor from the model reduces significantly the model fit.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
Model 1: Choice ~ Trial*Task + Order + Stimuli + (1|Bee)	7	768.7	-360.4		
Model 2: Choice ~ Trial + Task + Order + Stimuli + (1|Bee)	6	767.6	-360.8	0.9	0.35
Model 3: Choice ~ Trial + Task + Order + (1|Bee)	5	747.3	-368.6	15.7	0.61
Model 4: Choice ~ Trial + Task + (1|Bee)	4	746.2	-369.1	0.9	0.34
Model 5: Choice ~ Trial + (1|Bee)	3	744.8	-369.4	0.6	0.43
Model 6: Choice ~ 1 + (1|ID)	**2**	**744.5**	**-370.3**	**1.7**	**0.19**

Table S3. GLMM Analysis on honeybees' performance in the acquisition phases of the Experiment 3 (comparison of performance between an elemental and non-elemental Visual tasks). The model presenting the best fit to the data (lowest AIC) is highlighted in bold. P-values represent the comparison with the model with one more level of complexity. Significant p-values mean that removing the corresponding interaction or factor from the model reduces significantly the model fit.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
Model 1: Choice ~ Trial*Task + Order + Stimuli + (1|Bee)	7	1044.7	-498.3		
Model 2: Choice ~ Trial + Task + Order + Stimuli + (1|Bee)	6	1044.6	-499.3	1.9	0.17
Model 3: Choice ~ Trial + Task + Order + (1|Bee)	5	1019.6	-504.8	10.9	0.90
Model 4: Choice ~ Trial + Task + (1|Bee)	**4**	**1017.6**	**-504.8**	**0.05**	**0.82**
Model 5: Choice ~ Trial + (1|Bee)	3	1030.6	-512.3	15.0	<0.001***
Model 6: Choice ~ Task + (1|ID)	3	1024.6	-509.3	9.0	0.003**

4 Chapter 2: Individual consistency in the cognitive abilities of honey bees: Cognitive specialization within sensory and reinforcement modalities

Animal Cognition
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ORIGINAL PAPER

Individual consistency in the learning abilities of honey bees: cognitive specialization within sensory and reinforcement modalities

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Abstract
The question of whether individuals perform consistently across a variety of cognitive tasks is relevant for studies of comparative cognition. The honey bee (*Apis mellifera*) is an appropriate model to study cognitive consistency as its learning can be studied in multiple elemental and non-elemental learning tasks. We took advantage of this possibility and studied if the ability of honey bees to learn a simple discrimination correlates with their ability to solve two tasks of higher complexity, reversal learning and negative patterning. We performed four experiments in which we varied the sensory modality of the stimuli (visual or olfactory) and the type (Pavlovian or operant) and complexity (elemental or non-elemental) of conditioning to examine if stable correlated performances could be observed across experiments. Across all experiments, an individual's proficiency to learn the simple discrimination task was positively and significantly correlated with performance in both reversal learning and negative patterning, while the performances in reversal learning and negative patterning were positively, yet not significantly correlated. These results suggest that correlated performances across learning paradigms represent a distinct cognitive characteristic of bees. Further research is necessary to examine if individual cognitive consistency can be found in other insect species as a common characteristic of insect brains.

Keywords Inter-individual variability · Insect cognition · Domain-general cognition · Domain-specific cognition · Cognitive repeatability · Honey bee

Introduction

Cognition has been defined as the ability of animals to acquire, process, store and use vital information from the environment (Shettleworth 2009). While inter-individual differences in the cognitive skills in humans provide the basis of psychometrics, studies on animal cognition have generally neglected these differences in their attempt to underline the capacity of a given species to pass decisive cognitive tests (Boogert et al. 2018). Consequently, only the success of the most skilled individuals is usually highlighted. Alternatively, the average performance derived from individual data is used as a representative measure, leaving aside inter-individual differences, which could be informative about cognitive variation within a group (Pamir et al. 2011). Indeed, the existence of consistent inter-individual variability in cognitive traits is now well-studied across vertebrate species as it offers novel perspectives to study the link between cognition and behavioral syndromes or fitness (Matzel et al. 2003; Healy et al. 2009; Sih and Del Giudice 2012; Herrmann and Call 2012; Thornton et al. 2014; Guenther and Brust 2017; Dougherty and Guillette 2018; Cauchoix et al. 2018). These questions are relatively new in invertebrate research despite the tractability of these organisms for behavioral and neurobiological studies on inter-individual behavioral variability (Scheiner et al. 2005; Muller and Chittka 2012; Honegger and de Bivort 2018; Honegger et al. 2019; Tait et al. 2019; Tait and Naug 2020; Finke et al. 2021; Smith et al. 2022).

Social insects offer a great opportunity to study inter-individual cognitive differences due to their impressive cognitive capabilities (Dornhaus and Franks 2008; Avarguès-Weber et al. 2011; Giurfa 2013, 2019; Chittka 2017; Perry et al.

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4.1 Abstract

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4.2 Introduction

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Social insects offer a great opportunity to study inter-individual cognitive differences due to their impressive cognitive capabilities (Dornhaus and Franks 2008; Avarguès-Weber et al. 2011a; Giurfa 2013, 2019; Chittka 2017; Perry et al. 2017; Howard et al. 2018; Simons and Tibbetts 2019). Inter- individual variability has been described in a wide range of behaviors and is considered as a major factor for their ecological success, adding to division of labor and flexible responses to environmental changes (Thomson and Chittka 2001; Chittka and Muller 2009; Jeanson and Weidenmüller 2014; Bengston and Jandt 2014; Jandt and Gordon 2016; Walton and Toth 2016; Jeanson 2019).Variability in the learning abilities of bees has been connected to task allocation (Ray and Ferneyhough 1999; Ben-Shahar et al. 2000; Scheiner and Amdam 2009; Scheiner et al. 2017). For example, nectar and pollen foragers show inter-individual differences in their response thresholds to sucrose, correlating positively with differences in appetitive associative learning performances (Scheiner et al. 1999, 2001a, 2001b; Pankiw and Page 1999). However, only a few studies have examined whether inter-individual differences in learning proficiency remain consistent over time and across different contexts. Given that some bees are better learners than others, do they have general learning skills making them better in multiple tasks and contexts, or are they rather specialized in a given set of problems? We previously showed that learning proficiency is stable over time in forager bees, justifying that their pattern of performances could be defined as a cognitive profile. We also evidenced that the performance in an elemental visual discrimination correlates positively with the performance in a non-elemental visual relational concept learning task where subjects have to follow a rule based on spatial relations between objects independently of the physical properties of those objects (Finke et al. 2021). By contrast, no clear correlation was observed between the learning performances in the olfactory and visual modality, suggesting that cognitive consistency is modality-specific (Finke et al. 2021). Cognitive specialization i.e. increased ability for a given cognitive trait relatively to other functions within individuals and by comparison to the general population, was also found when comparing elemental appetitive and aversive learning (Junca et al. 2019) and between landmark learning and olfactory learning (Tait et al. 2019). However, it remains to be determined if cognitive specialization in bees would mostly depend on the type of reinforcement used to train animals or if specialization depends on distinct “cognitive modules” sensu Fodor (1983)., i.e. domain-specific and modality-

dependent conglomerates with a fixed neuronal architecture, which could operate separately or in conjunction according to the complexity of the learning task.

Here we aimed at testing whether individual performances correlate between different learning tasks relying on the same reinforcement and sensory modality or whether distinct cognitive modules mediate performance in these tasks. We tested bees in (i) a reversal learning task (Pavlov 1927) in which subjects are trained to discriminate a rewarded and a non-rewarded stimulus in two consecutive phases with a change of reward contingencies between phases (A+ vs. B− and then A− vs. B+), and (ii) a negative patterning discrimination in which subjects have to learn to respond to the presentation of single reinforced stimuli but not to their conjunctive presentation (e.g. C+ and D+ vs. CD−). Reversal learning has the advantage of using the first phase (A+ vs. B−) as a proxy for the animals' capacity to solve an elemental discrimination, and the second phase as a proxy of their flexibility to reverse this discrimination (Ben-Shahar et al. 2000; Hadar and Menzel 2010; Mota and Giurfa 2010; Boitard et al. 2015). The second phase induces indeed a transient stimulus ambiguity in terms of learned valence that needs to be overcome. Importantly, only individuals that learned the initial A+ B− association in the 1st phase of reversal learning can be evaluated for their ability to reverse this association in the 2nd phase (Mota and Giurfa 2010). The negative patterning discrimination (Whitlow and Wagner 1972) can only be solved if the compound stimulus is treated as being different from the sum of its components, which requires inhibiting stimulus summation and implementing different forms of processing such as configural processing (Deisig et al. 2001; Schubert et al. 2002; Devaud et al. 2015).Both learning paradigms were conducted using stimuli from different sensory modalities (visual or olfactory) and different set-ups involving conditioning protocols that involved either classical or operant type of conditioning to examine whether the patterns of individual consistency in performance between tasks are stable across these different contexts. In a series of four experiments (see Fig. 1 for an overview of the experiments), we tested bees consecutively in the two learning tasks explained in detail above. Two experiments with free-flying bees involved both a combination of operant (flying to the correct target) and classical (association between the CS and the reinforcement) learning either in the visual (experiment 1) or in the olfactory modality (experiment 2). The remaining two experiments involved pure classical conditioning of restrained bees and consisted of conditioning their proboscis extension reflex (PER) with either visual (experiment 3) or olfactory stimuli (experiment 4). Experiments on PER conditioning with restrained bees in the laboratory have the great advantage of providing standardized external (e.g., temperature and humidity) and experimental conditions (e.g., timing and duration of trials, stimuli illumination and

concentrations). Additionally, they allow testing of multiple bees per day and enable therefore the access to large sample sizes. However, they provide us only with restricted information of individuality in the test performances as performance is quantified as a binomial variable (response or no response), thus precluding fine- grain analyses of performance. Experiments with free-flying bees have the advantage of providing a more detailed grain analyses of individual data of test performances as learning can be quantified by the percentage of correct choices reached by each individual. On the contrary, these experiments require considerable time and focus on single individuals and restrict, in comparison, the sample sizes considerably. With such a portfolio of experiments differing in both procedure and sensory modality we aimed determining if individuals exhibit across-task consistency in their cognitive success, thus indicating the presence of abilities that would be independent of a specific experimental context. Our hypothesis was that within each experiment performances would correlate positively across all tasks and that we would find similar patterns of correlations in performances across the different experiments. We hypothesized this based on our recent findings showing that learning proficiency differs between individuals but remains consistent over time and across an elemental and an alternative non-elemental learning task as long as the stimuli were from the same sensory modality (Finke et al. 2021).

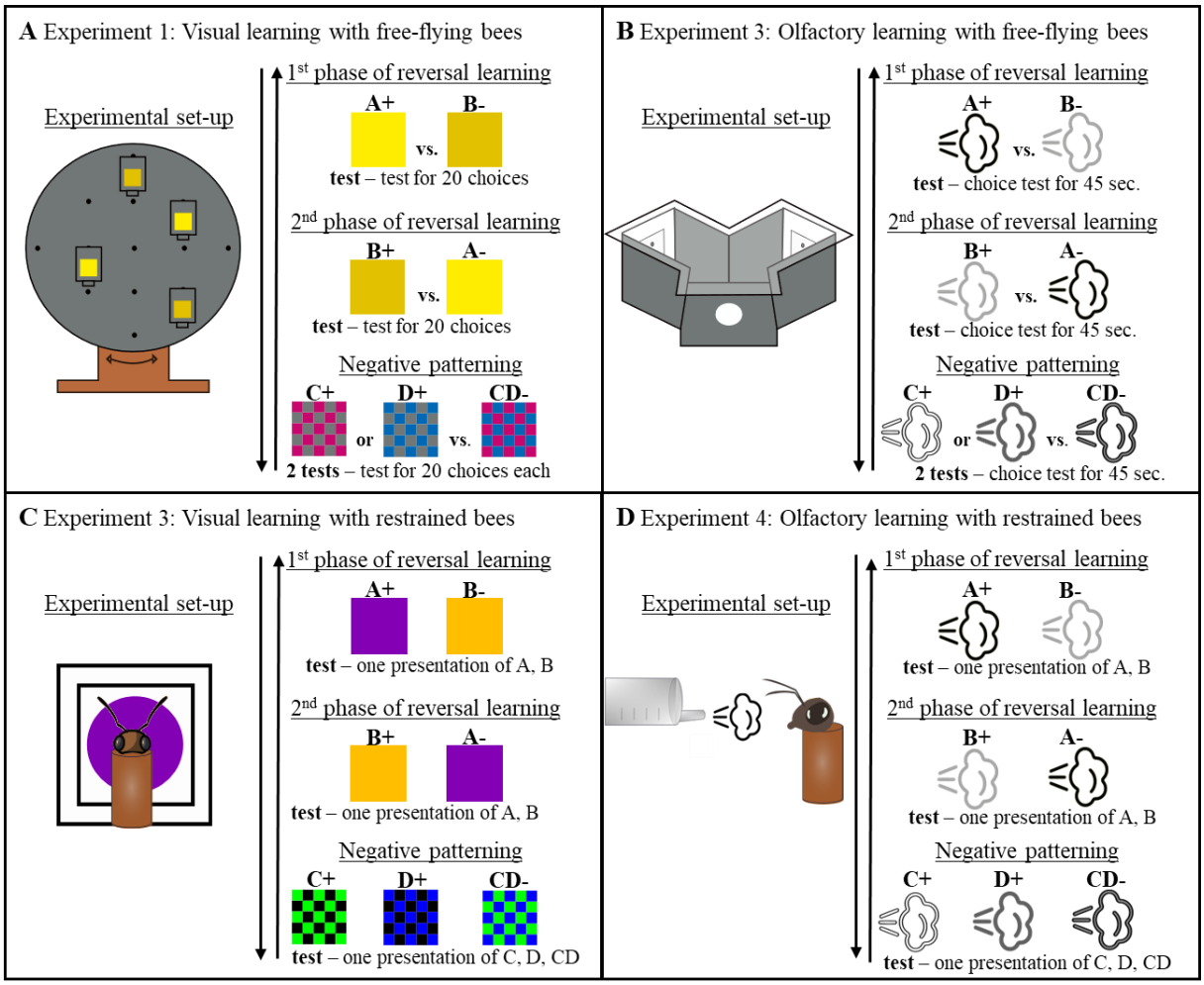

Fig. 1 Schematic overview of the experiments conducted. **A** Experiment 1: Visual learning with free-flying bees. The experimental set-up was a rotating screen apparatus where hangers could be attached to at various locations. The hangers displayed the stimuli during conditioning and testing. For reversal learning we used yellow and greenish-yellow cardboard squares as visual stimuli. For negative patterning we used checkerboard squares cut from pink or blue cardboards to create the single stimuli C/D and blue and pink cardboards to create the compound stimulus CD. Test performances were assessed by a 45 sec. choice tests during which all contacts of the bees with the respective stimuli were counted and a percentage of correct choices was then calculated for each test. **B** Experiment 2: Olfactory learning with free-flying bees. The experimental set-up was a Y-maze apparatus where the bees could fly through a hole to enter and get access to the inside where the olfactory stimuli are applied to filter papers on the backwalls of the maze. In reversal learning we used the odors linalool and 2-hexanone as A and B. For negative patterning we used limonene as stimulus C, 2-octanol as stimulus D and a mixture of these odors as CD. Test performances were assessed by a 20 choice tests during which all contacts of the bees with the respective stimuli were counted and a percentage of correct choices was then calculated for each test. **C** Experiment 3: Visual learning with restrained bees. The experimental set-up was a box with five chambers covered by movable red Plexiglas® ceilings preventing light stimulation. In reversal learning we used 400 nm and 600 nm monochromatic light discs as stimulus A and B. For negative patterning we used a blue or green checkerboard as single stimuli C/D and a blue-green checkerboard as compound stimulus CD. Test performances were assessed by a single presentation of each stimulus of the respective learning task. **D** Experiment 4: Olfactory learning experiments with restrained bees. The experimental set-up was also a movable box with compartments for ten bees in front of an exhaust fan. Odors were delivered through an airstream. In reversal learning we used the odors linalool and 2-hexanone as A and B. For negative patterning we used limonene as stimulus C, 2-octanol as stimulus D and a mixture of these odors as CD. Test performances were assessed by a single presentation of each stimulus of the respective learning task. (color figure online)

4.3 Experiment 1: visual learning in free-flying bees

4.3.1 Material and methods

4.3.1.1 General methods

The experiments were conducted within an indoor flight cage (~ 4 × 6 m) hosting a single colony. The flight cage was made from UV-transparent Plexiglas, thus providing light conditions that were similar to natural daylight conditions. The bees were provided with pollen *ad libitum*, a water source and an artificial gravity feeder containing sucrose solution (30% weight/weight). The bees used for the experiment were recruited from the feeder to the experimental set-up. The set- up consisted of a vertically mounted rotatable grey plastic screen (rotating screen; 50 cm in diameter), where hangers (6 × 8 cm) could be attached at various locations (see Fig. S1 for a schematic overview of the apparatus). The apparatus was achromatic for the bees. The hangers allowed to display stimuli (5 × 5 cm) and had a landing platform where the bees could land on to collect 10 µl of the reward (50% sucrose solution, weight/weight), punishment (quinine solution, 60 mM) or water. A punishment is commonly used in discrimination learning protocols to improve visual stimulus differentiation (Avarguès-Weber et al. 2010a). In the case of the negative patterning paradigm, as the compound stimulus opposed to the single rewarded stimuli should not be rein- forced, we provided water as neutral US stimulus (Deisig et al. 2001; Schubert et al. 2002). Bees (*n = 33*) were first pre-trained to collect ad libitum sucrose solution from the landing platforms of two hangers (see Fig. S1) in the absence of stimuli until they landed quickly after arriving at the set- up for at least five times. Once bees completed this pre- training, the learning protocols were initiated.

During training and testing only one bee, individually marked with a colored spot on the thorax (Uni-posca paint marker; Mitsubishi Pencil Co., Ltd.), was present at a time at the experimental set-up. Other bees approaching the setup were captured into cages to avoid them interfering with the focal bee. The total number of trials varied according to the learning tasks and are specified below in each case. During each trial, a choice for a given stimulus was recorded once the bee had landed on a platform and tasted the corresponding solution. If the bee made in incorrect choice, it was allowed to make further choices until a correct choice was scored. Having made a correct choice, the bee was transferred to a plexiglas spoon providing a sucrose solution, which was then moved 1 m away from the screen, while the screen was rotated to change the spatial positions of the stimuli. Subsequently, the solutions on the hangers were refilled. The bee was then allowed to make another choice. Bees usually made 3–5 choices per

foraging bout. When they returned to the hive, the hangers were cleaned with 50% ethanol and all solutions were refilled.

Each acquisition phase was directly followed by a non-reinforced test in which the bee had to choose between the trained stimuli. Each stimulus was presented twice as it occupied two hangers. Fresh stimuli and hangers were used during the test. After 20 choices a test was finished. A choice was defined as either landing or touching the landing plat- form or test stimulus. Half of the bees were first subjected to the reversal learning task and then to the negative patterning task while the task order was reversed for the other half. Once a learning paradigm was completed, the bee was allowed to collect sucrose solution on the hangers in the absence of any stimulus for three foraging bouts before the second learning paradigm started. Only highly motivated bees coming back to the experimental set-up regularly (with a maximum of 10 min between visits, usually 2–5 min) were kept for analysis. The whole procedure took 6–8 h per bee.

4.3.1.2 Reversal learning protocol

In the first phase of reversal learning one color was associated with a reward (A+) while a second color was associated with a punishment (B−). In the 2nd phase, the reward contingencies were reversed, so that the previously rewarded target stimulus became punished and vice versa (A− and B+). Each stimulus was presented on two hangers so that four hangers were presented during conditioning trials and in the tests. Both phases amounted to 30 trials in total and each phase was directly followed by a non-rewarded test presenting A and B in the absence of reinforcement. Colors used were squares cut from HKS-3N and HKS-68N card- board (5 × 5 cm; HKS-N pigment papers; Hostmann-Stein- berg K + E Druckfarben, H. Schmincke and Co., Germany) that appear yellow and greenish-yellow to the human eye (see Fig. S2 for the spectral reflectance curves of the stimuli and their positions in the hexagon color space, a model for color perception of bees, Chittka 1992). Half of the bees were initially conditioned with HKS-3N as stimulus A and HKS-68N as stimulus B while the other half experienced a reversed stimulus contingency. Color loci in the hexagon were separated by 0.07 hexagon units, which is sufficient to be discriminated by the bees (Chittka 1992; Dyer and Neumeyer 2005; Avarguès-Weber et al. 2010a). During acquisition and testing two correct and incorrect colored squares were displayed at the same time in varying positions and dispositions on the rotating screen.

4.3.1.3 Negative patterning protocol

The acquisition phase consisted of three different consecutive blocks of trials: Two blocks consisted of presenting at each trial four hangers displaying only one of the rewarded single stimuli (C+ or D+), a third block consisted of presenting the non-reinforced compound stimulus (CD−) on two hangers and a rewarding black-and-white checkerboard alternative (XY+) on two hangers. The addition of the checkerboard is necessary in the case of experiments with free-flying bees as presenting only the compound stimulus (CD−) in consecutive non-reinforced trials would result in a decrease of motivation and in the bees ceasing their foraging activities at the set-up. Presenting a rewarded neutral alternative (XY+) allows overcoming this problem while keeping the ambiguity of stimulus valence for C and D. This alternative was used successfully by Schubert et al. (2002) to study negative patterning in free-flying bees. The order of blocks throughout acquisition was pseudo-randomized so that each block was not conducted more than twice in a row. Each block lasted for one foraging bout, as stimuli were exchanged once a bee returned to the hive. Consequently, the number of trials in each block varied. Usually, the C+ and D+ blocks amounted to 3–6 trials and the CD− blocks to 4–8 trials per foraging bout. The experiment was completed when the bee reached 30 trials for both the C+ and D+ blocks and 60 trials for the CD− block, i.e. 120 trials in total. In this way, each bee experienced 60 rewarded and 60 non-rewarded experiences. Again, for each trial a choice was recorded once the bee landed on a platform and tasted the corresponding solution. The acquisition phase was followed by two non-rewarded tests where CD− and either C+ or D+, respectively, were presented together on two hangers each. None of the test stimuli provided reinforcement. The tests were completed when the bee performed 20 choices in total. The two tests were spaced by one refreshing foraging bout in which the reinforced trained stimuli were offered to maintain a high motivation. The square stimuli were cut from HKS-26N, HKS-44N, HKS-92N and HKS- 88N cardboards (5 × 5 cm; HKS-N pigment papers; Hostmann-Steinberg K + E Druckfarben, H. Schmincke and Co., Germany) and appeared pink, blue, grey and black to the human eye, respectively (see Fig. S2 for the spectral reflectance curves of the stimuli and their positions in the hexagon color space). The pink (26N) and blue stimuli (44N) were separated by 0.07 hexagon units, which is a perceptual distance sufficient to support discrimination (Chittka 1992; Dyer and Neumeyer 2005; Avarguès-Weber et al. 2010a). The two elemental stimuli (C and D) consisted of checkerboard patterns made of 1 × 1 cm squares of either the pink or blue cardboard on the HKS-92N background. The compound stimulus (CD) was thus a checkerboard pattern made of the pink and blue cardboards. The rewarding

alternative (XY) was a black and white checkerboard made of squares of the same size (i.e. 1 × 1 cm each). This design was adapted from (Schubert et al. 2002).

4.3.2 Statistical analysis

Test data were used to assess the individuals' learning performances as we did not observe, at the individual level, a sigmoidal increase of performance in the acquisition starting at a 50% random choice level and increasing significantly as classically observed at the group level. This is probably due to the stochasticity of choices, as bees have a 50% probability of making a correct choice at each trial, which may result by chance in unexpected high or low scores at the individual level (see Figs. S5 for analysis of the acquisition and test phases at the group level). We thus used the percent- age of correct choices in the non-reinforced tests to assess individual consistency across the three leaning tasks tested. Individual consistency across the different tasks was tested using Spearman rank correlations between test performances. Reversal learning ability can only be tested on individuals that successfully acquired the first A+B− association (Mota and Giurfa 2010). Most bees chose preferentially A in the test following the first phase and were consequently kept for analysis of their reversal learning ability (*n = 27 of 33*). We decided to use an arbitrary threshold of 60% correct choices in the test to consider a bee as learner or non-learner. To assess whether the order in which the learning tasks were conducted, or which stimulus was rewarded in reversal learning influenced test performances generalized linear mixed models (GLMM) were used. The models with a binomial error structure and logit-link function included the choices made (either correct scored as 1 or incorrect scored as 0) in the test as dependent variable and the order of the tasks (*order*) and the rewarded stimulus (*group_RL*) as fixed factors. The bees' identity (subject) was included as a random factor. Different models were performed where the factors were gradually removed and compared using an ANOVA. P-values from these comparisons were provided to account for each factor impact. The model with the lowest AIC value was chosen as most appropriate fit (S10-S12). All GLMMs were performed using R Statistical Software version 3.6.3 (R Core Team 2022) with the package lme4 (Bates et al. 2015). All other statistical analyses and graphs were performed using GraphPad prism version 9.0.0 (GraphPad Software Inc., San Diego, California, USA). The significance level was $\alpha = 0.05$ to account for our relatively small sample sizes (Lakens et al. 2018). For all correlations of the test performances across the three learning tasks, the null hypothesis was that the correlation coefficient rho was not different from zero.

4.3.3 Results

We analyzed whether individual performances of free-flying bees (*n = 33*) in a reversal learning problem correlated with performances in a negative-patterning problem, both established using visual stimuli. As reversal learning consists of two phases (A+ vs. B− → A− vs. B+), we performed separated analyses between negative patterning performances and performances in the 1st and 2nd phases of the reversal learning protocol. Only bees that successfully learned the initial discrimination of the reversal learning (≥ 60% correct choices in the test) were used for correlations including the 2nd reversal learning phase as successful reversal learning requires learning of the initial discrimination. Including bees that did not learn in the first phase goes against the definition of reversal learning as these bees did not have to overcome the transient stimulus ambiguity that characterizes the transition between phases. Importantly, neither the order in which reversal learning and negative patterning were conducted (order), nor the stimulus which was rewarded in reversal learning (group_RL) affected test performances (GLMM: *Order; n = 33,* **1st RL:** $\chi^2_{(1)}$= 0.32, p = 0.57, **2nd RL:** $\chi^2_{(1)}$ *= 0.04, p = 0.85,* **NP:** $\chi^2_{(1)}$ = 2.96, p = 0.09; *group_RL: n= 33,* **1st RL:** $\chi^2_{(1)}$*= 2.1, p = 0.08,* **2nd RL:** $\chi^2_{(1)}$ *= 1.41, p = 0.24,* NP: $\chi^2_{(1)}$ = 0.15, p = 0.70; tables S4-S6 in the supplementary).

Figure 2 shows that individual learning performances in the 1st discrimination phase of reversal learning correlated with performances in the 2nd phase of reversal learning (Spearman rank correlation, *n = 27, rho = 0.53, p = 0.005, R^2 = 0.34,* Fig. 2A, Table 1). Most bees (*n = 4 out of 6* non-learner bees in the 1st phase) that were excluded because they were considered as non-learners in the 1st phase also performed around a 50% chance level (ranging between 40 and 55% correct choices) in the 2nd phase of reversal learning. Two non-learner bees performed well (70 and 80% correct choices) in the 2nd phase of reversal learning but this cannot be seen as a case of reversal learning (see above) but rather as an elemental learning performance given that they did not learn to reverse the reinforcement contingency. Individual test performances were also positively correlated between the 1st phase of reversal learning and in the negative patterning procedure (*n = 33, rho = 0.42, p = 0.02, R^2 = 0.2,* Fig. 2B, Table 1). However, no significant correlation was observed between the 2nd phase of reversal learning and negative patterning (*n = 27, rho = 0.25, p = 0.201, R^2 = 0.06,* Fig. 2C, Table 1). Here, most bees (*n = 4 out of 6*) that were non-learners in the 2nd phase of reversal learning succeeded nevertheless in the negative patterning discrimination.

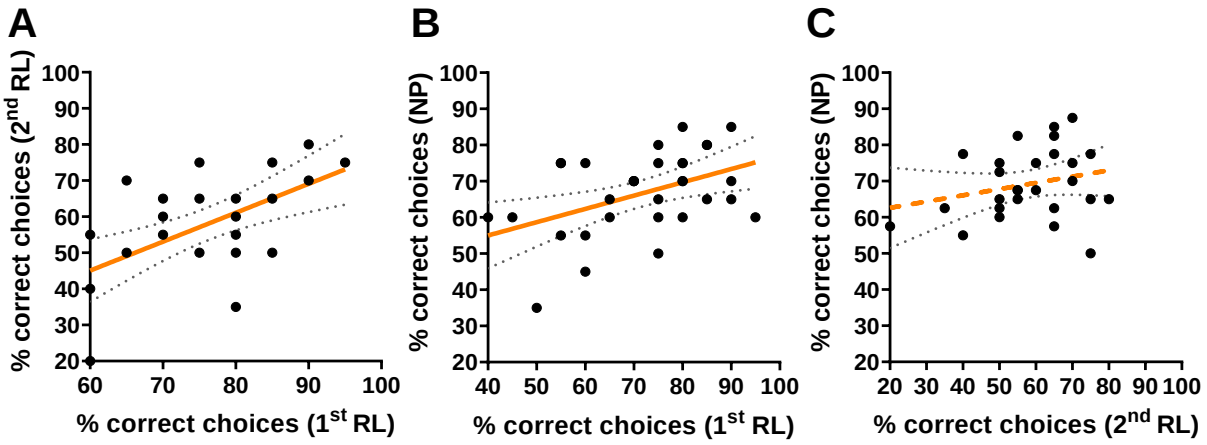

Fig. 2 Correlations of individual test performances in Experiment 1: Visual learning experiment with free-flying bees. Pairwise Spearman rank correlations between the test performances (Percent of correct choices) of individual bees in **A** the 1st phase of reversal learning (1st RL) and the 2nd phase of reversal learning (2nd RL; *n = 27, rho = 0.53, p = 0.005, R^2 = 0.34*), **B** the 1st phase of reversal learning and negative patterning (NP; *n = 33, rho = 0.42, p = 0.02, R^2 = 0.2*) and **C** the 2nd phase of reversal learning and negative patterning (n = 27, *rho = 0.25, p = 0.201, R^2 = 0.06*). Each dot represents data from one bee. The regression line is indicated in orange and the dotted grey lines show the 95%-confidence intervals of the regression. Solid regression lines indicate a significant correlation and dashed lines indicate a non-significant correlation (color figure online)

Table 1 Results of the Spearman rank correlations comparing the individual's test performances in the 1st phase of reversal learning (1st RL), the 2nd phase of reversal learning (2nd RL) and negative patterning (NP).

Experiment	Correlation	Rho	p-value
1	1st RL – 2nd RL	0.53	**
1	1st RL - NP	0.42	*
1	2nd RL - NP	0.25	ns
2	1st RL - 2nd RL	0.60	***
2	1st RL - NP	0.46	*
2	2nd RL - NP	0.19	ns
3	1st RL - 2nd RL	-	-
3	1st RL - NP	0.18	*
3	2nd RL - NP	0.18	ns
4	1st RL - 2nd RL	-	-
4	1st RL - NP	0.33	**
4	2nd RL – NP	0.15	ns

*ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001*

4.4 Experiment 2: olfactory learning in free-flying bees

4.4.1 Material and methods

4.4.1.1 General methods

We used a Y-maze apparatus placed on the garden of our apiary and protected by an umbrella from direct sunlight (see Fig. S3 for a schematic overview of the apparatus). The maze was illuminated by natural daylight and was composed of a sliding door allowing to control the exclusive access of a focal bee, an entrance arm leading to a decision chamber via a small aperture (6 cm in diameter) where the bees could choose between the two arms presenting the olfactory stimuli (arms dimensions: length: 40 cm, height: 20 cm, width: 20 cm). The backwalls (20 × 20 cm) of the two arms were placed at a distance from 15 cm to the decision chamber and coated with white copy paper. The odorant stimuli were applied onto a filter paper (5 × 5 cm) taped to a cardboard which was attached to the copy paper coating the backwalls. The whole Y-maze was covered by movable UV-transparent Plexiglas elements.

Bees (*n = 22*), marked individually with paint marker (Uni-posca paint marker; Mitsubishi Pencil Co., Ltd.) were recruited at a gravity feeder and pre-trained in a stepwise fashion to enter the Y-maze, fly through the entrance hole to access the decision chamber and collect a reward of sucrose solution from the backwalls where no odor stimulus was presented. Only one individual was trained and tested at a time. During acquisition phases, one odorant was rewarded with sucrose solution (50%, weight/weight) while a different odorant was punished with quinine solution (60 mM; for reversal learning) or associated with water (for negative patterning). Odorants were presented on a 5 × 5 cm filter paper onto which 10 µl of a pure odorant were applied. The odorant paper was attached with tape to the copy paper covering the maze backwalls. Reinforcement was delivered by means of transparent micropipettes tips located in the center of each backwall and odorant paper. The solution was not contaminated by the odors, as they were directly filled into the micropipette tips. The side of the rewarded stimulus was changed in a pseudo-random sequence to prevent positional learning. learning. In each acquisition trial, bees were required to enter the Y-maze, fly to the decision chamber and choose between odorants displayed at the two backwalls of the Y-maze. A correct choice led to an ad libitum reward of sucrose solution and an incorrect choice led to the tasting of quinine/water. In case of an incorrect choice, bees were allowed to collect subsequently sucrose solution from the alternative arm displaying the correct stimulus. Within each trial only the first choice of bees was recorded. A choice was scored once bees crossed an imaginary line that was

5 cm distant from the backwalls, i.e. from the odor stimuli. As bees received an *ad libitum* reward upon each correct choice, one trial amounted to one foraging bout. Between trials the Y-maze was cleaned with 50% ethanol and ventilated so that potentially remaining odors were removed. Then the paper cover of the backwalls was exchanged and fresh odorants were applied. Acquisition phases were immediately followed by non-reinforced tests with fresh stimuli. Each test was conducted twice to swap stimulus sides and lasted 45 s. During this period, all contacts with the stimuli were recorded. Between tests, bees were subjected to three reinforced conditioning trials ('refreshing trials') to maintain a high appetitive motivation. Half of the bees were first subjected to reversal learning and the other half to negative patterning. Each learning protocol was spaced by three foraging bouts where bees could collect sucrose solution at the entrance of the maze without any stimuli present. Only motivated foragers which completed both tasks and returned quickly to the experimental set-up (< 10 min, usually 2–5 min) were kept for analyses. The whole procedure took around 6 to 8 h per bee.

4.4.1.2 Reversal learning protocol

In the 1st phase of reversal learning, bees had to distinguish between two odorants, linalool and 2-hexanone (Sigma- Aldrich Chemie GmbH), one being associated with a reward of sucrose solution (A+) while the second odorant was associated with a punishment of quinine solution (B−). The odorants could be easily discriminated by bees (Laska et al. 1999). Half of the bees were trained with linalool as stimulus A and hexanone as stimulus B while odor identity was exchanged for the other half. Then in the 2nd phase the reward contingencies of the previous phase were reversed (A− vs. B+). Each phase amounted to 10 trials i.e. foraging bouts. Each acquisition phase was directly followed by two unreinforced retention tests (where the side of the stimuli were swapped between tests) presenting fresh stimuli.

4.4.1.3 Negative patterning protocol

The acquisition phase consisted of three types of trials presented in a pseudo-random order: C+ and D+ trials presented these rewarded odorants in both arms of the Y-maze. CD− trials offered the non-rewarded CD− compound vs. a rewarding alternative odorant X+, which was used to keep the bees coming to the setup (see above). Limonene was used as C+, 2-octanol as D+ and Nonanal a X+. All odor- ants were obtained from Sigma-Aldrich Chemie GmbH. These

odorants can all be well discriminated by the bees (Laska et al. 1999). The whole acquisition consisted of 20 trials, including 5 C+, 5 D+ and 10 CD-/X+ trials. After the acquisition phase, two non-reinforced tests were conducted, both in the absence of reinforcement, one presenting C vs. CD and the second presenting D vs. CD.

4.4.2 Statistical analysis

As in experiment 1 test data were used to assess the individuals' learning performances as we did not observe, at the individual level, a sigmoidal increase of performance in the acquisition starting at a 50% random choice level and increasing significantly as classically observed at the group level (see Fig S6 for analysis of the acquisition and test phases at the group level). We thus used the percent- age of correct choices in the non-reinforced tests to assess individual consistency across the three leaning tasks tested. Individual consistency across the different tasks was tested using Spearman rank correlations between test performances. Reversal learning ability can only be tested on individuals that successfully acquired the first A+B− association (Mota and Giurfa 2010). In both experiments, most bees chose preferentially A in the test following the first phase and were consequently kept for analysis of their reversal learning ability (*n = 20 of 22*). We decided to use an arbitrary threshold of 60% correct choices in the test to con- sider a bee as learner or non-learner. To assess whether the order in which the learning tasks were conducted or which stimulus was rewarded in reversal learning influenced test performances generalized linear mixed models (GLMM) were used (see the statistical analysis paragraph of experiment 1 for a detailed description of the GLMMs and model selection procedure, Tables S10–S12).

4.4.3 Results

We analyzed if individual performances of free-flying bees (*n = 22*) in a reversal learning discrimination correlated with performances in a negative-patterning problem, both established using olfactory stimuli. As before, we performed separated analyses between negative-patterning performances and performances in the 1st and 2nd phases of reversal learning. Only bees that successfully learned the initial discrimination of the reversal learning (≥ 60% correct choices in the test) were used for correlations including the 2nd reversal learning phase as successful reversal learning requires learning of the initial discrimination. There were no

significant effects of the sequence of problems trained (*order*) or of the stimulus which was rewarded in reversal learning (*group_RL*) on test performances (GLMM: *Order: n = 22*, **1st RL:** $\chi^2_{(1)} = 2.03$, $p = 0.15$, **2nd RL:** $\chi^2_{(1)} = 0.05$, $p = 0.85$, **NP:** $\chi^2_{(1)} = 0.04$, $p = 0.82$, tables S10-12; *group_RL: n = 22*, **1st RL:** $\chi^2_{(1)} = 0.17$, $p = 0.68$, **2nd RL:** $\chi^2_{(1)} = 0.35$, $p = 0.55$, **NP:** $\chi^2_{(1)} = 1$, $p = 0.32$, tables S10-S12).

Figure 3 shows that test performances remained consistent across the two phases of reversal learning (Spearman rank correlation, $n = 20$, $rho = 0.6$, $p = 0.006$, $R^2 = 0.25$, Fig. 3A, Table 1). The two bees that were excluded from this analysis, as they did not learn the initial discrimination, also did not show any sign of learning in the 2nd phase of reversal learning (38% and 50% correct choices). Test performances also remained consistent across the 1st phase of reversal learning and negative patterning ($n = 22$, $rho = 0.46$, $p = 0.03$, $R^2 = 0.25$, Fig. 3B, Table 1). As for the visual modality, no significant correlation was found between test performances of the 2nd phase of reversal learning phase and negative patterning ($n = 20$, $rho = 0.19$, $p = 0.41$, $R^2 = 0.03$, Fig. 3C, Table 1). Here one excluded bee also failed in negative patterning while the other just reached the learner threshold (62% correct choices).

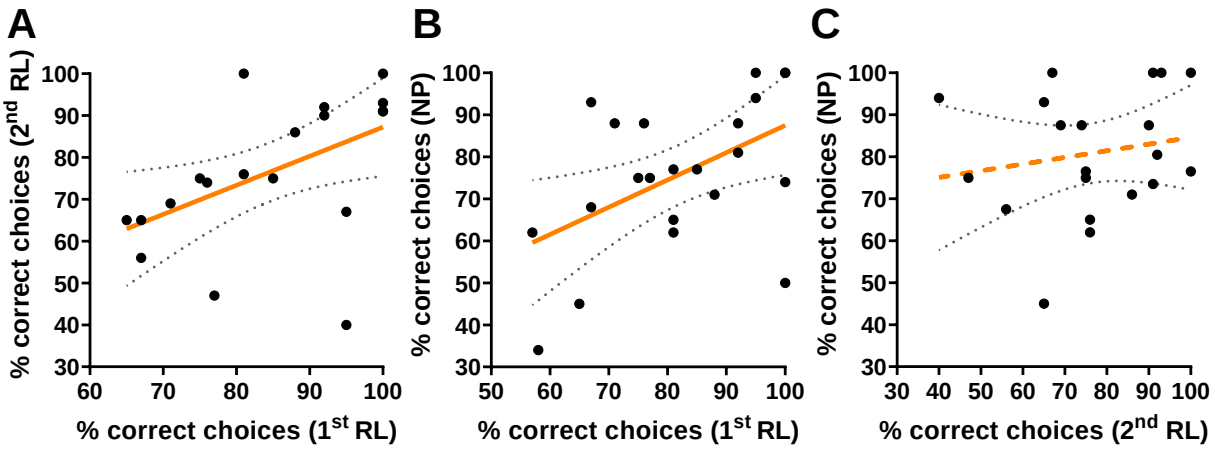

Fig. 3 Correlations of the individual test performances in Experiment 2: Olfactory learning experiment with free-flying bees. Pairwise Spearman rank correlations between test performances (Percent of correct choices) of individual bees in **A** the 1st phase of reversal learning (1st RL) and the 2nd phase of reversal learning (2nd RL; $n = 20$, $rho = 0.6$, $p = 0.006$, $R^2 = 0.25$), **B** the 1st phase of reversal learning and negative patterning (NP; $n = 22$, $rho = 0.46$, $p = 0.03$, $R^2 = 0.25$) and **C** the 2nd phase of reversal learning and negative patterning ($n = 20$, $rho = 0.19$, $p = 0.41$, $R^2 = 0.03$). Each dot represents the data of one bee. A regression line is indicated in orange and the dotted lines show the 95%-confidence intervals of the regression. Solid regression lines indicate a significant correlation and dashed lines indicate a non-significant correlation (color figure online)

4.5 Experiment 3: visual learning in restrained bees

4.5.1 Material and methods

4.5.1.1 General methods

The day before the learning experiments, returning non-pollen foragers from a single colony were individually caught in glass vials at the hive entrance. The vials were placed on crushed ice until the bees ceased their movements. They were then harnessed in plastic tubes with their heads fixed by two metal pins, allowing only minimal movements (Dobrin and Fahrbach 2012; Mancini et al. 2018). Thirty minutes after fixation, the bees were fed with 10 µl of sucrose solution (30% weight/weight) and stored in a dark and humid box at room temperature (~ 25 °C) for approximately 15 h. Experiments were performed in a dark room under weak red-light illumination, invisible for the bees, using the set-up described in detail by (Mancini et al. 2018). The experimental set-up consisted of a box with five chambers (10 × 10 × 10 cm) covered by movable red Plexiglas® ceilings preventing light stimulation between trials. In each chamber a bee was positioned vertically at 4 cm distance in front of a tracing paper screen (10 × 10 cm) onto which the visual stimuli were projected. Conditioned stimuli were different between protocols and are described below.

Bees (*n = 140*) were first tested for intact PER by stimulating the antennae with a sucrose solution (50% weight/weight). Only bees that fully extended their proboscis, i.e. showing high motivation for the reinforcement (Scheiner et al. 1999, 2005), were included in the experiments. Thirty minutes prior to the start of the experiment the bees were placed in the conditioning chambers to habituate to the set-up. Both learning protocols followed the same standardized protocol. Ten bees were conditioned in "parallel", i.e. they completed one trial, one after the other. Each trial lasted 30 s and the inter-trial interval was five minutes. In rewarded trials, the stimulus was presented for 16 s and a 50% sucrose solution was delivered 14 s after onset of stimulus presentation with two seconds overlap and two seconds of reward alone. In unrewarded trials the stimulus was presented for 16 s in the absence of reward. For the remaining 12–14 s of each trial the bee remained in its position without stimulation. Sucrose was delivered by touching the bees' antennae with a toothpick soaked in the sucrose solution to trigger the PER and allowing then the licking of the solution with their proboscis. Before US onset, the toothpick was always kept outside of the chamber to avoid responses to water vapor (Kuwabara 1957). Once the acquisition phase was completed, non-reinforced tests were conducted five minutes after the last trial. During tests, each trained stimulus was presented

once during 16 s without reinforcement. The order in which the stimuli were presented during acquisition and in the test was pseudo-randomized. For each acquisition and test trial the conditioned response to the colors (i.e. extension of the proboscis; 1 = response, 0 = no response) was recorded only during the 14 s of visual stimulation alone. Importantly, a response was scored differently compared to most studies on olfactory PER conditioning. Usually, a response is scored if the proboscis extends beyond a virtual line between the open mandibles (Deisig et al. 2002, 2003; Komischke et al. 2005; Matsumoto et al. 2012). However, such a strong response to the visual stimuli was almost never observed in pilot experiments (< 10%) even though the bees responded to the sucrose solution with a full PER. In consequence, a PER was scored as positive once the proboscis extended by 45° from its resting position, so once the proboscis reached a virtual line between the open mandibles. The reasons for this difference in PER strength to visual and olfactory stimuli are unclear but it may reflect the capacity and pertinence of PER to reflect learning for stimuli of both modalities. While visual information may guide distantly the bees' approach to a visual target, odorants might be more relevant at a closer range, for instance upon landing, and may thus act as triggers of proboscis extension.

After the retention test, PER integrity was checked again and bees that did not respond were discarded from the analyses (< 5%). Additionally, all bees that did not respond to the sucrose stimulation in any conditioning trial were also discarded. The bees were subjected to both learning protocols (reversal learning and negative patterning) on the same day spaced by one hour resting time to ensure a high appetitive motivation. Only bees that completed both tasks were kept for analysis. The order in which the bees were subjected to each protocol was randomized across test days. Conditioning and testing took 7.5 h for 10 bees.

4.5.1.2 Reversal learning protocol

In the 1st phase of reversal learning, one visual stimulus (A+) was associated with a sucrose reward while another visual stimulus was not reinforced (B−). The visual stimuli were colored discs (3 cm diameter) projected onto the tracing paper screen of the conditioning chamber via an optic fiber connected to a monochromator (Polychrome V®, Till Photonics, Germany). A custom-made software controlled the stimuli wavelengths, their intensity, the onset- and off-set of visual stimuli, and the inter-trial interval. Each colored disc subtended a visual angle of 40° to the bees' eye, ensuring perception of the chromatic properties of the stimuli (Giurfa et al. 1996b, 1997; Mancini et al. 2018). The stimuli were monochromatic lights peaking at either

400 or 600 nm and appeared violet and orange to the human's eye respectively (see Fig. S4 for the spectral reflectance curves of the stimuli and their positions in the hexagon color space). The colors of the stimuli were separated by 0.67 hexagon units which is sufficient to be discriminated by the bees (Chittka 1992; Dyer and Neumeyer 2005; Avarguès-Weber et al. 2010a). Half of the bees were trained with violet as stimulus A and orange as stimulus B, while the other half had color identity reversed. Once the 1st phase was completed, the bees remained in the experimental set-up for 30 min before the start of the 2nd phase. In this phase, the previously rewarded stimulus became unreinforced (A−) and the unreinforced stimulus became associated with a reward (B+). Both acquisition phases consisted of 16 trials in total, with the rewarded and the unrewarded stimuli presented eight times each in a pseudo-random sequence. Each acquisition phase was followed by two consecutive non-reinforced tests, each presenting once one of the two training stimuli.

4.5.1.3 Negative patterning protocol

The acquisition phase consisted of 32 trials which were divided into eight blocks of four trials. Each block contained one presentation of each of the two elemental stimuli (C+ and D+) which were rewarded with 50% sucrose solution and two non-reinforced presentations of the compound stimulus (CD−). The stimuli were striped patterns (5 × 5 cm) subtending 64° to the bees' eyes (Buatois et al. 2020). C+ and D+ consisted of either pure green or blue stripes respectively (RGB system: 0.255.0 and 0.0.255, see Fig. S4 for the spectral reflectance curves of the stimuli and their positions in the hexagon color space) on a black background while the compound CD- was composed of alternating blue and green stripes (Buatois et al. 2020). The colors of the stimuli were separated by 0.39 hexagon units, a color distance that granted color discrimination (Chittka 1992; Dyer and Neumeyer 2005; Avarguès-Weber et al. 2010a). All stimuli had 5 colored stripes, whereas the outer two stripes were 0.6 cm wide and the three inner stripes were 1.2 cm wide. The stripes subtended a visual angle of 17° to the bees' eyes, thus being perceived and discriminated based on their chromatic properties (Giurfa et al. 1996b, 1997; Hempel de Ibarra et al. 2002). Each of the three stimulus types (C+, D+ and CD−) had two variants with opposing stripe sequence to prevent learning based on fixed retinotopical images (Wehner 1972; Gould 1985; Giurfa et al. 1995a). Stimuli were projected on a tracing paper screen with a video projector (Acer K1351, Acer Inc., Taiwan). The acquisition phase was followed by three consecutive non-reinforced tests, each presenting once each of the three stimuli.

4.5.2 Statistical analysis

For individual analysis, a bee was characterized as 'learner', scored as 1 for the analysis, if it responded correctly in the test following each learning protocol (1st phase of reversal learning: response to A and not to B; 2nd phase of reversal learning: response to B and not to A; negative patterning: response to C and to D but not to CD, Mancini et al. 2018). All bees that exhibited other patterns of responses were considered as 'non-learners' and scored as 0 for the analysis. A more detailed analysis of the bees' group acquisition and test performances can be found in the supplementary (Figs. S7 and S8). Unfortunately, we were not able to establish a satisfactory learning score from the acquisition phase to allow performances comparison between individuals. Indeed, scoring "1" each correct PER to the CS+ is not sufficient to characterize learning as both for the reversal learning paradigm and negative patterning paradigm, an absence of response to the CS- is also mandatory. Any arbitrary scoring method considering e.g. +1 for a CS + response and −1 for a CS− response would lead to ambiguity in interpreting the resulting score. For example, a bee scored '0' could have been none responsive to any stimulus or responsive to all stimuli. Individual consistency in learning performance was analyzed using Spearman rank correlations. Only bees characterized as 'learners' (*n = 61 of 140* bees) in the test following the 1st phase of the reversal learning protocol were kept for analysis of the 2nd phase of reversal learning, as 'success' or 'failure' in the 2nd phase of reversal learning can only be assessed in bees that learned the initial association established in the 1st phase of reversal learning. Consequently, we could not correlate statistically the test performances of the 1st phase with those of the 2nd phase as correlations can only be performed when the data has more than one value (only learners of the first phase had to be used and in consequence all their responses were scored as 1). To assess if the order in which the experiments were conducted or the stimuli used (*group_RL*) had an influence on the test performances, GLMMs were used. The models with a binomial error structure and logit-link function included proboscis extensions made in the test (1 = PER, 0 = No PER) as dependent variable and the order of the tasks (*order*), the rewarded stimulus (*group_RL*) and the type of CS (*CS*) as fixed factors. The bees' identity (*subject*) was included as a random factor. Different models were calculated by gradually removing factors and compared with an ANOVA. P-values from these comparisons were provided to account for each factor impact. The model with the lowest AIC value was chosen as most appropriate fit (see tables S16-18).

All GLMMs were performed using R Statistical Software version 3.6.3 (R Core Team 2022) with the package lme4 (Bates et al. 2015). All other statistical analyses and graphs were

performed using GraphPad prism version 9.0.0 (GraphPad Software Inc., San Diego, California, USA). The significance level was $\alpha = 0.05$ to account for our relatively small sample sizes (Lakens et al. 2018). For all correlations of the test performances across the three learning tasks, the null hypothesis was that the correlation coefficient rho was not different from zero.

4.5.3 Results

We studied the learning performances of restrained bees (*n = 140*) conditioned with visual stimuli using a visual variant of the proboscis extension response (PER) protocol. Our goal was again to correlate performances across the two phases of reversal learning and between the reversal learning phases and negative patterning. Importantly, neither the order in which the learning tasks were trained (*order*), nor which stimulus was rewarded in reversal learning (*group_ RL*) had a significant effect on the test results (GLMM: *order: n = 140,* **1st RL:** $\chi^2_{(1)}$ *= 0.04, p = 0.8,* **2nd RL:** $\chi^2_{(1)}$ *= 0.002, p = 0.97,* **NP:** $\chi^2_{(1)}$ *= 0.04, p = 0.82, tables S10-12; group_RL: n = 140,* **1st RL:** $\chi^2_{(1)}$ *= 0.84, p = 0.3,* **2nd RL:** $\chi^2_{(1)}$ *= 3.74, p = 0.06,* **NP:** $\chi^2_{(1)}$ *= 1.58, p = 0.21,* tables S16-S18). As mentioned above, success ('learner') or failure ('non-learner') in the 2nd phase of reversal learning is only informative if the bees successfully acquired the initial A+ B− discrimination in the 1st phase of reversal learning. Due to this, we could only use learner bees of the 1st phase (bees with score = 1) for any correlation involving the performances in the 2nd phase of reversal learning. As correlation analyses require at least two different values within each data frame we were mathematically unable to correlate performances of the 1st and the 2nd phase of reversal learning. Nevertheless, we observed that the majority of learners in the 1st phase was also successful in the 2nd reversal phase (59%, Fig. 4A). In the case of the excluded bees that were non- learners in the 1st phase, 84% (*n = 66 out of 79* non-learners) in the 1st phase of reversal learning remained non-learners in the 2nd phase of reversal learning. Only 16% of the excluded non-learner bees (*n = 13*) learned to discriminate the two stimuli in the 2nd phase of reversal learning despite having failed to learn in the 1st phase. Individual test performances in the 1st phase of reversal learning and in negative patterning were significantly positively correlated (Spearman rank correlation; *n = 140, rho = 0.18, p = 0.03,* Fig. 4B, Table 1). No significant correlation was found between the test ranks of the 2nd phase of reversal learning and negative patterning (*n = 61, rho = 0.18, p = 0.17,* Fig. 4C, Table 1). This pattern of correlation could be intuited by the fact that only a minority of non-learner bees in the 1st phase of reversal learning was successful in the negative

patterning protocol (25%), suggesting that being able to solve an elemental task might be a prerequisite to be able to solve negative patterning. By contrast, half of the bees (51%) that failed in 2nd phase despite being successful in the 1st phase of reversal learning were nevertheless successful in the negative patterning discrimination (Fig. 4B and C).

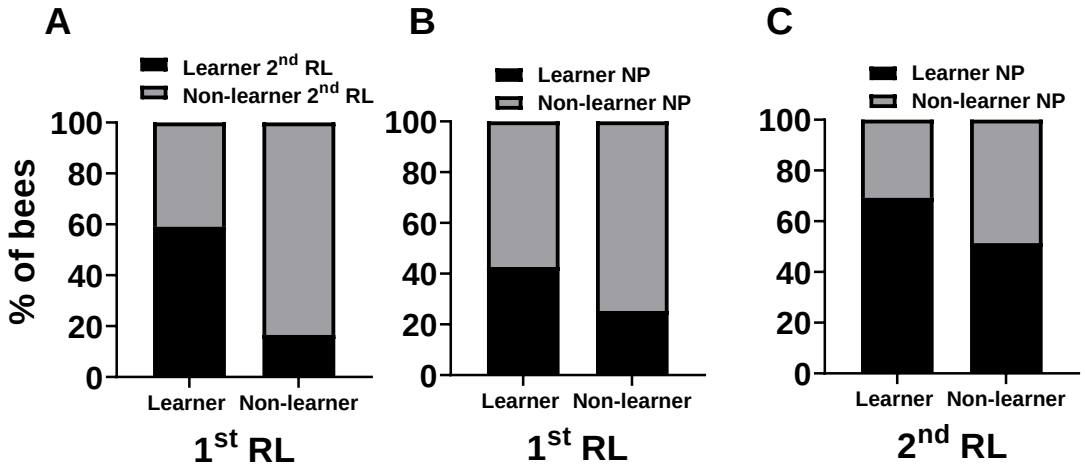

Fig. 4 Correlations of the individual test performances in Experiment 3: Visual learning experiments with restrained bees. A A direct statistical correlation could not be performed, as we could only use the learner bees in the 1st phase as successful reversal of reward contingencies in the 2nd phase of reversal learning prerequisites learning the initial discrimination. We still observed that a majority of the learners in the 1st phase of reversal learning (1st RL) were also successful in the 2nd phase of reversal learning (2nd RL; 59%). 84% of the bees that failed to learn in the 1st phase, and were thus not used for the correlation, remained non-learners in the 2nd phase of reversal learning. **B** The individual test performances were positively correlated between the 1st phase of reversal learning and negative patterning (NP; Spearman rank correlation; *n = 140, rho = 0.18, p = 0.03*). Indeed, the majority of learners (57%) and non-learners (75%) in the 1st phase of reversal learning remained in their category in the negative patterning paradigm. **C** The individual test performances of the 2nd phase of reversal learning were not significantly correlated with negative patterning (Spearman rank correlation;). While 69% of the learners in the 2nd phase of reversal learning were also successful in negative patterning, half of the bees (51%) that failed in the 2nd phase of reversal learning were nevertheless successful in negative patterning

4.6 Experiment 4: olfactory learning in restrained bees

4.6.1 Material and methods

4.6.1.1 General methods

Returning non-pollen foragers were caught at the entrance of a single hive in the morning of each experimental day. The bees were anaesthetized on crushed ice until they ceased their movements and were then harnessed individually in metal tubes so that only the mouthparts and antennae could be moved freely (Bitterman et al. 1983). The bees were fed with 2 µl of a sucrose solution (50%, w/w) and stored in a dark and humid box at room temperature for two

hours before the start of the learning experiments. The experimental set-up for the two learning paradigms consisted of a bee holder facing olfactory stimulation and an air extractor providing a constant airflow behind the bees to avoid odors to stagnate. The odor stimulation was done manually using 20 ml syringes containing a filter paper soaked with 4 µl of the concerning odor. The timing of odor and sucrose stimulation as well as the inter-trial interval was controlled by the custom-written software program "TimingProtocol" (Lichtenstein et al. 2018)

Before the start of the learning experiments, the bees were tested for an intact PER by touching their antennae with a sucrose solution (50%, w/w). Only bees (*n = 89*) that responded with an extension of the proboscis were kept for the experiments. Additionally, the bees were tested for spontaneous PER to all conditioned odors. Bees that showed such spontaneous responses were not used for the experiments. Both learning protocols followed the same standardized methodology. Each conditioning trial lasted for 30 s and 10 bees were conditioned in "parallel", i.e. they completed one trial, one after the other. In this way, the inter-trial-interval was five minutes. A trial started when a bee was positioned in front of the air extractor. After 14 s of familiarization with the experimental context, the odorant was delivered for four seconds. In rewarded trials, a toothpick soaked in sucrose solution was first delivered to the antennae to trigger the PER and then to the mouthparts so the bees could lick the solution for three seconds with one second overlap to odor stimulation. The toothpick was kept distant from bees before sucrose stimulation to avoid responses due to water vapor (Kuwabara 1957). During unrewarded trials, the timing remained identical, but no reward was given to the bees. After CS-US stimulation or CS stimulation only, the conditioned bee stayed in its position. Five minutes after completing each acquisition phase, two non-reinforced tests were conducted in which each CS was presented sequentially once without reinforcement. After the tests, the bees were checked again for intact PER. The order in which the stimuli were presented during the acquisitions and in the tests was pseudo-randomized. A conditioned response (a full extension of the proboscis beyond the imaginary line connecting the open mandibles) was recorded if the bee extended the proboscis (1 = response, 0 = no response) during the two seconds of odor stimulation. The two learning protocols were conducted on the same day spaced by one resting hour. Half of the bees were first subjected to the reversal learning protocol and then to negative patterning while the other half experienced the reversed sequence. Bees that did not respond to the sucrose solution with a proboscis extension during any acquisition trial were discarded from the analyses (*n = 1*). Overall, the conditioning and testing phases lasted 5.5 h for 10 bees.

4.6.1.2 Reversal learning protocol

The bees were first subjected to the 1st phase of reversal learning during which one odorant was presented in association with a sucrose reward (A+) while a second odorant was not reinforced (B−). Two non-reinforced tests presenting sequentially stimuli A and B once, where conducted after the first acquisition phase. Thereafter, the 2nd phase was initiated after a resting time of 30 min. In this phase, the previously rewarded stimulus was unrewarded (A−) while the previously unrewarded stimulus was rewarded (B+). The reversal learning phase was again followed by non-reinforced tests presenting both stimuli A and B once. Both phases consisted of 5 CS+ trials and 5 CS− trials in a pseudo-random sequence. The two odorants were pure solutions of 2-hexanone and linalool (Sigma-Aldrich Chemie GmbH). The choice of A and B identity was balanced between odorants and bees.

4.6.1.3 Negative patterning protocol

During the acquisition phase, the bees were subjected to 20 trials divided into five blocks of four trials. One block consisted of one presentation of the odorant limonene (C+) paired with a sucrose reward, one presentation of the odorant 2-octanol (D+) also paired with sucrose and two presentations of the mixture (CD−), which was not reinforced. All odorants were obtained from Sigma-Aldrich Chemie GmbH. The sequence in which stimuli were presented was pseudo- randomized across the six blocks of trials. The subsequent non-reinforced retention tests consisted of one pseudo-randomized presentation of C, D and CD each, one after the other.

4.6.2 Statistical analysis

For individual analysis, a bee was characterized as ‘learner’, scored as 1 for the analysis, if it responded correctly in the series of tests following each learning protocol (1st phase of reversal learning: response to A and not to B; 2nd phase of reversal learning: response to B and not to A; negative patterning: response to C and to D but not to CD, Mancini et al. 2018). All bees that exhibited other patterns of responses were considered as ‘non-learners’ and scored as 0 for the analysis. A more detailed analysis of the bees’ group acquisition and test performances can be found in the supplementary (Figs. S9 and S10). Unfortunately, we were not able to establish a satisfactory learning score from the acquisition phase to allow performances comparison between individuals. Indeed, scoring “1” each correct PER to the CS + is not sufficient to

characterize learning as both for the reversal learning paradigm and negative patterning paradigm, an absence of response to the CS- is also mandatory. Any arbitrary scoring method considering e.g. +1 for a CS + response and −1 for a CS− response would lead to ambiguity in interpreting the resulting score. For example, a bee scored '0' could have been none responsive to any stimulus or responsive to all stimuli. Individual consistency in learning performance was analyzed using Spearman rank correlations. Only bees characterized as 'learners' (*n = 42 of 89* bees) in the test following the 1st phase of the reversal learning protocol were kept for analysis of the 2nd phase of reversal learning, as 'success' or 'failure' in the 2nd phase of reversal learning can only be assessed in bees that learned the initial association established in the 1st phase of reversal learning. Consequently, we could not correlate statistically the test performances of the 1st phase with those of the 2nd phase as correlations can only be performed when the data has more than one value (only learners of the first phase had to be used and in consequence all their responses were scored as 1). To assess if the order in which the experiments were conducted or the stimuli used (*group_RL*) had an influence on the test performances, GLMMs were used. (see the statistical analysis paragraph of experiment 3 for a detailed description of the GLMMs and model selection procedure, Tables S22–S24).

4.6.3 Results

We subjected restrained bees (*n = 89*) to olfactory PER conditioning and determined if performances in the two phases of a reversal learning problem were correlated with performances in a negative-patterning problem. Again, the sequence in which the problems were trained (*order*) and the stimulus identity in reversal learning (*group_RL*) had no influence on the test performances (GLMM: *Order: n = 89,* **1st RL:** $\chi^2_{(1)}$ *= 3.07, p = 0.08,* **2nd RL:** $\chi^2_{(1)}$ *= 0.10, p = 0.75;* **NP:** $\chi^2_{(1)}$ *= 0.51, p = 0.47,* tables S22-S24*; group_RL: n = 89,* **1st RL:** $\chi^2_{(1)}$ *= 0.01, p = 0.9,* **2nd RL:** $\chi^2_{(1)}$ *= 0.76 p = 0.38,* **NP:** $\chi^2_{(1)}$ *= 0.13, p = 0.72,* tables S22-S24).

To analyze individual consistency across tasks including the 2nd phase of reversal learning, and for the reasons already explained, we used only bees that learned the discrimination of the 1st phase of reversal learning (*n = 41*). As in the experiment on visual PER conditioning, we could not correlate statistically the individual test performances of the 1st and 2nd phase of reversal learning in these bees. Yet, half of learners in the 1st phase (52%) were also successful in the 2nd phase of reversal learning (Fig. 5A). In the case of the bees that were excluded as non-learners due to their performance in the 1st phase, 91% (*n = 43 out of 47*)

remained non-learners in the 2nd phase of reversal learning. Test performances were significantly positively correlated in the 1st phase of reversal learning and in negative patterning (Spearman rank correlation; *n = 89, rho = 0.33, p = 0.002,* Fig. 5B, Table 1). However, there was no significant correlation between test performances in the 2nd phase of reversal learning and negative patterning (*n = 42, rho = 0.15, p = 0.36,* Fig. 5C, Table 1). As in the experiment on visual PER conditioning, this lack of correlation is mainly due to the fact that while only 17% of non-learners in the 1st phase of reversal learning learned successfully the negative patterning problem, 40% of the non-learner bees in the 2nd phase of reversal learning succeeded in negative patterning (Fig. 5B and C).

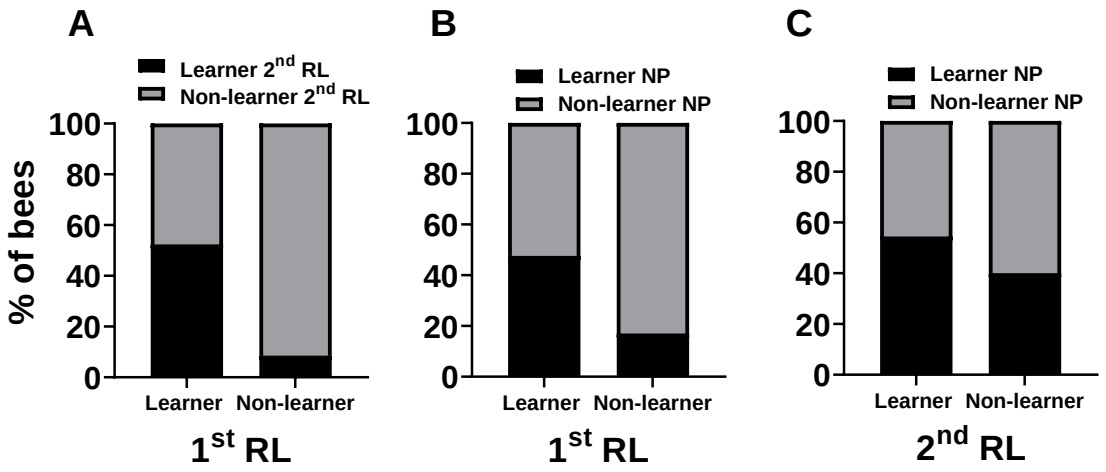

Fig. 5 Correlations of the individual test performances in Experiment 4: Olfactory learning experiments with restrained bees. A A direct statistical correlation could not be performed, as we could only use the learner bees in the 1st phase as successful reversal of reward contingencies in the 2nd phase of reversal learning prerequisites learning the initial discrimination. We still observed that many of the learners in the 1st phase of reversal learning (1st RL) were also successful in the 2nd phase of reversal learning (2nd RL; 53%). 91% of the bees that failed to learn in the 1st phase, and were thus not used for the correlation, were also non-learners in the 2nd phase of reversal learning. **B** The individual test performances were positively correlated between the 1st phase of reversal learning and negative patterning (2nd RL; Spearman rank correlation*; n = 89, rho = 0.33, p = 0.002*). Indeed, many learners (48%) and non-learners (83%) in the 1st phase of reversal learning remained in their category in the negative patterning paradigm. **C** The individual test performances of the 2nd phase of reversal learning were not significantly correlated with negative patterning (NP; Spearman rank correlation; *n = 42, rho = 0.15, p = 0.36*). While 55% of the learners in the 2nd phase of reversal learning were also successful in the negative patterning task, almost half of the bees (40%) that failed in the 2nd phase of reversal learning were nevertheless successful in the negative patterning task

4.7 Discussion

We focused on individual learning performances of honey bees to determine if learning proficiency is maintained at the individual level across learning tasks differing in cognitive complexity and processing (elemental or non-elemental discriminations requesting cognitive flexibility or configural abilities). We replicated this analysis using discrimination tasks involving different sensory modalities (olfaction or vision), distinct types of conditioning (Pavlovian, in the case of harnessed bees, or a combination of operant and Pavlovian in the case of free-flying bees) and various experimental set-ups and restricting or not freedom of movement (rotating screen or Y-maze for free-flying conditions; restrained conditions in PER conditioning experiments or free movement in free-flying experiments). Across these multiple scenarios, learning performances exhibit appreciable inter-individual variation. Interestingly, the individual performances remained consistent across some, but not all protocols tested. The individual bees' proficiency to solve an elemental association in the 1st phase of reversal learning (A+ vs. B−) correlated positively with the performance in the 2nd phase (A− vs. B+, Table 1) and negative patterning (C+ and D+ vs. CD−, Table 1). However, we did not find a significant correlation between performances in the 2nd phase of reversal learning and negative patterning, i.e. between the task that requires overcoming transient stimulus ambiguity and the configural task, respectively (Table 1). Interestingly, this pattern of correlation was stable irrespective of the training method and the sensory modality. We therefore conclude that the pattern of correlations observed is a real characteristic of the bees' cognitive profile.

Studies on bumble bees (*Bombus terrestris*) and fruit flies (*Drosophila melanogaster*) have also demonstrated a positive association between the 1st and 2nd phase of a reversal learning problem within a single modality (bumble bees, visual learning: Raine and Chittka 2012; fruit flies, olfactory learning: Smith et al. 2022). We now extend this conclusion to honey bees tested in two sensory modalities with either Pavlovian or operant-Pavlovian conditioning (i.e. using harnessed bees or free-flying bees, respectively). In a previous study (Finke et al. 2021), we also showed that the bees' performance in an elemental visual discrimination task was positively correlated with the performance in a higher- order visual task, a relational concept learning task. In the present study we found no significant association between the individuals' performances in the 2nd phase of reversal learning and negative patterning, suggesting that bees might specialize in some cognitive trait either independently or at the expense of other faculties. Indeed, a trade-off in the performances of bees has been found between appetitive and aversive learning (Junca et al. 2019) or between olfactory and landmark

learning (Tait et al. 2019). Moreover, no correlation, be it positive or negative, was found between visual and olfactory elemental learning (honey bees: Finke et al. 2021; bumble bees: Smith and Raine 2014).

Although cognitive specialization may account for the lack of significant correlation between reversal learning and negative patterning, an alternative hypothesis could be that different strategies are used by individual bees to solve non-elemental problems without being necessarily the consequence of an absence of competence (Komischke et al. 2003; Dyer et al. 2014). Additionally, modifications of motivational state and attention between the different experimental phases may lead to higher variability in the performances which may conceal individual consistency across these tasks. However, this last explanation should only act marginally as we found strong stability of performances across time within a given type of learning task in bees (Finke et al. 2021).

A long-standing question in cognitive sciences is whether cognition is composed of specialized modules that evolved independently of each other, or if and to what extent a general factor (termed 'factor g' by Charles Spearman) accounts for consistent inter-individual variability across multiple cognitive performances (Spearman 1904). The theory of general intelligence, which has been extensively studied in humans and other vertebrates, considers that the performances in multiple cognitive tests are highly correlated given that the g-factor accounts for a large proportion of inter-individual variability in these tests (Jensen 1998; Mackintosh 1998; Plomin and Spinath 2002; Matzel et al. 2003; Galsworthy et al. 2005; Brown and Price 2007; Herrmann and Call 2012). In humans, the g-factor has been correlated with brain parameters and function such as brain size, gray matter substance, cortical thickness, or processing efficiency (Jung and Haier 2007; Deary et al. 2010). These results suggest that mechanisms of general information processing represent an important part of multiple correlated cognitive performances (Deary et al. 2010). In recent years, the topic of domain-general cognition has gained increasing interest in the field of insect neurobiology although studies comparing the performances of individual insects across tasks with distinct cognitive demands are still missing (Simons and Tibbetts 2019). Besides the correlation between the performances in the 1st and 2nd phase of reversal learning found in bees and flies (see above), positive correlations across tasks have also been found between latent inhibition and reversal learning (Chandra et al. 2000) or between an elemental discrimination and a non-elemental concept learning task (Finke et al. 2021) and here between an elemental discrimination and reversal learning or configural learning. Additionally, in some insect species the learning

performance was consistent over time (honey bees: Finke et al. 2021; fruit flies: Smith et al. 2022) and across visual, olfactory and tactile elemental discriminations (bumble bees: Muller and Chittka 2012). Although our data do not provide enough evidence for significant positive correlations between the 2nd phase of reversal learning and negative patterning, it might still be possible that a g-factor accounts for a small proportion of inter-individual variability across the three learning tasks tested but being concealed by inter- individual variability caused by other experimental or intrinsic factors and low sample sizes. Inter-individual differences in cognitive performances have been related in some cases with inter-individual differences in neural anatomy and processing in insect brains (Li et al. 2017; Linneweber et al. 2020; Honegger et al. 2020) providing clear demonstrations of how individuality in behavior can be directly traced back to individual differences in neuronal processing in insects. An alternative explanation to the consistent performance observed within individuals despite the variability existing between individuals might refer to differences in reinforcement motivation. For example, in bees, sucrose responsive- ness, which is used to measure individual sensitivity to the sucrose reward, correlates positively with individual performances in elemental appetitive learning tasks, i.e. bees that show a higher responsiveness and thus responding to a broad spectrum of sucrose concentrations generally learn better than bees with lower responsiveness; Scheiner et al. 1999, 2001a, b, 2005). Since we used sucrose as reward in all our learning tasks, we cannot exclude that a proportion of the inter-individual variability in learning performances observed in our experiments could be attributed to differences in sucrose responsiveness. Yet, this factor cannot fully account for the consistent differences in individual learning performances reported here. Indeed, if it had played a major role, we would have found universal positive correlations across all tasks tested, which was not the case.

Contrary to the theory of general intelligence, the theory of domain-specific cognition postulates that cognition is modular, meaning that distinct mental or cognitive modules rely on specialized mechanisms used to solve specific problems evolving independently (Friederici 1990; Sperber 1994; Shettleworth 2000; Palmer and Palmer 2002). This hypothesis seems confirmed in honey bees by the existence of a trade-off between appetitive and aversive learning capabilities (Junca et al. 2019) despite previous negative results (Roussel et al. 2009) or between olfactory elemental learning and landmark learning and a lack of correlation between visual and olfactory learning (Finke et al. 2021) or here between reversal learning and negative patterning.

Finally, a third theory postulates the co-existence of domain-general and independent domain-specific cognitive modules (Plomin 2001; Brown and Price 2007). Indeed, in humans, some general properties of the brain (e.g. amount of grey matter, processing speed) have general effects on different brain regions and thereby lead to positive correlations among performances in different cognitive domains, even though their specific mechanisms are located in dis- tinct regions of the brain (Jensen 1993; MacLullich et al. 2002; Lee 2007). In honey bees, the adoption of this view was proposed by Menzel and Giurfa (2001), who referred these different levels of modularity to specific brain areas based on their cross-modality or insulation from other processing pathways. For instance, while some olfactory learning forms can be mediated by neural phenomena in pure olfactory regions and circuits (Faber et al. 1999; Rath et al. 2011), being therefore domain-specific, other learning forms require multi-modal regions such as the mushroom bodies, which can be seen as domain-general modules. Thus, domain-specific and domain-general mechanisms may interact to mediate the cognitive abilities in insects. We could thus hypothesize that general stimulus processing abilities within a given sensory modality, general brain structure and metabolism or attentional and working memory capacities mediate cognition in a domain-general manner, thereby deeply influencing performances in any learning task with a given sensory modality or reinforcement type such as found in humans (Chiappe and MacDonald 2005; Matzel and Kolata 2010; Kanai and Rees 2011; Völter et al. 2018). Different regions in the bee brain have been associated with different forms of learning based on the level of stimuli ambiguity: while the mushroom bodies are dispensable for solving elemental discriminations (Malun et al. 2002; Komischke et al. 2005; Devaud et al. 2007), they are indispensable for solving learning tasks with transient or permanent stimuli ambiguity (i.e. reversal learning: Devaud et al. 2007; Boitard et al. 2015; Negative patterning: Devaud et al. 2015). In parallel, the seemingly lack of correlation between reversal learning and negative performance may be due to individual differences in neuronal circuits specialized in these different tasks. While the mushroom bodies are necessary for acquiring non-elemental olfactory tasks both in a negative patterning paradigm (Devaud et al. 2015) and a reversal learning problem (Boitard et al. 2015), the specific neurons involved may be different.

An intriguing perspective of our findings is why stable individual differences in learning proficiency would be maintained within colonies. One potential explanation relies on the potential costs of cognitive functions (Mery and Kawecki 2003, 2004; Mery 2005): could hypothesize that increased ability for a cognitive trait might trade-off against the ability for another trait (Chittka et al. 2003; Hollis and Guillette 2015; Tait et al. 2019; Junca et al. 2019). Consequently, different cognitive abilities in different individuals could lead to task

specialization or specific adaptation to given environmental conditions. However, it remains understudied whether and to what extent inter-individual cognitive variability contributes to fitness and survival of animals (Thornton et al. 2014; Cauchoix and Chaine 2016; Cauchoix et al. 2018). It is often assumed that a mixture of individual strategies in behavioral traits could influence the flexibility of colonies to react to changing environmental conditions (Burns and Dyer 2008; Dyer et al. 2014; Jandt et al. 2014). However, evidence supporting that inter-individual variability in learning performance among workers accounts for differences in their foraging success or their foraging behavior (e.g. scouts and recruits ; Beekman et al. 2007) are still lacking. In bumble bees, colony variation in learning speed in an elemental visual discrimination task was correlated with their foraging success under natural conditions (Raine and Chittka 2008). However, Evans et al. (2017) found that fast and slow bumble bee learners had comparable rates of food collection and even that bees with higher learning proficiency foraged for shorter periods compared to those with lower learning abilities. These results might be the consequence of higher metabolic costs of increased learning proficiency. In any case, more research is thus necessary to link the cognitive abilities of individuals and their level of variability within a hive to their foraging performance (i.e. amount of food resources collected) in the field and under different scenarios of resources avail- ability and distribution.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s10071-022-01741-2

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Author contributions

Conceptualization: AAW, VF; methodology: VF, AAW, RS, MG; Formal analysis and investigation: AAW, VF, RS, MG; writing—original draft preparation: VF; writing—review and editing: VF, AAW, RS, MG; funding acquisition: AAW, VF; resources: RS, AAW, MG; supervision: AAW, RS, MG.

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Data availability

The raw data are available on figshare: https://doi.org/10.6084/m9.figshare.20473 113.v1

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Ethical approval

Our research involved honey bees from apiaries dedicated to research for which an approval of an ethical committee is not mandatory. The protocols comply with standard welfare practice in our field and a minimum number of individuals were used to study our scientific question. The animals were not harmed during the experimental procedures.

4.8 Supplementary material

Individual consistency in the learning abilities of honey bees: Cognitive specialization within sensory and reinforcement modalities

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Analysis of group performances

Statistical analysis

All acquisition performances were analyzed using generalized linear mixed models (GLMM) for repeated measurements with a binomial family including trial number, trial identity (reinforced/non-reinforced/punished for experiments 3 and 4 only), rewarded stimulus (group_RL) and which learning task was conducted first (negative patterning or reversal learning first; order) as fixed factors, subject as a random factor and the individual bee responses (correct = 1 or incorrect = 0) as dependent variable. We also included an interaction between trial identity and trial number. Different models were performed where the factors were gradually removed and compared using an ANOVA. The model with the lowest AIC value was chosen as most appropriate fit (Burnham and Anderson 1998; Panchal et al. 2010; see tables S1-S18). GLMMs were performed using R Statistical Software (v3.6.3; R Core Team 2022) with the package lme4 (Bates et al. 2015).

Experiments 1 & 2: Operant conditioning experiments with free-flying bees

Group acquisition curves of the learning tasks with free-flying bees were created by calculating the percentage of correct choices of all tested bees for each trial. For negative patterning, the acquisition curves consisted solely of the trials with the mixture since the trials with the single stimuli were conducted with absolute conditioning. A paired t-test was used to assess if the bees had learnt the task in each test. Test performances were also analyzed using GLMM with a binomial error structure and logit-link function. The responses (1 = correct, 0 = incorrect) of the bees in the 20 non-reinforced trials in the tests following each learning task were the dependent variable while rewarded stimulus (group_RL) and which learning task was conducted first (negative patterning or reversal learning first; order) as fixed factors. Subject identity was included as a random factor.

Experiments 3 & 4: Classical conditioning with restrained bees

The group acquisition curves of the learning protocols with restrained bees were generated by calculating the proportion of bees that showed the conditioned response upon stimulus presentation during each trial (reversal learning) or block of trials (negative patterning). For the test performances we calculated the percentage of bees showing PER upon stimulus presentation. To evaluate if the bees had learnt the task, test performances were analyzed using a McNemar test. Test performances were also analyzed using GLMM with a binomial error structure and logit-link function. The responses (PER = 1, no PER = 0) of each bee to the stimuli in the non-reinforced tests of each learning task were the dependent variable while rewarded stimulus (group_RL), which learning task was conducted first (negative patterning or reversal learning first; order) and the identity of the CS (CS) were included as fixed factors. Subject identity was included as a random factor.

The bees were also grouped according to their test performance: A bees' performance was characterized as 'correct response' if it responded fully correct in the test (1st phase of reversal learning: Response to A and not to B, 2nd phase of reversal learning: Response to B and not to A, Negative patterning: Response to C and D not to CD), as 'incorrect response' if it responded to all stimuli or only to the unrewarded stimulus and as 'no response' if it did not respond to any stimulus but showed an intact PER to sucrose after the test. Then, we compared the test groups of the bees in the different tests, by calculating the relative percentage of bees belonging to the three test categories in a given learning task in relation to their performance in an alternative task (Figures S6 for experiment 3 and S8 for experiment 4).

Results

Experiment 1: Visual learning experiments with free-flying bees

The bees' group acquisition performances improved significantly across the 1st phase of reversal learning (1st RL), the 2nd phase of reversal learning (2nd RL) and negative patterning (GLMM, *Trial*; N = 33; **1st RL**: χ^2 = 27.24, df = 1, p < 0.0001, Fig. S3A, Table S1; **2nd RL**: χ^2 = 27.1, df = 1, p < 0.0001, Fig. S3B, Table S2; **NP**: χ^2 = 64.60, df = 1, p < 0.0001, Fig. S3C, Table S3). Similarly, the bees' learnt to choose the rewarded stimuli significantly more often than the punished stimuli in both phases of reversal learning (Paired t-test, N = 33, **1st RL:** 71.36 ± 2.43 % of correct choices; t = 8.81, df = 32, p < 0.0001, Fig. S3A; **2nd RL**: 57.88 ± 2.4 % of correct choices; t = 3.28, df = 32, p = 0.003, Fig. S3B). As the performances of the bees in the two retention tests of the negative patterning protocol were not significantly different, they were pooled for analysis (Paired t-test; N = 33, 69.85 ± 2.05 % choices for the blue checkerboard C+ over CD- and 66.52 ± 1.98 % for the pink checkerboard D+ over CD; t = 1.89, df = 32, p = 0.07). The bees learnt to choose the single stimuli significantly more often than their mixture (Paired t-test, N = 33; 68.18 ± 1.81 % choices for C+/D+; t = 9.97, df = 32, p < 0.0001, Fig. S3C). The color used as target stimulus (68N or 3N) in reversal learning did not affect the acquisition performances nor the test performances (Acquisition: GLMM, *group_RL, N = 33, **1st RL:*** χ^2 = 0.01, df = 1, p = 0.91, Table S1; ***2nd RL:*** χ^2 = 0.04, df = 1, p = 0.85, Table S2; ***NP:*** χ^2 = 0.01, df = 1, p = 0.92, Table S3; Test: GLMM, *group_RL, N = 33, **1st RL:*** χ^2 = 2.1, df = 1, p = 0.08, Table S4; ***2nd RL:*** χ^2 = 1.41, df = 1, p = 0.24, Table S5; ***NP:*** χ^2 = 0.15, df = 1, p = 0.70, Table S6). The order in which the learning protocols were conducted had only a significant effect on the acquisition performances of the 2nd phase of reversal learning (GLMM; *order*, N = 33, ***1st RL:*** χ^2 = 3.29, df = 1, p = 0.07, Table S1; ***2nd RL:*** χ^2 = 6.27, df = 1, p = 0.01, Table S2; ***NP:*** χ^2 = 0.08, df = 1, p = 0.78, Table S3).

Experiment 2: Olfactory learning experiments with free-flying bees

Throughout the acquisition phases of the two learning protocols the bees' group performance increased significantly (GLMM; *trial*, N = 22; 1st RL: χ^2 = 38.71, df = 1, p < 0.0001, Fig. S4A; Table S7; 2nd RL: χ^2 = 45.11, df = 1, p < 0.0001, Fig. S4B, Table S8; NP: χ^2 = 28.92, df = 1, p < 0.0001, Fig. S4C, Table S9). In the retention tests of the two phases of the reversal learning paradigm the bees chose the correct stimulus significantly more often than the incorrect option (Paired t-test, N = 22; 1st RL: 81.95 ± 2.96 % of correct choices; t = 10.79, df = 21, p < 0.0001, Fig. S4A; 2nd RL: 73.45 ± 3.99 % of correct choices; t = 5.68, df = 21, p < 0.0001, Fig. S2B).

The performance of the bees in the two retention tests of negative patterning did not differ and were thus pooled for analysis (Wilcoxon signed rank test, N = 22; 76.05 ± 3.27 % choices for Limonene vs. the mixture and 75.09 ± 5.3 % choices for 2-Octanol vs. the mixture; W = 8, p = 0.86). The bees significantly preferred the single stimuli over their mixture in the non-reinforced tests (Paired t-test, N = 22; 75.57 ± 3.84 % choices for C+/D+; t = 6.46, df = 21, p < 0.0001, Fig. S4C). The rewarded stimulus used in reversal learning did not affect the bees' acquisition performances (Acquisition: GLMM, N = 22, *Stimuli;* 1st RL: χ^2 = 0.63, df = 1, p = 0.43, Table S7; 2nd RL: χ^2 = 0.37, df = 1, p = 0.53, Table S8; NP: χ^2 = 2.22, df = 1, p = 0.14, Table S9; Test: GLMM, N = 22, 1st RL: χ^2 = 0.17, df = 1, p = 0.68, Table S10; 2nd RL: χ^2 = 0.35, df = 1, p = 0.55, Table S11; NP: χ^2 = 1, df = 1, p = 0.32).

Experiment 3: Visual learning experiments with restrained bees

The bees' group performance increased significantly throughout the acquisition of all tasks (GLMM: *Trial*; N = 140; 1st RL: χ^2 = 66.04, df = 1, p < 0.0001, Fig. S5A Table S13; 2nd RL: χ^2 = 164.5, df = 1, p < 0.0001, Fig. S5B, Table S14; NP: χ^2 = 168.3, df = 1, p < 0.0001, Fig S5B, Table S15). In the non-reinforced tests following each phase of the reversal learning protocol the bees' showed the ability to discriminate the rewarded from the non-rewarded stimulus (McNemar test, N = 140; 1st RL: 62.1 % of the bees showing a PER to A+ and 21.4 % to B-; χ^2 = 48.25, df = 1, p < 0.0001, Fig S5A; 2nd RL: 60.7 % of the bees showing a PER to B+ and 38.6 % to A-; χ^2 = 13.43, df = 1, p < 0.0001, Fig. S5B). As bees did not respond differently to C and D in the test of the negative patterning protocol, they were pooled for analysis (McNemar test; N = 140; 66.4 % of the bees responding to C and 67.9 % responding to D; χ^2 = 0.06, df = 1, p = 0.81). Taking the pooled data into account the bees significantly preferred the single stimuli C/D over the compound CD in the acquisition and in the non-reinforced test of the negative patterning paradigm (Acquisition: GLMM, N = 140; *CS*: χ^2 = 188.5, df = 1, p < 0.0001, Fig. S5C, Table S15; Retention test: McNemar test; N = 140, χ^2 = 10.4, df = 1 p < 0.001, Fig. S5C). The order in which the learning paradigms were conducted had a significant effect on the acquisition performances of the 2nd phase of reversal learning only (Acquisition: GLMM, N = 140; *Order*: 1st RL: χ^2 = 0.12, df = 1, p = 0.73, Table S13; 2nd RL: χ^2 = 164.5, df = 1, p < 0.0001, Table S14; NP: χ^2 = 3.38, df = 1, p = 0.07, Table S15). There was a significant interaction effect between the trials, the stimuli used and CS across all acquisition phases (Acquisition: GLMM, N = 140, *trials*group_RL*CS*: 1st RL: χ^2 = 40.53, df = 1, p < 0.0001,

Table S13; 2nd RL: $\chi^2 = 164.5$, df = 1, $p < 0.0001$, Table S14; NP: $\chi^2 = 188.5$, df = 1, $p < 0.0001$, Table S15).

Experiment 4: Olfactory learning experiments with restrained bees

The bees' group performance increased significantly throughout the acquisition phases of all tasks (GLMM: N = 89; *Trial*; 1st RL: $\chi^2 = 274.1$, df = 1, $p < 0.0001$, Fig. S7A; 2nd RL: $\chi^2 = 291.8$, df = 1, $p < 0.0001$, Fig. S7B; NP: $\chi^2 = 293.9$, df = 1, $p < 0.0001$, Fig. S7C; Tables S19, S20 and S21). Similarly, the bees showed the ability to discriminate the two stimuli in the tests of the reversal learning protocol (McNemar test; N = 89; 1st RL: 62.92 % of bees responding to A+ and 17.98 % responding to B-; $\chi^2 = 34.57$, df = 1, $p < 0.0001$, Fig. S7A; 2nd RL: 61.8 % responding to B+ and 36 % to B-; $\chi^2 = 16.69$, df = 1, $p < 0.0001$, Fig. S7B). In the test of negative patterning the bees did not respond differently to the two single stimuli C and D and therefore the results were pooled for analysis (McNemar test, N = 89; 65.17 % of the bees responding to Limonene and 56.18 % responding to 2-Octanol; $\chi^2 = 3.36$, df = 1, $p = 0.07$). The pooled data shows that the bees significantly preferred the correct single stimuli C and D compared over the incorrect mixture CD in the test (60.67 % of bees responding to C+/D+ and 20.23 % responding to CD-; McNemar test; $\chi^2 = 29.26$, df = 1, $p < 0.0001$, Fig. S7C). The order in which the learning paradigms were conducted had a significant effect on the acquisition performances of the 1st phase of reversal learning only (Acquisition: GLMM, N = 89; *Order*: 1st RL: $\chi^2 = 5.34$, df = 1, $p = 0.03$, Table S19; 2nd RL: $\chi^2 = 3.83$, df = 1, $p = 0.06$, Table S20; NP: $\chi^2 = 1.6$, df = 1, $p = 0.21$, Table S21). There was a significant interaction effect between the trials, the stimuli used and CS across all acquisition phases (Acquisition: GLMM, N = 89, *trials*group_RL*CS*: 1st RL: $\chi^2 = 232.1$, df = 1, $p < 0.0001$, Table S19; 2nd RL: $\chi^2 = 239.9$, df = 1, $p < 0.0001$, Table S20; NP: $\chi^2 = 239$, df = 1, $p < 0.0001$, Table S21).

Supplementary figures

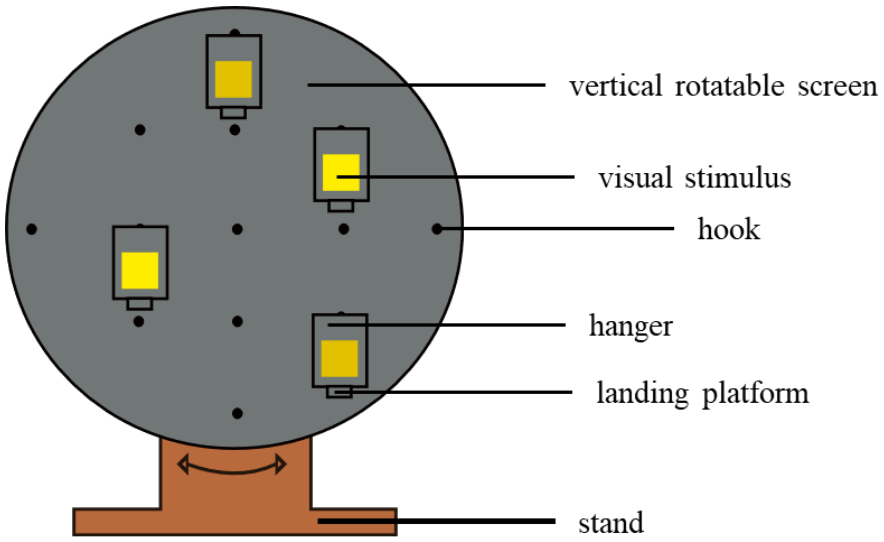

Fig. S1 Schematic overview of the rotating screen apparatus used in experiment 1: Visual learning experiments with free-flying bees. The rotating screen consisted vertical rotatable screen (50 cm in diameter) connected to a stand. The screen displayed hooks at various locations where hangers (6 x 8 cm) could be attached to. During conditioning and testing of the two learning tasks the hangers displayed the visual stimuli (5 x 5 cm). During conditioning the reinforcements of the stimuli could be collected by the bees from the landing platform of the respective hangers. Each stimulus was presented twice on the rotating screen.

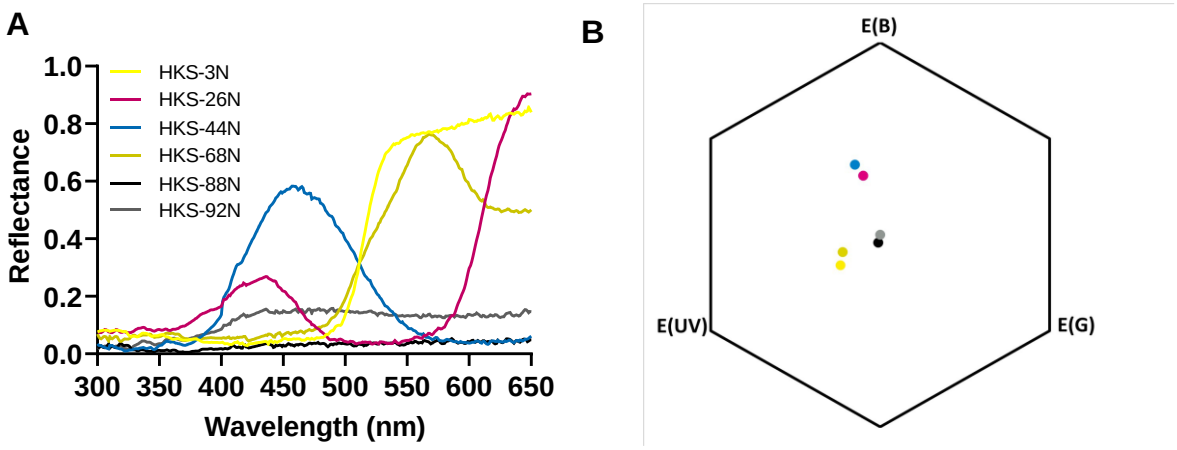

Fig. S2 Spectral properties of the colored stimuli used in reversal learning and negative patterning of experiment 1. The spectral curves were measured with a spectrophotometer (Avantes AvaSpec-ULS2048L). The reflectance curves for the HKS paper stimuli were measured with the Avalight Xenon source and FCR-7UV200-2-M2 optical fiber. The hexagon color space is a model representing color representation from photoreceptor excitation based on generalized color opponency. Perceptual discrimination between colors could be quantify via the Euclidean distance between colored stimuli (Chittka 1992). For the calculations of the hexagon distances of the stimuli, the spectral sensitivities of the honey bee photoreceptors (Peitsch et al. 1992), a standard daylight function D65 (Judd et al. 1964) and the grey background of the hangers were used. **A)** Spectral reflectance curves of the yellow (3N) and greenish-yellow (68N) stimuli used in reversal learning and of the pink (26N), blue (44N), black (88N) and grey (92) colors used to create the stimuli in negative patterning. **B)** Loci of the colored stimuli in a hexagon color space for the trichromatic color vision of honey bees. The distances of the stimuli used in reversal learning (3N and 68N) were 0.07 hexagon units and 0.07 for the colors used in negative patterning (26N and 44N). The mean distance between all stimuli used in experiment 1 was 0.30 ± 0.04 hexagon units.

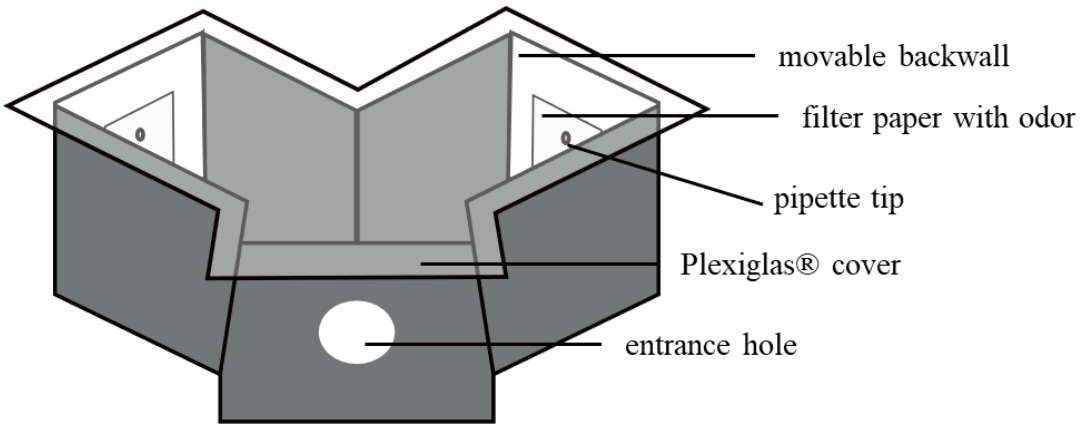

Fig. S3 Schematic overview of the Y-maze apparatus used in experiment 2: Olfactory stimuli and free-flying bees. The maze could be entered by free-flying bees through an entrance hole leading to the two arms of the maze (40 x 20 x 20 cm). The backwalls (20 x 20 cm), coated with white copy paper, displayed the stimuli during conditioning and testing. The stimuli in this experiment were different odors applied to a filter paper (5 x 5 cm) and attached to the backwalls. The reinforcement was provided inside micro pipette tips located in the middle of each backwall. The Y-maze was covered by a UV-transparent Plexiglas® ceiling.

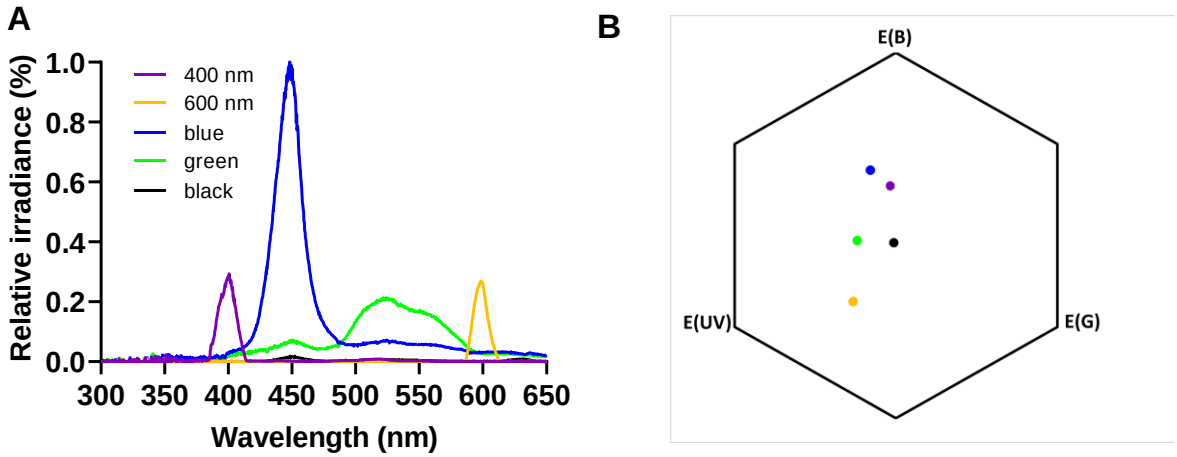

Fig. S4 Spectral properties of the colored stimuli used in reversal learning and negative patterning of experiment 3. The spectral curves were measured with a spectrophotometer (Avantes AvaSpec-ULS2048L). The irradiance curves for the coloured light stimuli were measured with the FC-UV400-2-SR optical fiber after calibration of the spectrophotometer with an halogen source (AvaLight-Hal-Cal-MINI), Ocean Optics SD2000 with a DT1000 mini light source (200–1,100 nm) and R400-7 UV/VIS optical fibre. The hexagon color space is a model representing color representation from photoreceptor excitation based on generalized color opponency. Perceptual discrimination between colors could be quantify via the Euclidean distance between colored stimuli (Chittka 1992). For the calculations of the hexagon distances of the stimuli, the spectral sensitivities of the honey bee photoreceptors (Peitsch et al. 1992) and the residual black light of the room were used. **A)** Normalized irradiance curves of the monochromatic stimuli (generated by a polychromator) used in reversal learning (400nm and 600 nm) and of the blue and green colors (generated by a video projector) used to create the stimuli in negative patterning. **B)** Loci of the colored stimuli in a hexagon color space for the trichromatic color vision of honey bees. The distances of the stimuli used in reversal learning (400 and 600 nm) were 0.67 hexagon units and 0.39 for the colors used in negative patterning (blue and green). The mean distance between all stimuli used in experiment 3 was 0.42 ± 0.01 hexagon units.

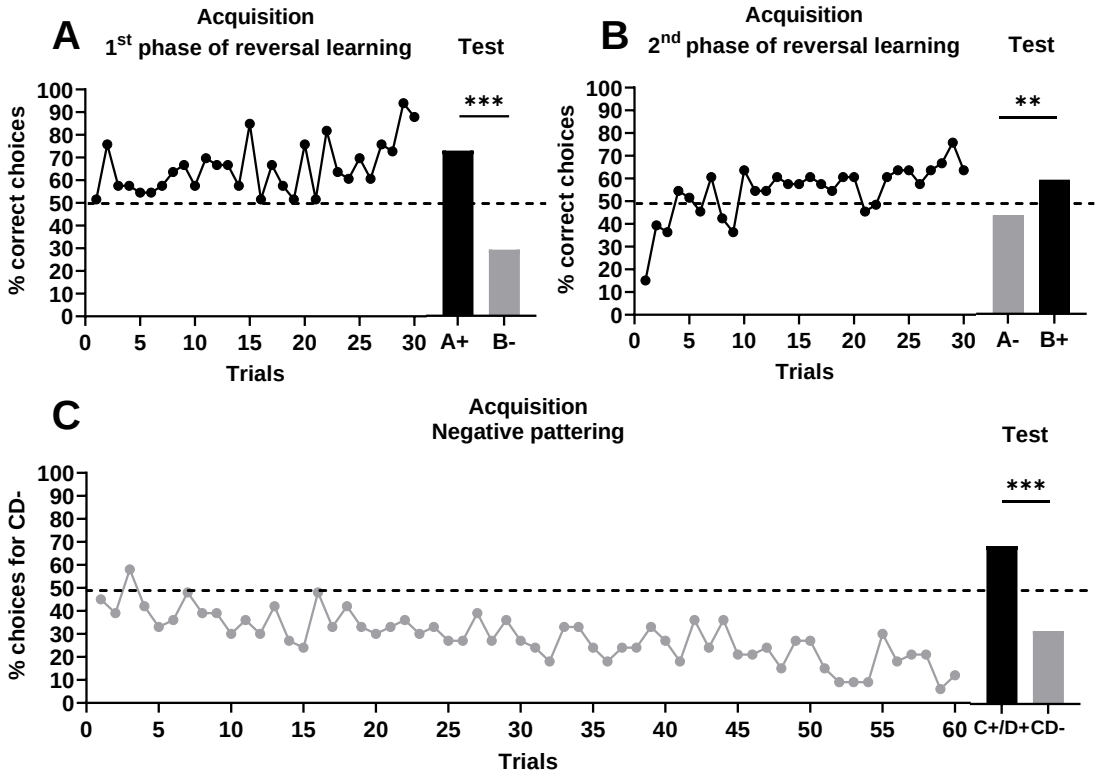

Fig. S5 Group acquisition curves and group test performances of the reversal learning (A+B) and negative patterning (C) protocols in experiment 1 (N =33). A) 1st phase of the reversal learning protocol. The curve shows the mean percentage of correct choices of the group for the rewarded stimulus (A+) during the 30 trials of the acquisition phase. The test performance shows the mean percentage of choices for the rewarded stimulus (A+; black bar) and the punished stimulus (B-; grey bar) during the 20 non-reinforced choices of the test, **B)** 2nd phase of the reversal learning protocol. The curve shows the mean percentage of correct choices of the group for the rewarded stimulus (B+) during the 30 trials of the acquisition. The histogram shows the mean percentage of choices for the correct (B+; black bar) and incorrect stimulus (A-; grey bar) during the 20 non-reinforced choices of the test and **C)** Negative patterning protocol. The curve shows the mean percentage of choices of the group for the incorrect mixture stimulus (CD-) throughout the 60 trials the acquisition phase. As the test performances of the two single stimuli (C+ and D+) did not differ from each other, they were pooled for analysis. The histogram shows the mean percentage of choices pooled for the correct two single stimuli (C+/D+; black bar) and the incorrect mixture stimulus (CD-; grey bar) during the 20 choices of the unreinforced tests. **$p \leq 0.01$, ***$p \leq 0.001$

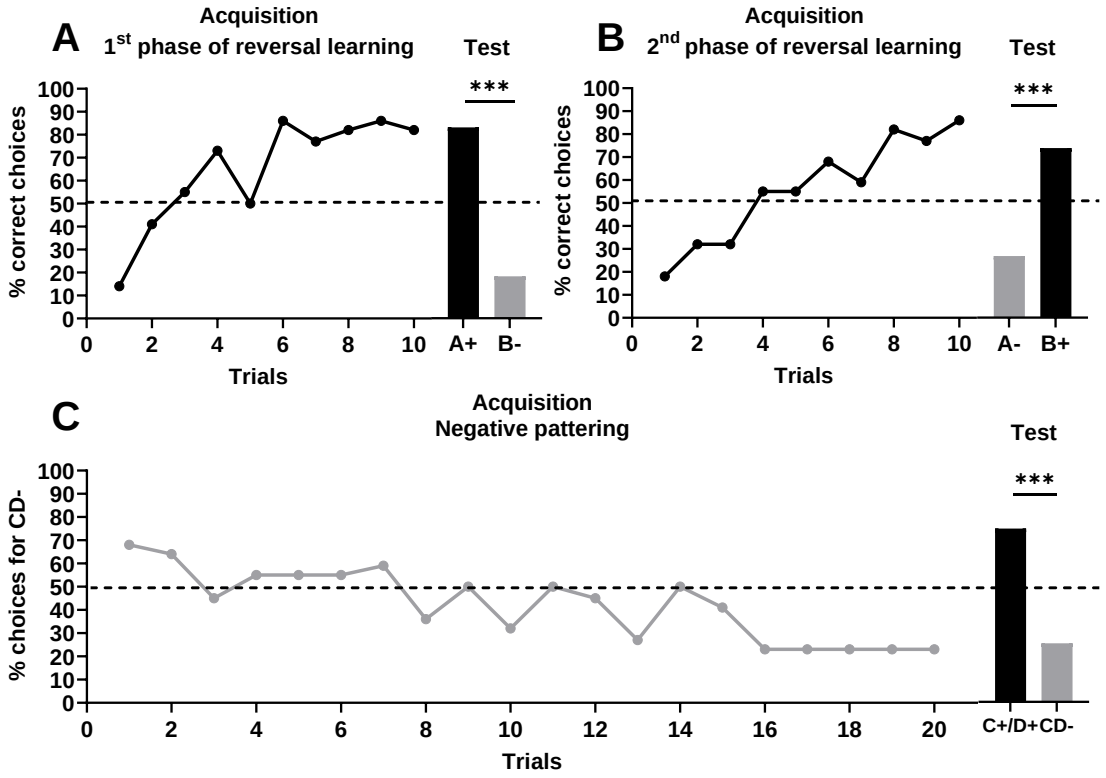

Fig. S6 Group acquisition curves and group test performances of the reversal learning (A+B) and negative patterning (C) protocols in experiment 2 (N=22). A) 1st phase of the reversal learning protocol. The curve shows the mean percentage of correct choices of the group for the rewarded stimulus (A+) during the 10 trials in the acquisition phase. The histogram shows the mean percentage of non-reinforced choices made during 45 seconds for the rewarded stimulus (A+; black bar) and the punished stimulus (B-; grey bar) in the test. **B)** 2nd phase of the reversal learning protocol. The curve shows the mean percentage of correct choices of the group for the rewarded stimulus (B+) during the 10 trials of the acquisition. The histogram shows the mean percentage of choices of the group made during 45 seconds in the non-reinforced test for the correct (B+, black bar) and incorrect stimulus (A-, grey bar) and **C)** Negative patterning. The curve shows the mean percentage of choices of the group for the incorrect mixture stimulus (CD-) throughout the 20 trials of the acquisition. As the test performances of the two single stimuli (C+ and D+) did not differ from each other, they were pooled for analysis. The histogram shows the mean percentage of choices pooled for the correct two single stimuli (C+/D+) and the incorrect mixture stimulus made during 45 seconds in the non-reinforced test. *** $p \leq 0.001$

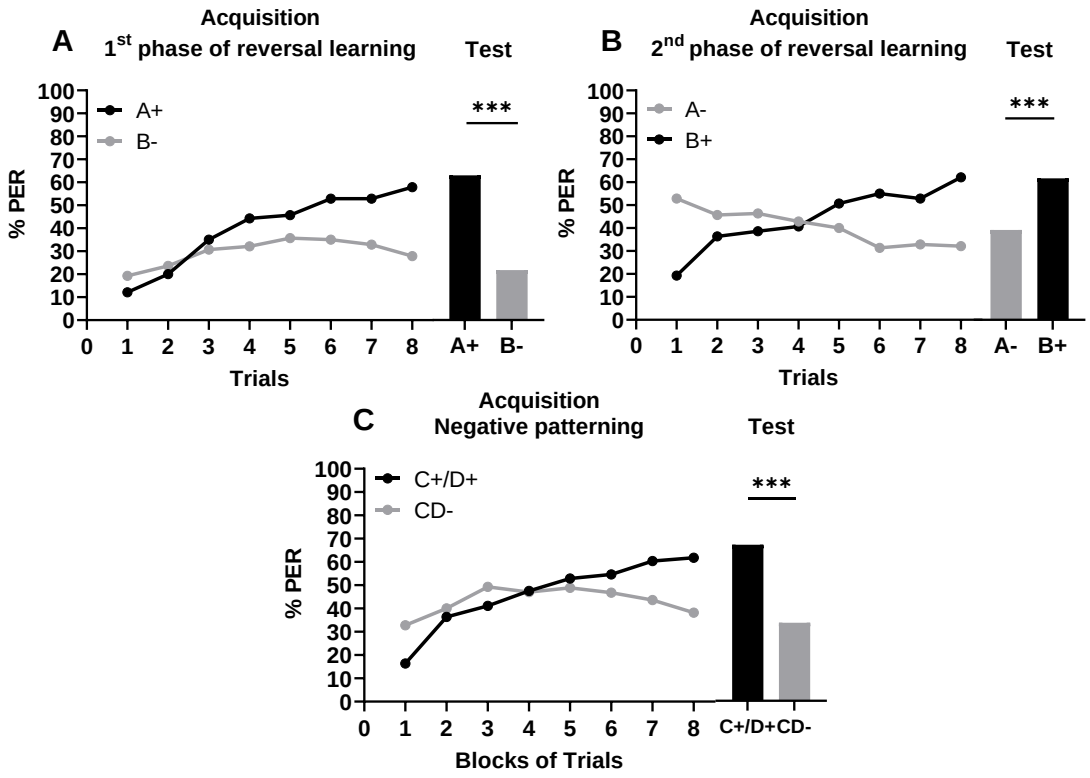

Fig. S7 Group acquisition curves and group test performances of reversal learning (A+B) and negative patterning (C) protocols in experiment 3 (N = 140). A) 1st phase of the reversal learning protocol. The curve shows the percentage of bees (N = 140) that responded with an extension of the proboscis (% PER) to either the correct stimulus (A+; black line) or the incorrect stimulus (B-; grey line) during the 8 trials each of the acquisition phase. The histogram shows the percentage of bees responding with an extension of the proboscis to the correct (A+; black bar) and incorrect stimulus (B-; grey bar) in the non-reinforced test. **B)** 2nd phase of the reversal learning protocol. The curves show the percentage of bees (N = 140) that responded with a PER to either the correct stimulus (B+; black line) or the incorrect stimulus (A-; grey line) during the 8 trials each of the acquisition. The histogram shows the percentage of bees responding with a PER to the correct stimulus (B+, black bar) and the incorrect stimulus (A-, grey bar) in the non-reinforced test. **C)** Negative patterning. The curve shows the percentage of bees responding with a PER to either the two correct single stimuli (C+/D+; black line) or their incorrect mixture (CD-, grey line) during the 8 blocks of trials of the acquisition. Each block consisted of one presentation of the two single stimuli and two presentations of the mixture. The histogram shows the percentage of bees responding with a PER to the two correct single stimuli (C+/D+, black bar) and to their incorrect mixture (CD; grey bar). The responses to the two single stimuli were not different in the acquisition, nor in the test and thus pooled for analysis. *** $p \leq 0.001$

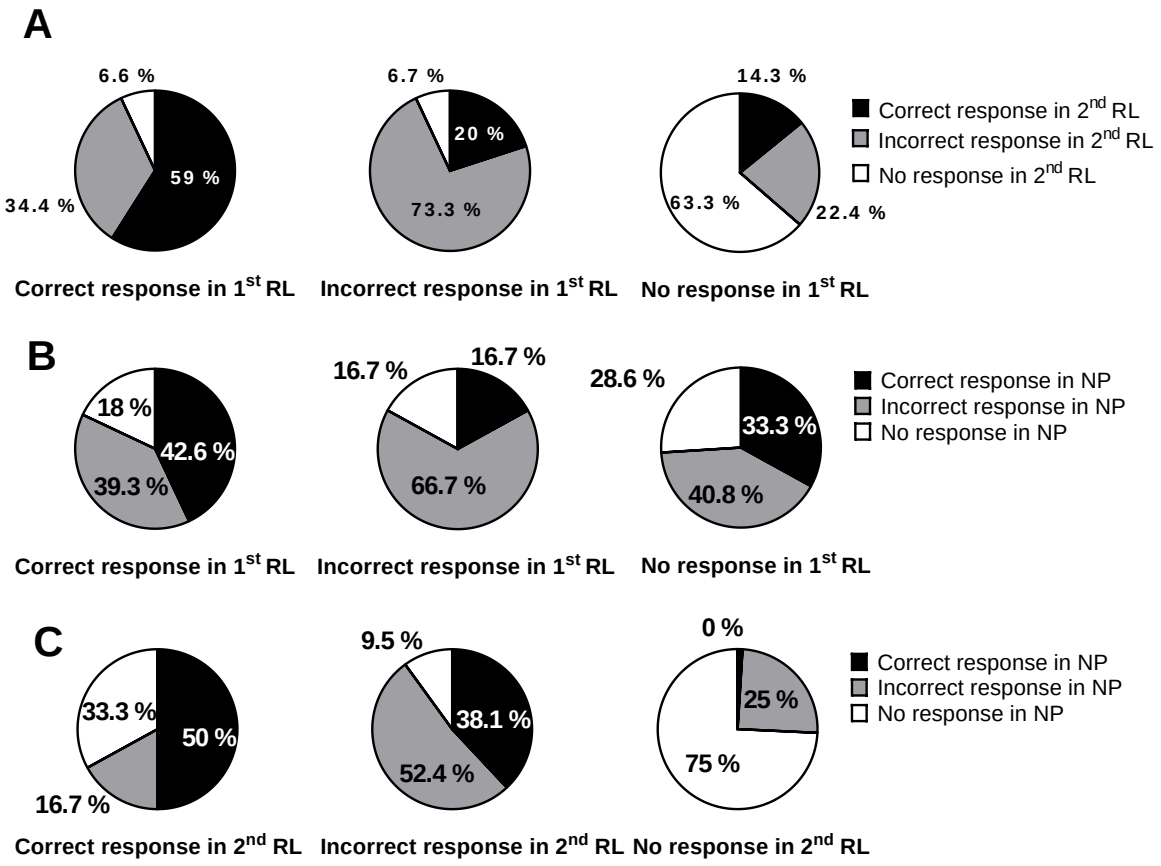

Fig. S8 Relative test performances of experiment 3: Visual learning experiments with restrained bees. The bees were assigned to groups, depending on their performances in the test. A 'correct response' was defined as being fully correct in the test (1st phase of reversal learning, 1st RL: Response to A and not to B; 2nd phase of reversal learning, 2nd RL: Response to B and not to A; Negative patterning, NP: Response to C and D but not to CD). A bee was scored as 'incorrect response' if it responded to the incorrect stimulus alone or to both stimuli and as 'no response' if it responded to none of the stimuli despite showing an intact PER. Relative test performances of the bees in the **A)** 2nd phase of reversal learning depending on their test performance in the 1st phase of reversal learning. **B)** negative patterning depending on their test performance in the 1st phase of reversal learning and **C)** negative patterning depending on their test performance in the 2nd phase of reversal learning

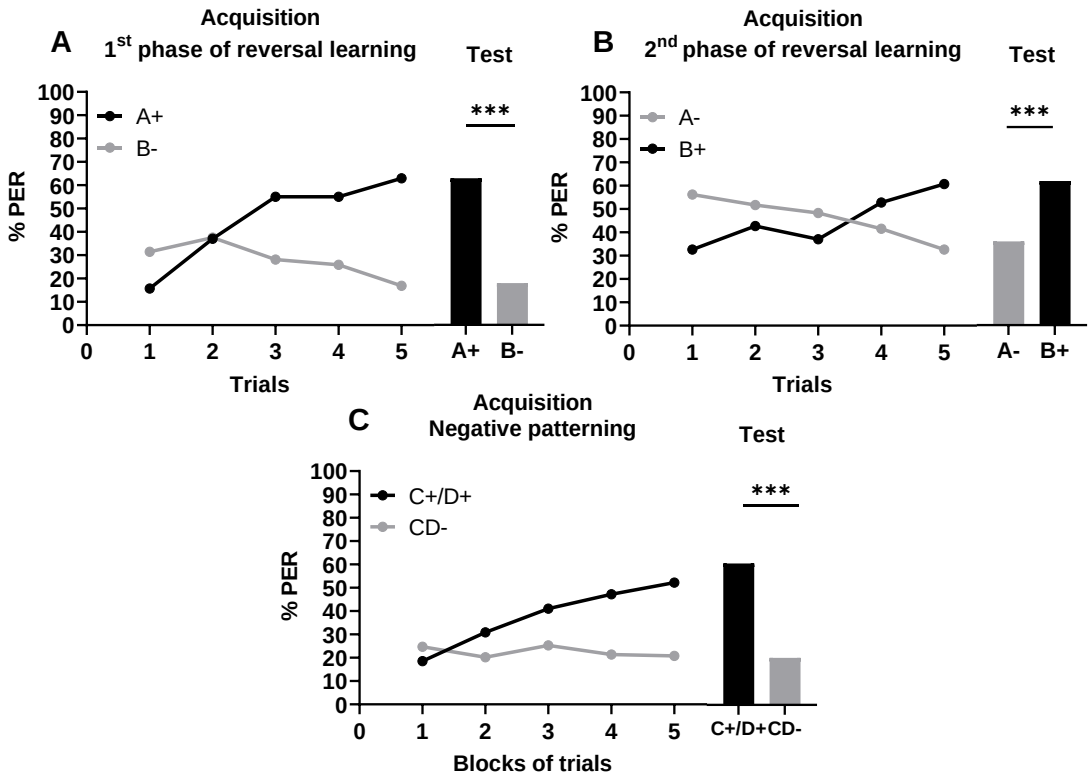

Fig. S9 Group acquisition curves and group test performances of reversal learning (A+B) and negative patterning (C) protocols in experiment 4. A) 1st phase of reversal learning. The curve shows the percentage of bees (N = 89) that responded with an extension of the proboscis (% PER) to either the correct stimulus (A+; black line) or the incorrect stimulus (B-; grey line) during the 5 trials each in the acquisition phase. The histogram shows the percentage of bees responding with a PER to the correct (A+; black bar) and incorrect stimulus (B-; grey bar) in the non-reinforced test. **B)** 2nd phase of reversal learning. The curve shows the percentage of bees (N = 89) that responded with a PER to either the correct stimulus (B+; black line) and the incorrect stimulus (A-; grey line) during the 5 trials each of the acquisition. The histogram shows the percentage of bees responding with a PER to the correct stimulus (B+, black bar) and the incorrect stimulus (A-, grey bar) in the non-reinforced test. **C)** Negative patterning paradigm. The curve shows the percentage of bees responding with a PER to either the two correct single stimuli (C+/D+; black line) and their incorrect mixture (CD-; grey line) during the 5 blocks of trials of the acquisition. Each block consisted of one presentation with the two single stimuli and two presentations of the mixture. The histogram shows the percentage of bees showing a PER to the two correct single stimuli (C+/D+; black bar) and to their incorrect mixture (CD; grey bar) in the non-reinforced test. The responses to the two single stimuli were not different and thus pooled for analysis. *** $p \leq 0.001$

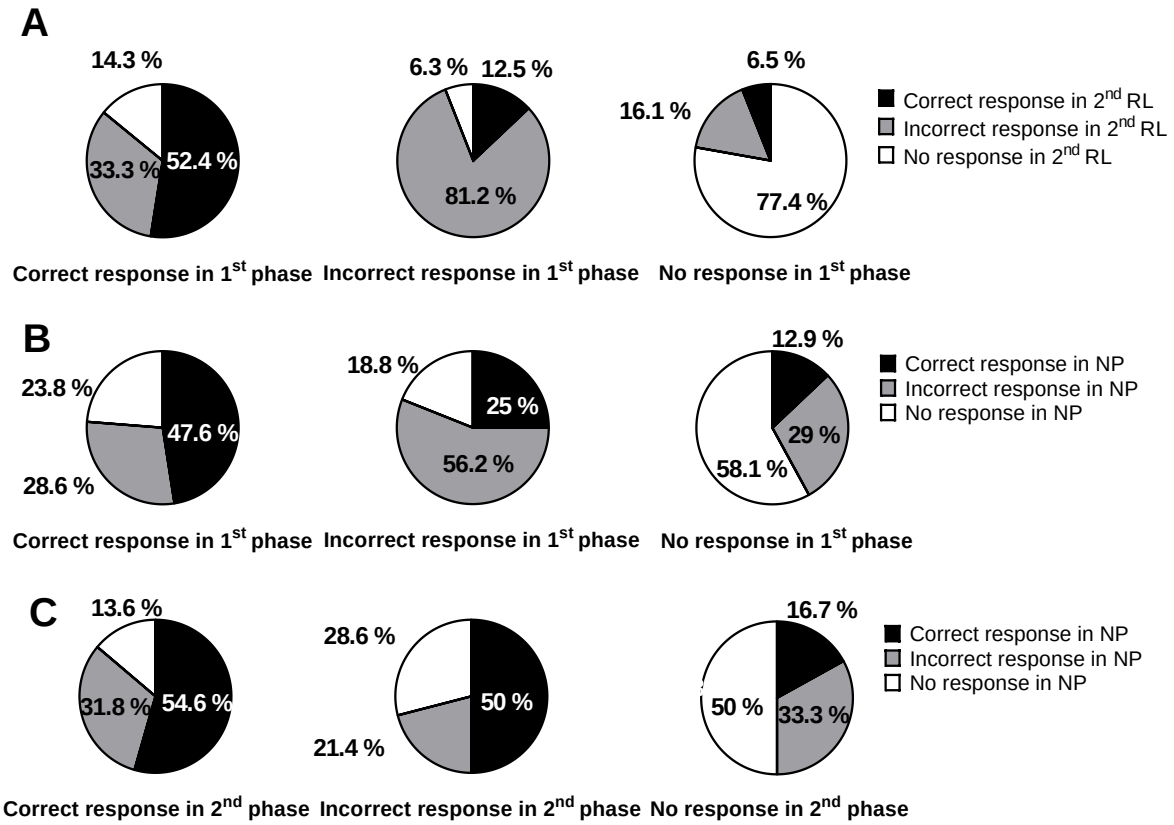

Fig. S10 Relative test performances of experiment 4: Olfactory learning experiments with restrained bees. The bees were assigned to groups, depending on their performances in the test. A 'correct response' was defined as being fully correct in the test (1st phase of reversal learning, 1st RL: Response to A and not to B; 2nd phase of reversal learning, 2nd RL: Response to B and not to A; Negative patterning, NP: Response to C and D but not to CD). A bee was scored as 'incorrect response' if it responded to the incorrect stimulus alone or to both stimuli and as 'no response' if it responded to none of the stimuli despite showing an intact PER. Relative test performances of the bees in the **A)** 2nd phase of reversal learning depending on their test performance in the 1st phase of reversal learning. **B)** the negative patterning paradigm depending on their test performance in the 1st phase of reversal learning and **C)** the negative patterning paradigm depending on their test performance in the 2nd phase of reversal learning.

Statistical tables

Table S1 GLMM analysis of the bees' performance in the acquisition of the 1st phase of reversal learning in experiment 1. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL + (1|subject)	9	1279.8	-630.88	-	-
model2: response ~ trial*order + group_RL + (1|subject)	6	1277.0	-632.51	3.27	0.35
model3: response ~ trial + order*group_RL + (1|subject)	6	1278.6	-633.32	4.88	0.18
model4: response ~ order + trial * group_RL + (1 | subject)	6	1278.6	-633.3	4.84	0.18
model5: response ~ trial + order + group_RL + (1 | subject)	5	1276.6	-633.32	4.88	0.30
model6: response ~ trial + group_RL + (1 | subject)	4	1277.9	-634.96	3.29	0.07
model7: response ~ trial + (1 | subject)	**3**	**1275.9**	**-634.97**	**0.01**	**0.91**
model8: response ~ (1 | subject)	2	1301.2	-648.59	27.24	< 0.0001

Table S2 GLMM analysis of the bees' performance in the acquisition of the 2nd phase of reversal learning in experiment 1. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial * order * group_RL + (1 |subject)	9	1337.1	-659.56	-	-
model2: response ~ trial * order + group_RL + (1 |subject)	6	1331.5	-659.72	0.34	0.95
model3: response ~ trial + order * group_RL + (1 |subject)	6	1331.9	-659.95	0.78	0.85
model4: response ~ order + trial * group_RL + (1 |subject)	6	1331.8	-659.91	0.7	0.87
model5: response ~ trial + order + group_RL + (1 |subject)	5	1329.9	-659.95	0.78	0.94
model6: response ~ trial + group_RL + (1 |subject)	4	1334.2	-663.08	6.27	0.01
model7: response ~ trial + order + (1 |subject)	**4**	**1327.9**	**-659.97**	**0.04**	**0.845**
model8: response ~ order + (1 |subject)	3	1353.0	-673.49	27.1	< 0.0001

Table S3 GLMM analysis of the bees' performances in the acquisition of negative patterning in experiment 1. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL + (1|subject)	9	2322.1	-1152.6	-	-
model2: response ~ trial*order + group_RL + (1|subject)	6	2317.3	-1152.6	1.15	0.76
model3: response ~ trial + order*group_RL + (1|subject)	6	2316.8	-1152.4	0.63	0.89
model4: response ~ order + trial * group_RL + (1 | subject)	6	2316.9	-1152.5	0.79	0.85
model5: response ~ trial + order + group_RL + (1 | subject)	5	2315.3	2343.3	1.17	0.88
model6: response ~ trial + group_RL + (1 | subject)	4	2313.4	-1152.7	0.08	0.78
model7: response ~ trial + (1 | subject)	**3**	**2311.4**	**-1152.7**	**0.01**	**0.92**
model8: response ~ (1 | subject)	2	2374.0	-1185.0	64.6	< 0.0001

Table S4 GLMM analysis of the bees' performances in the non-reinforced tests of the 1st phase of reversal learning in experiment 1. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL + (1 | subject)	5	785.87	-387.94	-	-
model2: response ~ order + group_RL + (1 | subject)	4	785.88	-387.94	0.01	0.94
model3: response ~ order + (1 | subject)	3	786.94	-390.47	2.1	0.08
model4: response ~ (1 | subject)	**2**	**785.26**	**-390.63**	**0.32**	**0.57**

Table S5 GLMM analysis of the bees' performances in the non-reinforced tests of the 2nd phase of reversal learning in experiment 1. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL + (1 | subject)	5	903.77	-446.88	-	-
model2: response ~ order + group_RL + (1 | subject)	4	902.33	-447.17	0.56	0.45
model3: response ~ order + (1 | subject)	3	901.74	-447.87	1.41	0.24
model4: response ~ (1 | subject)	**2**	**899.77**	**-447.89**	**0.04**	**0.85**

Table S6 GLMM analysis of the bees' performances in the non-reinforced tests of the negative patterning paradigm in experiment 1. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL + (1 | subject)	5	1658.0	-824.01	-	-
model2: response ~ order + group_RL + (1 | subject)	4	1656.1	-824.01	0.04	0.84
model3: response ~ order + (1 | subject)	3	1654.2	-824.11	0.15	0.70
model4: response ~ (1 | subject)	**2**	**1655.2**	**-825.59**	**2.96**	**0.09**

Table S7 GLMM analysis of the bees' performance in the acquisition of the 1st phase of reversal learning in experiment 2. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL + (1|subject)	9	262.39	-122.19	-	-
model2: response ~ trial*order + group_RL + (1|subject)	6	256.79	-122.40	0.41	0.82
model3: response ~ trial + order*group_RL + (1|subject)	6	257.30	-122.65	0.91	0.82
model4: response ~ order + trial * group_RL + (1 | subject)	6	257.64	-122.82	1.26	0.74
model5: response ~ trial + order + group_RL + (1 | subject)	5	255.72	-122.86	1.33	0.86
model6: response ~ trial + group_RL + (1 | subject)	4	254.76	-123.38	1.04	0.31
model7: response ~ trial + (1 | subject)	**3**	**253.38**	**-123.69**	**0.63**	**0.43**
model8: response ~ (1 | subject)	2	290.10	-143.05	38.71	< 0.0001

Table S8. GLMM analysis of the bees' performance in the acquisition of the 2nd phase of reversal learning in experiment 2. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL + (1|subject)	9	268.77	-125.39	-	-
model2: response ~ trial*order + group_RL + (1|subject)	6	264.25	-126.12	1.48	0.69
model3: response ~ trial + order*group_RL + (1|subject)	6	264.06	-126.03	1.29	0.73
model4: response ~ order + trial * group_RL + (1 | subject)	6	264.44	-126.22	1.67	0.64
model5: response ~ trial + order + group_RL + (1 | subject)	5	262.63	-126.31	1.86	0.76
model6: response ~ trial + group_RL + (1 | subject)	4	262.26	-127.13	1.63	0.20
model7: response ~ trial + (1 | subject)	**3**	**260.63**	**-127.32**	**0.37**	**0.54**
model8: response ~ (1 | subject)	2	303.75	-149.87	45.11	< 0.0001

Table S9. GLMM analysis of the bees' performance in the acquisition of the negative patterning paradigm in experiment 2. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL + (1|subject)	9	582.46	-282.23	-	-
model2: response ~ trial*order + group_RL + (1|subject)	6	578.16	-283.08	1.70	0.64
model3: response ~ trial + order*group_RL + (1|subject)	6	577.65	-282.83	1.19	0.76
model4: response ~ order + trial * group_RL + (1 | subject)	6	577.61	-282.81	1.15	0.77
model5: response ~ trial + order + group_RL + (1 | subject)	5	576.1	-283.09	1.71	0.79
model6: response ~ trial + group_RL + (1 | subject)	4	576.27	-284.14	2.10	0.15
model7: response ~ trial + (1 | subject)	**3**	**576.50**	**-285.25**	**2.22**	**0.14**
model8: response ~ (1 | subject)	2	603.42	-299.71	28.92	< 0.0001

Table S10. GLMM analysis of the bees' performances in the non-reinforced tests of the 1st phase of reversal learning in experiment 2. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order * group_RL + (1 | subject)	5	338.00	-164.00	-	-
model2: response ~ order + group_RL + (1 | subject)	4	336.26	-164.13	0.26	0.61
model3: response ~ order + (1 | subject)	3	334.44	-164.22	0.17	0.68
model4: response ~ (1 | subject)	**2**	**334.47**	**-165.24**	**2.03**	**0.15**

Table S11. GLMM analysis of the bees' performances in the non-reinforced tests of the 2nd phase of reversal learning in experiment 2. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order * group_RL + (1 | subject)	5	394.87	-192.43	-	-
model2: response ~ order + group_RL + (1 | subject)	4	394.87	-193.07	1.26	0.26
model3: response ~ order + (1 | subject)	3	392.48	-193.24	0.35	0.55
model4: response ~ (1 | subject)	**2**	**390.53**	**-193.26**	**0.05**	**0.83**

Table S12. GLMM analysis of the bees' performances in the non-reinforced tests of the negative patterning paradigm in experiment 2. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order * group_RL + (1 | subject)	5	353.05	-171.53	-	-
model2: response ~ order + group_RL + (1 | subject)	4	352.08	-172.04	1.03	0.31
model3: response ~ order + (1 | subject)	3	351.08	-172.54	1	0.32
model4: response ~ (1 | subject)	**2**	**349.11**	**-172.56**	**0.04**	**0.82**

Table S13. GLMM analysis of the bees' performance in the acquisition of the 1st phase of reversal learning paradigm in experiment 3. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial * order * group_RL * CS + (1 | subject)	17	2376.9	-1171.4	-	-
model2: response ~ trial * order * group_RL + CS + (1 | subject)	10	2428.9	-1204.5	66.04	< 0.0001
model3: response ~ trial * order * CS + group_RL + (1 | subject)	10	2388.4	-1204.5	40.53	< 0.0001
model4: response ~ trial * group_RL * CS + order + (1 | subject)	10	2374.5	-1177.3	0	1
model5: response ~ trial * group_RL*CS + (1 | subject)	**9**	**2372.5**	**-1177.3**	**0.12**	**0.73**

Table S14. GLMM analysis of the bees' performance in the acquisition of the 2nd phase of reversal learning in experiment 3. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial * order * group_RL * CS + (1 | subject)	**17**	**2396.1**	**-1181.0**	**-**	**-**
model2: response ~ trial * order * group_RL + CS + (1 | subject)	10	2546.6	-1263.3	164.5	< 0.0001
model3: response ~ trial * order * CS + group_RL + (1 | subject)	10	2413.7	-1196.9	31.69	< 0.0001
model4: response ~ trial * group_RL * CS + order + (1 | subject)	9	2546.6	-1263.3	164.5	< 0.0001

Table S15. GLMM analysis of the bees' performance in the acquisition of the negative patterning paradigm in experiment 3. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial * order * group_RL * CS + (1 | subject)	25	4814.6	-2382.2	-	-
model2: response ~ trial * order * group_RL + CS + (1 | subject)	11	4975.1	-2476.5	188.5	< 0.0001
model3: response ~ trial * order * CS + group_RL + (1 | subject)	**14**	**4812.7**	**-2392.4**	**168.3**	**< 0.0001**
model4: response ~ trial * group_RL * CS + order + (1 | subject)	14	4846.2	-2409.1	0	1
model5: response ~ trial * group_RL*CS + (1 | subject)	13	4847.6	-2410.8	3.38	0.07

Table S16. GLMM analysis of the bees' performances in the non-reinforced tests of the 1st phase of reversal learning in experiment 3. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ CS*order*group_RL + (1|subject)	9	168.10	-75.05	-	-
model2: response ~ CS*order + group_RL + (1|subject)	6	162.29	-75.15	0.19	0.9
model3: response ~ CS + order*group_RL + (1|subject)	6	162.10	-75.05	0	1
model4: response ~ order + CS* group_RL + (1 | subject)	6	162.29	-75.15	0.19	0.9
model5: response ~ CS + order + group_RL + (1 | subject)	5	160.29	-75.15	0.19	0.9
model6: response ~ CS + group_RL + (1 | subject)	4	158.34	-75.17	0.04	0.8
model7: response ~ CS + (1 | subject)	**3**	**157.18**	**-75.59**	**0.84**	**0.3**
model8: response ~ (1 | subject)	2	263.76	-129.88	108.6	< 0.0001

Table S17. GLMM analysis of the bees' performances in the non-reinforced tests of the 2nd phase of reversal learning in experiment 3. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ CS*order*group_RL + (1|subject)	9	292.12	-137.06	-	-
model2: response ~ CS*order + group_RL + (1|subject)	6	290.32	-139.16	4.20	0.24
model3: response ~ CS + order*group_RL + (1|subject)	6	291.91	-139.95	5.79	0.12
model4: response ~ order + CS* group_RL + (1 | subject)	6	290.26	-139.13	4.14	0.25
model5: response ~ CS + order + group_RL + (1 | subject)	5	290.09	-140.04	5.97	0.20
model6: response ~ CS + group_RL + (1 | subject)	4	288.09	-140.04	0.002	0.97
model7: response ~ CS + (1 | subject)	**3**	**288.09**	**-140.04**	**3.74**	**0.06**
model8: response ~ (1 | subject)	2	303.71	-149.85	15.88	< 0.0001

Table S18. GLMM analysis of the bees' performances in the non-reinforced tests of negative patterning in experiment 3. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ CS*order*group_RL + (1|subject)	13	381.93	-177.97	-	-
model2: response ~ CS*order + group_RL + (1|subject)	8	372.92	-178.46	0.99	0.96
model3: response ~ CS + order*group_RL + (1|subject)	8	370.53	-178.26	0.60	1
model4: response ~ order + CS* group_RL + (1 | subject)	8	372.87	-178.43	0.93	0.97
model5: response ~ CS + order + group_RL + (1 | subject)	6	369.10	-178.55	1.16	0.99
model6: response ~ CS + group_RL + (1 | subject)	5	367.12	-178.56	0.03	0.86
model7: response ~ CS + (1 | subject)	**4**	**366.70**	**-179.35**	**1.58**	**0.21**
model8: response ~ (1 | subject)	3	436.93	-216.47	74.23	< 0.0001

Table S19. GLMM analysis of the bees' performance in the acquisition of the 1st phase of reversal learning in experiment 4. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial * order * group_RL * CS + (1 | subject)	57	2842.2	-1364.1	-	-
model2: response ~ trial * order * group_RL + CS + (1 | subject)	15	3032.3	-1501.2	274.1	< 0.0001
model3: response ~ trial * order * CS + group_RL + (1 | subject)	**30**	**2830.2**	**-1385.1**	**232.1**	**< 0.0001**
model4: response ~ trial * group_RL * CS + order + (1 | subject)	30	2902.9	-1421.5	**0**	**1**
model5: response ~ trial * group_RL*CS + (1 | subject)	29	2906.3	-1424.1	5.34	0.03

Table S20. GLMM analysis of the bees' performance in the acquisition of the 2nd phase of reversal learning in experiment 4. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial * order * group_RL * CS + (1 | subject)	57	2851.5	-1368.8	-	-
model2: response ~ trial * order * group_RL + CS + (1 | subject)	15	3059.3	-1514.7	291.8	< 0.0001
model3: response ~ trial * order * CS + group_RL + (1 | subject)	**30**	**2849.4**	**-1394.7**	**239.9**	**< 0.0001**
model4: response ~ trial * group_RL * CS + order + (1 | subject)	30	2921.1	-1430.6	0	1
model5: response ~ trial * group_RL*CS + (1 | subject)	12	2923.0	-1432.5	3.83	0.06

Table S21 GLMM analysis of the bees' performance in the acquisition of the negative patterning paradigm in experiment 4. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial * order * group_RL * CS + (1 | subject)	**57**	**3053.0**	**-1469.5**	-	-
model2: response ~ trial * order * group_RL + CS + (1 | subject)	15	3262.9	-1616.5	293.9	< 0.0001
model3: response ~ trial * order * CS + group_RL + (1 | subject)	30	3235.6	-1496.9	239	< 0.0001
model4: response ~ trial * group_RL * CS + order + (1 | subject)	30	3133.1	-1536.6	0	1
model5: response ~ trial * group_RL*CS + (1 | subject)	**29**	**3132.7**	**-1537.4**	**1.6**	**0.21**

Table S22. GLMM analysis of the bees' performances in the non-reinforced tests of the 1st phase of reversal learning in experiment 4. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ CS*order*group_RL + (1|subject)	9	203.74	-92.87	-	-
model2: response ~ CS*order + group_RL + (1|subject)	6	205.99	-96.99	8.25	0.04
model3: response ~ CS + order*group_RL + (1|subject)	6	202.12	-95.06	4.38	0.22
model4: response ~ order + CS* group_RL + (1 | subject)	6	203.13	-98.56	3.43	0.33
model5: response ~ CS + order + group_RL + (1 | subject)	5	204.04	-97.02	8.31	0.08
model6: response ~ CS + group_RL + (1 | subject)	4	205.11	-98.56	3.07	0.08
model7: response ~ CS + (1 | subject)	**3**	**201.17**	**-94.59**	**0.01**	**0.9**
model8: response ~ (1 | subject)	2	244.23	-120.11	43.10	< 0.0001

Table S23. GLMM analysis of the bees' performances in the non-reinforced tests of the 2nd phase of reversal learning in experiment 4. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ CS*order*group_RL + (1|subject)	9	234.89	-108.44	-	-
model2: response ~ CS*order + group_RL + (1|subject)	6	230.56	-109.28	1.67	0.64
model3: response ~ CS + order*group_RL + (1|subject)	6	231.42	-109.71	2.53	0.47
model4: response ~ order + CS* group_RL + (1 | subject)	6	231.89	-109.95	3.01	0.39
model5: response ~ CS + order + group_RL + (1 | subject)	5	230.05	-110.03	3.16	0.53
model6: response ~ CS + group_RL + (1 | subject)	4	228.16	-110.08	0.10	0.75
model7: response ~ CS + (1 | subject)	**3**	**226.92**	**-110.46**	**0.76**	**0.38**
model8: response ~ (1 | subject)	2	243.76	-119.88	18.85	< 0.0001

Table S24. GLMM analysis of the bees' performances in the non-reinforced tests of negative patterning in experiment 4. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ CS*order*group_RL + (1|subject)	13	271.42	-122.71	-	-
model2: response ~ CS*order + group_RL + (1|subject)	8	264.29	-124.14	2.87	0.72
model3: response ~ CS + order*group_RL + (1|subject)	8	257.10	-121.55	0	1
model4: response ~ order + CS* group_RL + (1 | subject)	8	263.99	-124.00	2.57	0.77
model5: response ~ CS + order + group_RL + (1 | subject)	6	261.67	-124.83	4.25	0.75
model6: response ~ CS + group_RL + (1 | subject)	4	260.18	-125.09	0.51	0.47
model7: response ~ CS + (1 | subject)	**3**	**258.31**	**-125.16**	**0.13**	**0.72**
model8: response ~ (1 | subject)	2	346.93	-171.47	92.62	< 0.0001

5 Chapter 3: The impact of genetic diversity on consistent individual differences in the cognitive proficiency of honey bees

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5.1 Abstract

The honey bee is a suitable model to examine the impact of genetic diversity on inter-individual cognitive variability and consistency. They live in colonies where different patrilines co-exist due to extreme polyandry of the queen. This natural genetic diversity can be easily manipulated by artificial insemination of the queen. Additionally, bees have developed cognitive abilities which can be studied in multiple elemental and non-elemental tasks and individuals show distinct consistent cognitive profiles. Here we studied the impact of genetic diversity on inter-individual variability and consistency across elemental and non-elemental learning tasks in honey bees. We subjected free-flying bees originating either from a hive with a naturally mated polyandrous queen (high genetic diversity) or with a monandrous super-sister queen (low genetic diversity) to a simple visual discrimination, reversal learning and negative patterning. Our results revealed that the degree of inter-individual variability in the performances of the three learning tasks was not different between bees from the high and low genetic diversity hives. However, the group-level performances of bees from the monandrous hive showed an overall reduced cognitive performance across the tasks tested. Similarly, when we correlated the individual performances of bees across the tasks tested, the pattern of correlated performances was different between the two groups: while the individual test performances of bees from the polyandrous hive showed the same pattern of individually correlated performances across the three tasks as we have previously evidenced for naturally mated hive (i.e. simple discrimination correlating positively with reversal learning and negative patterning, no significant correlation between reversal learning and negative patterning), the performances of bees from the monandrous hive were positively and significantly correlated across all tasks tested. We discuss our results in terms of the possible importance of genetically determined inter-individual variability in cognitive proficiency for task specialization and allocation.

5.2 Introduction

A major question in the study of cognition is to what extent variation in cognitive processes is adaptive. For natural selection to act on cognitive traits and their underlying neural mechanisms, these traits must show heritable variation within a population. In humans, it is generally accepted that individuals often perform consistently throughout diverse cognitive tests, referred to as ‘general intelligence’ or ‘domain-general cognitive ability’ (Spearman 1904; Carroll and Maxwell 1979; Jensen 1998; Mackintosh 1998). Since this discovery, the research of the

heritability of general cognitive ability in humans has increased tremendously, especially due to the nature vs. nurture debate in the social sciences (Pinker 2003). Indeed, 'big family studies', twin studies and adoption studies have provided a vast amount of evidence that consistent individual differences in intelligence are highly heritable (Bouchard and McGue 1981, 2003; Jensen 1998; McGue et al. 2004). Meta-analyses have revealed that about 50 % of inter-individual variability in measures of intelligence can be attributed to inherited DNA sequence differences (Knopik et al. 2017). Domain-general cognitive ability has been described as one of the most heritable traits in humans with heritability estimates ranging from moderate to high (h^2 = 0.26-0.86; Plomin and Spinath 2002). Similarly, heritable general cognitive ability has also been evidenced in non-human primates and mice (Galsworthy et al. 2002, 2005; Hopkins et al. 2014). The study of inter-individual variation in cognitive skill in invertebrates, in contrast, has only emerged recently (Tait et al. 2019; Tait and Naug 2020; Honegger et al. 2020; Finke et al. 2021, 2023; Smith et al. 2022). It has largely remained unknown whether and to what extent general learning ability is inherited in these species.

Insects, predominantly the honey bee (*Apis mellifera*) and the fruit fly (*Drosophila melanogaster*), are well-established models for the study of learning, memory and cognition (Menzel 1990; Giurfa 2003; McGuire et al. 2005; Keene and Waddell 2007; Bellen et al. 2010; Avarguès-Weber et al. 2011a). Selection experiments in *Drosophila* have shown that populations of flies evolved an increased ability to associate and memorize the taste or smell of an oviposition medium with an aversive reinforcement after about 15 generations (Mery and Kawecki 2002). In honey bees, the expression of latent inhibition i.e. the relative difficulty to associate a familiar neutral stimulus with a novel reinforcement tendency to ignore stimuli which have recently been associated with the absence of reward is heritable (Chandra et al. 2000; Cook et al. 2020). The authors tested the ability for latent inhibition in drones and queens, allowing to artificially select lineages of bees with high and low proficiency. The worker progeny of the selected lineages showed similar performances to those of their parents independent of the social environment they experienced (Cook et al. 2020). Similar results have been obtained for olfactory elemental associative learning (Brandes 1988; Bhagavan et al. 1994; Benatar et al. 1995). First, the learning performance in an olfactory discrimination task (one stimulus A associated with a reward vs. a second stimulus B is associated a punishment) of drones was evaluated and selective breeding with virgin queens produced high- and low-learning performance lineages. Consequently, the learning performances of the workers progeny could be predicted based on the father's phenotype (Benatar et al. 1995). Although estimates of heritability are rare in animal research (Dukas 2004), associative learning

proficiency was shown to have low to moderate heritability in insects (honey bees: Brandes, 1988; fruit flies: Lofdahl et al., 1992).

Within honey bee colonies, an important source of genetic variation arises due to extreme polyandry of the queen as she mates on average with 17 different males (Tarpy et al. 2012). Such high genetic diversity within the colony leads to higher productivity and foraging efficiency compared to colonies with genetically uniform workers (Mattila and Seeley 2007, 2014; Wray et al. 2011). Bees from different patrilines exhibit genetically determined differences in their sensitivity to task related stimuli (Beshers & Fewell, 2001; Erber et al., 2006; Fewell & Page, 1993; Page et al., 1998; Scheiner & Arnold, 2010). As a consequence, bees from different patrilines show a high-degree of inter-individual variability across various behaviors such as the individual evaluation of sucrose (Scheiner et al. 1999, 2001b, a; Scheiner 2004; Scheiner and Arnold 2010), associative learning (Brandes and Menzel 1990; Brandes 1991; Bhagavan et al. 1994; Benatar et al. 1995; Laloi and Pham-Delegue 2010; Junca et al. 2014), latent inhibition (Chandra et al. 2000; Ferguson et al. 2001) and foraging specialization (Robinson and Page 1988, 1989a) among others.

Here, we investigated the impact of multiple patrilines on group-level cognitive performances and the degree of inter-individual variability in honey bee foragers. Our control were worker bees from a queen which had been artificially inseminated by one drone. Bees were tested in a reversal learning paradigm and a negative patterning paradigm. We hypothesized that polyandry should result in greater variability among worker bee learning performances. We further hypothesized that group-level learning performances of the ‘low genetic diversity’ hive would either be increased or decreased in comparison to the ‘high genetic diversity’ hive depending on the (unknown) learning phenotype of the drone used for artificial insemination.

5.3 Material and methods

5.3.1 Honey bee colonies

The experimental colonies were artificially created by introducing the respective queens into mini plus hives (outer dimensions: 30 x 30 cm) with six empty frames together with a shook swarm of 500 gram of worker bees. The two queens were super sisters, sharing 75 % of their DNA. One queen was allowed to mate naturally with an unknown number of drones, thereby

generating offspring originating from different patrilines ('high-genetic diversity' hive). The other queen was artificially inseminated with the sperm of a single drone and thus produced only super sisters as offspring ('low-genetic diversity' hive) (Cobey et al., 2013). The two hives were installed at the same location at approximately 10 meters distance, experiencing the same environmental conditions.

Since the queens were introduced into shook swarms, all newly emerged bees were surely the offspring of the respective queen. Once the first bees emerged, the two hives were opened every day and all newly emerged bees were caught. They were then painted with a unique color code (color pigments of various colors dissolved in shellac) that allowed to determine the hive origin and the date of marking. Then they were re-introduced into their respective hives. One week after emergence of the first bees, bees from both hives were trained to collect a sucrose solution (10 %, weight/weight) from an artificial gravity feeder located outside approximately 20 m from the hives. While normally honey bees only start foraging at the age of two to three weeks (Seeley 1982), bees initiate foraging much earlier in these small mini plus hives (Schilcher et al. 2022). All bees used in the experiment were obtained from this feeder and only bees with clearly identifiable color code were used.

5.3.2 Pre-training

The assessment of the cognitive performances followed the same procedure as in experiment 1 in Finke et al. (2023). Before the bees were subjected to the learning paradigms, they were pre-trained to the experimental set-up. The experimental set-up was located on an outside table at two meters distance from the artificial gravity feeder and consisted of a grey vertical rotatable screen (50 cm in diameter) with hooks where hangers could be attached to at multiple locations. The grey hangers (6 x 8 cm) displayed the visual stimuli and had a small landing platform that displayed the reinforcement during conditioning. For pre-training bees were recruited from the feeder to the experimental set-up with a Plexiglas® spoon dipped in a sucrose solution (50 % w/w) and transferred to the landing platform of a plain hanger with sucrose solution. The bees were then allowed to consume the solution *ad libitum* before flying back to the hive. Once returning to the experimental set-up the bees were then required to find the landing platform on the rotating screen by themselves to collect the sucrose reward. Upon landing and consuming the solution the bees were marked with another colored spot on the abdomen to allow individual identification. This course of action was repeated at least five times until the bees reliably

returned to the set-up and learned where to find the reinforcement. Then the conditioning of the learning paradigms was initiated. Only one individually marked bee was trained at a time and recruited bees were removed from the set-up and transferred into cages for the duration of the experiment.

5.3.3 General conditioning procedure

Visual stimuli (6 cm x 6 cm) were attached to the hangers and 10 µl of either a sucrose solution (50% w/w) for the correct visual target or a quinine solution (1.6 mM) as punishment or water for the incorrect visual target were applied to the corresponding landing platform. In each trial, a bee had to choose between the correct and incorrect visual targets, whereas each target was represented twice on the rotating screen. A correct choice was recorded once a bee landed on the platform of the correct visual target and consumed the sucrose reward. An incorrect choice was scored once a bee landed on the platform of the incorrect visual target and tasted either quinine or water (depending on the learning task). After this first choice, the bee was carefully transferred to a Plexiglas® spoon providing a small droplet of sucrose solution (~ five µl) and moved away from the set-up. The screen was then rotated to avoid positional learning and then the bee had to approach the screen for the next trial. A bee made approximately three to five choices before returning to the hive to empty her honey sac. While the bee was absent, all hangers were cleaned with 50 % ethanol to remove potential odor signatures and the screen was again rotated to exchange the position of the visual targets and all solutions were refilled. Each conditioning phase amounted to a defined number of trials and once this number was reached the training was terminated. A non-reinforced test followed each conditioning phase. New hangers and visual stimuli were used to assure that no scent markings were present. During the tests the stimuli of the corresponding conditioning phase were presented without reinforcement or water and the bees had to choose between the targets for a total of 20 choices. Since no reinforcement was presented, a choice was here defined as touching or landing on either the stimuli or the corresponding platform. For each test the percentage of correct choices was calculated for each bee.

Once the conditioning and testing of one learning paradigm was terminated, the stimuli were removed from the hangers and the bee was subjected to the procedure of the pre-training for three foraging bouts before the conditioning of the other learning paradigm was initiated. The order in which the learning paradigms were conducted was counterbalanced across all bees

tested. Only bees that completed both learning tasks and returned reliably to the experimental set-up throughout the whole procedure were kept for statistical analysis, because they had the motivation to participate in the experiment from beginning to end.

5.3.4 Reversal learning

The reversal learning paradigm consisted of two phases: in the 1st phase of reversal learning (1st RL) one visual target was associated with a sugar reward (A+) and a second visual target was associated with a quinine solution as punishment (B-). In the 2nd phase of reversal learning, the previously rewarded stimulus was punished, and the previously punished stimulus became rewarded (2nd RL; A- and B+). Each phase was conducted for 30 trials in total. A non-rewarded test followed each phase. The stimuli used were colored pigment paper squares (yellow: HKS-3N and greenish yellow: HKS-68N, Hostmann-Steinberg K+E Druckfarben, H. Schmincke and Co., Germany). Hexagon color loci of the two stimuli were separated by 0.07 hexagon units, a distance sufficient to be discriminated by the bees (see Finke et al. 2023). During the whole procedure two correct stimuli and two incorrect stimuli were attached to the rotating screen. The stimuli used as rewarded target were counter-balanced across all tested bees.

5.3.5 Negative patterning

In the negative patterning paradigm two single stimuli (C+ and D+) were rewarded while their compound was not reinforced (CD-). For this task three different blocks of trials were conducted in a pseudo-random sequence throughout conditioning. Two blocks followed an absolute conditioning procedure where four hangers of either single stimulus (C+ or D+) were presented to the bees in association with a sucrose reward. In the third block, the non-reinforced compound (CD-) associated with water and a rewarding black-and-white checkerboard alternative (XY+) were presented on two hangers each. As purely non-reinforced trials cannot be conducted with free-flying bees as multiple non-reinforced trials would decrease the bees' motivation, the traditional protocol for negative patterning which was developed for classical conditioning of harnessed bees had to be changed by adding the rewarding alternative (XY+) for the block with the compound presentation. The acquisition lasted for 120 trials, 30 each with C+ and D+ and 60 with CD-. Each block of trials was conducted for one foraging bout and once the bees returned to the hive, all hangers were cleaned with ethanol, and the stimuli of the

new block were attached to the rotating screen. Again, the acquisition phase was followed by non-reinforced tests where stimuli were presented without any solutions present. Here, two tests had to be conducted, one with either C+ or D+ presented together with the compound stimulus CD-, to examine if the bees learned to prefer the single stimuli over their compound. Between the tests, refreshing trials of the acquisition were performed for one foraging bout, to ensure that the bees had a high motivation during the second test.

The stimuli were adapted from (Schubert et al. 2002). They were made using HKS-26N, HKS-44N, HKS-92N and HKS-88N pigment papers (HKS-N; Hostmann-Steinberg K+E Druckfarben, H. Schmincke and Co., Germany) that appear pink, blue, grey and black to the human eye respectively. The pink (26N) and blue (44N) colors were separated by 0.07 hexagon units, a perceptual distance which supports color discrimination (Finke et al. 2023). All stimuli were handmade in a 6 x 6 cm checkerboard pattern. The two single element stimuli were created using 1 cm x 1 cm squares of either the pink or blue cardboard on grey background. The compound stimulus was analogously manufactured by using HKS-26N and HKS-44N cardboards and the rewarding alternative for the compound trials was prepared with HKS-88N cardboard on a white copy paper background.

5.4 Statistical analysis

All basic statistical analyses and graphs were done using GraphPad prism 9.2.0 (GraphPad Software Inc., San Diego CA). All generalized linear mixed models (GLMMs) were performed with R version 4.0.2 (R Core Team 2022) with the package lme4 (Bates et al. 2015). All acquisition performances were analyzed using GLMMs for repeated measurements with a binomial family. The order in which the two learning tasks were conducted (*order*), the trial number (*trial*), the age (*age*) and the stimulus used as rewarded one in reversal learning (*group_RL*) were entered as fixed factors. When comparing the performances between the two groups, the hive identity (*hive)* was also included as fixed factor. The subject's identity (*1*|*subject*) was added as random factor. Different models were performed by gradually removing factors or interactions between factors. Those models were then compared with an ANOVA and the model with the lowest AIC value was chosen as best fit (Burnham and Anderson 1998; Panchal et al. 2010) (see tables S1-3, S7-9, S13-15 in the supplementary material). Test performances were also analyzed with GLMMs using the choices in the test (1 = correct, 0 = incorrect choice) as dependent variable. Order, age, and group_RL were included

as fixed factors and subject identity was included as a random factor. When comparing the performances between the two groups, the hive identity (*hive)* was also included as fixed factor. Again, the model with the lowest AIC value was considered as most appropriate fit (see tables S4-6, S10-12, S16-18 in the supplementary material). As percentages of correct choices were used as measure for the individual's test performances, Wilcoxon matched pairs signed rank sum tests were used to test if the bees within each of the two groups learned to discriminate the two stimuli of the respective learning task. In the case of negative patterning where two retention tests were conducted (one for each of the single stimuli: C+ vs. CD- and D+ vs. CD-). Wilcoxon matched pairs signed rank sum tests were used to assess if the bees within a group performed differently in these two tests. As no significant differences were found between these tests, they were pooled for analysis. Individual consistency across the different tasks were assessed by means of the test performances with Spearman rank correlations. Importantly, only bees that successfully learned in the 1st phase of reversal learning phase (1^{st} RL; > 60 % correct choices in the test) were used for all correlations involving performances in the 2nd phase of reversal learning (2^{nd} RL). This is because the ability for reversal learning prerequisites the success to acquire the initial stimulus-reward association established in the 1st phase (Mota and Giurfa 2010). The relationship between the age of the individuals and their acquisition performances (i.e. percent of all correct choices made during each acquisition phase) and test performances were further analyzed with Spearman rank correlations. To compare the age of the individuals from the two groups we used a Mann-Whitney U test. To descriptively characterize the inter-individual variability in the performances of the bees in both the acquisition phases and in the tests, the coefficient of quartile variation (cqv) was calculated with following the formula:

$$cqv = \left(\frac{q_3 - q_1}{q_3 + q_1}\right) \times 100$$

where q_3 and q_1 are the third (i.e. 75^{th} percentile) and first quartile (i.e. 25^{th} percentile) respectively.

However, due to the lack of statistical tests to compare the cqv of two or more samples we used the Fligner-Killeen test to check for homogeneity of variances between the two groups, as this test uses the median as opposed to the mean for its calculation and is thus the most robust test to compare variances when data does not follow a normal distribution (Conover et al. 1981).

5.5 Results

5.5.1 Low genetic diversity hive

The performances of the bees increased significantly in each task throughout all acquisition phases (GLMM: *trial*; see table 1 for the test statistics, Fig. 1A-C and tables S1-S3 respectively). The bees learned to discriminate between the two stimuli in the non-reinforced retention test of the 1st phase of reversal learning (A+ vs. B-: median ± standard error: 65 ± 3.4 % correct choices; Wilcoxon matched-pairs signed rank test, *n = 30*, W = 364, P < 0.0001 Fig. 1A) and of the 2nd phase of reversal learning (B+ vs. A-:57.5 ± 3.7 %; *N = 30, W = 242 p = 0.005*, Fig. 1B). As the performances of the bees in the two retention tests of negative patterning did not significantly differ, they were pooled for further analysis (C+ vs. CD-: 62.5 ± 3.9 % correct choices; D+ vs.CD-: 65 ± 4.0 % correct choices; *n = 30, W = 30, p = 0.74*). The bees preferred the single stimuli (C+, D+) over their compound (C+/D+ vs. CD-: 62.5 ± 3.7 % correct choices; *n = 30, W = 311, p = 0.0004*, Fig. 1C). The order in which the two learning paradigms were conducted had no significant on the acquisition and test performances of all tasks (GLMM: *order*, see table 1 for the test statistics, tables S1-6). The stimulus which was rewarded in the reversal learning task had only a significant effect on the acquisition performance of the first phase of reversal learning but not on all other acquisition and test performances (GLMM; *group_RL*, see table 1 for the test statistics, tables S1-6). Interestingly, neither the performances in the acquisition phases nor in the non-reinforced tests were influenced by the age (median = 32 ± 2.7 days, Fig. S1) of the individuals (GLMM; *age*, see table 1 for the test statistics, tables S1-6). Consequently, the age of the individuals was not significantly correlated with neither the acquisition performances (Spearman rank correlation; *n = 30*; **1st RL:** *rs = 0.26, p = 0.16*, **2nd RL:** *rs = -0.09, p = 0.65*, **NP:** *rs = 0.17, p = 0.37*, Fig. 2A-C) nor the test performances in any task (*n = 30*, **1st RL:** *rs = 0.0006, p = 0.997*, **2nd RL:** *rs = -0.04, p = 0.84*, **NP:** *rs = -0.1, p = 0.6*, Fig. 2D-F).

The performance of individual bees in the non-reinforced tests correlated positively across all three tasks tested. The individuals' performances were correlated across the two phases of reversal learning (Spearman rank correlation: *N = 23, rs = 0.47, p = 0.02*, Fig. 4A) and across the 1st phase of reversal learning and negative patterning (*N = 30, rs = 0.48, p = 0.006*, Fig. 4B). Furthermore, there was a significant association between the performances of individuals in the 2nd phase of reversal learning and negative patterning (*N = 23, rs = 0.45 p = 0.03*, Fig. 4C). The correlation between the 2nd phase of reversal learning and negative

patterning contrasts previous findings revealing no correlation when bees from unmanipulated hives were tested (Finke et al. 2023).

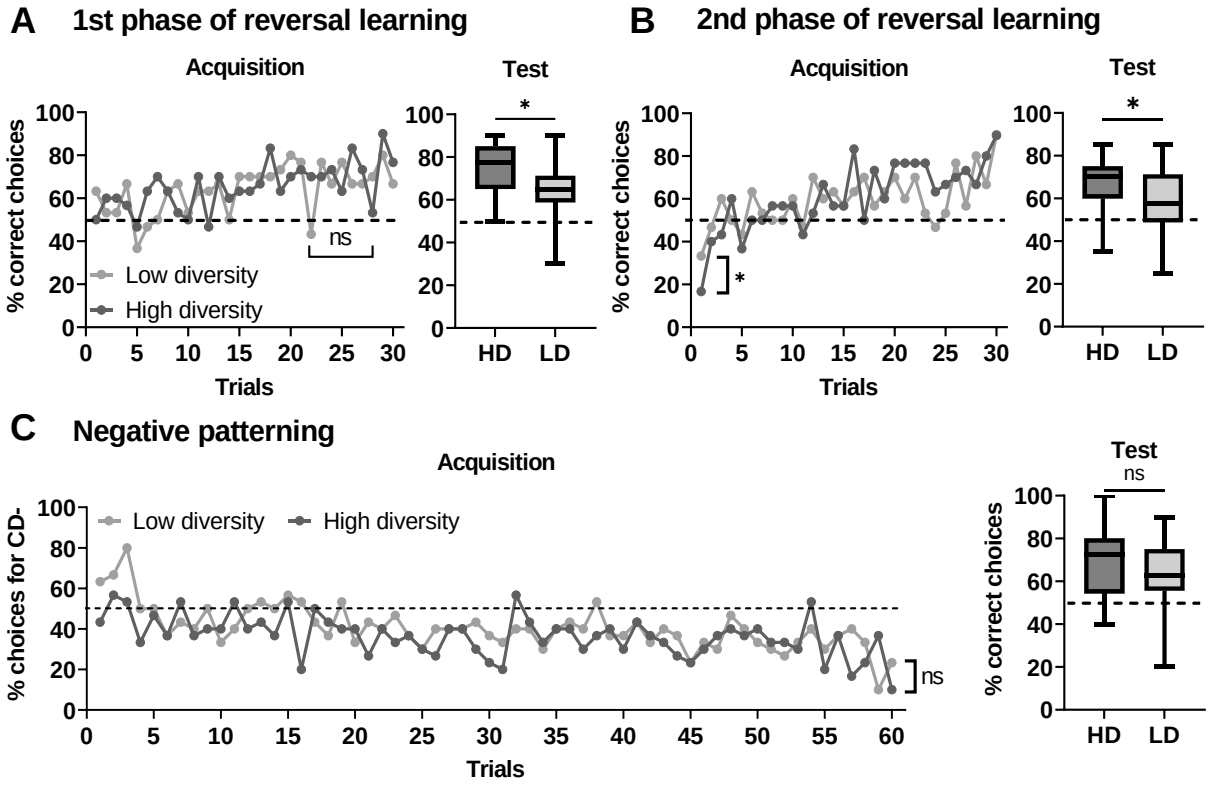

Figure 1. **Comparison of the group acquisition and test performances of the bees from high (HD; dark grey; N = 30) and low (LD; light grey; N = 30) genetic diversity hive in the 1st phase of reversal learning (A), the 2nd phase of reversal learning (B) and negative patterning (C).** All curves show the mean percentages of choices made by the bees during the acquisition phases for a given stimulus of the respective learning task. The dashed lines indicate a random choice level of 50 %. All boxplots show the median percentage of choices for the correct stimulus in the test of the respective task. The boxes represent the 25th-75th percentiles, the solid lines indicate medians, and the bars show the minima and maxima. **A)** The curves show the acquisition performances in the 1st phase of reversal learning (A+ vs. B-). There was no difference in the acquisition performances of the elemental learning phase between the bees from the high and low genetic diversity hives. The boxplots show the test performances of the bees from both groups in the 1st phase of reversal learning as measured by the percentage of correct choices for the rewarded stimulus A+ during the 20 choices in the non-reinforced test. Here, a significant difference was found in the test performances of the 1st phase of reversal learning with bees from the high genetic diversity hive performing better compared to the low genetic diversity hive. **B)** The curves show the acquisition performances in the 2nd phase of reversal learning (A- vs. B+) as measured by the mean percentage of correct choices for the rewarded stimulus B+ throughout the 30 trials of acquisition. The acquisition performances of the reversal learning phase from the two groups differed significantly. The boxplots show the test performances of the bees from both groups in the 2nd phase of reversal learning as measured by the percentage of correct choices for the rewarded stimulus B+ made during the 20 choices in the non-reinforced test. The test performances of the 2nd phase of reversal learning differed significantly between the two groups, whereas the bees from the high genetic diversity hive performed better compared to those from low genetic diversity hive. **C)** The curves show the acquisition performances in negative patterning (C+, D+ vs. CD-) as measured by the mean percentages of choices for the non-reinforced compound stimulus CD- throughout the 60 trials of acquisition. There was no difference in the acquisition performances of the negative patterning paradigm between the bees from both groups. The boxplots show the test performances of the bees from both groups in negative patterning as measured by the percentage of correct choices for the rewarded two single stimuli C+ and D+ combined during the 20 choices in each of the two retention tests. No significant difference was found in the test performances of the negative patterning paradigm. * < 0.05, ns: not significant.

Table 1 Test statistics for the GLMMs of the data from the low genetic diversity hive.

factor	task	phase	N	χ^2	p-value
trial	1st RL	acquisition	30	15.33	< 0.0001
	2nd RL	acquisition	30	20.62	< 0.0001
	NP	acquisition	30	43.84	< 0.0001
order	1st RL	acquisition	30	0.63	0.43
	2nd RL	acquisition	30	0.83	0.36
	NP	acquisition	30	0.04	0.85
	1st RL	test	30	0.03	0.42
	2nd RL	test	30	1.15	0.28
	NP	test	30	0.05	0.82
group_RL	1st RL	acquisition	30	8.88	0.003
	2nd RL	acquisition	30	2.60	0.11
	NP	acquisition	30	2.43	0.12
	1st RL	test	30	1.30	0.25
	2nd RL	test	30	0.25	0.62
	NP	test	30	4.09	0.16
age	1st RL	acquisition	30	3.50	0.06
	2nd RL	acquisition	30	1.23	0.27
	NP	acquisition	30	0.18	0.68
	1st RL	test	30	0.67	0.87
	2nd RL	test	30	2.67	0.10
	NP	test	30	0.83	0.37

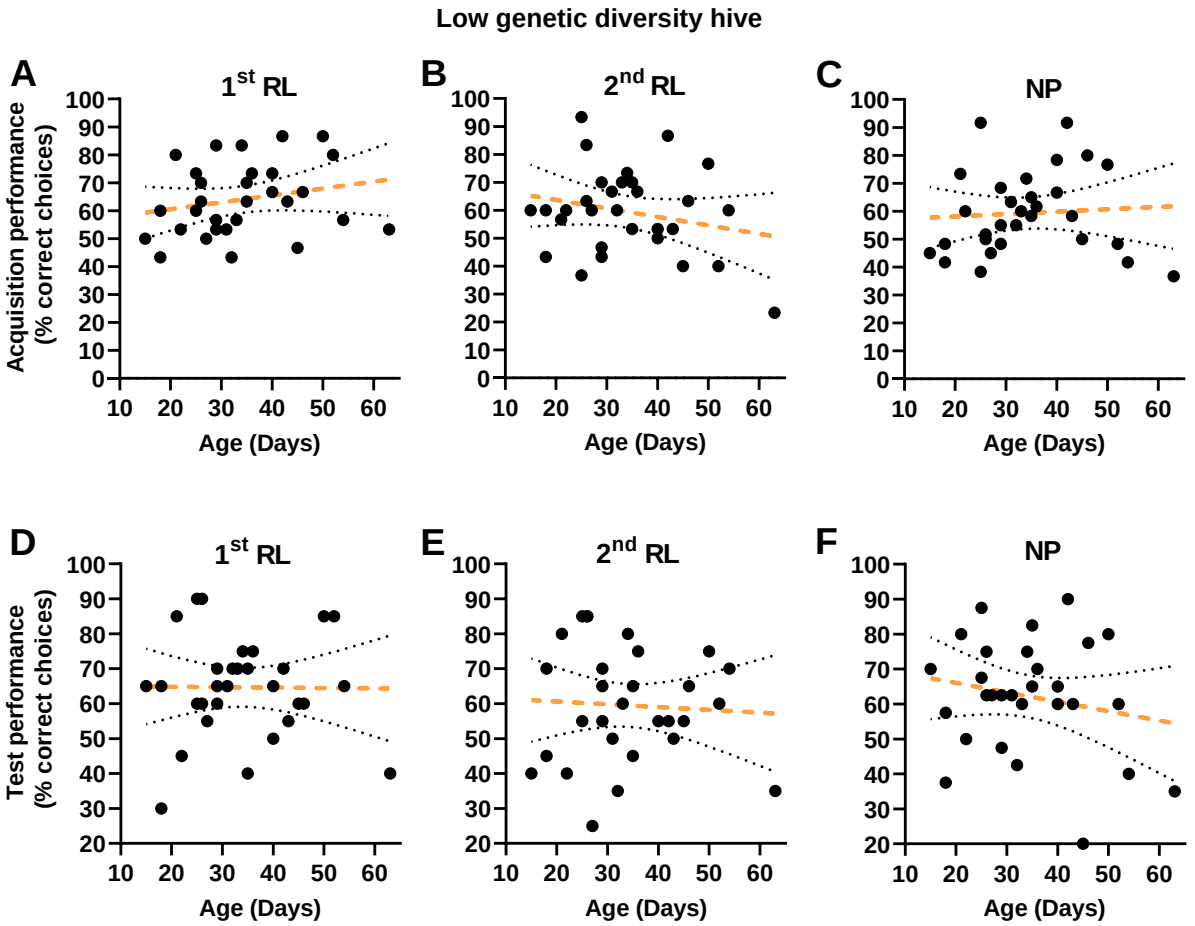

Fig. 2 Relationship between the age (in days) of the bees from the low genetic diversity hive and their performances in the acquisitions and non-reinforced tests of the 1st (A, D) and 2nd phase of reversal learning (B, E) and negative patterning (C, F). Acquisition performances were measured as percentage of correct choices made during the acquisition phase of each task. The test performances were measured as the percentages of correct choices the bees made during the 20 choices in the non-reinforced test of each task. In all cases, no significant relationship between the bees' age and their performances were evidenced. The orange lines indicate regression lines and the black dashed lines indicate the confidence intervals of the regression. Each dot represents the data of a single bee.

5.5.2 High genetic diversity hive

The performances of the bees increased significantly throughout the acquisition phases of the three tasks (GLMM: *trial*; see table 2 for the test statistics, Fig. 1A-C and tables S7-9 respectively). The bees also learned to discriminate between the two stimuli in the non-reinforced test of the 1st phase of reversal learning (A+ vs. B-: 77.5 ± 2.7 % correct choices; Wilcoxon matched-pairs signed rank sum test; $n = 30$, $W = -378$ $p < 0.0001$, Fig. 1A) and learned the reversed reward contingencies in the 2nd phase of reversal learning (A- vs. B+: 70 ± 2.8 % correct choices; $n = 30$, $W = 383$ $p < 0.0001$, Fig.1B). The performances in the two tests of negative patterning did not significantly differ from each other (C+ vs. CD-: 70 ± 3.4

% correct choices; D+ vs. CD-: 75 ± 4.2 % correct choices; *N = 30, W= 33, p = 0.65*) and were thus pooled for analysis. The bees chose the two single stimuli (C+, D+) significantly more often compared to the compound stimulus (C+/D+ vs. CD-; *N = 30, W = 378 p < 0.0001*, Fig. 1C). The performances throughout all tasks were not influenced by the order in which the tasks were conducted (GLMM: *order*; see table 2 for the test statistics, tables S7-12). Similarly, the stimulus which was rewarded in reversal learning had no significant effect on neither acquisition or test performances (GLMM: *group_RL*; *n =30*, see table 2 for the test statistics, tables S7-12). The age (median of 33 ± 2.7 days, Fig. S1) of the individuals did not affect either the acquisition performances or the accuracy in the tests of any task (GLMM: a*ge*; see table 2 for the test statistics, tables S7-12). Thus, there were no significant correlations between the age of the individuals and their acquisition performances (Spearman rank correlation; *n = 30*; **1st RL:** *rs = 0.3, p = 0.11*, **2nd RL:** *rs = -0.01, p = 0.18*, **NP:** *rs = 0.23, p = 0.22*, Fig. 3A-C) or test performances (*n = 30*; **1st RL:** *rs = -0.19, p = 0.31*, **2nd RL:** *rs = 0.07, p = 0.71*, **NP:** *rs = 0.04, p = 0.82*, Fig. 3D-F).

Here, the test performances of individual bees correlated positively across the two phases of reversal learning (Spearman rank correlation; *N = 26, rs = 0.57, p = 0.002*, Fig. 4D). Similarly, the test performances also correlated between the 1st phase of reversal learning and negative patterning (*n = 30, rs = 0.6, p = 0.0004*, Fig. 4E). No correlation was found between the test performances of the 2nd phase of reversal learning and negative patterning (*n = 26, rs = 0.34, p = 0.1*, Fig. 4F). This pattern of correlated and independent performances corresponds to results we obtained in an earlier study conducted with natural honey bee colonies (Finke et al. 2023).

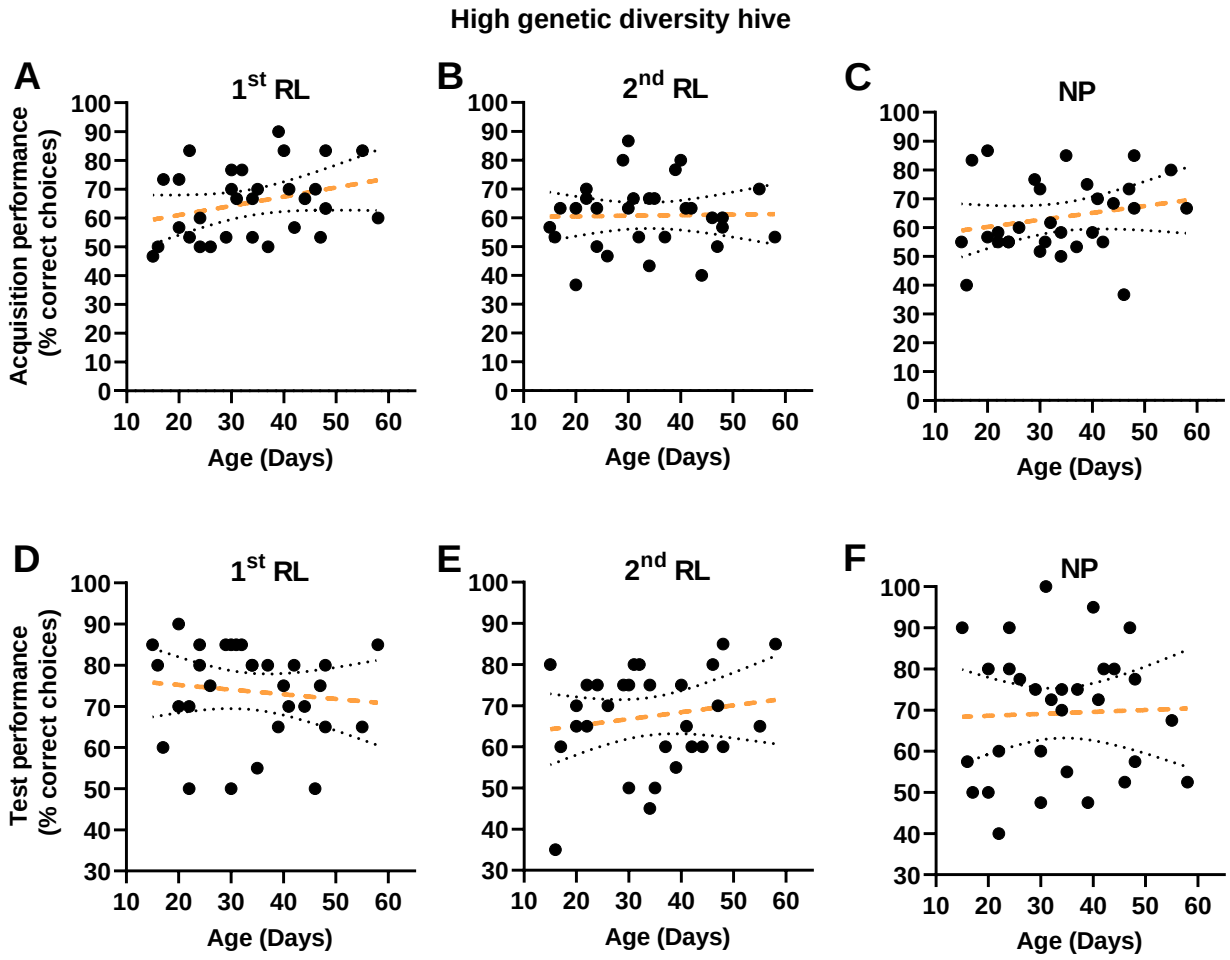

Fig. 3. Relationship between the age (in days) of the bees from the high genetic diversity hive and their performances in the acquisitions and non-reinforced tests of the 1st (A, D) and 2nd phase of reversal learning (B, E) and negative patterning (C, F). Acquisition performances were measured as percentage of correct choices made during the acquisition phase of each task. The test performances were measured as the percentages of correct choices the bees made during 20 choices in the non-reinforced test of each task. In all cases, no significant relationship between the bees' age and their performances were evidenced. The orange lines indicate regression lines and the black dashed lines indicate the confidence intervals of the regression. Each dot represents the data of a single bee.

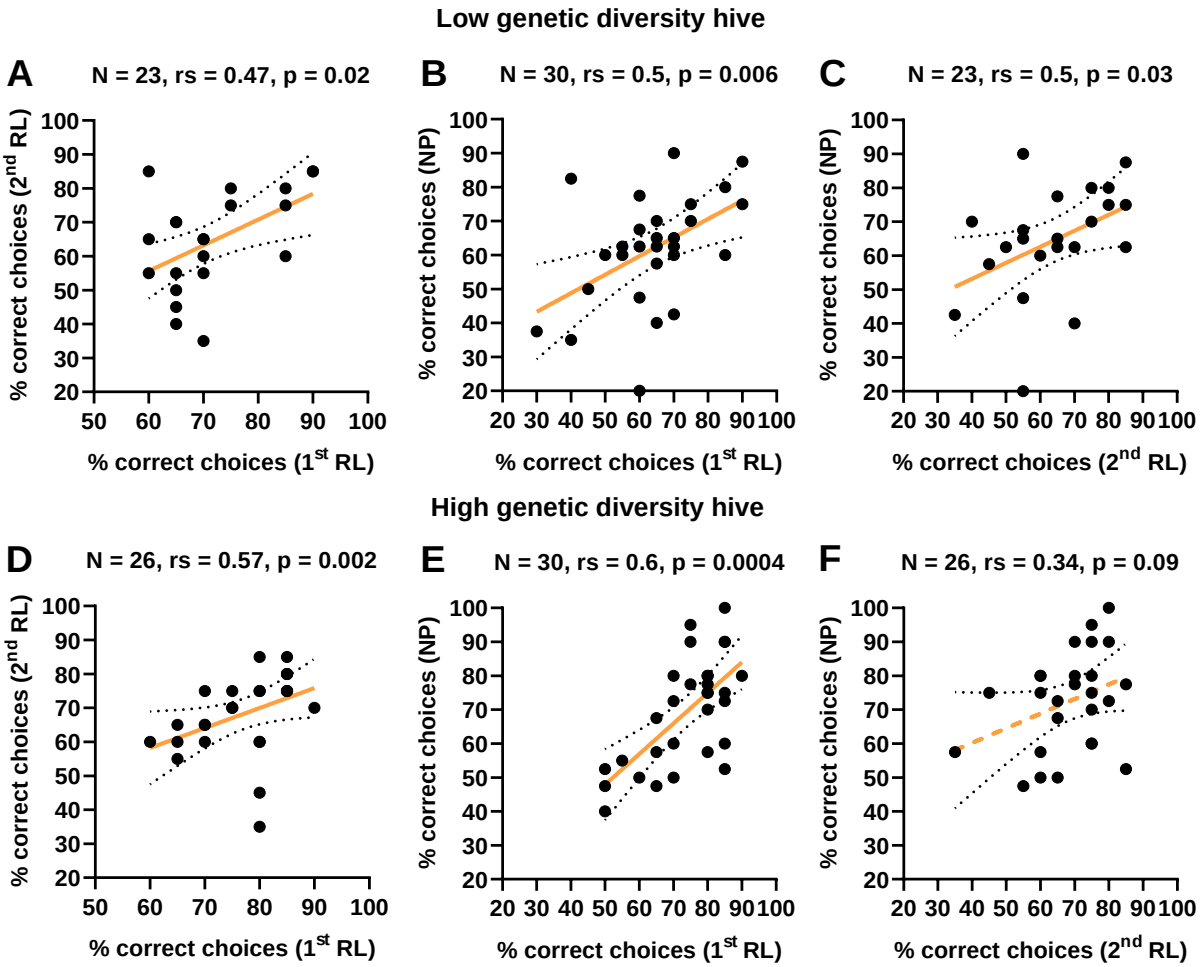

Fig. 4. Correlations between the individual test performances in the different tasks of the bees from the low (A-C) and high genetic diversity hives (D-F). The individual test performances of the bees from the low genetic diversity hive correlated positively across **A)** the 1st phase (1st RL: A+ vs. B-) and the 2nd phase of reversal learning (2nd RL: B+ vs. A-) of the reversal learning paradigm, **B)** the 1st phase of reversal learning (1st RL) and negative patterning (NP: C+/D+ vs. CD-) and **C)** the 2nd phase of reversal learning (2nd RL) and negative patterning (NP). Similarly, the individual test performances of the bees from the high genetic diversity hive were also positively correlated across **D)** both phases of the reversal learning and **E)** the 1st phase of reversal learning phase and negative patterning. **F)** Here, the individual test performances of the reversal learning phase and negative patterning revealed no significant correlation. Each dot represents the data of a single bee. The orange lines indicate the regression lines, and the dashed lines indicate the 95 % confidence intervals of the regression. The results of the Spearman rank correlations are presented above each panel.

Table 2 Test statistics for the GLMMs of the data from the high genetic diversity hive.

factor	task	phase	N	χ^2	p-value
trial	1st RL	acquisition	30	18.83	< 0.0001
	2nd RL	acquisition	30	63.31	< 0.0001
	NP	acquisition	30	20.21	< 0.0001
order	1st RL	acquisition	30	2.29	0.13
	2nd RL	acquisition	30	0.02	0.88
	NP	acquisition	30	0.10	0.75
	1st RL	test	30	0.67	0.42
	2nd RL	test	30	0.06	0.81
	NP	test	30	1.97	0.16
group_RL	1st RL	acquisition	30	0.40	0.53
	2nd RL	acquisition	30	1.06	0.30
	NP	acquisition	30	0.08	0.78
	1st RL	test	30	0.08	0.78
	2nd RL	test	30	1.41	0.24
	NP	test	30	0.36	0.55
age	1st RL	acquisition	30	0.61	0.44
	2nd RL	acquisition	30	0.004	0.95
	NP	acquisition	30	0.005	0.95
	1st RL	test	30	0.59	0.44
	2nd RL	test	30	1.03	0.31
	NP	test	30	0.05	0.83

5.5.3 Comparison of cognitive performances

There was no effect of hive origin on the acquisition performances of the 1st phase of reversal learning (GLMM; h*ive*: $n = 60$, $\chi^2 = 0.72$, $p = 0.4$, Fig. 1A, table S13) and the negative patterning paradigm (*hive:* $n = 60$, $\chi^2 = 1.21$, $p = 0.27$, Fig. 1C, table S15). However, there was a significant interaction effect between the trials of the acquisition and hive origin (*trial*hive*) in the 2nd phase of reversal learning only (GLMM; *hive*: $n = 60$, $\chi^2 = 6.49$ $p = 0.04$, Fig. 1B, Table S14). Nevertheless, the acquisition performances in the 2nd phase of reversal learning (i. e. the percentage of correct choices made during the 30 trials of the acquisition) were not significantly different between the two groups (Low genetic diversity hive: 60.0 ± 3.5 % correct choices; High genetic diversity hive: 63.3 ± 2.7 % correct choices; Mann-Whitney U test; $n = 60$, $U = 424$, $p = 0.70$, Fig. 1B). This indicates that although the bees from the different groups

made a similar number of correct choices, the bees showed different dynamics of learning throughout the acquisition of the 2nd phase of reversal learning (see Fig. 1B for both acquisition curves). Conversely, the bees from the two groups differed significantly in their test performances of both phases of reversal learning (GLMM; *hive*: *n = 60*, **1st RL:** $\chi^2 = 6.44$, $p = 0.01$, **2nd RL:** $\chi^2 = 6.38$, $p = 0.01$, Fig. 1A-B, Table S16-17). The bees from the 'high genetic diversity hive' performed better compared to those from the 'low genetic diversity hive' (**1st RL:** median of 77.5 ± 2.7 % vs. 65 ± 3.4 % correct choices; **2nd RL:** median of 70 ± 2.8 % vs. 57.5 ± 3.7 % correct choices). However, the test performances of negative patterning did not significantly differ between the two groups ('Low genetic diversity' hive: 62.5 ± 3.7 %; 'High genetic diversity' hive: 72.5 ± 3.7 % correct choices; GLMM; $n = 60$, $\chi^2 = 2.34$, $p = 0.13$, Fig. 1C, Table S18). Thereby, the age of the tested individuals did not significantly differ between the two groups (Low genetic diversity hive: 32.5 ± 2.6 days; High diversity group: 33 ± 2.7 days; Mann-Whitney U test, $n = 60$, $U = 443$, $p = 0.92$, Fig. S3).

The results demonstrate that the inter-individual variability in the test performances did not significantly differ between the bees from the high and low genetic diversity hive. The coefficient of quartile variation of the test performances in the learning phase amounted to 13.3 % for the 'high genetic diversity' group and 9.6 % for the 'low genetic diversity' group indicating homogeneity of variances between both groups (Fligner-Killeen test, $n = 60$, $\chi^2 = 0.05$, $df = 1$, $p = 0.83$). The same result was found for the test performances of the reversal learning phase (cqv_{HD} = 18.75 %, cqv_{LD} = 11.11 %, $n = 60$, $\chi^2 = 2.17$, $df = 1$, $p = 0.14$) and negative patterning (cqv_{HD} = 14.83 %, cqv_{LD} = 19.07 %; $n = 60$, $\chi^2 = 0.12$, $p = 0.73$).

5.6 Discussion

In this study we compared the cognitive capacities of bees originating either from a naturally mated queen ('high genetic diversity') or from her sister queen which had been mated with one drone only (low genetic diversity) in two complex associative learning paradigms. We asked whether genetic diversity among worker bees would have an impact on (1) inter-individual variability in learning performance, (2) group-level performance or (3) consistent individual differences in performance. In the 'low genetic diversity' hive all workers share 75 % of their genes, on average, as they belong to a single patriline. However, genetic recombination, developmental and environmental factors may contribute to individual learning performance. A queen naturally mates on average with 17 males (Blanchetot 1991; Estoup et al. 1994; Tarpy

et al. 2012). Her daughters therefore share between 28% and 33% of their genes. Inter-patriline variability in different behaviors is well documented (Robinson and Page 1989a; Page et al. 2000; Chapman et al. 2007; Seeley and Tarpy 2007; Mattila and Seeley 2007; Mattila et al. 2008; Scheiner and Arnold 2010; Eckholm et al. 2011). Differences in behaviors among workers of a patriline has been proposed to occur frequently in social insects (Frumhoff and Baker 1988; Page and Robinson 1991) although it is thought to be less distinct compared to inter-patriline differences (Oldroyd and Fewell 2007). However, the degree of intra-patriline variability in different behaviors remains poorly understood. In a study by Laloi & Pham-Delegue (2010), the proportion of variability in an olfactory discrimination task explained by differences among workers within a patriline was several times higher (88.4 %) compared to those explained by differences between patrilines within a colony (11.2 %), suggesting a small effect of patriline on learning performance in honey bees. In another study on olfactory PER conditioning the authors found that different patrilines showed differences in sucrose responsiveness and thus also in associative learning but they found no independent effect of patrilines on learning performances (Scheiner and Arnold 2010). Similarly, we found hardly any differences in the degree of inter-individual variability in the learning performances of bees from the 'high genetic diversity' hive and those from the 'low genetic diversity' hive. At the colony level, (genetically determined) consistent inter-individual variability in learning proficiency may contribute to task specialization and allocation and thus concur to the colonies' ability to flexibly adapt to environmental changes (Robinson 1992).

We further hypothesized that there would be differences in the group-level learning performances of bees from the high and low genetic diversity hives. Learning is a highly heritable trait. It has been shown that the learning performance of the worker bee progeny in several tasks can be predicted based on the drones' phenotype (appetitive and aversive olfactory discrimination: Benatar et al., 1995; Bhagavan et al., 1994; Brandes, 1988, 1991; Junca et al., 2014, 2019; Appetitive visual discrimination: Brandes & Menzel, 1990 Reversal learning and latent inhibition: Chandra et al., 2000). Consequently, we expected that the group-level learning performances of the low genetic diversity hive would either be increased or decreased in comparison to the high genetic diversity hive depending on the (unknown) learning phenotype of the drone used for artificial insemination. Indeed, we found that the group-level test performances of the bees from the low genetic diversity hive in the 1st phase (A+ vs. B-; Median of 77.5 % vs. 65 % correct choices) and the 2nd phase of reversal learning (A- vs. B+; Median of 70 % vs 57.5 % correct choices) were significantly lower compared to the performances of bees from the high genetic diversity hive although no significant difference was found between

the performances of both groups in the negative patterning paradigm (C+, D+ vs. CD-; Median of 72.5 % vs. 62.5 % correct choices). These differences in learning performances might also be explained by the age of the individuals rather than their colony origin as several studies have provided evidence that younger bees show lower learning proficiency compared to older bees (Pham-Delègue et al. 1990; Ray and Ferneyhough 1997; Laloi et al. 2001). However, the median age of the tested individuals from the two hives was not significantly different in our experiment (median of 32.5 vs 33 days of age), indicating that the differences in the group-level performances we found are most likely not due to differences in the age structure of the two different hives. Additionally, we found no overall effect of age on either the acquisition or the test performances in the simple discrimination, reversal learning or negative patterning. We therefore conclude that the differences in learning proficiency we observed could mainly be attributed to differences in the degree of genetic diversity between the two worker groups. At colony level, another explanation for the increased learning ability of bees from the 'high genetic diversity' hive is conceivable, independent on the learning phenotype of the drones. Genetically diverse colonies are generally healthier (i.e. have a lowered risk of diseases Tarpy and Seeley 2006; Seeley and Tarpy 2007) and have a higher fitness (Mattila and Seeley 2007; Wray et al. 2011), Indeed, genetically diverse colonies are more productive, show increased foraging efficiency (Mattila and Seeley 2007) and foraging-related communication compared to genetically uniform colonies (Mattila et al. 2008) which could reduce the potential costs of cognitive functions (Mery and Kawecki 2003, 2004; Mery 2005).

Finally, we investigated the potential impact of genetic diversity on individual consistency in the cognitive proficiency across the three learning tasks tested here. We found that bees from the 'high genetic diversity' hive displayed a performance in the 1st phase (A+ vs. B-) which was significantly positively correlated with the performance in the 2nd phase of reversal learning (A- vs. B+) and in negative patterning (C+, D+ vs. CD-), while the performances in reversal learning and in negative patterning were not significantly correlated. These results agree with those we obtained in an earlier study where we found this pattern of correlated individual performances (Finke et al. 2023). However, when we tested bees from the 'low genetic diversity' hive, the pattern of correlated performances was different. Here we found a significant positive correlation across the individual performances over all three learning tasks. These results indicate that the pattern of individual consistency we describe here and in Finke et al. (2023) may only account for naturally mated bee colonies with workers from multiple patrilines. A recent study by Smith et al. (2022) examined whether genetically identical *Drosophila*, reared under identical environmental laboratory conditions, show consistent

individual differences in their learning ability. The authors evidenced that isogenic flies still exhibited consistent individual differences in their olfactory discrimination performance which generalized across two aversive sensory modalities (electric shock and bitter taste). Furthermore, they also demonstrated a strong positive correlation between the individual fly's performance in an olfactory discrimination and subsequent reversal learning tasks. Interestingly, this correlation was largely independent of the odors and aversive reinforcements used and remained stable for two consecutive days. These results are in line with our findings and highlight the existence of individuality even though the flies were isogenic and experienced the same environmental conditions. It would be interesting to additionally test isogenic and genetically diverse flies in a negative patterning task, which has been recently established for *Drosophila* (Durrieu et al. 2020), to see if the pattern of individual consistency in learning ability also accounts for other insect species and thus might represent a more general cognitive characteristic of insects.

It is well-established that the individual responsiveness to the unconditioned stimulus (appetitive: sugar solution; aversive: bitter taste, electric shock) is genetically determined, at least partially, and can predict the performance in elemental learning tasks (Page et al. 1998; Pankiw and Page 1999; Scheiner et al. 2001a, b, 2004; Junca et al. 2014, 2019). Furthermore, the sucrose responsiveness of individual honeybees was found to correlate with the responsiveness to olfactory and tactile stimuli which serve as conditioned stimuli in classical conditioning as well as their phototactic behavior (Scheiner et al. 2004, 2005; Erber et al. 2006). Although the relationship between the sucrose responsiveness and the learning performance of bees in a free-flying context remains understudied (but see Mujagic and Erber 2009; Mujagic et al. 2010). Indeed, it could be possible that the bees from the low genetic diversity hive originated from a patriline with low sensitivity to sucrose, which could explain why they performed worse compared to the high genetic diversity hive. Thus, further research is clearly necessary to examine the contribution of subjective evaluation of the conditioned and unconditioned stimulus to consistent individual differences in the learning proficiency in honey bees.

Author Contributions

Conceptualization: AAW, RS; Methodology: VF, RS; AAW, MG; Data analysis: VF; Writing—original draft preparation: VF; Writing—review and editing: VF, AAW, RS and MG; Visualization: VF; Supervision: RS, AWW, MG; Project administration: AAW, RS; Funding acquisition: AAW, VF.

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Conflict of Interest

The authors declare no conflict of interest.

5.7 Supplementary material

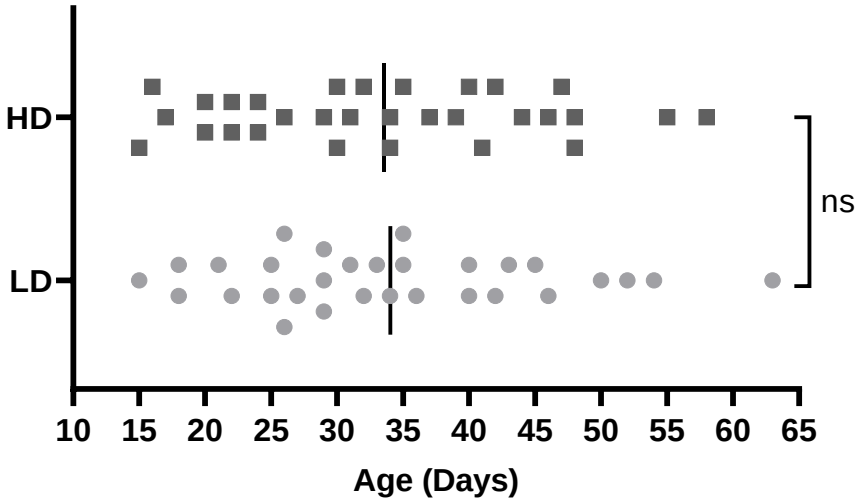


Figure S1. Comparison of the age (in days) of the tested bees from the high (HD, dark grey) and low (LD, light grey) genetic diversity hive. The median age of the bees from both groups was not significantly different (Median of 33 days for the high diversity group vs. 32.5 days for the low genetic diversity group: Mann-Whitney U test, N = 60, U = 443, p = 0.92). Each dot represents the age of a single tested bee. The black line indicates the median age of the bees from the two different hives.

Table S1. GLMM analysis of the bees' performance from the low genetic diversity group in the acquisition of the 1st phase of the reversal learning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL*age + (1|subject)	17	1165.2	-565.61	-	-
model2: response ~ trial*order*group_RL + age + (1|subject)	10	1154.3	-567.13	3.05	0.88
model3: response ~ trial*order*age + group_RL + (1|subject)	10	1154.6	-567.29	3.36	0.85
model4: response ~ order*age*group_RL + trial + (1|subject)	10	1154.3	-567.13	3.05	0.88
model5: response ~ trial*age*group_RL + order + (1|subject)	10	1152.9	-566.45	1.68	0.98
model6: response ~ trial*order + group_RL + age + (1|subject)	7	1150.1	-568.03	4.85	0.90
model7: response ~ trial*age + group_RL + order + (1|subject)	7	1150.1	-568.03	4.85	0.90
model8: response ~ order*age + group_RL + trial + (1|subject)	7	1149.5	-567.73	4.24	0.94
model9: response ~ group_RL*trial + order + age + (1|subject)	7	1148.8	-567.39	3.57	0.97
model10: response ~ age*group_RL + order + trial + (1|subject)	7	1149.0	-567.51	3.80	0.96
model11: response ~ trial + order + group_RL + age + (1|subject)	6	1148.3	-568.18	0.90	0.34
model12: response ~ trial + order + group_RL + (1|subject)	5	1149.8	-569.93	3.50	0.06
model13: response ~ trial + order + (1|subject)	4	1156.7	-574.37	8.88	0.003
model14: response ~ trial + group_RL + (1|subject)	**4**	**1148.5**	**-570.24**	**0.63**	**0.43**
model15: response ~ group_RL + (1|subject)	3	1161.8	-577.91	15.33	< 0.0001

Table S2. GLMM analysis of the bees' performance from the low genetic diversity group in the acquisition of the 2nd phase of the reversal learning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL*age + (1|subject)	17	1185.4	-575.68	-	-
model2: response ~ trial*order*group_RL + age + (1|subject)	10	1181.4	-580.70	10.04	0.19
model3: response ~ trial*order*age + group_RL + (1|subject)	10	1180.6	-580.3	9.267	0.23
model4: response ~ order*age*group_RL + trial + (1|subject)	10	1180.1	-580.05	8.74	0.27
model5: response ~ trial*age*group_RL + order + (1|subject)	10	1180.3	-580.15	8.94	0.26
model6: response ~ trial*order + group_RL + age + (1|subject)	7	1178.0	-581.98	12.60	0.25
model7: response ~ trial*age + group_RL + order + (1|subject)	7	1176.6	-581.32	11.27	0.34
model8: response ~ order*age + group_RL + trial + (1|subject)	7	1176.1	-581.04	10.71	0.38
model9: response ~ group_RL*trial + order + age + (1|subject)	7	1177.4	-581.70	12.04	0.28
model10: response ~ age*group_RL + order + trial + (1|subject)	7	1177.9	-581.95	12.53	0.25
model11: response ~ trial + order + group_RL + age + (1|subject)	**6**	1176.0	-581.99	1.91	0.17
model12: response ~ trial + order + group_RL + (1|subject)	5	1175.2	-582.61	1.23	0.27
model13: response ~ trial + order + (1|subject)	4	1175.8	-583.90	2.60	0.11
model14: response ~ trial + (1|subject)	**3**	**1174.6**	**-584.32**	**0.83**	**0.36**
model15: response ~ (1|subject)	2	1193.3	-594.63	20.62	< 0.0001

Table S3. GLMM analysis of the bees' performance from the low genetic diversity group in the acquisition of the negative patterning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL*age + (1|subject)	17	2315.2	-1140.6	-	-
model2: response ~ trial*order*group_RL + age + (1|subject)	10	2317.8	-1148.9	16.51	0.02
model3: response ~ trial*order*age + group_RL + (1|subject)	10	2310.4	-1145.2	9.16	0.24
model4: response ~ order*age*group_RL + trial + (1|subject)	10	2310.9	-1145.4	9.63	0.21
model5: response ~ trial*age*group_RL + order + (1|subject)	10	2316.9	-1148.4	15.62	0.03
model6: response ~ trial*order + group_RL + age + (1|subject)	7	2312.0	-1149.0	16.74	0.08
model7: response ~ trial*age + group_RL + order + (1|subject)	7	2312.5	-1149.	17.29	0.07
model8: response ~ order*age + group_RL + trial + (1|subject)	7	2308.7	-1147.3	13.46	0.20
model9: response ~ group_RL*trial + order + age + (1|subject)	7	2313.6	-1149.8	18.32	0.05
model10: response ~ age*group_RL + order + trial + (1|subject)	7	2313.4	-1149.7	18.16	0.05
model11: response ~ trial + order + group_RL + age + (1|subject)	**6**	2311.8	-1149.9	0.40	0.53
model12: response ~ trial + order + group_RL + (1|subject)	5	2310.0	-1150.0	0.18	0.68
model13: response ~ trial + order + (1|subject)	4	2310.4	-1151.2	2.43	0.12
model14: response ~ trial + (1|subject)	**3**	**2308.4**	**-1151.2**	**0.04**	**0.85**
model15: response ~ (1|subject)	2	2350.3	-1173.2	43.84	< 0.0001

Table S4. GLMM analysis of the bees' performance from the low genetic diversity group in the test of the 1st phase of the reversal learning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL*age + (1|subject)	9	781.43	-381.71	-	-
model2: response ~ order*group_RL + age + (1|subject)	6	776.69	-382.34	1.26	0.74
model3: response ~ age*group_RL + order + (1|subject)	6	777.09	-382.54	1.66	0.65
model4: response ~ order*age + group_RL + (1|subject)	6	777.34	-382.67	1.91	0.59
model5: response ~ order + group_RL + age + (1|subject)	5	775.99	-383.00	2.57	0.63
model6: response ~ order + group_RL + (1|subject)	4	776.57	-386.29	0.67	0.87
model7: response ~ order + (1|subject)	3	775.99	-383.00	1.30	0.25
model8: response ~ (1|subject)	**2**	**774.02**	**-383.01**	**0.03**	**0.42**

Table S5. GLMM analysis of the bees' performance from the low genetic diversity group in the test of the 2nd phase of the reversal learning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL*age + (1|subject)	9	803.37	-392.68	-	-
model2: response ~ order*group_RL + age + (1|subject)	6	802.05	-395.02	4.68	0.20
model3: response ~ age*group_RL + order + (1|subject)	6	804.33	-396.16	6.96	0.07
model4: response ~ order*age + group_RL + (1|subject)	6	802.99	-395.50	5.62	0.13
model5: response ~ order + group_RL + age + (1|subject)	5	802.4	-396.21	7.05	0.13
model6: response ~ order + group_RL + (1|subject)	4	803.09	-397.54	2.67	0.10
model7: response ~ order + (1|subject)	3	801.34	-397.67	0.25	0.62
model8: response ~ (1|subject)	**2**	**800.49**	**-398.2**	**1.15**	**0.28**

Table S6. GLMM analysis of the bees' performance from the low genetic diversity group in the test of the negative patterning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL*age + (1|subject)	9	1536.6	-759.29	-	-
model2: response ~ order*group_RL + age + (1|subject)	6	1535.9	-761.94	5.30	0.15
model3: response ~ age*group_RL + order + (1|subject)	6	1535.1	-761.54	4.50	0.21
model4: response ~ order*age + group_RL + (1|subject)	6	1534.6	-761.31	4.02	0.26
model5: response ~ order + group_RL + age + (1|subject)	5	1534.0	-761.98	5.37	0.25
model6: response ~ order + group_RL + (1|subject)	4	1533.8	-762.39	0.83	0.37
model7: response ~ order + (1|subject)	3	1534.9	-764.44	4.09	0.16
model8: response ~ (1|subject)	**2**	**1532.9**	**-764.46**	**0.05**	**0.82**

Table S7. GLMM analysis of the bees' performance from the high genetic diversity group in the acquisition of the 1st phase of the reversal learning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL*age + (1|subject)	17	1160.3	-563.14	-	-
model2: response ~ trial*order*group_RL + age + (1|subject)	10	1149.1	-564.56	2.85	0.90
model3: response ~ trial*order*age + group_RL + (1|subject)	10	1148.6	-564.29	2.31	0.94
model4: response ~ order*age*group_RL + trial + (1|subject)	10	1148.6	-564.29	2.31	0.94
model5: response ~ trial*age*group_RL + order + (1|subject)	10	1147.7	-563.83	1.38	0.99
model6: response ~ trial*order + group_RL + age + (1|subject)	7	1144.2	-565.12	3.97	0.95
model7: response ~ trial*age + group_RL + order + (1|subject)	7	1143.3	-564.65	3.03	0.98
model8: response ~ order*age + group_RL + trial + (1|subject)	7	1144.3	-565.14	4.00	0.95
model9: response ~ group_RL*trial + order + age + (1|subject)	7	1144.2	-565.07	3.87	0.95
model10: response ~ age*group_RL + order + trial + (1|subject)	7	1142.8	-564.40	2.52	0.99
model11: response ~ trial + order + group_RL + age + (1|subject)	**6**	1142.3	-565.15	**4.01**	**0.97**
model12: response ~ trial + order + group_RL + (1|subject)	5	1140.9	-565.45	0.61	0.44
model13: response ~ trial + order + (1|subject)	4	1139.3	-565.65	0.40	0.53
model14: response ~ trial + (1|subject)	**3**	**1139.6**	**-566.79**	**2.29**	**0.13**
model15: response ~ (1|subject)	2	1156.4	-576.21	18.83	< 0.0001

Table S8. GLMM analysis of the bees' performance from the high genetic diversity group in the acquisition of the 2nd phase of the reversal learning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL*age + (1|subject)	17	1156.8	-561.41	-	-
model2: response ~ trial*order*group_RL + age + (1|subject)	10	1153.5	-566.73	10.65	0.16
model3: response ~ trial*order*age + group_RL + (1|subject)	10	1145.4	-562.72	2.63	0.92
model4: response ~ order*age*group_RL + trial + (1|subject)	10	1147.5	-563.76	4.71	0.70
model5: response ~ trial*age*group_RL + order + (1|subject)	10	1152.1	-566.07	9.33	0.23
model6: response ~ trial*order + group_RL + age + (1|subject)	7	1148.2	-567.11	11.41	0.33
model7: response ~ trial*age + group_RL + order + (1|subject)	7	1146.9	-566.46	10.11	0.44
model8: response ~ order*age + group_RL + trial + (1|subject)	7	1143.9	-564.95	7.08	0.72
model9: response ~ group_RL*trial + order + age + (1|subject)	7	1150.1	-568.03	13.24	0.21
model10: response ~ age*group_RL + order + trial + (1|subject)	7	1149.7	-567.84	12.86	0.23
model11: response ~ trial + order + group_RL + age + (1|subject)	6	1148.1	-568.03	13.24	0.28
model12: response ~ trial + order + group_RL + (1|subject)	5	1146.1	-568.03	0.004	0.95
model13: response ~ trial + order + (1|subject)	4	1145.1	-568.56	1.06	0.30
model14: response ~ trial + (1|subject)	**3**	**1143.1**	**-568.57**	**0.02**	**0.88**
model15: response ~ (1|subject)	2	1204.5	-600.22	63.31	< 0.0001

Table S9. GLMM analysis of the bees' performance from high genetic diversity group in the acquisition of the negative patterning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL*age + (1|subject)	17	2310.4	-1138.2	-	-
model2: response ~ trial*order*group_RL + age + (1|subject)	10	2302.3	-1141.2	5.89	0.55
model3: response ~ trial*order*age + group_RL + (1|subject)	10	2301.8	-1140.9	5.30	0.62
model4: response ~ order*age*group_RL + trial + (1|subject)	10	2301.7	-1140.9	5.27	0.63
model5: response ~ trial*age*group_RL + order + (1|subject)	10	2302.8	-1141.4	6.36	0.50
model6: response ~ trial*order + group_RL + age + (1|subject)	7	2297.5	-1141.8	7.04	0.72
model7: response ~ trial*age + group_RL + order + (1|subject)	7	2297.4	-1141.7	6.96	0.73
model8: response ~ order*age + group_RL + trial + (1|subject)	7	2296.0	-1141.0	5.55	0.85
model9: response ~ group_RL*trial + order + age + (1|subject)	7	2297.2	-1141.6	6.71	0.75
model10: response ~ age*group_RL + order + trial + (1|subject)	7	2297.5	-1141.7	7.04	0.72
model11: response ~ trial + order + group_RL + age + (1|subject)	**6**	2295.5	-1141.8	7.05	0.80
model12: response ~ trial + order + group_RL + (1|subject)	5	2293.5	-1141.8	0.005	0.95
model13: response ~ trial + order + (1|subject)	4	2291.6	-1141.8	0.08	0.78
model14: response ~ trial + (1|subject)	**3**	**2289.7**	**-1141.8**	**0.10**	**0.75**
model15: response ~ (1 |subject)	2	2307.9	-1152.0	20.21	< 0.0001

Table S10. GLMM analysis of the bees' performance from the high genetic diversity group in the test of the 1st phase of the reversal learning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL*age + (1|subject)	9	707.2	-344.6	-	-
model2: response ~ order*group_RL + age + (1|subject)	6	705.2	-346.6	4.00	0.26
model3: response ~ age*group_RL + order + (1|subject)	6	705.11	-346.55	3.91	0.27
model4: response ~ order*age + group_RL + (1|subject)	6	702.93	-345.47	1.74	0.63
model5: response ~ order + group_RL + age + (1|subject)	5	703.29	-346.64	4.09	0.39
model6: response ~ order + group_RL + (1|subject)	4	701.88	-346.94	0.59	0.44
model7: response ~ order + (1|subject)	3	699.96	-346.98	0.08	0.78
model8: response ~ (1|subject)	**2**	**698.62**	**-347.3**	**0.67**	**0.42**

Table S11. GLMM analysis of the bees' performance from the high genetic diversity group in the test of the 2nd phase of the reversal learning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL*age + (1|subject)	9	753.20	-367.61	-	-
model2: response ~ order*group_RL + age + (1|subject)	6	749.99	-368.99	2.77	0.43
model3: response ~ age*group_RL + order + (1|subject)	6	749.93	-368.97	2.71	0.44
model4: response ~ order*age + group_RL + (1|subject)	6	749.53	-368.77	2.31	0.51
model5: response ~ order + group_RL + age + (1|subject)	5	747.99	-368.99	2.77	0.60
model6: response ~ order + group_RL + (1|subject)	4	747.02	-369.51	1.03	0.31
model7: response ~ order + (1|subject)	3	746.43	-370.21	1.41	0.24
model8: response ~ (1|subject)	**2**	**744.49**	**-370.24**	**0.06**	**0.81**

Table S12. GLMM analysis of the bees' performance from the high genetic diversity group in the test of the negative patterning paradigm. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	$p(>\chi^2)$
model1: response ~ order*group_RL*age + (1|subject)	9	1424.7	-703.34	-	-
model2: response ~ order*group_RL + age + (1|subject)	6	1423.5	-705.75	4.81	0.17
model3: response ~ age*group_RL + order + (1|subject)	6	1423.3	-705.65	4.60	0.20
model4: response ~ order*age + group_RL + (1|subject)	6	1421.5	-704.76	2.84	0.42
model5: response ~ order + group_RL + age + (1|subject)	5	1421.6	-705.78	4.87	0.30
model6: response ~ order + group_RL + (1|subject)	4	1419.6	-705.80	0.05	0.83
model7: response ~ order + (1|subject)	3	1418.0	-705.98	0.36	0.55
model8: response ~ (1|subject)	**2**	**1417.9**	**-705.98**	**1.97**	**0.16**

Table S13. GLMM analysis of the bees' performance in the acquisition of the 1st phase of the reversal learning paradigm for both groups combined. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	$p(>\chi^2)$
model1: response ~ trial*order*group_RL*hive*age + (1|subject)	33	9275.5	-4604.7	-	-
model2: response ~ trial*order*group_RL*hive + age + (1|subject)	18	9270.2	-4617.1	24.66	0.06
model3: response ~ trial*order*age*hive + group_RL + (1|subject)	18	9264.4	-4614.2	18.94	0.22
model4: response ~ order*age*hive*group_RL + trial + (1|subject)	18	9260.7	-4612.3	15.18	0.44
model5: response ~ trial*age*hive*group_RL + order + (1|subject)	18	9269.4	-4616.7	23.88	0.07
model6: response ~ trial*age*hive + group_RL + order + (1|subject)	11	9261.6	-4619.8	30.16	0.12
model7: response ~ trial*order*group_RL + hive + age + (1|subject)	11	9260.9	-4619.4	29.4	0.37
model8: response ~ trial*order*age + hive + group_RL + (1|subject)	11	9255.0	-4616.5	23.54	0.37
model9: response ~ order*age*hive + group_RL + trial + (1|subject)	11	9255.2	-4616.6	23.67	0.36
model10: response ~ hive*group_RL*trial + order + age + (1|subject)	11	9256.8	-4617.4	25.32	0.28
model11: response ~ trial*age + hive + group_RL + order + (1|subject)	8	9255.7	-4619.9	6.71	0.08
model12: response ~ trial*order + group_RL + hive + age + (1|subject)	8	9256.0	-4620.0	7.01	0.07
model13: response ~ trial + order + group_RL + hive + age + (1|subject)	7	9254.0	-4620.0	7.01	0.14
model14: response ~ trial + order + group_RL + hive + (1|subject)	6	9252.2	-4620.1	0.13	0.72
model15: response ~ trial + order + group_RL (1|subject)	5	9250.9	-4620.4	0.72	0.4
model16: response ~ trial + order + (1|subject)	4	9250.4	-4621.2	1.51	0.22
model17: response ~ trial + (1|subject)	**3**	**9248.9**	**-4621.5**	**0.56**	**0.46**
model17: response ~ (1|subject)	2	9343.6	-4669.8	96.67	< 0.0001

Table S14. GLMM analysis of the bees' performance in the acquisition of the 2nd phase of the reversal learning paradigm for both groups combined. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	$p(>\chi^2)$
model1: response ~ trial*order*group_RL*hive*age + (1|subject)	33	2342.6	-1138.3	-	-
model2: response ~ trial*order*group_RL*hive + age + (1|subject)	18	2332.8	-1148.4	20.26	0.16
model3: response ~ trial*order*age*hive + group_RL + (1|subject)	18	2328.7	-1146.3	16.12	0.37
model4: response ~ order*age*hive*group_RL + trial + (1|subject)	18	2332.2	-1148.1	19.70	0.18
model5: response ~ trial*age*hive*group_RL + order + (1|subject)	18	2329.9	-1146.9	17.33	0.30
model6: response ~ trial*age*hive + group_RL + order + (1|subject)	11	2322.5	-1150.2	23.95	0.35
model7: response ~ trial*order*group_RL + hive + age + (1|subject)	11	2332.8	-1155.4	34.21	0.06
model8: response ~ trial * order * age + hive + group_RL + (1 | subject)	11	2326.7	-1152.4	28.19	0.17
model9: response ~ order * age * hive + group_RL + trial + (1 | subject)	11	2327.0	-1152.5	28.41	0.16
model10: response ~ hive * group_RL * trial + order + age + (1 | subject)	11	2323.5	-1150.8	24.99	0.30
model11: response ~ trial * age + hive + group_RL + order + (1 | subject)	8	2327.9	-1156.0	11.44	0.01
model12: response ~ trial * hive + group_RL + order + age + (1 | subject)	8	2321.9	-1152.9	5.37	0.15
model13: response ~ trial + order + group_RL + hive + age + (1 | subject)	7	2326.2	-1156.1	6.32	0.012
model14: response ~ trial * hive + group_RL + order + (1 | subject)	7	2320.6	-1153.3	0.78	0.38
model15: response ~ trial * hive + group_RL + (1 | subject)	6	2319.2	-1153.6	0.58	0.45
model16: response ~ trial * hive + (1 | subject)	**5**	**2317.6**	**-1153.8**	**0.41**	**0.52**
model17: response ~ trial + (1 | subject)	3	2320.1	-1157.1	6.49	0.04
model18: response ~ hive + (1 | subject)	3	2398.2	-1196.1	84.54	< 0.0001

Table S15. GLMM analysis of the bees' performance in the acquisition of the negative patterning paradigm for both groups combined. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ trial*order*group_RL*hive*age + (1|subject)	33	4623.7	-2278.8	-	-
model2: response ~ trial*order*group_RL*hive + age + (1|subject)	18	4616.7	-2290.3	22.96	0.09
model3: response ~ trial*order*age*hive + group_RL + (1|subject)	18	4610.0	-2287.0	16.35	0.36
model4: response ~ order*age*hive*group_RL + trial + (1|subject)	18	4610.8	-2287.4	17.08	0.31
model5: response ~ trial*age*hive*group_RL + order + (1|subject)	18	4616.3	-2290.1	22.56	0.09
model6: response ~ trial*age*hive + group_RL + order + (1|subject)	11	4606.4	-2292.2	26.69	0.22
model7: response ~ trial*order*group_RL + hive + age + (1|subject)	11	4608.1	-2293.1	28.41	0.16
model8: response ~ trial*order*age + hive + group_RL + (1|subject)	11	4603.2	-2290.6	23.54	0.37
model9: response ~ order*age*hive + group_RL + trial + (1|subject)	11	4602.7	-2290.3	23.01	0.40
model10: response ~ hive*group_RL*trial + order + age + (1|subject)	11	4605.5	-2291.8	25.79	0.26
model11: response ~ trial*age + hive + group_RL + order + (1|subject)	8	4603.6	-2293.8	6.40	0.09
model12: response ~ trial*order + group_RL + hive + age + (1|subject)	8	4603.1	-2293.6	5.88	0.12
model13: response ~ trial + order + group_RL + hive + age + (1|subject)	7	4601.9	-2294.0	0.83	0.36
model14: response ~ trial + order + group_RL + hive + (1|subject)	6	4600.0	-2294	0.08	0.78
model15: response ~ trial + order + group_RL (1|subject)	5	4599.2	-2294.6	1.21	0.27
model16: response ~ trial + order + (1|subject)	4	4598.2	-2295.1	0.95	0.33
model17: response ~ trial + (1|subject)	**3**	**4596.2**	**-2295.1**	**0.004**	**0.95**
model18: response ~ (1|subject)	2	4656.0	-2326.0	61.87	< 0.0001

Table S16. GLMM analysis of the bees' performance in the test of the 1st phase of the reversal learning paradigm for both groups combined. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL*hive * age + (1|subject)	17	1486.9	-726.44	-	-
model2: response ~ order*group_RL*hive + age + (1|subject)	10	1478.4	-729.20	5.53	0.60
model3: response ~ order*age*hive + group_RL + (1|subject)	10	1481.0	-730.52	8.15	0.32
model4: response ~ age*hive*group_RL + order + (1|subject)	10	1478.4	-729.18	5.49	0.60
model5: response ~ order*group_RL*age + hive + (1|subject)	10	1480.4	-730.21	7.54	0.38
model6: response ~ order*group_RL + hive + age + (1|subject)	7	1477.1	-731.56	10.25	0.42
model7: response ~ order*age + hive + group_RL + (1|subject)	7	1475.4	-730.72	8.57	0.57
model8: response ~ hive*group_RL + order + age + (1|subject)	7	1473.6	-729.80	6.71	0.75
model9: response ~ order*hive + age + group_RL + (1|subject)	7	1477.5	-731.77	10.65	0.39
model10: response ~ age*hive + group_RL + order + (1|subject)	7	1477.4	-731.70	10.52	0.40
model11: response ~ age*group_RL + hive + order + (1|subject)	7	1477.3	-731.67	10.47	0.40
model12: response ~ order + group_RL + hive + age + (1|subject)	6	1475.5	-731.77	10.65	0.47
model13: response ~ order + group_RL + hive + (1|subject)	5	1473.9	-731.94	0.35	0.56
model14: response ~ order + group_RL + (1|subject)	4	1478.3	-735.16	6.44	0.01
model15: response ~ order + (1|subject)	4	1474.5	-733.26	2.64	0.10
model16: response ~ (1|subject)	**3**	**1473.8**	**-733.91**	**1.30**	**0.25**

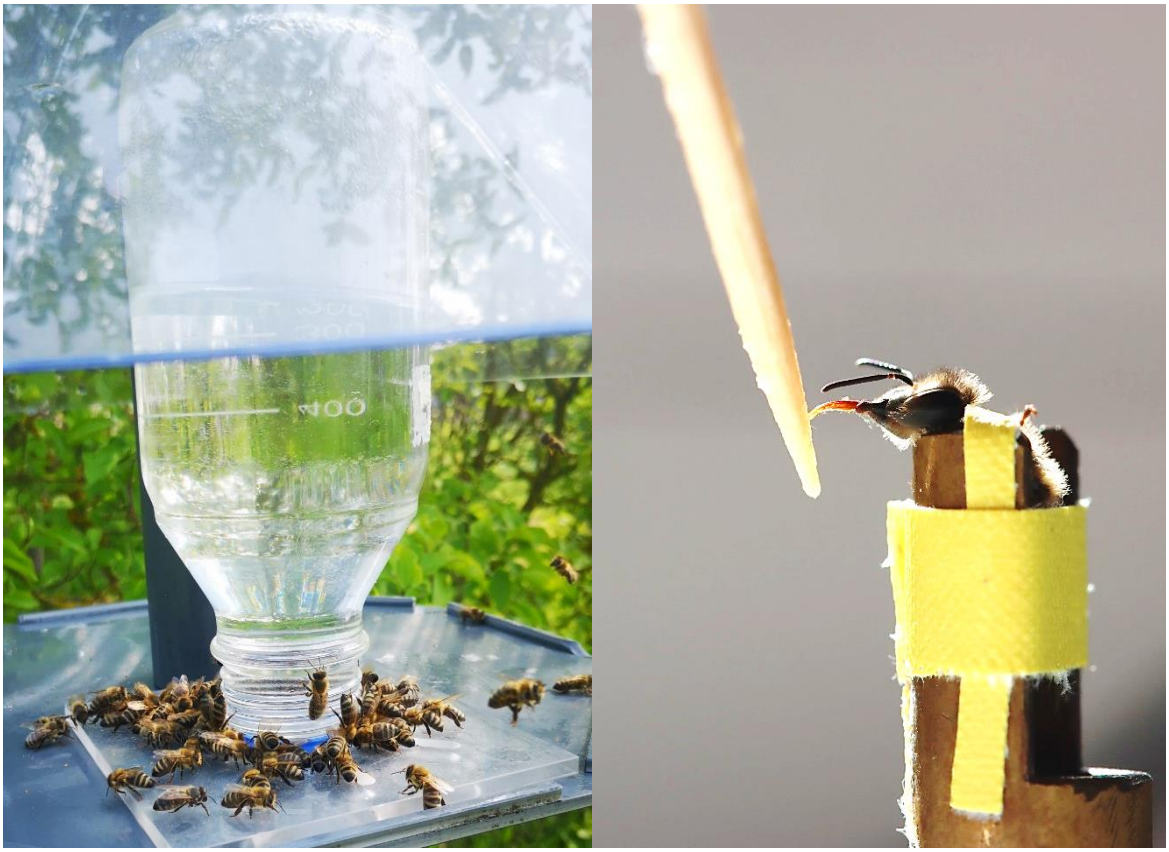
Table S17. GLMM analysis of the bees' performance in the test of the 2nd phase of the reversal learning paradigm for both groups combined. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL*hive*age + (1|subject)	17	1557.0	-761.52	-	-
model2: response ~ order*group_RL*hive + age + (1|subject)	10	1554.1	-767.07	11.10	0.13
model3: response ~ order*age*hive + group_RL + (1|subject)	10	1550.7	-765.36	7.69	0.36
model4: response ~ age*hive*group_RL + order + (1|subject)	10	1553.7	-766.82	10.62	0.16
model5: response ~ order*group_RL*age + hive + (1|subject)	10	1551.1	-765.54	8.10	0.33
model6: response ~ order*group_RL + hive + age + (1|subject)	7	1550.2	-768.11	13.19	0.21
model7: response ~ order*age + hive + group_RL + (1|subject)	7	1550.7	-768.35	13.66	0.19
model8: response ~ hive*group_RL + order + age + (1|subject)	7	1550.3	-768.16	13.29	0.21
model9: response ~ order*hive + age + group_RL + (1|subject)	7	1551.1	-768.54	14.05	0.17
model10: response ~ age*hive + group_RL + order + (1|subject)	7	1549	-767.50	11.96	0.29
model11: response ~ age*group_RL + hive + order + (1|subject)	7	1551.5	-768.75	14.47	0.15
model12: response ~ order + group_RL + hive + age + (1|subject)	6	1549.5	-768.77	14.50	0.21
model13: response ~ order + group_RL + hive + (1|subject)	5	1547.8	-768.88	0.23	0.63
model14: response ~ order + group_RL + (1|subject)	4	1552.1	-772.07	6.38	0.01
model15: response ~ order + (1|subject)	3	1545.8	-768.93	0.09	0.76
model16: response ~ (1|subject)	**2**	**1544.9**	**-769.43**	**1.02**	**0.31**

Table S18. GLMM analysis of the bees' performance in the test of the negative patterning paradigm for both groups combined. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order*group_RL*hive*age + (1|subject)	17	2960.8	-1463.4	-	-
model2: response ~ order*group_RL*hive + age + (1|subject)	10	2957.5	-1468.8	10.77	0.15
model3: response ~ order*age*hive + group_RL + (1|subject)	10	2957.3	-1468.7	10.55	0.16
model4: response ~ age*hive*group_RL + order + (1|subject)	10	2957.3	-1468.6	11.12	0.13
model5: response ~ order*group_RL*age + hive + (1|subject)	10	2957.9	-1468.9	11.12	0.13
model6: response ~ order*group_RL + hive + age + (1|subject)	7	2955.8	-1470.9	15.05	0.13
model7: response ~ order*age + hive + group_RL + (1|subject)	7	2952.5	-1469.3	11.77	0.30
model8: response ~ hive*group_RL + order + age + (1|subject)	7	2952.8	-1469.4	12.01	0.29
model9: response ~ order*hive + age + group_RL + (1|subject)	7	2955.0	-1470.5	14.26	0.16
model10: response ~ age*hive + group_RL + order + (1|subject)	7	2955.4	-1470.7	14.71	0.14
model11: response ~ age*group_RL + hive + order + (1| subject)	7	2954.6	-1470.3	13.85	0.18
model12: response ~ order + group_RL + hive + age + (1|subject)	6	2953.8	-1470.9	15.08	0.18
model13: response ~ order + group_RL + hive + (1|subject)	5	2951.9	-1470.9	0.06	0.81
model14: response ~ order + group_RL + (1|subject)	4	2952.2	-1472.1	2.34	0.13
model15: response ~ order + (1|subject)	3	2951.1	-1472.5	0.89	0.35
model16: response ~ (1|subject)	**2**	**2950.3**	**-1473.2**	**1.21**	**0.27**

6 Chapter 4: Relationships between sensory responses and learning performances are context-dependent in honey bees



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6.1 Abstract

The honey bee is a traditional insect model for studying learning, memory and cognition. Learning capacities play a fundamental role in the life of honeybees, especially in the context of foraging for profitable food sources such as sugar solutions provided by flowering plants in the form of nectar. This is the main source of carbohydrates for food provisioning of the colony. Honeybees need to remember the way to rich floral resources and back to their colony. In addition, they have been shown to memorize the shapes, colors and odors of profitable flowers very easily. This learning behavior can be employed for controlled experiments under laboratory, semi-natural and near-natural conditions aiming at understanding general principles of learning motivation and elucidating mechanistic reasons for individual differences in learning performance. Honey bees can be easily trained to solve a variety of different elemental and non-elemental learning tasks by pairing a conditioned stimulus (CS) with sucrose as unconditioned stimulus (US). Laboratory studies with restrained bees demonstrated that sucrose responsiveness correlates positively with appetitive elemental learning performances. Furthermore, sucrose responsiveness also correlates with responsiveness to different CS such as odors and visual stimuli. Here we have tested for the first time how responsiveness to sucrose and to light (phototaxis) is related to performance in elemental and non-elemental forms of learning under free-flying conditions. Three visual learning tasks of different complexity were studied: differential conditioning, reversal learning and negative patterning. Subsequently, the bees were tested for their sucrose and visual responsiveness. Importantly, the extensive conditioning procedures did not alter the sucrose responsiveness of the bees, as we did not find any differences between trained bees and bees randomly caught from a feeder. Our results do not point to a relationship between sensory responsiveness and one of the learning tasks under free-flying conditions. We also did not find a positive relationship between sucrose responsiveness and visual responsiveness as had been demonstrated earlier. These results indicate that relationships between sensory responsiveness and learning proficiency established under restrained conditions in the laboratory might not hold true for the natural behavior of bees in the field.

6.2 Introduction

The honey bee has been a long-standing model organism for learning, memory and cognition (Menzel and Müller 1996; Menzel et al. 2007; Giurfa 2007; Srinivasan 2010; Avarguès Weber et al. 2012). In appetitive learning experiments with honey bees, a sucrose solution is commonly used as positive reinforcement and paired with a conditioned stimulus (CS). In the well-established protocol of classical olfactory conditioning of the proboscis extension response (PER; Takeda 1961), sucrose solution is used as unconditioned stimulus (US) which evokes an innate response, the extension of the proboscis once the tarsi or antennae are stimulated. The odorant is used as CS and presented in close temporal association with the sucrose reward. When the bees have learned this association, they will show a PER if only the CS is presented without reinforcement. Learning proficiency is influenced by numerous factors among which the perception of the US and the CS play a major role (Annau and Kamin 1961; Rescorla and Wagner 1972; Scheiner et al. 2005). Sucrose is a crucial stimulus for honey bees because it is their main source of carbohydrates. Associative learning is essential for foragers to learn the location and characteristics of profitable food sources providing nectar (Menzel and Müller 1996).

Within a honey bee colony there is considerable inter-individual variation in the behavioral responses to sucrose. Individual sucrose responsiveness varies not only between bees of different ages, castes, sex (Pankiw and Page 1999), genotypes (Scheiner et al. 2001a, b; Scheiner and Arnold 2010), feeding states (Scheiner et al. 2003b) and foraging experience (Scheiner et al. 1999), but is also influenced by external factors such as season (Scheiner et al. 2003a). Interestingly, sucrose responsiveness correlates with elemental olfactory and tactile learning capabilities in classical and operant conditioning with restrained bees in the laboratory. Bees with higher responsiveness show an increased learning and memory performance compared to bees with low responsiveness (Scheiner et al. 1999, 2001 a,b, 2005). However, even when bees received equal subjective rewards during tactile conditioning inter-individual variability in learning performances were still observed, albeit only small ones (Scheiner et al. 2005). This indicates that the individual salience and perception of the CS also accounts for inter-individual variability in learning performances (Scheiner et al. 2005). For example, flower color preferences occur frequently in bees and might affect their learning performances (Lunau and Maier 1995; Giurfa et al. 1995b; Ings et al. 2009).

The relationship between learning proficiency and sucrose responsiveness has mainly been studied by analyzing elemental learning capabilities in restrained bees exposed to

differential conditioning procedures. Here, one stimulus is unambiguously associated with a reward (A+) and a second stimulus is not reinforced (B-). Such experiments have the great advantage of providing constant external conditions (such as temperature, humidity, and illumination) and defined timings of CS and US presentations that are the same for all tested bees. On the other hand, bees can also be subjected to operant-Pavlovian conditioning under free-flying conditions in their natural environment. Obviously, this form of conditioning has the disadvantage of fluctuating external conditions. However, the bees are free to leave the experimental set-up, and in contrast to the laboratory, they have a high intrinsic motivation to participate in the experiment. Furthermore, the conditioning of free-flying bees mirrors the natural behavior of bees while foraging for nectar and allows to reveal fine inter-individual differences in learning proficiency (Finke et al. 2023). Additionally, besides learning elemental associations which are characterized by the linear formation of unambiguous links between a stimulus (e.g. visual, olfactory or tactile) and a reinforcement (Rescorla and Wagner 1972), bees can also learn non-elemental associations which are non-linear and ambiguous (Rudy and Sutherland 1989; Giurfa 2003).

Whether sucrose responsiveness is similarly positively correlated with elemental and non-elemental learning performances under free-flying conditions is an unresolved question. Mujagic and Erber (2009) compared the sucrose acceptance of free-flying bees at an artificial feeder with their sucrose responsiveness measured under restrained conditions in the laboratory using the PER assay (Scheiner et al. 2013). They showed that it is not possible to extrapolate the sucrose acceptance in the field from responsiveness in the laboratory. The authors concluded that the environmental and behavioral context may therefore be an important factor modulating sucrose responsiveness. Indeed, a key feature of nervous systems is the context-dependent processing of sensory information. Recent studies in rodents contrasting inactive and active behavioral states strongly suggest that the behavioral context plays a prominent role in sensory processing. Arousal which is associated with increased locomotion (McGinley et al. 2015) enhances visually evoked neural activity and visual processing in the primary visual cortex (Niell and Stryker 2010; Erisken et al. 2014; Dadarlat and Stryker 2017; Pakan et al. 2018), increases spatial resolution (Mineault et al. 2016), reduces surround suppression (Ayaz et al. 2013; Erisken et al. 2014) and increases the detection of low contrast stimuli (Bennett et al. 2013). Thereby, arousal and locomotion seem to have complementary roles in the enhancement of visual processing (Vinck et al. 2015). Taken together these results suggest a more accurate encoding of visual stimuli during increased arousal and locomotion in vertebrates. Intriguingly, increased locomotion was associated with better performances in an associative learning task

in mice, suggesting that the behavioral context could modulate learning performances (Albergaria et al. 2018). In insects, some evidence for the modulation of sensory processing by behavioral context exists. Locomotion modulates neuronal processing for example by increasing in the gain of motion-sensitive neurons in *Drosophila melanogaster* (Chiappe et al. 2010; Maimon et al. 2010) and it also modulates sensory processing at the level of photoreceptors in bumble bees resulting in an increase of visual processing speed (Rother et al. 2022). However, the relationship between the modulation of sensory processing by behavioral state and learning remain largely unknown.

Within the last few years, the study of insect learning starts to shift from evaluating the average group performance to focusing on inter-individual variability in learning performance and the question of whether individuals remain stable in their proficiency over time and across different cognitive tasks (Tait et al. 2019; Junca et al. 2019; Tait and Naug 2020; Finke et al. 2021, 2023; Smith et al. 2022). Individual free-flying honey bees were shown to be consistent in their proficiency to learn an appetitive discrimination task over time (Finke et al. 2021). Similarly, the individual's proficiency to solve an appetitive, elemental discrimination was positively correlated with the ability to solve an appetitive non-elemental concept learning task under free-flying conditions (Finke et al. 2021). Considering these positive relationships between different learning performances it seems reasonable to assume that major factors contributing to such individual consistency in learning proficiency are sucrose and visual responsiveness. However, we recently provided evidence that restrained bees in the laboratory and free-flying bees in the field remain consistent across some, but not all learning tasks tested. While the proficiency to solve a simple discrimination (A+ vs. B-) was positively correlated with the ability to reverse the reward contingencies in a non-elemental reversal learning task (A- vs. B+) and a non-elemental negative patterning task (i.e. the ability to treat single elements differently from their mixture; C+ and D+ vs. CD-), the performances in two non-elemental tasks was not significantly correlated (Finke et al. 2023). These results challenge the hypothesis that CS and US responsiveness could be the decisive factors underlying consistent individual differences in cognitive proficiency.

The main goal of this study was to examine whether the relationships between sensory responsiveness and cognitive performance evidenced with restrained bees in the laboratory also applies to free-flying bees in the field. If similar relationships between responsiveness and cognitive performances can be found in restrained and free-flying bees, this would point towards general basic mechanisms underlying cognitive proficiency in bees. Demonstrating

distinct differences in these relationships, however, would indicate that the internal evaluation of sensory input is dependent on the bees' behavioral context. We also studied the effects of sucrose and visual responsiveness on elemental and non-elemental learning performances of free-flying bees in the field. We hypothesized that sucrose responsiveness could explain a proportion of the variability we observe in the consistent individual differences in the learning proficiency across reversal learning and negative patterning. We further hypothesized that the effect of sucrose responsiveness would decrease with increasing complexity of the task. Therefore, we repeated an earlier experiment (Finke et al. 2023) and sequentially subjected free-flying bees to a visual reversal learning and negative patterning task to correlate individual performances across these tasks. Reversal learning consists of two consecutive phases with a switch of reward contingencies between phases: The first phase of reversal learning tests the elemental learning proficiency of a subject by differential conditioning (A+ vs. B-). In the second phase of reversal learning the reward contingencies are switched (A- vs. B+), thereby creating transient stimulus ambiguity, which is a characteristic of non-elemental learning (Giurfa 2003, 2007). In a purely non-elemental negative patterning task (Whitlow and Wagner 1972) subjects have to learn that two single stimuli are rewarded (C+ and D+) when presented in isolation, but are not reinforced when presented in conjunction (CD-). After the bees had been tested in both learning tasks, they were subsequently assayed for their sucrose responsiveness and to light (Erber et al. 2006; Tsuruda and Page 2009).

6.3 Material and methods

A single mini plus colony (280 x 280 mm) was introduced into an outdoor flight cage (4 x 6 m) with an artificial gravity feeder providing 20 % (weight/weight) sucrose solution, a pollen and water source *ad libitum*. All bees used for this experiment were nectar foragers which were exclusively obtained from the artificial gravity feeder.

6.3.1 Learning tasks – General procedure

We used the same conditioning protocol as previously described (Finke et al. 2023). Before the bees (*n = 30*) were subjected to the learning tasks, they were first individually pre-trained to the experimental set-up. The experimental set-up was a rotating screen (50 cm in diameter) on a table where hangers (6 cm x 8 cm) could be attached at various locations. They displayed the

visual stimuli (see Fig. 1). A small landing platform on the bottom of the hanger displayed the reinforcement during pre-training and conditioning. Both the rotating screen and the hangers had the same grey color which was achromatic for the bees. For pre-training a bee was obtained from the artificial feeder and placed on the landing platform of a hanger displaying no stimuli, where it was allowed to collect an *ad libitum* reward of sucrose solution (50 % w/w). Once an unmarked bee landed by itself on the landing platform, it was individually marked with a colored spot on the thorax (Uni posca paint marker, Mitsubishi Pencil Co., Ltd.). Then this marked bee had to land on the landing platform to collect the reward for at least five consecutive foraging bouts until it quickly landed on a platform after arriving at the experimental set-up. Only one bee was conditioned at a time and recruited bees were caught and maintained in a cage to avoid any disturbance of the focal bee. Both learning tasks followed the same general procedure, but the visual stimuli, the type of reinforcement and the number of conditioning trials varied between tasks and are specified below. Once the bee completed pre-training it was fed *ad libitum* with sucrose solution, so it had to return to its colony.

While the bee was absent from the experimental set-up, the visual stimuli of the respective learning task (see specifications below) were taped to the hangers, attached to the rotating screen and 10 µl of the corresponding reinforcement was pipetted onto each landing. Each visual stimulus was represented two times on the rotating screen. For each trial during conditioning, a bee was required to approach the rotating screen and choose between the visual targets, land on the corresponding platform, consume (sucrose solution 50 % w/w in case of a correct choice) or taste (quinine or water in case of an incorrect choice) the reinforcement and then this choice was scored. If the bee made a correct choice, it was transferred to a big plexiglas spoon containing a small droplet of sucrose solution and moved one meter away from the screen. Meanwhile, the screen was rotated to alter the relative position of the hangers and the sucrose solution was refilled. If a bee made an incorrect choice, it could make further choices until a correct choice was made. Bees usually made three to five choices per foraging bout. While the bee was returning to the colony, all hangers were cleaned with 50 % ethanol to remove potential odor markings. The positions of the hangers were altered and all solutions were refilled. This course of action was repeated until the number of conditioning trials of the respective task (see below) was reached. Non-reinforced tests directly followed once the conditioning phase of the respective task was completed. New hangers and stimuli were used during tests and the visual stimuli were presented on the hangers without reinforcement. Each test lasted for 20 choices in total, whereas due to the absence of reinforcement a choice was

scored once a bee landed or touched a landing platform or stimulus. From this data we calculated a percentage of correct choices made by each bee in each non-reinforced test.

Half of the bees were first subjected to reversal learning followed by negative patterning and the other half experienced the reversed order. After the non-reinforced test of one learning task, the bee could collect sucrose reward *ad libitum* placed on the hangers displaying no visual stimuli for three foraging bouts. Afterwards, the conditioning phase of the alternative task started as described above. We used highly motivated bees which returned to the experimental set-up reliably within a short time frame after returning to the colony (usually within two to five minutes, a maximum of 10 minutes was tolerated one time during conditioning) and only bees that completed the conditioning and test phases of both learning tasks were kept for statistical analysis.

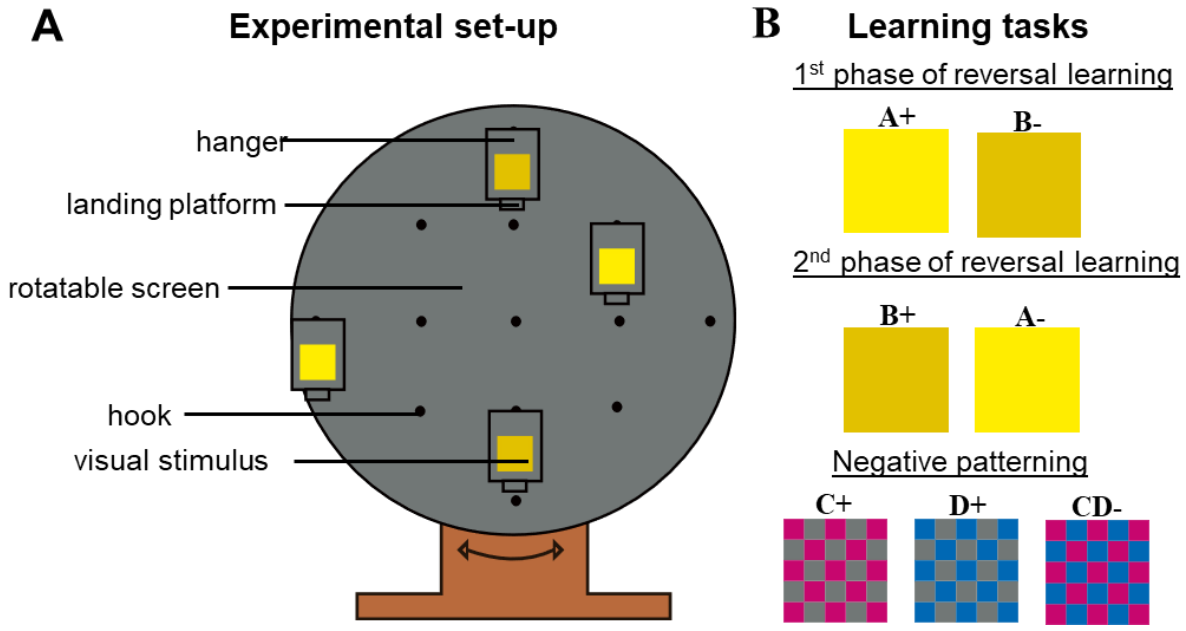

Fig. 1 Schematic overview of A) the experimental set-up and B) the learning tasks tested. **A)** The rotating screen apparatus was a vertical rotatable screen on a stand. The hangers displayed the stimuli of the respective learning tasks (5 x 5 cm squares) during conditioning and testing. They could be attached via hooks to the screen at various locations. The hangers had small landing platforms where the reinforcements could be collected by the bees during conditioning. **B)** Overview of the learning tasks tested. In the first phase of reversal learning one stimulus A+ was rewarded with a sucrose solution and a second stimulus B- was punished with a bitter quinine solution. In the second phase of reversal learning, the reward contingencies of the first phase were reversed, so that stimulus A- was now punished and B+ was rewarded. The stimuli used were HKS-3N and HKS-68N cardboards that appeared yellow and greenish yellow to the human eye, respectively. Half of the bees were initially trained with HKS-3N as stimulus A and HKS-68N In the negative patterning task the bees had to learn that two single stimuli C+ and D+ were rewarded with a sucrose solution while their compound CD- was not reinforced. The single stimuli were checkerboard squares cut from HKS-26N cardboards (C+, pink) and HKS-44N (D+, blue) on a grey background which was achromatic to the bees (HKS-92N). The CD- stimulus was produced with HKS-26N and HKS-44N cardboards.

6.3.2 Reversal learning

The reversal learning protocol involved two phases: The first phase (1st RL) is a differential conditioning where one visual stimulus (A+) was associated with a reward (50 % sucrose solution w/w) and a second stimulus (B-) was associated with a punishment (60 mM quinine solution). In the second phase, the reward contingencies of the first phase were reversed, so that the previously rewarded stimulus was now associated with the punishment (A-) and the other became associated with a sucrose reward (B+). Importantly, reversal learning ability can only be evaluated in individuals that have previously learned the initial discrimination in the first phase of reversal learning (Ben-Shahar et al. 2000; Hadar and Menzel 2010; Mota and Giurfa 2010; Boitard et al. 2015). The stimuli used as A and B were colored squares cut from HKS-3N and HKS-68N cardboards (5 x 5 cm HKS-N pigment papers; Hostmann-Steinberg K+E Druckfarben, H. Schmincke and Co., Germany) that appeared yellow and greenish yellow to the human eye respectively. Each of the two phases involved conditioning for 30 trials followed by a non-reinforced test for 20 choices and after the test of the first phase, the conditioning of the second phase was started after one foraging bout. During conditioning and testing the screen always displayed four hangers in total, two with stimulus A and two with stimulus B. Half of the bees were initially conditioned using HKS-3N as stimulus A and HKS-68N as stimulus B while it was switched for the other half.

6.3.3 Negative patterning

In negative patterning, bees must learn that two colors are rewarded when presented in isolation (C+ and D+) but are not reinforced when presented together (CD-). Such a task can only be solved when the compound stimulus is treated differently than the sum of its components and requires configural processing abilities (Deisig et al. 2001; Schubert et al. 2002; Devaud et al. 2015). The protocol and the design of the stimuli were adapted and modified from Schubert et al. (2002). The conditioning phase consisted of three different blocks of trials: two where either of the stimuli C+ or D+ were presented in isolation (absolute conditioning) with 10 µl of a 50 % sucrose solution on four hangers. In the third block the non-reinforced compound stimulus CD- was associated with 10 µl of water and presented together with a rewarding alternative (XY+, differential conditioning) which offered a 10 µl sucrose reward on two hangers each. In the block with the compound stimulus, it was necessary to add a rewarding alternative because too many un-rewarding trials cause a decrease in the bees' motivation and ultimately in the

'loss' of the bee, because it would stop foraging on the experimental set-up. The different blocks of trials were pseudo-randomized throughout conditioning and lasted for one foraging bout. Then, when a bee returned to the hive, the stimuli were exchanged and another block was initiated. The whole conditioning phase amounted to 120 trials in total, 30 each with the single stimuli C+ or D+ and 60 with the compound CD- and the rewarding alternative XY+. Two non-reinforced tests consisting of 20 trials each directly followed the conditioning procedure where either C+ or D+ were presented together with CD- on two hangers each. Between tests, the bees were subjected to reinforced refreshing trials for one foraging bout to ensure a high motivation for the second test. The stimuli used were all checkerboard squares (5 x 5 cm) cut from different HKS-N cardboards (Hostmann-Steinberg K+E Druckfarben, H. Schmincke and Co., Germany). The single stimuli consisted of 1 x 1 cm squares of either HKS-26N (C+, pink) or HKS-44N (D+, blue) on a grey background (HKS-92N) and the compound stimulus CD- consisted of 1 x 1 cm squares of HKS-26N and HKS-44N (see Fig. 1). The rewarding alternative XY+ consisted of black 1 x 1 cm squares (HKS-88N) on white copy paper background.

6.3.4 Sucrose responsiveness assay

Once a bee completed the non-reinforced test of the second learning task it was directly captured in a small glass vial. In the laboratory, the bee was immobilized by placing the vial in crushed ice until all movements ceased (~ two to three minutes). Once immobilized, the bee was carefully harnessed in small metal tubes, so that only the mouthparts could be moved freely. The head was fixed with a small stripe of adhesive tape and the body remained inside the tube and was also stabilized with adhesive tape. As the bees had to be removed from the harness after the assay, all parts of the adhesive tape that made direct contact with the bee was coated with soft tissue. After fixation, the bee was fed with five µl of sucrose solution (30 % w/w) and placed in a dark and humid chamber at room temperature for 30 minutes. Then the bees' responsiveness to different sucrose concentrations of 0 (water), 0.1, 0.3, 1, 3, 10 and 30 % (w/w) was tested (Scheiner et al. 2004; Scheiner and Arnold 2010; Scheiner et al. 2013). Briefly, the bees' antennae were touched with a toothpick soaked in the corresponding solution. If the bee perceived the sucrose in the solution, it extended its proboscis upon antennal stimulation. The assay started with water and continued with ascending sucrose concentrations. The inter-trial interval was two minutes between sucrose stimulations to avoid sensitization effects (Scheiner et al. 2003b). We then calculated a gustatory response score (GRS) for each bee by summing up all proboscis extensions to water and the six different sucrose concentrations. The GRS could

consequently range between ‘0’ and ‘7’, while ‘0’ indicates no responsiveness to sucrose and ‘7’ indicates high responsiveness. After a bee had completed this assay, it was very carefully removed from the harness and placed in a petri dish for the phototaxis assay. Classically, the sucrose responsiveness assay is performed before learning proficiency is tested (Scheiner et al. 1999, 2001a, 2003a). We therefore randomly collected bees from the artificial gravity feeder at two time points (beginning and end of the experiment) and measured their GRS to evaluate whether the participation of the bees in the learning tasks altered their sucrose responsiveness.

6.3.5 Phototaxis assay

Phototactic behavior or responsiveness to light was assessed directly after sucrose responsiveness. First the bees had to adapt to the dark and were placed in a petri dish (diameter = 85 mm) in constant darkness for 15 minutes. The phototaxis assay was conducted according to an established and standardized protocol (Erber et al. 2006; Thamm et al. 2010; Scheiner et al. 2014; Schilcher et al. 2021). We used the same set-up as described by Schilcher et al. (2021). After dark adaptation, a bee was placed inside the phototaxis arena (diameter = 35 cm) in darkness to quantify its mean velocity while walking in the arena for two minutes. This served as a measure of its general locomotor activity.

Then, the 12 green light-emitting diodes (LEDs, wavelength = 527 nm) with different relative light intensities (3.125 %, 6.2 5%, 12.5 %, 25 %, 50 % and 100 % intensity; maximum light intensity: 2.61×10^{14} photons/cm^2) were successively switched on, beginning with the lowest intensity and then in ascending order. Two LEDs of the same light intensity were located on opposing sides in the arena. Once an LED was switched on, it was recorded how long a bee needed to walk towards it. When a bee reached the LED, the light was switched off and the opposing LED was switched on. This procedure ensured that the bees had to walk a minimum of 35 cm to reach the opposing light source. This course of action was repeated four times for each light intensity. The behavior of the bees was monitored and recorded via an infra-red camera mounted on top of the arena and connected to a computer, where the walking time of each bee was recorded with a computer stopwatch. For every light intensity we calculated the mean walking time from the four trials for each bee. To determine the locomotor activity of the bees during the dark walks we used the software UMA tracker (Yamanaka and Takeuchi 2018) which allowed us to measure the distance walked during one minute and from that we calculated

the mean velocity (m/s). Bees that were not motivated to walk in the arena for five minutes were discarded from analysis (*n = 6 of 30*).

6.4 Statistical Analysis

Individual consistency across the different performances in the learning tasks, i.e. the percentages of correct choices made in the non-reinforced tests, was investigated using Spearman rank correlations. For all correlations including the second phase of reversal learning we performed correlations only including bees that had earlier learned in the first phase. This is important, because the ability to reverse a previously learned association in the second phase of reversal learning prerequisites successful learning of the initial discrimination task (A+B-, Mota and Giurfa 2010, *n = 26 of 30* bees in total). We used an arbitrary threshold of ≥ 60 % correct choices to consider bees as a learner in the first phase of reversal learning (Finke et al. 2023). To assess if the order in which the learning tasks were conducted or the rewarding color used in reversal learning (*group_RL*) had an influence on the test performances of the three learning tasks we used generalized linear mixed models (GLMM). The models had a binomial error structure with a logit-link function and included the percentage of correct choices made in each non-reinforced test as dependent variable. All models included the order in which the learning tasks were conducted and the rewarding color used in reversal learning as fixed factors and the bees' identity as random factor. We performed different models, starting with the most complex model including all factors and then one factor was gradually removed and compared with an ANOVA. The most appropriate fit was selected based on the lowest AIC value (Burnham and Anderson 1998; Panchal et al. 2010, Tab. S1-S3). The GLMMs were performed using R Statistical Software (v3.6.3; R Core Team 2022) with the package lme4 (Bates et al. 2015).

To evaluate the relationship between the individual test performances in reversal learning and negative patterning, locomotor activity, visual and gustatory responsiveness we performed Spearman rank correlations. The GRS of the tested bees were compared to control bees caught at the artificial feeder using Mann-Whitney U tests to evaluate whether having been subjected to the learning tasks altered the bees' sucrose responsiveness. These statistical analyses and the graphs were made with GraphPad prism version 9.4.0 (GraphPad Software Inc., San Diego, California, USA). To account for our relatively small sample size, we chose a significance level of $\alpha = 0.05$.

6.5 Results

6.5.1 Individual consistency in learning performances

We tested all bees sequentially in the two learning tasks reversal learning (1st RL, 2nd RL) and negative patterning (NP) to examine if the proficiency to solve one task correlated with the proficiency to learn an alternative task. Only bees (*n = 26*) that successfully learned in the first phase of reversal learning (≥ 60 % correct choices in the test), were used for all correlations including the second phase of reversal learning. The order in which the learning tasks were conducted (either reversal learning or negative patterning being conducted first respectively) did not affect test performances in any task (GLMM*; order; n = 30,* ***1st RL:*** $\chi^2_{(1)}$ *= 0.89, p = 0.35,* ***2nd RL:*** $\chi^2_{(1)}$ *= 1.07, p = 0.30,* ***NP:*** $\chi^2_{(1)}$ *= 0.29, p = 0.59).* Similarly, the color identity of the rewarded stimulus (yellow or greenish-yellow cardboard) used in reversal learning did not affect test performances in any task (GLMM*; group_RL: n = 30,* ***1st RL:*** $\chi^2_{(1)}$ *= 0.11, p = 0.74,* ***2nd RL:*** $\chi^2_{(1)}$ *= 2.93, p = 0.09,* ***NP:*** $\chi^2_{(1)}$ *= 0.28, p = 0.59).* The data were thus pooled for further statistical analysis.

Individual test performances in the first phase of reversal learning were positively correlated with performances in the second phase of reversal learning (Spearman rank correlation, *n = 26, rho = 0.62, p = 0.0008,* Fig. 2A). We also found a significant positive correlation between test performance in the first learning phase of reversal learning and negative patterning (*n = 30, rho = 0.61, p = 0.0003,* Fig. 2B). The correlation between the individual test performances in the second phase of reversal learning and negative patterning showed a positive trend which, however, was not significant (*n = 26, rho = 0.33, p = 0.1, Fig. 2C*). This pattern of individual correlated performances was the same as previously described by Finke et al. (2023).

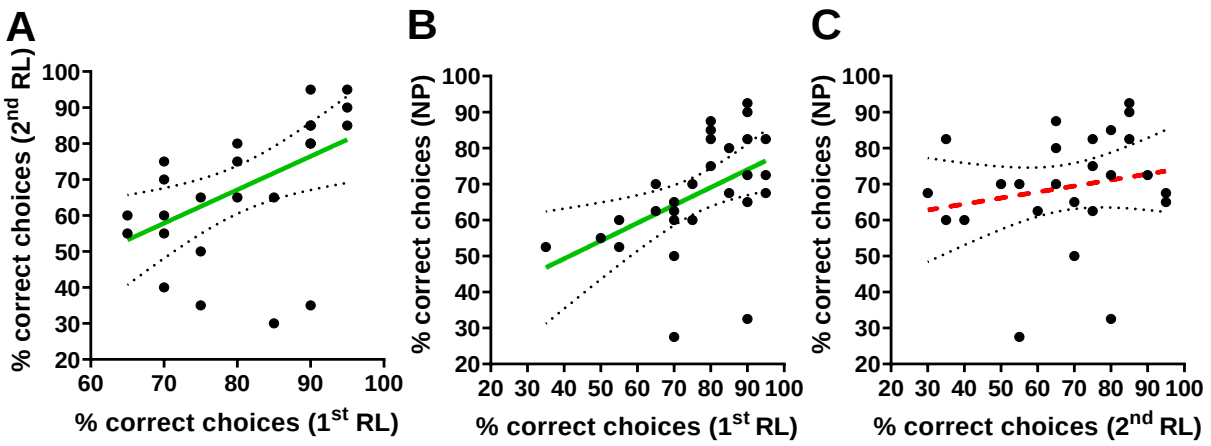

Fig. 2 Individual consistency in the test performances of the first and second phase of reversal learning (1st and 2nd RL) and negative patterning (NP). The graphs show the Spearman rank correlations of the individual test performances measured as percentage of correct choices made during the 20 choices in the non-reinforced test of the respective learning task. For all correlations including the 2nd phase of reversal learning, only bees (n = 26) that successfully learned in the 1st phase of reversal learning (≥ 60 % correct choices in the test), as reversal learning ability can only be assessed in individuals that have learned the initial A+B- association established in the 1st phase. The individual bees' test performances were significantly positively correlated across the 1st phase of reversal learning and **A)** the 2nd phase of reversal learning (*n = 26, rho = 0.62, p = 0.0008, R^2 = 0.26*) and **B)** negative patterning. (*n = 30, rho = 0.61, p = 0.0003, R^2 = 0.22*). **C)** The individual test performances in the 2nd phase of reversal learning and negative patterning revealed a positive but non-significant correlation (*n = 26, rho = 0.33, p = 0.1, R^2 = 0.04*). Each dot represents the data of a single bee. The lines indicate a linear regression with the 95 % confidence intervals shown as grey dotted lines. Green solid lines indicate a significant positive correlation and red dotted lines indicate positive but non-significant correlations.

6.5.2 Effect of locomotor activity, visual and gustatory responsiveness on learning performances

The bees tested were generally highly responsive to sucrose, displaying a median GRS of '6'. Untrained control bees collected at the start of the experiment ('control 1') and those collected at the end ('control 2') of the experiment did not differ in their GRS from trained bees testes in their GRS after training (Two-way Mann-Whitney U test; **control 1 vs. trained bees**: $n_{untrained}$ *= 19,* $n_{trained}$ *= 29, U = 189, p = 0.06;* ,**control 2 vs. trained bees:** $n_{untrained}$ *= 30,* $n_{trained}$ *= 29, U = 374, p = 0.35,* Fig. S1B). Thus, we conclude that performing the extensive learning protocols did not alter the responsiveness to sucrose. We next examined the relationship between GRS and the learning performances of the bees in the different tasks. There was no significant correlation between GRS and test performances in the first phase of reversal learning (Spearman rank correlation; *n = 29, rho = -0.08, p = 0.68,* Fig. 3A) in the second phase of reversal learning (*rho = 0.11, p = 0.55,* Fig. 3B*)* and in negative patterning (*rho = -0.11, p = 0.55,* Fig. 3C). This indicates that sucrose responsiveness tested after training does not correlate with learning performance in free-flying honeybees.

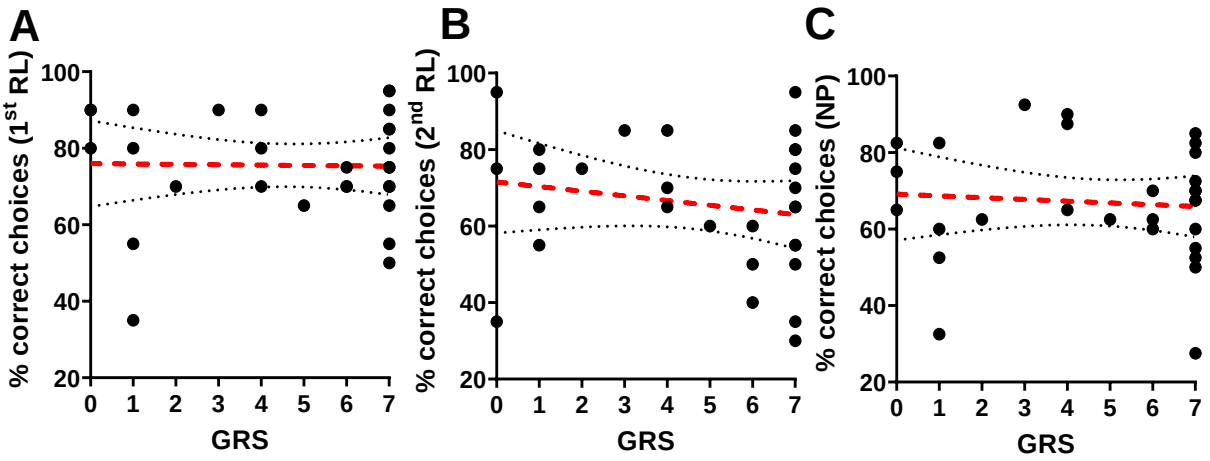

Fig. 3 Relationship between the sucrose responsiveness of the bees and their test performances in reversal learning and negative patterning. Sucrose responsiveness was measured with the gustatory response score (GRS) i.e. the number of probiscis extensions upon antennal stimulation with water and six ascending sucrose concentrations. The test performances were measured as percentage of correct choices during the 20 choices in the non-reinforced test of the respective learning task. The GRS was not significantly correlated with the test performances in **A)** the 1st phase of reversal learning (1st RL: Spearman rank correlation; *n = 29, rho = -0.08, p = 0.68, R^2 = 0.0003*), **B)** the 2nd phase of reversal learning (2nd RL: *n = 29, rho = -0.15, p = 0.43, R^2 = 0.04*) and **C)** negative patterning (NP: *n = 29, rho = -0.12, p = 0.55, R^2 =0.006*). Each dot represents the data of a single bee. The lines indicate a linear regression with the 95 % confidence intervals shown as grey dotted lines. Red dashed lines represent non-significant correlations.

We further assessed phototaxis, i.e. visual responsiveness of honey bees, after training to colors with the three learning tests. Walking time significantly increased with increasing light intensity (Spearman rank correlation; *n = 24, rho = -0.35, p < 0.0001,* Fig.S2). However, test performances in the first and second phases of reversal learning and in negative patterning did not correlate with phototactic responses at different light intensities (Fig. 4, Fig. S3 and S4, see Tab. 1 for correlation coefficients and p-values). Intriguingly, we found a tendency for a positive correlation (Spearman rank correlation; *rho = 0.39, p = 0.06* in both cases) for the two highest intensities (50 % and 100 %), indicating that bees which were slower in walking towards the light stimulus performed better in the test of the first phase of reversal learning. Locomotor behavior did not correlate with test performances in any of the three learning tasks (Spearman rank correlation; *n = 29*; first phase reversal learning: *rho = 0.08, p = 0.69*; second phase of reversal learning: *rho = 0.02, p = 0.92*, negative patterning: *rho = 0.114, p = 0.49*, Fig. S5).

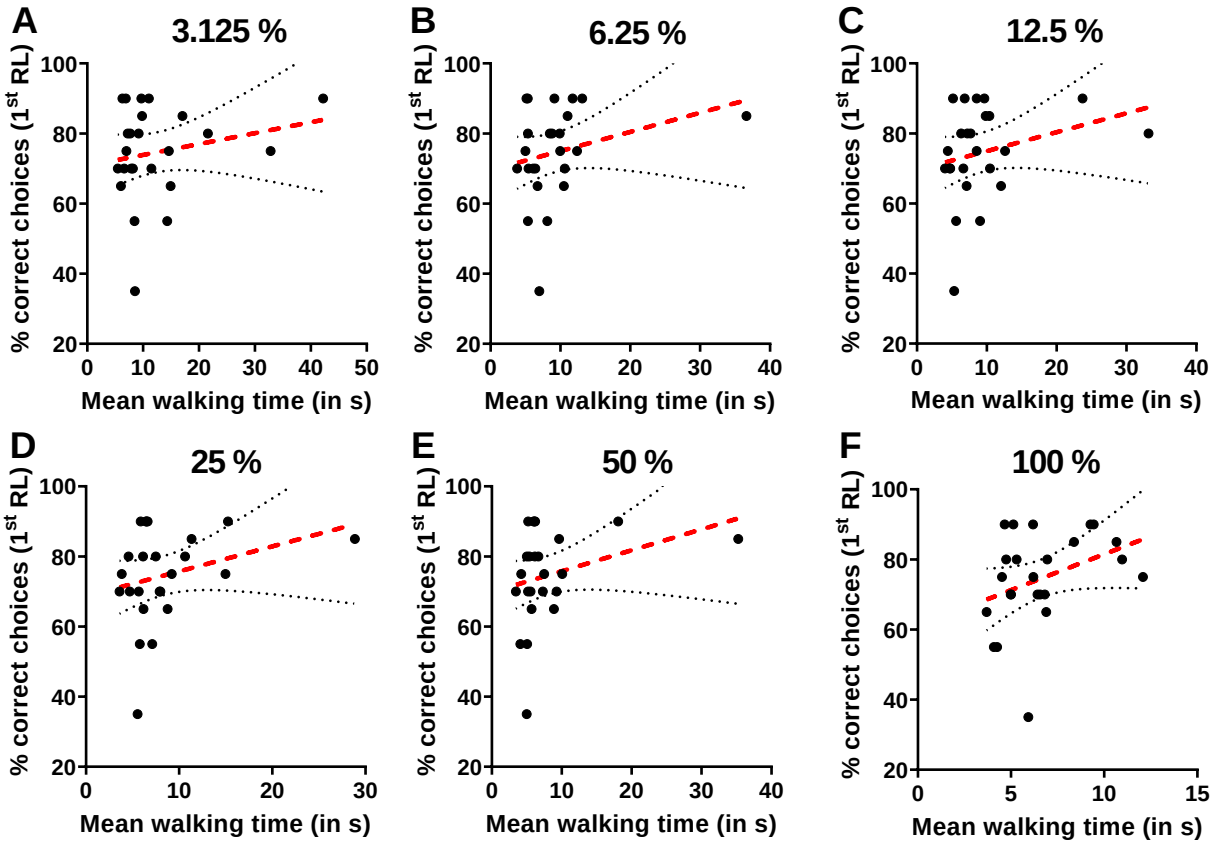

Fig. 4 Relationship between the individual bees' test performances in the 1st phase of reversal learning (1st RL) and visual responsiveness. The visual responsiveness was measured by the mean walking times (in seconds) in the phototaxis assay for different relative light intensities. The test performances were measured as percentage of correct choices made in the non-reinforced test during 20 choices in total. The graphs show the Spearman rank correlations of the GRS and the mean walking times at light different intensities (*n = 24*): **A)** 3.125 %: *rho = 0.13, p = 0.56, R^2 = 0.04*; **B)** 6.25 %: *rho = 0.26, p = 0.21, R^2 = 0.07*; **C)** 12.5 %: *rho = 0.27, p = 0.2, R^2 =0.07*; **D)** 25 %: *rho = 0.30, p = 0.16, R^2 = 0.08*; **E)** 50 %: *rho= 0.39, p = 0.06, R^2 =0.08*; **F)** 100 *%: rho = 0.39, p = 0.06, R^2 = 0.13*. Each dot represents the data of a single bee. The lines indicate a linear regression with the 95 % confidence intervals shown as grey dotted lines. Red dashed lines indicate non-significant correlations.

Table 1 Results of the Spearman rank correlations analyzing the relationship between the test performances in the two phases of reversal learning (1st RL, 2nd RL) and negative patterning (NP) with the mean walking time (in s) of the different light intensities in the phototaxis assay.

learning task	relative light intensity	rho	p-value
1st RL	3.125 %	0.13	0.56
1st RL	6.25 %	0.26	0.21
1st RL	12.5 %	0.27	0.20
1st RL	25 %	0.30	0.16
1st RL	50 %	0.39	**0.06**
1st RL	100 %	0.39	**0.06**
2nd RL	3.125 %	0.08	0.70
2nd RL	6.25 %	0.25	0.24
2nd RL	12.5 %	0.25	0.23
2nd RL	25 %	-0.009	0.97
2nd RL	50 %	0.12	0.59
2nd RL	100 %	0.13	0.53
NP	3.125 %	-0.09	0.68
NP	6.25 %	0.11	0.60
NP	12.5 %	0.32	0.13
NP	25 %	0.21	0.34
NP	50 %	0.25	0.24
NP	100 %	0.27	0.20

6.5.3 Relationship between gustatory and visual responsiveness

We did not find a significant correlation between GRS and mean walking times towards each light intensity (Spearman rank correlation; *n = 24*; **3.125 %***: rho = 0.04, p = 0.84*; **6.25 %**: *rho = 0.05, p = 0.82*; **12.5 %**: *rho = -0.008, p = 0.97*; **25 %**: *rho = -0.11, p = 0.62*; **50 %**: *rho = -0.11, p = 0.62*; **100 %**: *rho = -0.15, p = 0.48*, Fig. 5).

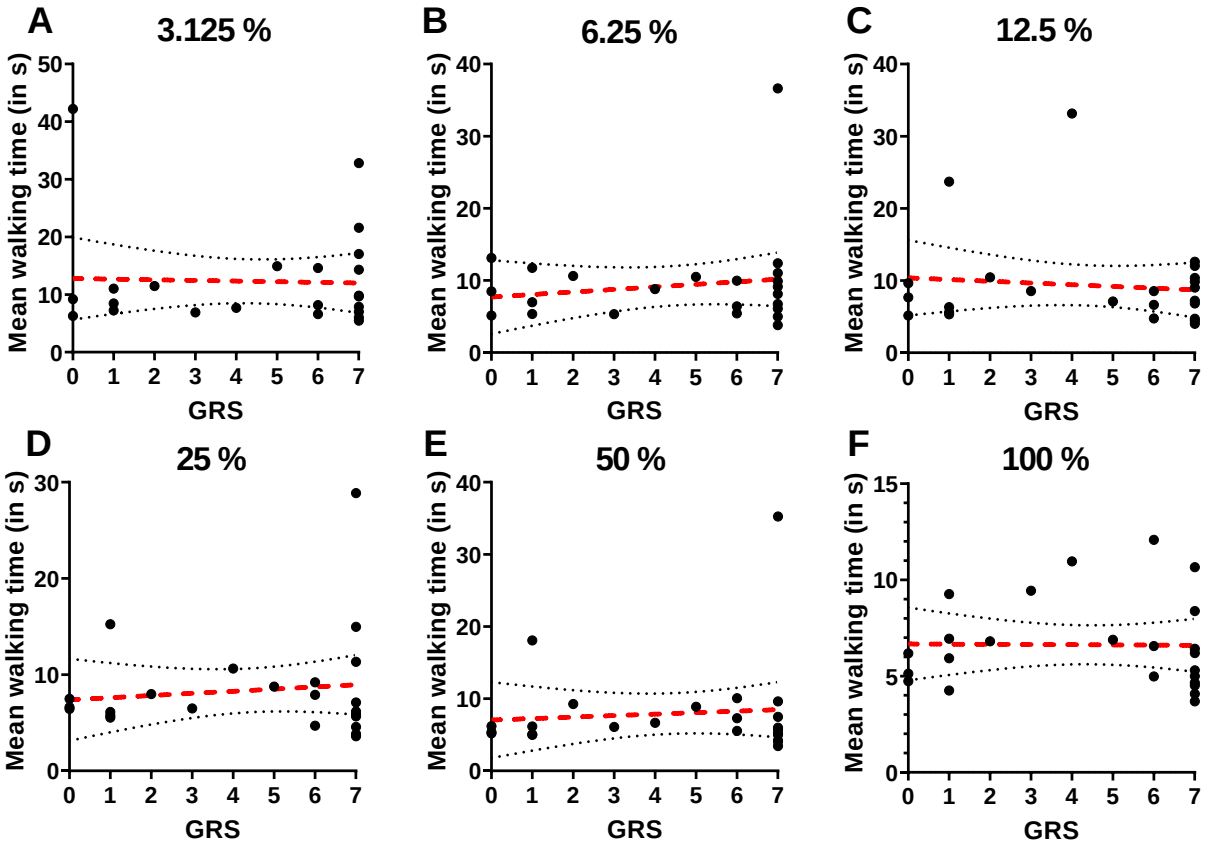

Fig. 5 Relationship between sucrose responsiveness and visual responsiveness for different light intensities. Sucrose responsiveness was assessed with the gustatory response score (GRS) i.e. the number of proboscis extensions to water and six different sucrose concentrations delivered to the bees' antennae. Visual responsiveness was assessed in a phototaxis assay by measuring the mean walking times (in seconds) the bees needed to reach a colored light stimulus. The graphs show the Spearman rank correlations of the GRS and the mean walking time at different light intensities (*n = 24*): **A)** 3.125 %: *rho = 0.04, p = 0.84, R^2 = 0.001*, **B)** 6.25 %: *rho = 0.05, p = 0.82, R^2 = 0.02*, **C)** 12.5 %: *rho = -0.008, p = 0.97, R^2 = 0.01*, **D)** 25 %: *rho = -0.11, p = 0.62, R^2 = 0.01*, **E)** 50 %: *rho = -0.11, p = 0.62, R^2 = 0.008*, **F**) 100 %: *rho = -0.15, p = 0.48, R^2 = 0.0001*. Each dot represents the data of a single bee. The lines indicate a linear regression with the 95 % confidence intervals shown as grey dotted lines. Red dotted lines indicate positive but non-significant correlations.

6.6 Discussion

In this study we examined the relationships between sensory responsiveness and proficiency in learning tasks of different complexity in free-flying bees. Our goal was to gain a better understanding of factors underlying consistent individual differences in the cognitive skills of honey bees. We have recently demonstrated that the proficiency of individual honey bees to solve an elemental discrimination was positively correlated with non-elemental learning performances in reversal learning and negative patterning, while the two non-elemental tasks were not significantly correlated (Finke et al. 2023). These results indicate that sucrose responsiveness might have different effects on the performances in elemental and non-elemental learning tasks with free-flying bees. Numerous studies have demonstrated a strong positive correlation between elemental learning performances in the laboratory and sucrose responsiveness (Scheiner et al. 1999, 2001a, b, 2003a, 2005; Junca et al. 2019). Here we replicated the visual learning experiment as described in Finke et al. (2023) and consecutively tested free-flying honey bees in visual reversal learning and negative patterning, allowing us to assess the proficiency of bees in elemental learning (first phase of reversal learning) and non-elemental learning (second phase of reversal learning and negative patterning). We then determined sucrose and visual responsiveness of the bees in the laboratory. First, we correlated the individual test performances of bees across the three learning tasks to determine whether we could replicate our previous results (Finke et al. 2023, see above). Indeed, our results comply with our earlier findings, i.e. the individual performances in the first phase of reversal learning (A+ vs. B-) positively correlated with performances in the second phase of reversal learning (A- vs. B+) and negative patterning (C+ and D+ vs. CD-), while no significant correlation was evidenced between the second phase of reversal learning and negative patterning. These results further strengthen our hypothesis that this pattern of correlations represents a distinct characteristic of the cognitive profile of honey bees.

We then investigated the relationship between test performances in the three learning tasks and sucrose responsiveness. We hypothesized differential effects of sucrose responsiveness on learning performances in a complexity-dependent manner. We expected that the positive relationship between sucrose responsiveness and learning performances would be highest in the elemental task (first phase of reversal learning), intermediate in the second phase of reversal learning and lowest in negative patterning. Our results, however, did not show any significant correlations between GRS and test performances in the three tasks. These results are in stark contrast with those obtained from PER conditioning studies, where sucrose

responsiveness strongly correlated with elemental learning performances (Scheiner et al. 1999, 2001a,b, 2003, 2005; Junca et al. 2019). Importantly, we compared the GRS of bees randomly collected from an artificial feeder with the GRS of the experimental bees to test the hypothesis that the long conditioning procedures (between six and eight hours per bee) might have altered the bees' evaluation of sucrose. Generally, sucrose responsiveness is tested prior to PER conditioning in laboratory experiments (Scheiner et al. 1999, 2001b, a, 2003a, 2005). As we tested the bees in free-flying conditions this was not possible in our experiments. However, the GRS of the experimental bees was not different from those captured from an artificial feeder, indicating that the conditioning procedure did not alter their sucrose responsiveness. The median GRS of the bees amounted to '6' with more than half of the bees (*n = 16*) being highly responsive to sucrose (GRS of 6-7), while fewer bees showed an intermediate score (GRS 4-5: *n = 6*; GRS 2-3: n = 2) or a low gustatory score (GRS 0-1: n = 7). Consequently, the variability of GRS in our experiment was relatively low compared to other studies which reported an equal distribution across these GRS classes (see e.g. Erber et al. 2006). It might thus be possible that the low variability of GRS in our sample of bees biased the correlation analysis. Intriguingly, all experimental bees accepted the 20 % sucrose solution which was offered at the artificial feeder before they were recruited to the experimental set-up. However, some bees did not extend their proboscises upon stimulation with a 30 % sucrose solution during the sucrose responsiveness assay, which indicates that the PER assay in the laboratory might not reflect which sucrose concentrations are accepted by the bees in the field during foraging. Mujagic and Erber (2009) studied the relationship between sucrose responsiveness measured via the PER assay in the laboratory and sucrose acceptance at an artificial feeder under free-flying conditions. Their results showed that the bees accepted lower sucrose concentrations at the feeder compared to those eliciting a PER response in the laboratory. They concluded that it is not possible to extrapolate sucrose acceptance in the field from sucrose responsiveness measured in the laboratory, which seems to be confirmed by our experiments.

Due to previous results showing a positive correlation between sucrose responsiveness and visual responsiveness measured in a phototaxis assay (Erber et al. 2006) we wished to examine 1) the effects of visual responsiveness on elemental and non-elemental learning performances in free-flying conditions and 2) the relationship between sucrose responsiveness and visual responsiveness. Generally, the walking times of bees decreased with increasing light intensities, a result that has been shown repeatedly (Erber et al. 2006; Scheiner et al. 2014; Schilcher et al. 2021). We performed correlation analyses between the test performances in the learning tasks and the mean walking times in the phototaxis assay for the six different light

intensities. The correlations between the mean walking times at the two highest light intensities (50 and 100 %) and the test performances in the first phase of reversal learning (elemental discrimination) were both positive and close to significance ($\alpha = 0.06$), while all the other correlations tested remained non-significant. Bees that were slower in walking towards the colored light stimulus had the tendency to perform better in the first phase of reversal learning. This tendency was independent of the general locomotor activity, as measured by the mean velocity of the bees during two-minute dark walks in the phototaxis arena. Locomotion did not correlate with test performances in reversal learning. These results indicate that visual responsiveness might have effects on elemental learning performances in visual elemental learning in free-flying conditions. Clearly, more research is necessary to test this hypothesis. Similar results have been reported by Scheiner et al. (2014) who demonstrated that pollen foragers, which generally perform better in elemental PER conditioning than nectar foragers due to their increased sucrose responsiveness (Scheiner et al. 1999, 2001b, 2003a), had lower visual responsiveness compared to nectar foragers. However, other studies found a positive relationship between responsiveness to sucrose and that to light (Erber et al. 2006; Tsuruda and Page 2009). In our experiments, GRS and mean walking times towards different light intensities did not correlate, suggesting no tight relationship between responsiveness to gustatory stimuli and light. These differences in results might be attributed to differences in the methodology. First, we did not use the same LED light stimulus as in Erber et al. (2006), although we used a similar green light (here: 527 nm; Erber et al. 2006: 520 nm), thus the illuminance of the LEDs were different. Furthermore, we subjected the bees to extensive free-flying learning protocols before the responsiveness tests, which might have altered their sensory responsiveness. We tested this possibility at least for sucrose responsiveness and found no evidence that being subjected to the learning tasks altered the bees' sucrose responsiveness (see above). However, it might still be possible that the bees' visual responsiveness was altered by the extensive conditioning protocols, which we did not test for.

Mujagic and Erber (2009) hypothesized that because the behavioral context of free-flying and harnessed bees are entirely different, it might be possible that behavioral context might alter the importance of sensory responsiveness when the bees are tested in the field or in the laboratory. This hypothesis has important implications for the study of learning, memory and cognition in insects, as it might challenge the assumption that results established in the laboratory are also valid for behaviors shown under natural conditions. For example, when harnessed bees are subjected to learning tasks in the laboratory, they are "forced" to participate in the experiment, which might have important effects on the bees' intrinsic motivation. In this

respect, one could hypothesize that when restrained bees are subjected to learning tasks within the laboratory and are consequently "forced' to participate in the experiment independent of their intrinsic motivation, rather "basic" mechanisms such as response thresholds and appetitive motivation may drive the bees' behavior. However, learning experiments with free-flying bees simulate the natural foraging behavior. Thereby, the animals are free to leave the experiment at any time, and consequently it seems reasonable to assume that they have a high intrinsic motivation to participate in the learning task. In that situation, other factors such as attentional processes may play a more dominant role than individual sensory response thresholds. Indeed, recent studies contrasting the sensory processing of active (free walking), and inactive (fixed) mice evidenced a modulation of sensory processing by behavioral states. Generally, increased locomotion and arousal in active individuals were associated with a more robust and accurate visual encoding (Niell and Stryker 2010; Ayaz et al. 2013; Erisken et al. 2014; Dadarlat and Stryker 2017; Pakan et al. 2018). This behavioral state-dependent modulation of sensory processing has recently been shown to affect associative learning performances in mice (Albergaria et al. 2018). Although modulatory effects of locomotion on neuronal processing (Chiappe et al. 2010; Maimon et al. 2010; Weir et al. 2014) and sensory processing (Rother et al. 2022) have also been evidenced in insects, the link between behavioral state, sensory processing and associative learning remains unknown. It seems plausible that the behavioral context is distinctly different in free-flying and restrained honey bees. Free-flying conditions mimic the natural foraging behavior. While searching for rewarding flowers, stimuli of different sensory modalities guide the bees' behavior in a distance-dependent manner (von Frisch 1965). Flower colors provide a mid-range signal to facilitate detection during the bees' search flights (von Frisch 1965). A sucrose reward is offered once the bees have landed on the flower inducing associative learning of its physical characteristics (color, shape and odor) to facilitate detection and discrimination during future foraging trips. Thus, color signals might play a more dominant role during foraging flights, while odors and sucrose responsiveness guides the bees' behavior while walking or standing still. This could also explain why in restrained bees almost universal positive correlations were found between sucrose responsiveness and responsiveness to light, odors and pollen (Scheiner et al. 2004; Erber et al. 2006). For a trapped animal, i.e. a restrained bee, a likely response is escape behavior which could result in increased general sensory responsiveness to various stimuli. Since we observed clear differences in the relationship between associative learning and the responsiveness to sucrose and visual stimuli in free-flying bees in contrast to those reported in restrained bees, it seems reasonable to assume that

behavioral states might also have modulatory effects on sensory processing during associative learning.

A potential neural mechanism underlying behavioral context-dependent modulation of sensory responsiveness could involve octopaminergic neurons mediating arousal (Bacon et al. 1995; Stern 1999). Octopamine is a crucial neurotransmitter, neurohormone and neuromodulator in insects (Beninger 1983; Roeder 1999; Schulz et al. 2002; Scheiner et al. 2006; Farooqui 2007). In honey bees, octopamine enhances sensory responsiveness to sucrose and visual stimuli by modulating the sensitivity of receptors and interneurons (Page and Erber 2002; Scheiner et al. 2002; Schilcher et al. 2021) and also enhances associative learning performances (Behrends and Scheiner 2012). Interestingly, octopamine also enhances locomotor and flight activities in bees (Akasaka et al. 2010; Mezawa et al. 2013; Watanabe and Sasaki 2022). A recent study in fruit flies showed that octopaminergic neurons can stimulate specific central dopaminergic neurons which are active during flight (protococerebral anterior medial, PAM) projecting to GABAergic MBONs and that prolonged flight bouts require disinhibition of these MBONs via octopaminergic signaling (Manjila et al. 2019). In honey bees, MB-dependent modulation of flight behavior has been observed as well (Kiya et al. 2007; Lutz and Robinson 2013). It is thus tempting to speculate that the prolonged flight activity (6 to 8 hours training) of the bees in our free-flying experiment led to higher octopamine titers in the central brain facilitating learning independent of the US processing pathway. At this stage it is premature to conclude on behavioral context-dependent sensory processing, but our results suggest that this a fruitful topic for future research.

Author Contributions

Conceptualization: RS; VF; Methodology: RS, VF, AAW, MGT; Data analysis: VF; Writing—original draft preparation: VF; Writing—review and editing: VF, AAW, RS and MG; Visualization: VF; Supervision: RS, AWW, MG; Project administration: AAW; Funding acquisition: VF.

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Conflict of Interest

The authors declare no conflict of interest.

6.7 Supplementary material

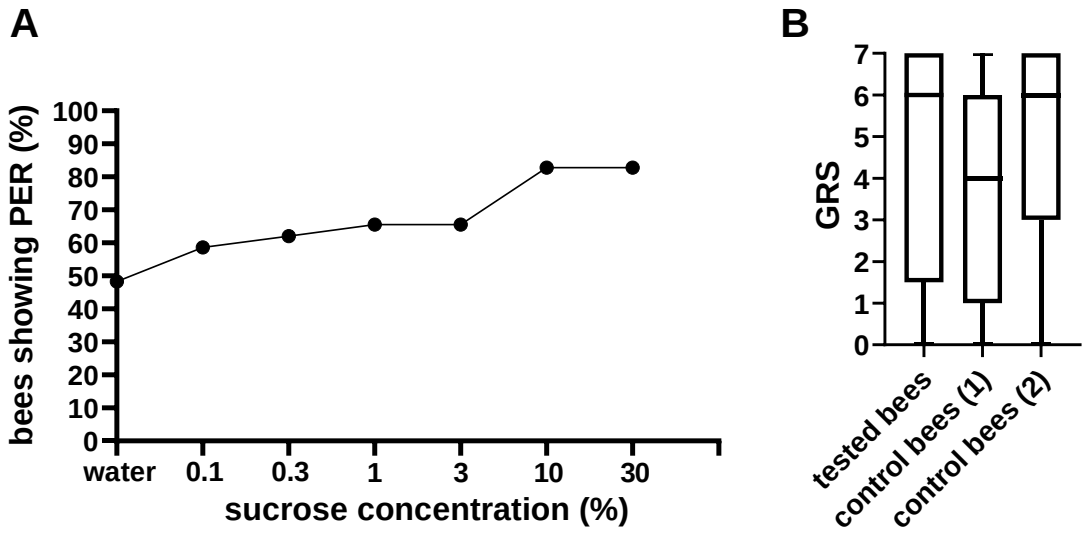

Fig. S1 A) Percentages of tested bees (*n = 29*) showing proboscis extension responses upon stimulation with water and different sucrose concentrations. **B)** Boxplots of the gustatory response scores (GRS) of the tested bees (n = 29) which were subjected to the learning tasks and bees which were not subjected to any learning task and collected from the artificial gravity feeder at the start (control 1, *n = 19*) and end (control 2, *n = 30*) of the experiment. The black lines in the boxplots depict the median GRS of the different groups. The median GRS of the tested bees did not significantly differ from those of the control bees 1 and 2 (Mann-Whitney U test; control 1: *n = 19, U = 189, p = 0.06*; control 2: *n = 30, U = 374, p = 0.35*).

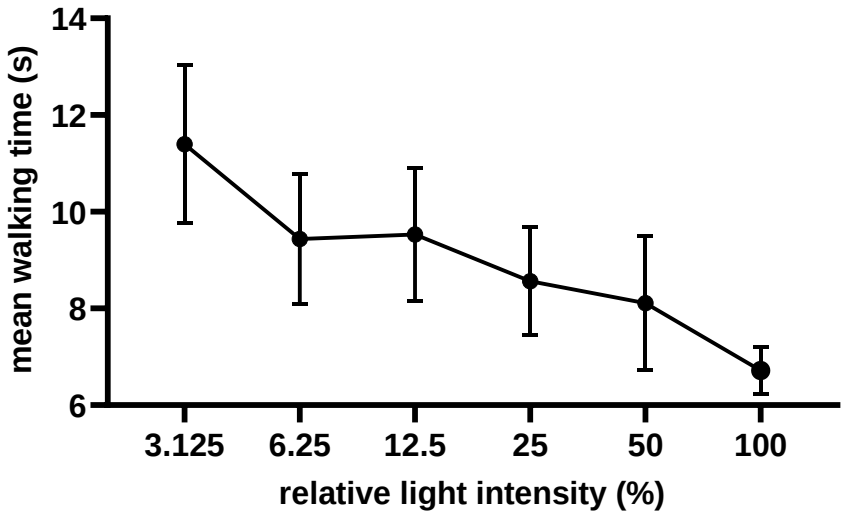

Fig. S2 Mean walking times (s) of the bees for the different relative light intensities during the phototaxis assay. The mean walking times decreased with increasing relative light intensities. We found a significant negative correlation between mean walking times and the relative light intensity (Spearman rank correlation; *n = 24, rho = -0.35, p < 0.0001*). The bees walked faster towards a colored light stimulus of higher intensity. Means and standard errors (S.E.M) are shown.

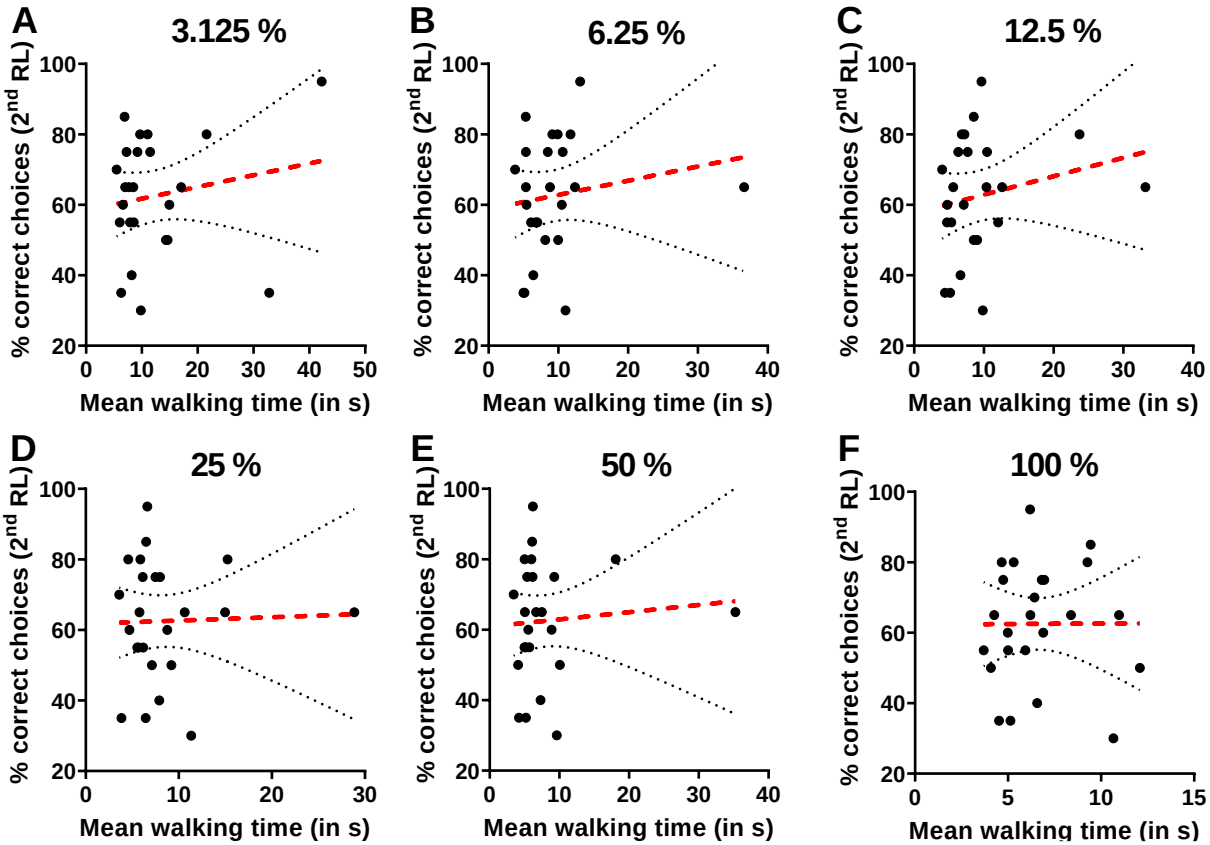

Fig. S3 Relationship between the individual bees' test performances in the second phase of reversal learning (2nd RL) and visual responsiveness. Visual responsiveness was measured by the mean walking time (in seconds) in the phototaxis assay for different relative light intensities (3.125, 6.25, 12.5,25, 50 and 100 %). The test performances were measured as percentage of correct choices made in the non-reinforced test during 20 trials. The Spearman rank correlations were non-significant in all cases (n = 24): **A)** rho = 0.08 p = 0.70, R^2 = 0.03; **B)** rho = 0.25, p = 0.24, R^2 = 0.02; **C)** rho = 0.25, p = 0.23, R^2 = 0.04; **D)** rho = -0.009, p = 0.97, R^2 = 0.0008; **E)** rho= 0.12, p = 0.59, R^2 = 0.006; **F)** rho = 0.13, p = 0.53, , R^2 = 0.0002. Each dot represents the data of a single bee. The lines indicate a linear regression with the 95 % confidence intervals shown as grey dotted lines. Red dashed lines indicate non-significant correlations.

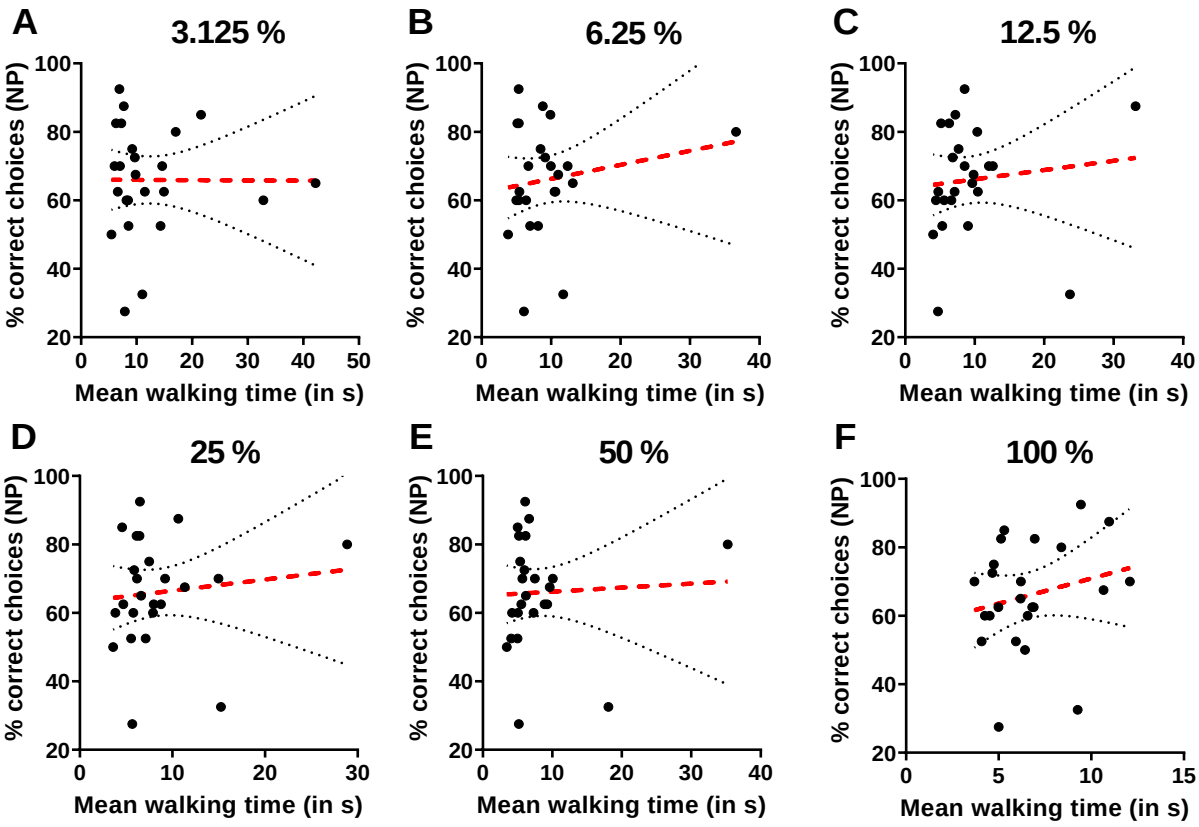

Fig. S4 Relationship between the individual bees' test performances in negative patterning (NP) and visual responsiveness. Visual responsiveness was measured by the mean walking time (in seconds) in the phototaxis assay for different relative light intensities (3.125, 6.25, 12.5,25, 50 and 100 %). The test performances were measured as percentage of correct choices made in the non-reinforced test during 20 trials. The Spearman rank correlations were low and non-significant in all cases ($n = 24$): **A)** *rho = -0.09 p = 0.68, R^2 = 0.0004*; **B)** *rho = 0.11, p = 0.6, R^2 = 0.03*; **C)** *rho = 0.32, p = 0.13, R^2 = 0.01*; **D)** *rho = 0.21, p = 0.34, R^2 = 0.01*; **E)** *rho= 0.25, p = 0.24, R^2 = 0.002*; **F)** *rho = 0.27, p = 0.2, R^2 = 0.05*. Each dot represents the data of a single bee. The lines indicate a linear regression with the 95 % confidence intervals shown as grey dotted lines. Red dashed lines indicate non-significant correlations.

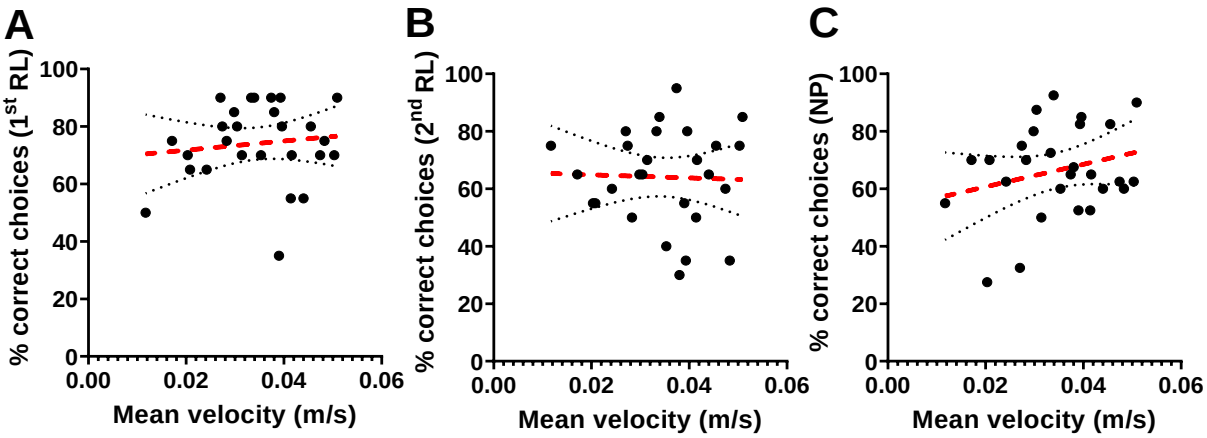

Fig. S5 Relationship between the general locomotor activity of the bees and their test performances in reversal learning and negative patterning. The locomotor activity was assessed via the mean velocity (in m/s) of the bees during 2-minute dark walks in the phototaxis arena. The test performances were measured as percentage of correct choices made in the non-reinforced test during 20 trials. The GRS was not significantly correlated with the test performances in **A)** the first phase of reversal learning (1st RL: Spearman rank correlation; *n = 29, rho = 0.08, p = 0.69, R^2 = 0.01*), **B)** the second phase of reversal learning (2nd RL: *n = 29, rho = 0.02, p = 0.92,, R^2 = 0.001*) and **C)** negative patterning (NP: *n = 29, rho = 0.114, p = 0.49, R^2 = 0.06*). Each dot represents the data of a single bee. The lines indicate a linear regression with the 95 % confidence intervals shown as grey dotted lines. Red dashed lines represent non-significant correlations.

Table S1. GLMM analysis of the bees' performances in the non-reinforced tests of the 1st phase of reversal learning. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order * group_RL + (1 | subject)	5	538.70	-264.35	-	-
model2: response ~ order + group_RL + (1 | subject)	4	536.74	-264.37	0.04	0.84
model3: response ~ order + (1 | subject)	3	534.85	-264.42	0.11	0.75
model4: response ~ (1 | subject)	**2**	**533.73**	**-264.87**	**0.89**	**0.35**

Table S2. GLMM analysis of the bees' performances in the non-reinforced tests of the 2nd phase of reversal learning. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order * group_RL + (1 | subject)	5	626.80	-308.40	-	-
model2: response ~ order + group_RL + (1 | subject)	4	625.39	-308.69	0.59	0.44
model3: response ~ order + (1 | subject)	3	626.32	-310.16	2.9	0.09
model4: response ~ (1 | subject)	**2**	**625.39**	**-310.70**	**1.07**	**0.3**

Table S3. GLMM analysis of the bees' performances in the non-reinforced tests of negative patterning. The model with the best fit is highlighted in bold. The p-value indicates the comparison of the concerning model with the model including one level of higher complexity.

Models	df	AIC	Log-Lik	χ^2	p(>χ^2)
model1: response ~ order * group_RL + (1 | subject)	5	1158.1	-574.05		-
model2: response ~ order + group_RL + (1 | subject)	4	1156.1	-574.05	0.004	0.95
model3: response ~ order + (1 | subject)	3	1154.4	-574.19	0.28	0.59
model4: response ~ (1 | subject)	**2**	**1152.7**	**-574.33**	**0.29**	**0.59**

7 General Discussion

7.1 Are some bees smarter than others?

Are some bees smarter than others? - This was the central question underlying the research presented in this thesis. I applied a novel view on inter-individual variability in the cognitive proficiency of honey bees by focusing on the individual level and not the average group performance frequently of interest in the field of animal cognition. Classically, studies on cognition in animals aim to investigate whether a certain species is capable to solve a certain cognitive task. The experimental framework includes testing a certain sample of individuals and then the individual data are pooled to calculate an average group performance allowing to determine the capability for this trait for the whole species. Inter-individual variability in this case, is undesirable and regarded as noise around an optimal behavioral mean. The existence of individuality in cognition has been profoundly ignored in invertebrate species, probably due to the widespread opinion of invertebrates as 'reflex machines' being only capable of simple forms of learning. The honey bee, among other eusocial insects, is an outstanding invertebrate species due to its surprisingly sophisticated cognitive capabilities that were not thought to occur in invertebrates. I have here concentrated on the cognitive capabilities of individual bees and the question of whether individuals would perform consistently over time, across contexts and across tasks requiring different cognitive abilities.

Some researchers have started to explore these questions in insects mainly in honey bees, bumble bees and fruit flies (Raine and Chittka 2012; Muller and Chittka 2012; Smith and Raine 2014; Tait et al. 2019; Junca et al. 2019; Smith et al. 2022). They focused predominantly on the question of whether individuals perform consistent across simple (elemental) learning tasks involving either stimuli belonging to different sensory modalities or including different types of reinforcement (appetitive vs. aversive). In contrast, I focused on individual consistency across elemental learning and several forms of higher-order (non-elemental) learning, a viewpoint that has - to my knowledge - never been tackled before in insects. Furthermore, most of these studies performed their learning experiments in the laboratory with restrained animals. This approach allows to precisely control for different experimental parameters such as temperature, humidity and stimulus presentation and test a high number of individuals in a short amount of time. However, it remains to be determined whether results established in the

laboratory with restrained individuals are also valid for learning abilities shown under natural conditions. I mainly focused on testing the bees under free-flying conditions, a procedure that is very time-consuming as training a single bee took up to eight hours. Additionally, I replicated some experiments conducted with free-flying bees under restrained conditions to examine the general validity of my findings.

The research presented here provides the first comprehensive investigation of individual consistency in the cognitive proficiency of honey bees. A prerequisite for studying individual consistency in cognitive proficiency was to determine whether individuals show temporal repeatability of their cognitive proficiency (Griffin et al. 2015). I show that individual bees remained consistent in their proficiency to solve an elemental discrimination task over three consecutive days (chapter 1, Finke et al. 2021, Fig. 1). The next step was to examine individual consistency across elemental discrimination tasks involving stimuli from different sensory modalities i.e. achromatic visual patterns or odors respectively, because existing studies on bumble bees yielded contradictory results (positive correlation: Muller and Chittka 2012; no correlation: Smith and Raine 2014). The individual performances of bees revealed no association between visual and olfactory learning, the first evidence that individual bees might show a cognitive specialization (chapter 1, Finke et al. 2021). These results all future experiments were conducted using stimuli from the same sensory modality to explore individual consistency across elemental and non-elemental learning tasks.

To begin examining individual consistency across performances in learning tasks of different complexity and cognitive requirements I chose an elemental discrimination and one of the most complex non-elemental tasks honey bees are capable of, a relational concept learning task (Avarguès-Weber and Giurfa 2013 for review). Intriguingly, free-flying bees that performed well in the elemental task also showed increased ability in concept learning (chapter 1, Finke et al. 2021, Fig. 3). These results were a strong indicator to further examining the relationship between elemental and different non-elemental learning tasks, especially given the importance of such findings for the question of brain modularity in honey bees. I thus chose to focus on two distinct non-elemental learning tasks reversal learning and negative patterning. These two tasks can be performed both with free-flying bees in the field (Schubert et al. 2002; Dyer et al. 2014) and with restrained bees in the laboratory (Deisig et al. 2001; Devaud et al. 2007), enabling me to test bees in both conditions to examine the general validity of my results across seminatural and unnatural learning scenarios. Additionally, given the cognitive specialization between olfactory and visual learning I further wished to examine if similar

results can be obtained when the tasks are conducted with either the use of visual or olfactory stimuli. However, visual learning with restrained bees was still in its infancy, so there were no established protocols available to study non-elemental learning (Avarguès-Weber and Mota 2016). I consequently spent several months to establish protocols allowing to reliably test visual reversal learning and negative patterning in restrained bees by altering several experimental factors such as the inter-trial interval, length of stimulus presentation and the duration of starvation among others. Finally, I succeeded in achieving robust learning performances in reversal learning and negative patterning with restrained bees (see Fig. S7 in chapter 2 for the group-level performances) (Finke et al. 2023). Nevertheless, fewer bees learned the visual stimuli compared to olfactory PER conditioning, a result that has been found for elemental learning as well (Avarguès-Weber and Mota 2016). Astonishingly, the same pattern of individually correlated performances was found across all four experiments: The individual performances in the 1st phase of reversal learning were positively correlated with performances in the 2nd phase of reversal learning and negative patterning. However, individual performances in the 2nd phase of reversal learning and negative patterning revealed no significant correlation (chapter 2, Finke et al. 2023). Consequently, if the experimental context and the sensory modality of the stimuli is kept constant across learning tasks within one experiment, the same pattern of correlated performances can be observed across experiments. These findings suggests that this pattern of correlated performances might represent a characteristic of the bees' cognitive profile. These findings have important implications for the question of brain modularity in honey bees which I will discuss in detail in section 7.2 of the general discussion.

A second major goal of this thesis was to identify factors underlying consistent individual cognitive skills. Given the conclusive evidence of a heritable genetic influence on inter-individual variability in the learning abilities of bees (Brandes and Menzel 1990; Brandes 1991; Bhagavan et al. 1994; Benatar et al. 1995; Chandra et al. 2000; Ferguson et al. 2001; Junca et al. 2019) I examined if a genetic component contributes to consistency in cognitive proficiency. The honey bee is perfectly suited to study the genetic influence on consistency in cognitive proficiency as the genetic diversity among individuals within a colony can be easily manipulated via artificial insemination of the queen (Cobey et al. 2013). Therefore, I subjected free-flying bees originating from either a polyandrous ('high-genetic diversity') or a monandrous super-sister queen ('low-genetic diversity') to reversal learning and negative patterning. The pattern of individually correlated performances across the two phases of reversal learning and negative patterning were different in the two groups. This strongly

indicates that the genetic diversity naturally occurring in honey bee colonies contributes to the existence of consistent individual differences in cognitive proficiency. These findings are discussed in section 7.3 with respect to the possible benefits of such genetically determined consistent inter-individual variability in cognitive skill for honey bee colonies.

Another factor that is known to substantially cause inter-individual variability in learning performances is the individual evaluation of the CS and US (Annau and Kamin 1961; Rescorla 1972; Scheiner et al. 2005). It has been demonstrated repeatedly in restrained honey bees that inter-individual differences in elemental learning performances can be mainly attributed to differences in the responsiveness to sucrose solution which is used as US in appetitive learning tasks (Scheiner et al. 1999, 2001b, a, 2004, 2005; Scheiner and Arnold 2010). Additionally, evidence strongly suggests that sucrose responsiveness correlates positively with responsiveness to odors or visual stimuli used as CS (Scheiner et al. 2004; Erber et al. 2006; Tsuruda and Page 2009). I provide evidence, however, that neither sucrose responsiveness nor visual responsiveness can account for the inter-individual variability observed in differential conditioning, reversal learning and negative patterning (chapter 4). These results indicate that the relationship between sensory responsiveness and learning performances of bees are dependent on the behavioral context, which is discussed in detail in section 7.4 of the general discussion.

7.1.1 ‘Learners’ and ‘non-learners’ in free-flying and restrained conditions

A major issue in the study of cognitive abilities is that they cannot be measured directly but have to be assessed via behavioral responses of an individual confronted with a task (Mery 2013; Boogert et al. 2018). In conditioning experiments with bees several behavioral responses could be used as measure for their proficiency. One possibility is to use the responses made during the acquisition phase. For example, one can calculate a ‘learning score’ by summing up all correct responses made by an individual in the acquisition phase. This approach was used by Junca et al. (2019) to evidence a negative correlation between individual performances in an appetitive and an aversive discrimination task under restrained conditions. A second possibility is to fit a sigmoidal curve to individual learning curves obtained from acquisition performances to assess the speed with which an individual learned during acquisition (Smith and Raine 2014). Otherwise, it is also possible to use the responses in the non-reinforced test following the acquisition phase, a method employed by Muller and Chittka (2012) to examine individual

cognitive consistency in free-flying bumble bees. In the free-flying experiments presented here the individual performances during the acquisition did not show a sigmoidal increase in performance, as usually observed at the group level (Pamir et al. 2011) but also at the individual level (Smith and Raine 2014). This can be explained by the experimental design of the learning experiments: In the free-flying learning experiments, the probability to make a correct choice by chance amounted to 50 % at each trial. This could have resulted in a learning score that did not represent the actual learning ability of the bees. Indeed, I could not find any association between individual acquisition and test performances either in the elemental or the non-elemental tasks. Consequently, I used the percentage of correct choices made by an individual in the non-reinforced tests to assess cognitive consistency. For the restrained experiments, using test data is not optimal, given that every CS is only presented once in the test which precludes the quantification of performances as for the free-flying experiments. Instead, here the test data only allows to classify the performance of the bees qualitatively i.e. a bee responding to the CS+ but not to be CS- can be classified as 'learner' while a bee responding to both stimuli could be considered a 'generalist' or 'non-learner' for example (Mancini et al. 2018; Finke et al. 2023). Due to that situation, I tried several possibilities to calculate 'learning scores' from individual acquisition performances. I considered calculating the 'learning score' by Junca et al. (2019), i.e. the sum of responses to the CS+ during conditioning. However, even for a simple discrimination using only the sum of the correct responses is, in my opinion, not sufficient to capture the behavior shown by the bees. For example, a bee that responded only to CS+ but not to any CS- presentation during conditioning could be considered as a 'perfect learner'. Another bee responded to all CS+ and CS- presentations, thus it may have learned something but was unable to differentiate between the stimuli, an ability that is specifically tested during differential conditioning. These two bees would then receive the same learning score and would thus be considered as being 'equally well learners' in the statistical analysis. In the case of reversal learning and negative patterning the situation is even more complex as successful learning requires, per definition, not responding to the CS-. I further considered using an arbitrary score by scoring '+1' for a correct response and '-1' for an incorrect response. These scores would also be hard to interpret as a single score could have multiple meanings: a bee scored with a '0' could either have responded to all stimuli or to none of the stimuli which, again, are two distinctly different learning outcomes. In the end, I decided to use the test performances of the bees and classified a bee as 'learner' if it responded fully correct in the test while bees with other responses were considered as 'non-learner' (chapter 2, Finke et al. 2023). This qualitative measure precludes fine grain analysis of individuality in comparison to the

quantitative measure of cognitive performance obtained from the free-flying experiments. These examples show that the use of free-flying experiments should be favored in order to study individually correlated performances especially across higher-order learning tasks.

7.1.2 The motivation to learn – a comparison of free-flying and restrained conditions

The concordance of correlated performances across free-flying and restrained bees (chapter 2, Finke et al. 2023) is remarkable given the distinct differences between these learning scenarios. For learning experiments in the field, I preselected the bees because a prerequisite for testing free-flying bees is that they learn to handle the experimental apparatus during pre-training. Even after prolonged pre-training some bees were not able to learn how to handle the rotating screen or the Y-maze (personal observation). It goes without saying that these bees could not be tested for their learning ability. Furthermore, only highly motivated foragers i.e., only bees that returned reliably and swiftly to the experimental set-up were used for analysis. Across all experiments I conducted only a small proportion of bees (between 5 % and a maximum of 10 %) did not fulfill this criterion. This is quite impressive, given that the conditioning of the bees usually took six to eight hours for one individual. On the contrary, non-pollen foragers used for the experiments under restrained conditions were randomly caught at the hive entrance. Here, preselection concerned only bees that showed innate PER to the CS before the start of conditioning or that did not extend their probosces to antennal stimulation with the sucrose solution used as reward during conditioning i.e. showing a lack of appetitive motivation. Nevertheless, in the restrained conditions the bees were ‘forced’ to participate in the experiments while the bees participated voluntarily in the free-flying conditions as they were free to leave the experimental set-up at any time during the whole procedure. It thus seems reasonable to assume that the intrinsic motivation, to participate in the restrained experiments could differ between individuals in addition the appetitive motivation. This is also reflected in the data obtained: in the free-flying conditions only a few bees (between 10 % to 18 %, depending on the type of task) performed around chance level in the non-reinforced tests following conditioning (50 % correct choices) and thus could be considered as ‘non-learner’ (chapter 2, Finke et al. 2023). In the restrained conditions, almost one third (between 27 % and 30 % depending on the type of task) of the bees could be considered as ‘non-learners’, because they did not show any response to either CS+ or CS- during conditioning, despite proper appetitive motivation. Additionally, another 16-23 % of the bees (depending on the type of task)

responded to the CS+ and CS- during the non-reinforced tests and could thus also be considered as 'non-learners' (chapter 2, Finke et al. 2023). As consequence, a high proportion of the concordance between performances of the restrained bees can be attributed to the consistent absence of competence across different learning tasks. Indeed, most bees that showed no PER responses remained non-responsive throughout the experiments (chapter 2, Finke et al. 2023, see Figs. S8 and S10). I am consequently convinced that although the same results were obtained under seminatural and unnatural training conditions, free-flying experiments are better suited to further examine the behavioral relationship between individual performances in a variety of learning tasks. However, the restrained conditions offer a great tool to study the neurobiological underpinnings of individuality in cognitive skill (Matsumoto et al. 2012; Giurfa and Sandoz 2012).

7.2 Modularity of cognition in honey bees and other insects

A traditional question originating from studies on human intelligence concerns the structure underlying cognition. Is cognition comprised of independent specialized modules designated to solve specific problems, or does a general factor explain performances across a wide array of different cognitive abilities? A pioneering study to tackle this question was conducted by Spearman (1904). He subjected humans to multiple cognitive test batteries and found a high variability in performances across individuals, but they were strongly positively correlated in the same individual. A high proportion of this variability was attributable to a general factor ('g factor') underlying the success in all tested cognitive abilities, also referred to as 'general intelligence'. These findings lead to the emergence of the theory of 'general intelligence' or domain-general cognition. A contrasting theory originally developed by Fodor (1983) postulates that cognition depends on distinct 'cognitive modules'. Each module is represented by specialized neuropils with a fixed neuronal structure that operate domain-specific by responding only to certain stimuli. Cognitive modules are informationally encapsulated, meaning that they operate independently without information transfer between different modules. Fodor had an intermediate view on the structure of cognition by postulating that domain-specificity is manifested at the level of input systems while higher-level cognitive processes are non-modular (Fodor 1983). Other researchers view cognition as being entirely or primarily modular (Sperber 1994; Shettleworth 2000; Palmer and Palmer 2002; Carruthers 2003).

The question of modularity in cognition is only starting to emerge in invertebrate research despite the importance to study to what extent invertebrate and vertebrate cognition co-evolved or evolved independently to gain a better understanding of how selection has shaped cognition across diverse taxa (Simons and Tibbetts 2019). Social insects were suggested to offer a great possibility to examine these questions as they show complex behaviors comparable to vertebrates such as cooperation. navigation, communication and central-place foraging (Dyer 1998; Sheehan and Tibbetts 2011; Simons and Tibbetts 2019). It has indeed been hypothesized that the interactions between domain-general and domain-specific processes may underlie cognition in insects (Menzel and Giurfa 2001): the authors characterize different levels of modularity in the insect brain with respect to distinct brain areas and their cross-modality or insulation from other processing pathways. Some olfactory learning tasks, for example, require only processing in purely olfactory neuronal circuits (Faber et al. 1999; Malun et al. 2002; Devaud et al. 2007; Rath et al. 2011), which could thus be viewed as being a domain-specific module (Menzel and Giurfa 2001). In contrast, other tasks especially higher-order forms of learning require multi-modal integration in the MBs (Devaud et al. 2007, 2015; Boitard et al. 2015) which could be regarded as domain-general module (Menzel and Giurfa 2001).

With the results presented here, this hypothesis seems to be confirmed at least for honey bees. Contrary to humans, I did not evidence universal positive correlations across the different tasks tested. This points towards an interaction of domain-general and domain-specific processes underlying cognition in honey bees. For example, the performances of honey bees were not consistent across visual and olfactory learning (chapter 1, Finke et al. 2021). These results seem to be confirmed in bumble bees (Smith and Raine 2014; but see Muller and Chittka 2012). Given the lack of correlation between visual and olfactory learning, it seems reasonable to assume that they are represented by different cognitive modules. Indeed, the neuronal processing of visual and olfactory information is dedicated to distinct uni-modal neuropils in the insect brain before multi-modal integration in the MBs (Leonard and Masek 2014). However, presence of an olfactory cue can increase color learning and vice versa (Gerber and Smith 1998; Kunze and Gumbert 2001) which might reflect higher-order processes (Giurfa 2007; Leonard and Masek 2014). Interestingly, when honey bee strains were artificially selected for high olfactory PER learning ability, they also showed an increased proficiency for visual learning under free-flying conditions (Brandes and Menzel 1990). A similar result has been found in the wasp *Nasonia vitripennis* where selected lines for increased visual learning ability showed increased learning proficiency independent of the context or CS used (Liefting et al. 2018). These results show that selection for increased domain-specific learning ability

might lead to the simultaneous selection of increased domain-general processes which could then interact with different domain-specific modules. Similarly, two distinct cognitive modules seem to underlie appetitive and aversive elemental learning: individual bees trade-off performances in an appetitive and an aversive elemental learning task i.e., bees that performed well in appetitive learning performed poorly in aversive learning and vice versa (Junca et al. 2019). The cause of this trade-off was attributed to genetically determined biases between different patrilines, meaning that some patrilines showed higher responsiveness to the appetitive stimuli and lower responsiveness to the aversive stimuli while it was reversed for other patrilines. These results indicate that cognitive specialization exists in individual bees i.e., increased ability in a certain trait at the expense of alternative traits or functions. Indeed, the natural behavioral context underlying appetitive and aversive learning abilities are totally different. Appetitive behaviors are associated with foraging activities, while aversive behaviors are associated with defensive activities (Giray et al. 2000; Roussel et al. 2009; Junca et al. 2019). The theory that appetitive and aversive learning are represented by different cognitive modules seems to be confirmed by their distinct processing pathways in the brain. Processing of appetitive reinforcements such as different sugars or water are mediated by octopaminergic neurons in the brain (Hammer and Menzel 1998). For example, the activity of a single octopaminergic neuron, the VUMmx1 mediates the US pathway during appetitive olfactory PER conditioning in bees (Hammer 1993). Aversive reinforcements are mediated via dopaminergic neurons (Vergoz et al. 2007). Intriguingly, a cognitive specialization was also found between elemental olfactory learning and landmark learning, abilities which are both required in the same behavioral context i.e. during foraging (Tait et al. 2019).

Some results of my thesis also point towards domain-general processes underlying the cognitive abilities of honey bees. Honey bees performed consistently across elemental and non-elemental forms of learning. Individual honey bees that performed well in an elemental discrimination task also performed well in concept learning (chapter 1, Finke et al. 2021). Similarly, elemental learning proficiency was also positively correlated with performances in reversal learning and negative patterning (chapter 2, Finke et al. 2023). These positive correlations were replicated in four independent experiments using either visual or olfactory stimuli and different conditioning contexts (free-flying or restrained) (chapter 2, Finke et al. 2023). These finding indicate that if learning occurs within a given cognitive module (visual/olfactory and possibly free-flying/restrained) the same patterns of individually correlated performances can be evidenced across elemental and non-elemental learning abilities. Concerning the modularity of cognition these results admit two hypotheses. The first

hypothesis is that similar brain circuitry underlies the proficiency in elemental and non-elemental tasks and consequently belong to the same cognitive module. The second hypothesis is that differences in domain-general processing abilities, attentional processes and working memory have a decisive influence on the proficiency to solve learning tasks of different cognitive complexity, similar to what has been found in humans and rodents (Matzel and Kolata 2010; Deary et al. 2010 for review). In the honey bee brain, elemental and non-elemental learning capabilities are segregated into distinct neuropils of the brain. Elemental olfactory and tactile learning require only intact ALs while the MBs seem to be dispensable (Scheiner et al. 2001c; Malun et al. 2002; Boitard et al. 2015). Both olfactory reversal learning and negative patterning require intact MB function (Boitard et al. 2015; Devaud et al. 2015). Although, it remains currently unclear if a similar segregation in neural processing during elemental and non-elemental learning exists in the visual modality (Avarguès-Weber and Mota 2016). The existence of tremendous afferences from the primary visual centers to the MBs strongly suggest that this is the case (Ehmer and Gronenberg 2002; Paulk and Gronenberg 2008). The first hypothesis can most likely be dismissed and thus it seems reasonable to assume that the MBs act as domain-general cognitive module in the insect brain, as it has been previously postulated (Menzel and Giurfa 2001). Indeed, evidence in insects suggests the existence of selective attentional-processes and working memory mediated by higher-order brain areas including the MBs (Zhang et al. 2005; van Swinderen 2007, 2011; Menzel 2009; Nityananda 2016). Their role in the ability to solve learning tasks of different complexity remain currently unknown. A finding that seems to contradict this hypothesis and standing alone rather points towards cognitive specialization across different non-elemental learning tasks is the absence of correlation I observed between two non-elemental tasks reversal learning and negative patterning (chapter 2, Finke et al. 2023). However, it might nevertheless be still possible that a proportion of the inter-variability observed between the three tasks tested in these experiments is attributable to a ‘g-factor’ but being concealed by variability caused by the experimental design and low sample sizes. Another explanation might be that different strategies are used by individual bees to solve complex tasks, which does not necessary account for an absence of competence (Komischke et al. 2003; Dyer et al. 2014). For example, it has been shown that the use of elemental or non-elemental strategies to solve a visual learning task in free-flying conditions depends on the training duration i.e. the experience of the bees with the stimuli presented (Giurfa et al. 2003).

Although some of my results point towards domain-general and domain-specific processes underling the cognitive capacities of honey bees, clearly more research is necessary

to conclude on the modularity of honey bee and insect cognition. Studying explicitly the involvement of a 'g-factor' in the cognitive abilities of bees would be of high value for future research.

7.3 The significance of inter-individual variability and consistency in cognitive performances for honey bee colonies

It is generally accepted that cognitive proficiency is an adaptation to reduce the unpredictability of the ever-changing environment (Dukas 1998; Shettleworth 2009). A central question in the study of cognition is whether and to what extent inter-variability in cognitive traits are adaptive and shaped by natural selection (Cole et al. 2012; Hopkins et al. 2014; Thornton et al. 2014; Rowe and Healy 2014). For natural selection to act on cognitive traits three criteria need to be fulfilled: the traits under consideration are 1) variable across individuals, 2) heritable and 3) the inter-individual variability in these traits are linked to fitness consequences (Darwin 1871). Inter-individual variability in a variety of cognitive tasks exists in honey bees (Pham-Delègue et al. 1990; Ray and Ferneyhough 1997; Scheiner et al. 1999; Ben-Shahar et al. 2000; Ferguson et al. 2001; Laloi and Pham-Delegue 2010; Finke et al. 2021, 2023) and have a heritable component (Brandes 1988, 1991; Benatar et al. 1995; Chandra et al. 2000; Junca et al. 2014). Concerning the third criterion, it is usually assumed that inter-individual variability in cognitive traits has fitness consequences (Johnston 1982; Dukas 1998; Healy 2012). However, studies examining directly how individual differences in cognitive traits translate to differences in fitness under natural conditions are rare in animal research (Raine and Chittka 2008; Cole et al. 2012; Isden et al. 2013; Evans et al. 2017, 2021; Madden et al. 2018).

Unravelling the link between inter-individual variation in behavioral traits and fitness is especially complex in insect societies because the reproduction is restricted to a few individuals (i.e. the queen and multiple drones). Consequently, natural selection should predominantly act on colony-level (between colonies) rather than on the individual-level (between individuals of the same colony) (Korb and Heinze 2004; Bergmüller et al. 2007). It is well understood that increased inter-individual variability in behavioral traits based on genetic diversity within honey bee hives enhances the productivity and fitness of honey bee colonies (Mattila and Seeley 2007; Wray et al. 2011) Evidence from bumble bees suggests that a cognitive trait can be directly linked to foraging success, a robust measure for fitness, under natural conditions (Raine and Chittka 2008). Colonies hosting individuals that learned faster in

a discrimination task collected more nectar compared to colonies with a higher number of slow-learning individuals (Raine and Chittka 2008). It might thus be appealing to speculate that natural selection should favor higher learning speed.

However, individual bumble bees and honey bees within a colony remain consistent in their propensity to make either 'fast-inaccurate' or 'slow-accurate' choices during discrimination tasks, referred to as speed-accuracy trade-offs (Chittka et al. 2003; Burns and Dyer 2008). These two cognitive strategies seem to be favorable in different environmental situations. The 'fast-inaccurate' bees showed increased efficiency in food collection in a fluctuating environment, while the 'slow-accurate' individuals outperformed the 'fast-inaccurate' bees in a constant environment (Burns and Dyer 2008). Consequently, it seems that maintaining a mixture of individuals which differ consistently in behavioral strategies could influence the flexibility of colonies to react to changing environmental conditions (Mattila and Seeley 2007; Burns and Dyer 2008; Jandt et al. 2014). Indeed, enhanced cognitive proficiency may not always be associated with fitness benefits. For example, fast-learning bumble bees foraged for a shorter time-span compared to slow-learning bumble bees, providing evidence that enhanced learning ability is associated with costs (Evans et al. 2017). Cognitive function is associated with high metabolic and operating costs (Mery and Kawecki 2004; Burns et al. 2011). Individuals trade-off enhanced ability in one cognitive trait at the expense of alternative cognitive traits (Hollis and Guillette 2015; Tait et al. 2019; Junca et al. 2019) or other traits such as reproduction, immune system function and longevity (Dukas 1999; Mery and Kawecki 2003, 2004; Mallon et al. 2003; Burger et al. 2008; Jaumann et al. 2013).

It seems thus reasonable to speculate that at the colony level, consistent individual differences in cognitive proficiency leads to a more fine-tuned task specialization of workers. For example, the 'best-learning' foragers within a honey bee colony could perform more complex tasks and allocate their foraging effort towards the scouting for novel profitable food sources, referred to as 'scouts' (Biesmeijer and De Vries 2001; Beekman et al. 2007). 'Poor-learning' foragers might be 'recruits' that follow the information provided by the scouts (Biesmeijer and De Vries 2001; Beekman et al. 2007). Some evidence indeed suggests that scouts perform better in associative and non-associative learning in comparison to recruits (Carr-Markell and Robinson 2014; Cook et al. 2019). Intriguingly, these differences in learning proficiency were not attributable to differences in sucrose responsiveness (Carr-Markell and Robinson 2014) but rather to differences of biogenic amine titers in the brain (Cook et al. 2019). Similarly, this could also explain why individual bees show a trade-off in appetitive and

aversive learning capabilities (Junca et al. 2019). While individuals that perform better in appetitive learning compared to aversive learning might specialize in foraging tasks, individuals that show the reversed cognitive profile might specialize in guarding the nest entrance and colony defense (Roussel et al. 2009).

7.4 Context-dependent importance of sensory responsiveness during learning

For all animals, a crucial step for survival is to make the 'correct' decision at the right moment (Siju et al. 2021). Consequently, behavioral responses predominantly depend on the type of sensory cue the response is directed to and on the context the cue is encountered in. Additionally, the internal state of the animal as well as the previous experience and innate preferences are also integrated during the decision-making of which behavior to perform (McFarland 1977). A vast majority of behaviors are shown in a behavioral context-dependent manner. For example, when a satiated individual is trapped, a droplet of sugar solution might not be the most relevant cue to escape. However, for a starved individual foraging in the environment the same droplet of sucrose solution has probably a much higher relevance than for the satiated trapped individual. This example illustrates that the behavioral context is critical to show the appropriate behavioral response in any situation. In general, the perception of sugars is of major importance for bees while foraging in the field. Foragers assess the quality of the nectar they collect and alter their foraging behavior depending on several nectar parameters such as volume and sugar concentration (von Frisch 1965; Banschbach 1994). Thereby, this assessment is dependent on the individual evaluation of the sucrose solution offered. The individual evaluation of sucrose can be tested by stimulating a restrained bees' antennae with ascending sucrose concentrations (Page et al. 1998; Pankiw and Page 1999; Scheiner et al. 2013). Bees with a low responsiveness respond with a PER only to higher concentrations of sucrose solution, while bees with a high responsiveness respond also to lower concentrations because they give a higher value to a given sucrose concentration in comparison to bees with lower responsiveness (Scheiner et al. 2005).

Importantly, an individuals' sucrose responsiveness is not constant but can be modulated by several factors such as feeding states (Scheiner et al. 2003b) and individual experience (Scheiner et al. 1999; Pankiw et al. 2001) among others. Within honey bee colonies there is appreciable inter-individual variability in the responsiveness to sucrose, a mechanism

that leads to further behavioral specialization of foragers to collect nectar, pollen or water (Pankiw and Page 1999, 2000; Page et al. 2006). For example, bees with the higher responsiveness usually specialize in collecting pollen or water while bees with lower responsiveness usually specialize in collecting nectar. Intriguingly, sucrose responsiveness correlates positively with learning capabilities in a way that bees with higher responsiveness perform better in olfactory and tactile elemental learning under restrained conditions compared to bees with a lower responsiveness (Scheiner et al. 1999, 2001a, b, 2005). It has remained unknown whether sucrose responsiveness also correlates positively in a different behavioral context i.e. in free-flying conditions. Consequently, I examined this question in chapter 4 of this thesis.

Restrained conditions in the laboratory offer the great possibility to study learning ability of bees with precise control of the external conditions such as the illumination, the temperature and humidity as well as of experimental parameters such as the duration of CS and US presentation (Matsumoto et al. 2012; Giurfa and Sandoz 2012). However, the bees are in an inactive behavioral state as they cannot move freely and are 'forced' to participate in the experiment. In free-flying conditions bees are in an active state as are allowed to fly freely and show a high intrinsic motivation to participate in the experiment as they are free to leave the experimental set-up during any time. It seems thus reasonable to conclude that these two conditions indeed represent two distinctly different behavioral contexts. Some evidence suggests that the sensory perception might be different between these two behavioral contexts: When free-flying bees were tested for their acceptance of different sucrose solutions at an artificial feeding site, the concentration which they were willing to consume was lower compared to the responsiveness determined subsequently with the PER assay under restrained conditions (Mujagic and Erber 2009). The authors consequently showed that it is not possible to conclude on which sucrose solutions are accepted by bees in the field from results obtained in the laboratory. They concluded that the behavioral and environmental context might play an important role in the modulation of sensory responsiveness to task relevant stimuli (Mujagic and Erber 2009). This questions the often-made assumption that findings established in the laboratory also apply for behaviors expressed in natural scenarios and might thus have important implications for the study of learning in insects. This hypothesis seems to be confirmed by the fact that I could not evidence a positive association between sucrose responsiveness and learning performances in elemental and non-elemental learning tasks in free-flying conditions (chapter 4), despite convincing evidence that elemental learning

proficiency is strongly positively correlated with sucrose responsiveness in laboratory conditions (Scheiner et al. 1999, 2001a, b, 2005).

It is indeed a principal characteristic of nervous systems to process sensory information in a context-dependent manner (Sayin et al. 2018; Anton and Rössler 2021; Siju et al. 2021; Oram and Card 2022). By contrasting active (free-walking) and inactive (fixed) mice researchers evidenced that visual processing is modulated as such that free-walking individuals showed an enhanced neuronal encoding of visual information in comparison to the fixed individuals (Niell and Stryker 2010; Ayaz et al. 2013; Erisken et al. 2014; Dadarlat and Stryker 2017; Pakan et al. 2018). Additionally, locomotion increases spatial resolution (Mineault et al. 2016) and the detection of low contrast stimuli (Bennett et al. 2013) while also reducing surround suppression (Ayaz et al. 2013; Erisken et al. 2014). This enhancement of neuronal encoding was attributed to increased arousal which is associated with locomotion (Niell and Stryker 2010; Erisken et al. 2014; McGinley et al. 2015; Dadarlat and Stryker 2017). Arousal can be defined as a state of heightened physiological activity and reactivity (Eysenck 1982). Interestingly, recent evidence suggests that free-walking mice performed better in a visual associative learning task compared to fixed individuals, which provides some evidence that the behavioral context modulates not only sensory perception but also learning proficiency (Albergaria et al. 2018).

In insects, locomotion-dependent modulation of visual information was also evidenced. For example, locomotion increases the gain of motion sensitive neurons in the fruit fly (Chiappe et al. 2010; Maimon et al. 2010) and the processing speed of photoreceptors in bumble bees (Rother et al. 2022). However, the relationship between behavioral context-dependent modulation of sensory processing and learning proficiency has remained mostly unknown in insects. In honey bees it is known that aversive substances such as quinine seem to have a different valence depending on the behavioral context: while ‘bitter’ substances such as quinine are used in free-flying conditions as aversive reinforcement to improve discrimination performances, because bees avoid them (Avarguès-Weber et al. 2010a), they are readily consumed by bees in restrained conditions even if they die afterwards due to post-ingestional malaise (Ayestaran et al. 2010). A recent study on honey bees demonstrated that the behavioral context modulates the perception of aversive substances during learning. The authors subjected bees to an appetitive-aversive discrimination task conducted either in a restrained or free-walking context (De Brito Sanchez et al. 2015). These groups of bees were subsequently subjected to non-reinforced retention tests in both behavioral contexts. Interestingly, bees

trained in the restrained context transferred their choice to the free-walking context while this was not the case for the bees trained in the free-flying context. Furthermore, quinine was found to have an intermediate aversive effect during conditioning in the restrained context, while it had a high aversive effect in the free-walking context (De Brito Sanchez et al. 2015). Taken together with my results showing a dichotomy in the importance of sucrose responsiveness in the restrained and free-flying context (chapter 4), these results demonstrate that the perception of the US can be modulated by behavioral context which has important consequences for the design of learning experiments in these two contexts.

This behavioral-context dependent importance of sensory responsiveness also makes sense from an ecological perspective. When bees are foraging in the environment to search for profitable food sources, they encounter stimuli of multiple sensory modalities guiding their behavior in a distance-related manner (von Frisch 1965). Colors of flowers provide mid-range signals to enable their detection by a foraging bee (von Frisch 1965). Once a bee has detected the flower, it lands on the flower and a nectar reward is offered to the bee to enable associative learning of the flowers' physical features in order to facilitate discrimination and detection during future foraging trips. It might thus be possible that color signals play a more dominant role to guide the bees' behavior during flight while odors and sucrose responsiveness might be more important while walking or standing still. On one side, this could explain why in restrained individuals almost universal positive correlations were observed between the responsiveness to sucrose and odors or pollen (Scheiner et al. 2004). On the other side, this could also explain why the PER responses I observed in response to visual stimulation were generally not as strong compared to those observed in response to olfactory stimulation during PER conditioning (chapter 2, Finke et al. 2023). Furthermore, the restrained context corresponds to the bee being trapped and consequently a probable response is escape behavior in order to survive. This escape response might lead to a general increase of responsiveness to stimuli of multiple sensory modalities which could again explain the positive associations between responsiveness of the bees to various stimuli.

Neuromodulation is known to be an excellent tool to attain neuronal flexibility resulting in behavioral modifications by integrating multiple internal and external signals (Bargmann 2012; Marder 2012). Such modulation is achieved via neuromodulators such as neurotransmitters, neuropeptides and biogenic amines which can alter the synaptic properties of neurons (Marder 2012). For example, octopamine is a crucial neurotransmitter, neuromodulator and neurohormone in insects (Beninger 1983; Roeder 1999; Schulz et al. 2002;

Scheiner et al. 2006; Farooqui 2007). Octopamine leads to the enhancement of several behaviors such as associative learning (Behrends and Scheiner 2012), locomotion and flight activities in honey bees (Akasaka et al. 2010; Mezawa et al. 2013; Watanabe and Sasaki 2022). Furthermore, octopaminergic neurons are involved in arousal mechanisms in insects (Bacon et al. 1995; Stern 1999) and also enhance the sensory responsiveness to sucrose and light by modulating the sensitivity of receptors and interneurons (Page and Erber 2002; Scheiner et al. 2002; Schilcher et al. 2021). Recent evidence in fruit flies provided evidence that prolonged flight activity requires disinhibition of certain MBONs via octopaminergic signaling (Manjila et al. 2019). Similarly, MB-dependent modulation of flight activity also occurs in honey bees (Kiya et al. 2007; Lutz and Robinson 2013). It can thus be speculated that a potential neural mechanism underlying the modulation of sensory responsiveness in dependence of the behavioral context involves octopaminergic signaling. However, clearly future research is necessary in order to test this hypothesis.

7.5 Final Conclusion and Outlook

The present doctoral thesis provides the first comprehensive overview of consistent inter-individual variability in the cognitive proficiency of honey bees. I have shown that the honey bee offers a unique opportunity to study individual 'cognitive profiles' due to their impressive and versatile cognitive proficiency to gain a deeper understanding of the mechanisms underlying cognition.

In **chapter 1** I established that individual free-flying bees remained consistent in their discrimination performance over a time span of three days and that increased proficiency in the elemental discrimination task was associated with increased ability to solve a highly complex concept learning task. However, these positive associations were only found when the stimuli belonged to the same sensory modality, because individuals show a cognitive specialization for either visual or olfactory learning.

In **chapter 2** I examined more closely the relationship between elemental and non-elemental learning capabilities in individual bees. I show that elemental learning proficiency is positively correlated with performances in two non-elemental learning tasks (reversal learning and negative patterning), while the non-elemental tasks revealed no clear association. This pattern of individually correlated and independent performances was the same across four experiments using stimuli from different sensory modalities (either only visual or olfactory

stimuli) and different behavioral contexts (free-flying or restrained conditions). I thus conclude that this pattern of correlated proficiency represents a real characteristic of the bees' 'cognitive profile'.

In the next two chapters (**chapters 3 and 4**) I wished to determine the mechanisms by which consistent cognitive proficiency is maintained within honey bee colonies. I demonstrated in **chapter 3** that the 'cognitive profile' evidenced in chapter 2 can, at least partially, be attributed to the genetic diversity which naturally exists in honey bee colonies due to extreme polyandry of honey bee queens (Tarpy et al. 2012). This was evidenced by contrasting individuals originating from either a polyandrous or a monandrous colony. Finally, I show in **chapter 4** that neither the individual evaluation of the US (sucrose responsiveness) nor of the CS (visual responsiveness) could predict the performances of free-flying bees in an elemental discrimination, reversal learning or negative patterning. This result is especially interesting, because sucrose responsiveness reliably predicts elemental learning performances when the bees are tested in restrained conditions (Scheiner et al. 1999, 2001b, a, 2005) indicating that sensory responsiveness might be modulated in a behavioral-context dependent manner.

Although a number of questions have been elucidated by the research presented here, other questions have remained poorly understood. Still, the molecular and mechanistic causes underlying individual cognitive skills in honey bees are remain mostly unknown. I have shown that some 'basic mechanisms' such as the individual evaluation of the CS and US stimuli or the age of the individuals could not explain consistent inter-individual variability across elemental and non-elemental learning tasks. A factor that seems likely to contribute to consistent cognitive skill in individual bees is the sensory experience during early development and adult maturation. Across several species, growing up in an enriched environment leads to changes in neuronal anatomy and function associated with learning and memory (Withers et al. 1993; Heisenberg et al. 1995; Fahrbach et al. 1998; Eckert and Abraham 2012; Cabirol et al. 2017). For example, bees that matured in an impoverished environment during early adulthood had fewer numbers of synapses in the MBs compared to bees which matured in an enriched in-hive environment (Cabirol et al. 2017). Interestingly, the impoverished-environment bees were incapable of reversal learning, a MB-dependent learning task (Cabirol et al. 2017). However, artificially reared bees (impoverished-environment) did not differ from their in-hive sisters in their elemental performance, a MB-independent learning task (Devaud et al. 2007), despite having smaller MBs (Steijven et al. 2017). It might thus be possible that development in impoverished environments disrupts the here presented individual consistency across elemental

and non-elemental learning tasks. However, this hypothesis needs to be tested by contrasting the elemental and non-elemental learning performances of bees reared artificially or in the natural in-hive environment.

Another fruitful area for future concerns the question of whether individual cognitive profiles have an adaptive significance for honey bee colonies. It is indeed a common hypothesis that the diversity of individual strategies in various behaviors fine-tunes the division of labor and thus improves colony fitness and survival (Mattila and Seeley 2007; Burns and Dyer 2008; Eckholm et al. 2011; Wray et al. 2011; Dyer et al. 2014). In honey bees, it is known that individuals vary in their foraging efficiency (Dukas and Visscher 1994) but less is known how individual cognitive skill relates to individual foraging efficiency. Are the consistently 'good learners' also those that forage the most and collect more resources? Does the resource collection of 'good' and 'poor' learners show differences under distinct resource distributions? Another intriguing perspective would be to determine if the highly skilled individuals are actually those that perform more complex foraging tasks and scout for resources while the less skilled individuals follow the information provided by the scouts. Such experiments can be relatively easily conducted with honey bees as the foraging environment can be precisely controlled in flight cages (Schilcher et al. 2022). Furthermore, the foraging behavior of individual bees and the whole colony can be monitored via radio frequency identification (RFID) (Sumner et al. 2007; Hesselbach et al. 2020; Kablau et al. 2020; Schilcher et al. 2022). It is possible to couple RFID with a scale that weighs the bees before and after leaving the colony. This allows to calculate the amount of nectar brought back to the colony, from which the individuals' and the colonies' foraging efficiency can be extrapolated (Feltham et al. 2014).

Finally to gain a mechanistic understanding of individual cognitive proficiency, a prerequisite is the identification of the underlying brain structures and how they change in relation to cognitive performances. Honegger and colleages (2019) showed in an elegant experiment combining behavioral analysis with *in-vivo* calcium imaging that odor preferences of individual flies remained consistent over time and were related to individual differences in neural coding of PNs across glomeruli in the AL. In another study, isogenic flies that were reared identically remained consistent in their performances in aversive discrimination learning tasks (Smith et al. 2022). Taken these two results together it seems that the biological basis for consistent individual differences in learning performance originates from stochastic physiological variation in neurons conveying general US signals (Smith et al. 2022). Similar calcium imaging studies have been conducted in honey bees, where *in-vivo* calcium imaging of

PNs in the glomeruli of the AL was coupled with olfactory PER learning to investigate the effect of associative learning on early sensory processing (Rath et al. 2011). As the technique used by Honegger et al. (2019) is also available in bees, a first step towards the understanding of how individuality in behavior is encoded in the brain would be to replicate their experiments with bees to see if individual differences in neural coding can be traced back to individual odor preferences. Bees could thus be measured for several days to determine their individual odor preferences. Then, simultaneous in-vivo calcium imaging in the glomeruli of the Als of an individual while presenting its preferred odor would allow to see if individual patterns of neuronal activity to that odor can be found.

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Affidavit

I hereby confirm that my thesis entitled “Are some bees smarter than others? An examination of consistent individual differences in the cognitive abilities of honey bees” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Sind manche Bienen schlauer als andere? Eine Untersuchung von konsistenten individuellen Unterschieden in den kognitiven Fähigkeiten von Honigbienen“ eigenständig, d.h. insbesondere eigenständig und ohne Hilfe eines kommerziellen Promotionsberaters angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift

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"Friendship is the hardest thing in the world to explain. It's not something you learn in school. But if you haven't learned the meaning of friendship, you really haven't learned anything." – Muhammed Ali

This quote expresses my gratitude to all my friends especially to Kristina Müller, Yannic Gehlen, Jannik Peters, Julia Böse, Stefan Häußner and Fabio Hedrich.

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In memory of Dieter Müller

Appendix

Statement of individual author contributions and of legal second publication rights to manuscripts included in the dissertation

Manuscript 1 (complete reference): Finke, V., Baracchi, D., Giurfa, M., Scheiner, R., Avarguès-Weber, A. (2021) Evidence of cognitive specialization in an insect: proficiency is maintained across elemental and higher-order visual learning but not between sensory modalities in honey bees. Journal of Experimental Biology, 224(24):jeb242470.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	AAW AAW	VF VF			
Data Collection	VF				
Data Analysis and Interpretation	VF	AAW	DB	MG	RS
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 VF VF VF VF	 AAW AAW AAW AAW	 MG MG MG	 RS RS RS	 DB DB DB

Manuscript 2 (complete reference): Finke, V., Scheiner, R., Giurfa, M., Avargués-Weber, A. (2023) Individual consistency in the learning abilities of honey bees: cognitive specialization within sensory and reinforcement modalities. Animal Cognition.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	AAW VF	VF AAW	RS* RS*	MG* MG*	
Data Collection	VF				
Data Analysis and Interpretation	VF	AAW	RS	MG	
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 VF VF VF VF	 AAW AAW AAW	 RS* RS* RS*	 MG* MG* MG*	

Explanations (if applicable):* these authors contributed equally

Manuscript 3 (complete reference): Finke, V., Avarguès-Weber, A., Giurfa, M. and Scheiner, R. (unpublished) The impact of genetic diversity on consistent individual differences in the cognitive proficiency of honey bees

unpublished manuscript to be submitted in 2023

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	RS VF	VF RS	AAW AAW	MG MG	
Data Collection	VF				
Data Analysis and Interpretation	VF	RS	AAW	MG	
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 VF VF VF VF	 AAW AAW AAW	 RS RS RS	 MG MG MG	

Manuscript 4 (complete reference): Finke, V., Avarguès-Weber, A., Giurfa, M. and Scheiner, R. (unpublished) Relationships between sensory responses and learning performances in honey bees are context-dependent.

unpublished manuscript to be submitted in 2023

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	RS VF	VF RS	AAW AAW	MG MG	
Data Collection	VF				
Data Analysis and Interpretation	VF	RS	AAW	MG	
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 VF VF VF VF	 AAW AAW AAW	 RS RS RS	 MG MG MG	

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Valerie Kuklovsky

Doctoral Researcher's Name	Date	Place	Signature

Prof. Dr. Ricarda Scheiner 14/03/2023 Würzburg

Primary Supervisor's Name	Date	Place	Signature

Statement of individual author contributions to figures/tables of manuscripts included in the dissertation

Manuscript 1 (complete reference): Finke, V., Baracchi, D., Giurfa, M., Scheiner, R., Avarguès-Weber, A. (2021) Evidence of cognitive specialization in an insect: proficiency is maintained across elemental and higher-order visual learning but not between sensory modalities in honey bees. Journal of Experimental Biology, 224(24):jeb242470.					
Figure	**Author Initials,** Responsibility decreasing from left to right				
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Manuscript 2 (complete reference): Finke, V., Scheiner, R., Giurfa, M., Avargués-Weber, A. (2023) Individual consistency in the learning abilities of honey bees: cognitive specialization within sensory and reinforcement modalities. Animal Cognition.					
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Manuscript 3 (complete reference): Finke, V., Avarguès-Weber, A., Giurfa, M. Scheiner, R. (unpublished) The impact of genetic diversity on consistent individual differences in the cognitive proficiency of honey bees

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Table	**Author Initials,** Responsibility decreasing from left to right				
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Explanations (if applicable):

I also confirm my primary supervisor's acceptance.

Valerie Kuklovsky

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Doctoral Researcher's Name Date Place Signature