UNIVERSITÀ
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Honeybee cognition:

From numbers to extraction of regularities

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The bees' life is like a magic well:
The more you draw from it,
The more it fills with water.

(Karl von Frisch)

L'ape è piccola
tra gli esseri alati
ma il suo prodotto
ha il primato
fra i dolci sapori.

(Siracide, 11,3)

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Bortot, M., Regolin, L., & Vallortigara, G. (2021). A sense of number in invertebrates. *Biochemical and Biophysical Research Communications*, *564*, 37-42. https://doi.org/10.1016/j.bbrc.2020.11.039

Chapter 1

Bortot, M., Stancher, G., & Vallortigara, G. (2020). Transfer from number to size reveals abstract coding of magnitude in honeybees. *iScience*, *23*(5). https://doi.org/10.1016/j.isci.2020.101122

Chapter 2

Bortot, M., & Vallortigara G. (2023). Transfer from continuous to discrete quantities in honeybees. *iScience*, *26*(10). https://doi.org/10.1016/j.isci.2023.108035.

Chapter 3

Bortot, M. & Vallortigara, G. (2023). An investigation of the odor sequence learning in honeybees using a PER classical conditioning paradigm. *In preparation*.

Not included in this thesis:

Bortot, M., Agrillo, C., Avarguès-Weber, A., Bisazza, A., Miletto Petrazzini, M. E., & Giurfa, M. (2019). Honeybees use absolute rather than relative numerosity in number discrimination. *Biology Letters*, *15*(6), 20190138. https://doi.org/10.1098/rsbl.2019.0138

Zanon, M., Potrich, D., **Bortot**, M., & Vallortigara, G. (2022). Towards a standardization of non-symbolic numerical experiments: GeNEsIS, a flexible and user-friendly tool to generate controlled stimuli. *Behavior Research Methods*, *54*(1), 146-157. https://doi.org/10.3758/s13428-021-01580-y

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GLOSSARY

absolute numerical value unique numerical value of an array

continuous quantity uncountable quantity

discrete quantity countable quantity

magnitude physical feature of an object (e.g., numerousness, size)

number sense perception of the approximate number of items

numerosity quality of being numeric

prelinguistic before language onset

prothetic dimension dimension that could be either larger or smaller if compared

proto-counting primitive form of counting without sophisticated numerical system

quantity property that can be measured, counted and compared; it can represent discrete and continuous aspects

relative numerical value non-unique numerical value defined by the comparison with other arrays

GENERAL ABSTRACT

Insects are not mere reflex machines. Instead, they adapt their behaviour flexibly to changing environmental contingencies. Among the insects, honeybees (*Apis mellifera*) possess an impressive repertoire of cognitive abilities, despite their limited number of neurons. Thanks to the standardization of behavioral, neurobiological, neuroimaging, and genetic methods, bees became a widely used invertebrate model in research. Importantly, the study of their capacities allows us to integrate evidence from an invertebrate species into broader scientific frameworks - often based on vertebrate studies - supporting a deeper understanding of the evolution of certain cognitive mechanisms and their universality.

Honeybees can process different information from their environment, such as the numerousness of an array or the relationships – both perceptual and abstract – between objects. Once identified, such relationships allow bees to form distinct categories to which they will refer to implement adaptive choices. An ongoing debate focused on whether numerical abilities in bees are supported by a unified neural mechanism – as for vertebrates - or if multiple segregated mechanisms are involved. Additionally, there is interest in further expanding our knowledge about the extent of bees' categorization capacities in different contexts. This thesis aims to address these questions, providing evidence that can shed light on the neural organization and limits of honeybees' cognitive abilities, as well as on potential similarities or differences with other species.

In the first two studies, the existence of a general mechanism for the estimation of quantity in honeybees was investigated. Specifically, I addressed the issue of whether bees' numerical abilities are supported by a general magnitude mechanism that estimates

continuous (e.g., space, time, size) and discrete (i.e., number) quantities. In the first study, we investigated the bees' ability to transfer learning from numerical to size dimension. Using appetitive-aversive conditioning, independent groups of free-flying foragers were trained to discriminate between larger and smaller visual numerousness (i.e., 2 *vs.* 4, 2 *vs.* 3, 4 *vs.* 8, 4 *vs.* 6; 0.5 or 0.67 ratio difference). We then tested the bee's generalization ability with a comparison between stimuli with different sizes and identical numerosity (e.g., 4 larger elements *vs.* 4 smaller elements). Honeybees spontaneously chose the congruent size with respect to their training. No effect of numerical contrast and ratio difference experienced was found as bees previously reinforced toward the larger numerosity, chose the relatively larger size, and vice versa. These results demonstrated the ability of this insect species to make a transfer from the numerical to the size dimension.

Given the possibility of asymmetric relationships between magnitudes, we sought to explore whether honeybees possess the capacity to make the reverse transfer as well, from a continuous (size) to a discrete (number) dimension. Similar to the previous study, free-flying foragers were trained to discriminate between relatively larger *vs.* smaller squares or diamonds. Their generalization ability over novel shapes (i.e., circles) and novel dimensions (i.e., number) was subsequently tested. Our results confirmed the ability of bees to transfer size discrimination to novel shapes. Moreover, when presented with a 4 *vs.* 8 elements comparison, bees spontaneously selected the congruent numerosity with respect to their training (i.e., bees trained to select the smaller/larger size, selected the smaller/larger numerosity, respectively). To check for any perceptual cue involvement in bees' decision-making, different continuous variables covarying with numerosity were controlled for (i.e., total area, contour length, stimulus size, convex hull). Subsequent analyses also revealed no role of spatial frequency in the bees’ behavior. The results revealed a bee’s capacity to transfer between numerical and size dimensions, suggesting the universality of the magnitudes coding mechanism and highlighting the presence of a unified circuit supporting discrete and continuous quantity processing.

The second aim of this thesis was to enlarge our knowledge of the ability of bees to spontaneously encode regularities from the physical world. To this purpose, I tested bees' ability to extrapolate the structure of temporally defined odor sequences. In a series of six experiments, the spontaneous and trained ability of bee foragers to learn, memorize, and generalize an odor sequence composed of three distinct odors was tested. A *proboscis extension response* (PER) conditioning paradigm was employed (i.e., absolute, differential, and generalization). The first two experiments investigated honeybees’ ability to learn an arbitrary odor sequence. Bees were trained to respond to a specific sequence of three odors and then tested for their spontaneous ability to generalize their response to novel sequences with a similar structure but composed of novel odors and to reject novel configurations although composed of familiar odors. The role of a particular odor position in the sequence, the odor-reward temporal closeness, and their possible effects on memory were also investigated in the third experiment. The fourth and fifth experiments aimed to understand the effect of differential conditioning on bees’ learning ability. Lastly, we determined whether a conditioning procedure favouring a generalization strategy could lead to the spontaneous encoding of the internal sequence structure. In general, the results highlighted an early tendency of bees to encode the single odor properties, instead of learning the entire sequence structure, together with a significantly increased response towards the novel odor configurations composed of familiar odors. No effect of the odor’s position or temporal closeness with the reward was apparent. During absolute and differential conditioning, bees likely employed two strategies to memorize the dyad of the first and second elements of the sequence, together with a more general response to novelty. However, the use of a transfer paradigm potentially revealed a weak spontaneous generalization over similar structures one hour after the training, irrespective of the single-element properties. Overall, these results shed light on the strategies employed by bees to solve an odor abstraction task, highlighting the crucial role of the type of conditioning to let them emerge.

Altogether, the thesis provides new evidence on honeybees' cognition. The findings have implications not only for the study of bees' behavior but also for broader investigations into the universal development of basic cognitive mechanisms and the convergent evolution of similar abilities in small and large brains.

CHAPTER 1

GENERAL INTRODUCTION

This chapter contains a modified version of the following scientific article under the permission of the copyright holder: *Bortot, M., Regolin, L., & Vallortigara, G. (2021). A sense of number in invertebrates. Biochemical and Biophysical Research Communications, 564, 37-42.* The Journal Author Rights are reported in Appendix A.1.

Insects live a short but complex life in a challenging environment. They need to orient and navigate the surroundings, find food or mates, cooperate, and communicate efficiently with conspecifics living in the same colony. Most importantly, during their life they need to learn regular contingencies and retain that knowledge in their memory for a convenient amount of time to make adaptive choices and maximize their chance to survive and reproduce. Indeed, the insects' ability to use efficient cognitive strategies to maximize the costs/benefits ratio of being equipped with a limited number of neurons is of great scientific interest.

A cognitive perspective about insects' behavior was proposed for the first time by Charles Henry Turner (1867 – 1923), which also addressed memory as the fundamental property supporting their navigation and learning strategies (Turner, 1907a, 1907b, 1908b, 1908a, 1918; see Giurfa and de Brito Sanchez, 2020). He anticipated some of the more recent cognitive interpretations on insect behavior, advising a new vision where insects "*are much more than mere reflex machine; they are self-acting creatures guided* *by memories of past individual (ontogenetic) experience*" (Turner, 1907b; and see Giurfa and de Brito Sanchez, 2020).

1.1 The honeybee as an animal model system in cognitive research

Among insects, honeybees (*Apis mellifera*) are the most known and easy commercially accessible social species worldwide. They have been investigated for several decades, paving the way for the study of insects' memory, learning, and cognition. The study of bee cognition first involved ethological observations of their natural behavior (Turner, 1910; von Frisch, 1914), gradually moving towards more controlled experimental paradigms developed in the field (Avarguès-Weber and Giurfa, 2014) and laboratory conditions (Matsumoto et al., 2012; Schultheiss et al., 2017).

As argued by Menzel and Giurfa (2006) the rich repertoire of behaviors supported by an accessible nervous system, together with the possibility of having bees undergo controlled training and testing procedures, are the three main reasons that encouraged the use of this insect species to study cognitive complexity. Among these reasons, the latter is of crucial importance in research, allowing scientists the creation of complex paradigms to test the bees perceptual and cognitive capacities in a controlled manner (Menzel and Giurfa, 2006).

Briefly, different experimental procedures have been standardized to study honeybees' cognitive capacities using classical and operant conditioning (Giurfa, 2003). Exploiting classical or Pavlovian conditioning, bees can be trained to show spontaneous aversive (i.e., sting eversion; *sting extension reflex*, SER; Vergoz et al., 2007) or appetitive (i.e., ligula eversion; *proboscis extension response*, Takeda, 1961) reflexes when presented with a punishment (e.g., electric shock) or reinforcement (e.g., a drop of sucrose solution). The conditioning of the spontaneous ligula eversion of restricted bees (Proboscis Extension Response, PER; Matsumoto et al., 2012) entails the presentation of a stimulus (e.g., odor, Giurfa & Sandoz, 2012; tactile stimulation, Giurfa & Malun, 2004; colored light, Avarguès-Weber and Mota, 2016) to harnessed bees that have to associate it with a positive reinforcement delivered to their antennae (i.e., sucrose solution eliciting the spontaneous eversion of the ligula) in a contingent manner. When bees learn this association, they will spontaneously show the PER behavior following the unconditioned stimulus presentation (Takeda, 1961; Fig. 1).

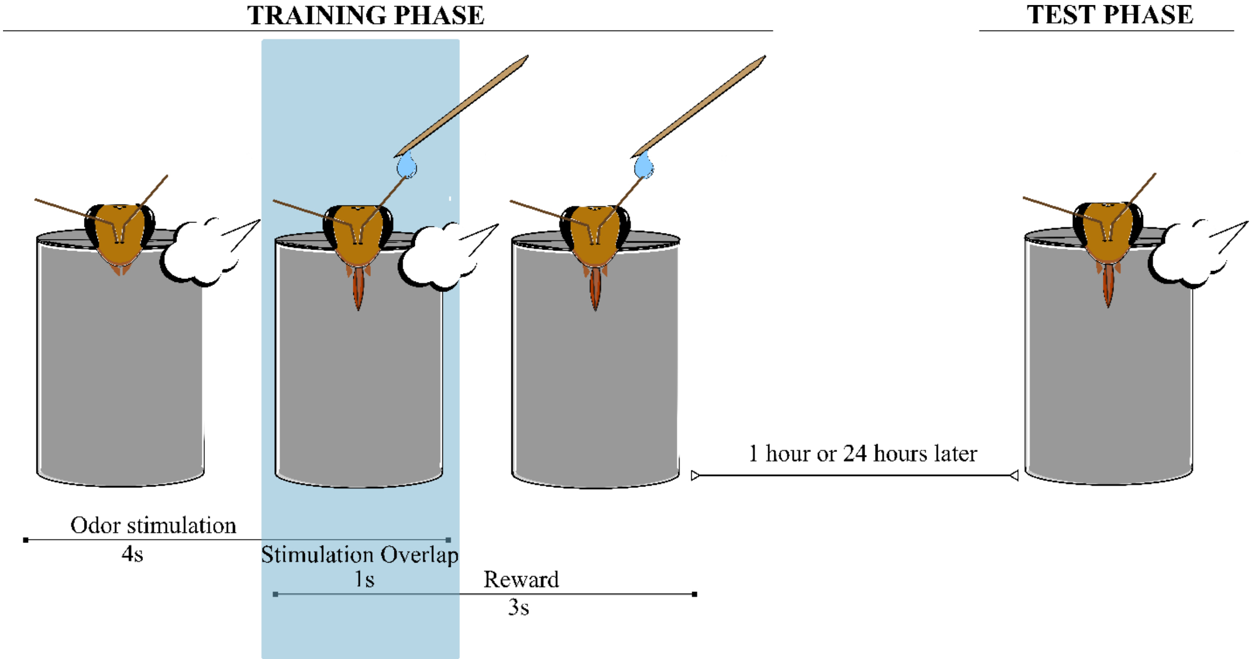
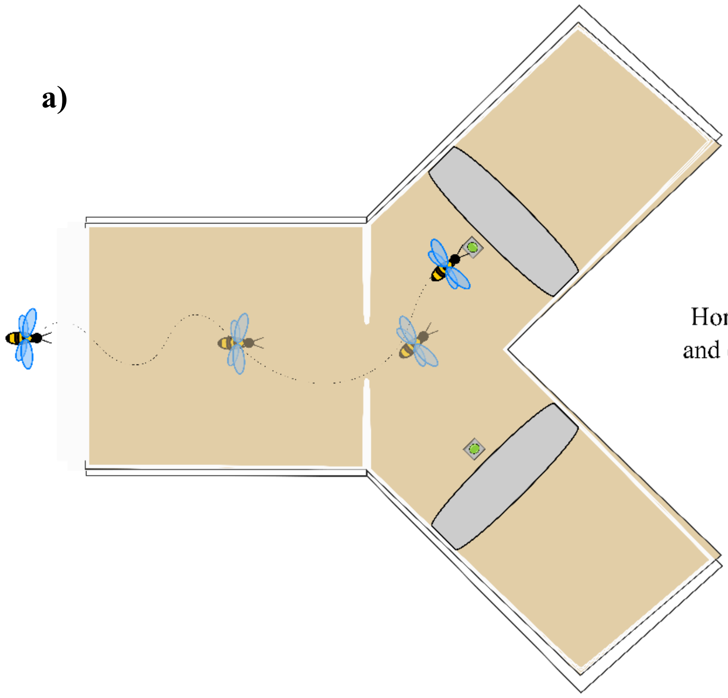


FIGURE 1: Schematic representation of Proboscis Extension Response (PER).

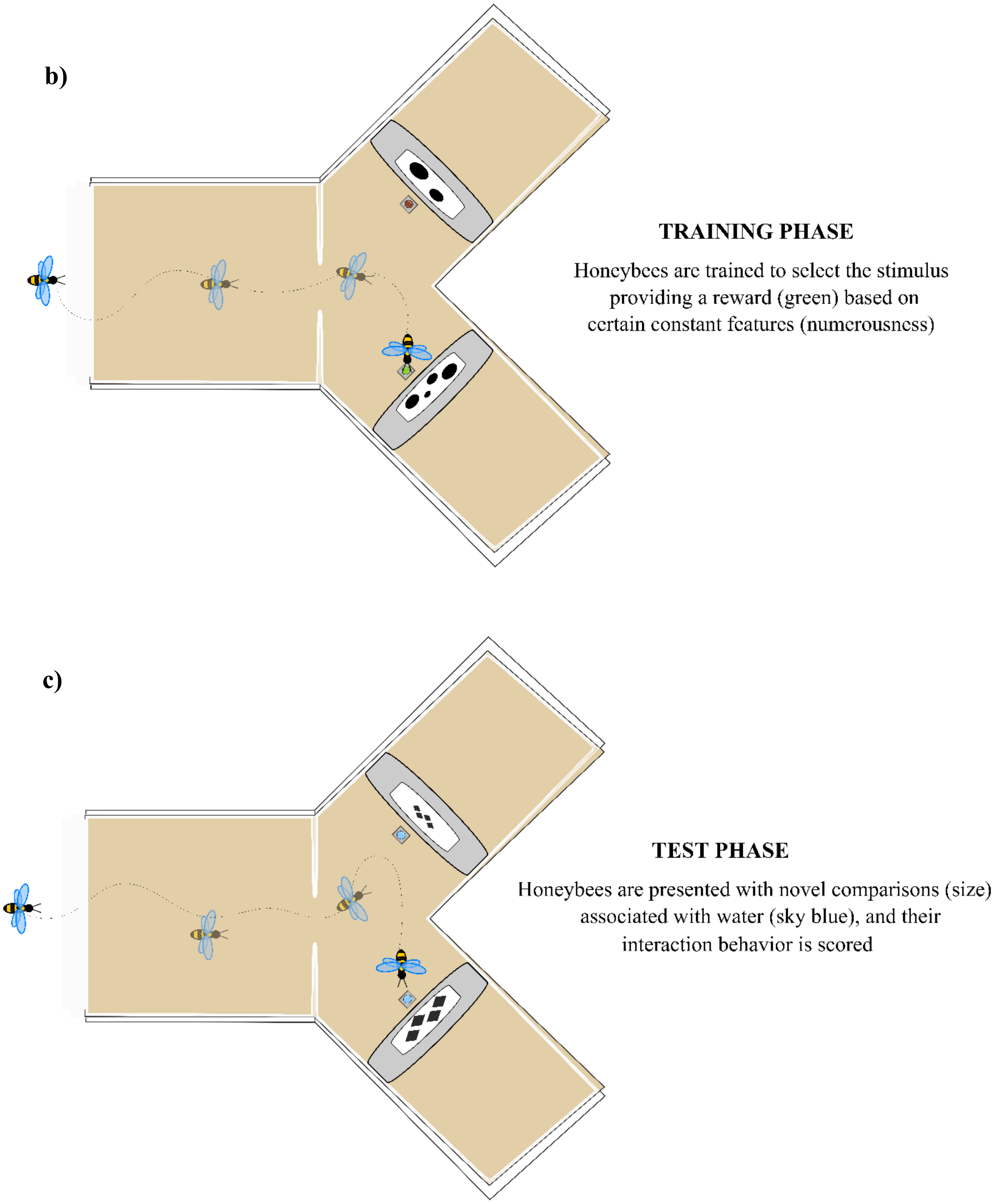
This technique has allowed the study of honeybees' olfactory discrimination abilities (Paoli and Galizia, 2021), highlighting the existence of different memory types (Menzel and Müller, 1996; Paoli et al., 2023). Combined with neuroimaging techniques, this paradigm unveiled the anatomical (Galizia and Szyszka, 2008; Paoli and Galizia, 2021), functional (Galizia and Szyszka, 2008; Szyszka et al., 2011, 2005), and biological underpinnings of olfactory learning in honeybees (Villar et al., 2020). More than 60 years of research allowed us to establish the PER as one of the most reliable experimental paradigms to study Pavlovian association from a behavioral and neurobiological perspective in different insect species (honeybees: Giurfa & Sandoz, 2012; bumblebees: Riveros & Gronenberg, 2009).

Honeybees can be also trained to associate a visual stimulus with a reward, following the procedures of operant conditioning. To this purpose, free-flying bee foragers learn to visit an apparatus (e.g., Y-maze, rotating disk) and select a visual target having specific perceptual characteristics to get a food reward (Menzel & Giurfa, 2006; but see also von Frisch, 1967). This approach was used for the first time to study honeybees' color vision (Turner, 1910; von Frisch, 1914), and was then further developed to study other visual capacities (Srinivasan, 2010). Generally, bees are first trained to approach the apparatus in the absence of any stimulation (i.e., habituation phase; Fig. 2a), followed by a training phase where subjects acquire knowledge about the task in a trial-and-error manner (Fig. 2b), and a test phase (Fig. 2c) where researchers analyze bees' ability to show and generalize the acquired learning in absence of any reward (Bortot et al., 2019 for an example). Usually, this training procedure follows differential appetitive-aversive conditioning with the correct stimulus presented in association with a positive reward (i.e., sucrose solution), while the incorrect counterpart is presented with a punishment (i.e., bitter quinine solution). This type of conditioning has been proven to enhance bees' visual performance, with the punishment acting as an aversive input at the gustatory sensory level (Avarguès-Weber et al., 2010). Taking advantage of honeybees' foraging behavior, which comprises a high motivation to maximize food intake and minimize energy costs (Stabentheiner and Kovac, 2016), this procedure allowed the investigation of foragers' visual discrimination abilities based on perceptual (Giurfa et al., 1996) or abstract cues (Avargues̀-Weber et al., 2012; Avarguès-Weber et al., 2011; Giurfa et al., 2001). Despite being highly ecologically relevant, because the animal moves freely in the environment, this method does not allow the simultaneous investigation of the neural substrates supporting visual abilities in bees. In recent years, the use of virtual reality (VR) to investigate bees' visual abilities in laboratory conditions has offered new possibilities (Schultheiss et al., 2017).



HABITUATION PHASE

Honeybees are trained to enter the apparatus and collect food from one of the two providers



TRAINING PHASE

Honeybees are trained to select the stimulus providing a reward (green) based on certain constant features (numerousness)

TEST PHASE

Honeybees are presented with novel comparisons (size) associated with water (sky blue), and their interaction behavior is scored

FIGURE 2: Schematic representation of visual operant conditioning in a Y-maze

For instance, using this technique which implies the presentation of computer-controlled visual stimulation to tethered walking or flying bees in association with electrophysiological recordings, Paulk and colleagues demonstrated the activation of the medulla (i.e., the central neuropile of the optic lobe) preceding the behavioral response (i.e., turning towards the stimulus; Paulk et al., 2014).

Thanks to their impressive learning abilities and versatility, along with the development of behavioural and neuroimaging techniques, honeybees have been employed to uncover the relationship between the existence of complex behavior arising from a relatively scarce neural substrate (Avarguès Weber et al., 2012; Menzel, 2012; Menzel and Giurfa, 2006; Srinivasan, 2010).

In this thesis, I used both behavioral paradigms, namely PER (*Proboscis Extension Response*) and operant conditioning, to investigate the cognitive abilities of honeybees. With the first technique, I explored bees' capacity to infer temporal structure regularities from odor sequences. Additionally, by employing operant conditioning with free-flying honeybees, I studied cross-transferring ability between numerical and size dimensions, adding an invertebrate species to the pool of evidence supporting the existence of a general cognitive mechanism allowing simultaneous processing of countable and non-countable dimensions in vertebrates (see Gazes et al., 2023 for a review).

1.2 A theory of magnitude: Insight from honeybees

1.2.1 Numerical competence in the invertebrate subphylum

The non-symbolic and non-verbal capacity to deal with the numerical value of an array has been documented in the majority of animal species, both vertebrates and invertebrates. It is though not any longer considered a prerogative of human beings (see e.g., for some recent reviews Butterworth et al., 2018; Giurfa, 2019; Nieder, 2019; Vallortigara, 2015, 2017, 2018).

The ability to process numerical information was, for a long time, thought to be linked to language, but several studies demonstrated the existence of differential neuronal patterns in participants involved in lingual *vs.* numerical tasks (Amalric and Dehaene, 2018). Developmental research also demonstrated that numerical cognition is present already in newborns and infants, suggesting the existence of numerical abilities at a prelinguistic stage of development (Di Giorgio et al., 2019; Lipton and Spelke, 2003; Izard et al., 2009), as well as in human traditional societies with a restricted abstract vocabulary (Gordon, 2004; Pica et al., 2004).

Discrimination of numerosities appears highly adaptive (see e.g., Haun et al., 2010; Nieder, 2020). Animals can use their *sense of number* to make choices that enhance their chance of survival, e.g., avoiding predation risks (Gómez-Laplaza & Gerlai, 2011; Hager & Helfman, 1991; Buckingham et al., 2007; Wong & Rosenthal, 2006), maximizing foraging intake or hunting success (Hanus and Call, 2007; Hauser et al., 2000; Panteleeva et al., 2013; Watts and Mitani, 2002), succeeding in intergroup conflicts (Benson-Amram et al., 2018; Grinnell et al., 1995; Heinsohn, 1997; Kitchen, 2004; McComb et al., 1994; Wilson et al., 2001), increasing their mating opportunities (Agrillo et al., 2008), maintaining the social contact with the larger group of conspecifics (Rugani et al., 2009), and reducing brood parasitism (Lyon, 2003). It has also been argued that an animal ability to discriminate different numerical items seems to be in place at birth, as demonstrated in domestic chicks (Lemaire et al., 2021; Rugani et al., 2010).

These abilities would be supported by a non-verbal *number sense* or *Approximate Number System (ANS),* which has been described as a capacity to estimate numerosity (Butterworth et al., 2018; Dehaene, 2011; Gallistel and Gelman, 2000, 1992; Vallortigara, 2017). The *number sense* hypothesis is "*the peculiar idea that we owe our mathematical intuitions to an inherited capacity that we share with other animals, namely, the rapid perception of approximate numbers of objects*" (Dehaene, 2011). The ANS allows estimation and discrimination of discrete quantities with an accuracy which depends on the numerical ratio of the comparison (e.g., discrimination of 5 *vs.* 10 items is easier than discrimination of 10 *vs.* 15 items; Feigenson et al., 2004), obeying to Weber's Law (i.e., the just noticeable difference between two elements depends on the ratio between their magnitudes, rather than their absolute difference; see Gallistel & Gelman, 2000). The *numerical distance* effect and the *numerical size* effect represent the basic key signatures of the ANS system. The *numerical distance* effect describes the increasing accuracy in the ability to discriminate between two quantities when their numerical distance increases (e.g., to discriminate 6 *vs.* 9 is easier than to discriminate 6 *vs.* 7; Nieder, 2019). The *numerical size* effect describes the decrease in accuracy in discriminating larger quantities with equal numerical distance (e.g., it is easier to discriminate 5 *vs.* 6 items than 10 *vs.* 11 items, in spite of the numerical distance being the same; Nieder, 2019).

Nowadays, we are fully aware of the ability of humans and other animals to use numerical information to deal with cognitive tasks, both in ecological and laboratory settings. However, some behavioral (Merritt et al., 2010) and neurobiological (Piazza et al., 2007) evidence also support the view that the ability to deal with numerosities would be part of a more general system dealing with quantity (or magnitude), underlying the ability of processing also continuous dimensions e.g., space and time. Gallistel (1989) was the first to suggest the existence of a "*common mental currency*" that would enable the representation of discrete (e.g., numbers) and continuous (e.g., space and time) dimensions in the brain (Gallistel, 1989). Later evidence from behavioral and brain studies demonstrated the existence of mutual associations between number and time (Meck and Church, 1983), number and space (Dehaene et al., 1993), and space and time (De Corte et al., 2017; Sarrazin et al., 2004) in humans and other animal species. Walsh (2003) also proposed *A Theory Of Magnitude* (ATOM) suggesting that a common prelinguistic framework would allow organisms the encode of different "*prothetic dimensions"* (i.e., dimensions that can be "more than" or "less than"), such as numbers, space, time, brightness or length (Walsh, 2003). According to the ATOM theory, the simultaneous processing of different magnitudes would lead to symmetrical interference across dimensions, as in the case of studies with human infants that process similarly numerical, temporal, and spatial dimensions (Lourenco and Longo, 2010). In their study, Merritt and colleagues (2010) compared the interaction between space and time in human adults and monkeys. Subjects had to judge either the length or the temporal duration of a line presented on a computer screen. At test, they were faced with lines having a different combination of features (e.g., longer length and shorter temporal duration and vice versa). The results showed a prevalent interference of the spatial over the temporal dimension in humans, while monkeys showed a mutual interference of the two magnitudes, without a significantly larger effect of spatial over temporal information (Merritt et al., 2010). An association between space and time is also found in pigeons (De Corte et al., 2017), providing further support to the hypothesis that a general system for magnitude representations is observed even in non-mammals.

For a long time, invertebrates have been virtually neglected in the study of cognitive abilities, and chiefly so for what concerns numerical cognition, mainly due to their smaller brains (Menzel, 2012) and different behavioral, anatomical, and ecological traits (Mikhalevich and Powell, 2020). Notwithstanding, several studies have now clearly demonstrated that information based on numerosity can provide an advantage in terms of fitness also to invertebrates (Skorupski et al., 2018; Fig. 3).

FIGURE 3: Evidence of the presence of a *sense of number* has been documented in various invertebrate species: ***1)*** mealworm beetle (*Tenebrio molitor*) prefer the substrate bearing the odors of the larger number of females. ***2)*** Ladybirds (*Coccinellidae*) choose the best laying spot according to the number of conspecific larvae and quantity of food (i.e., aphids) available. ***3)*** When they have lost the higher number of prey, spiders (*Nephila clavipes*) spend a higher amount of time searching for their food. ***4)*** Bumblebees (*Bombus terrestris*) can discriminate between visual sets using numerical information, and transfer their learning to novel stimuli differing in shape and color. ***5)*** Cuttlefish (*Sepia pharaonis*) prefer the larger number of shrimps when presented with dual-choice tasks, even when the numerical comparison involves fractions (e.g., 1 *vs.*1.5 shrimps). ***6)*** Solitary bees (*Eucera*) seem to use also numerical information to adjust their foraging departure strategy, avoiding returns to nectaries already visited. ***7)*** When an ant (*Formica polyctena*) returns to the nest, its antennal contact with the nestmates lasts longer if the ant has passed a high number of branches on the way back. ***8)*** Honeybees (*Apis mellifera*) use numerical information during navigation, process the zero quantity, perform simple arithmetic operations, and transfer a particular learning across dimensions (i.e., from number to space). Adapted from *Bortot, M., Regolin, L., & Vallortigara, G. (2021). A sense of number in invertebrates. Biochemical and Biophysical Research Communications, 564, 37-42.* Journal Rights are reported in Appendix A.1.

Numerical quantities have an important adaptive value for all animals, likewise, several species of invertebrates may rely on a primitive ability to approximate object's number to make more sensible choices. For example, mealworm beetles might use numerical information during mating to choose the substrate bearing odors of the larger number of females (Carazo et al., 2012, 2009). Honeybees and ants use proto-counting during navigation (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008; Reznikova and Ryabko, 1996; Wittlinger et al., 2006). Bumblebees and solitary bees, spiders and ladybirds seem to be able to also consider the additional information concerning the number of competitors or the number of food sources during foraging, egg laying, and hunting activities (Bar-Shai et al., 2011a, 2011b; Hemptinne et al., 1992; Nelson and Jackson, 2012; Rodríguez et al., 2015). Last but not least, ants are more likely to increase their aggressive behavior if they are part of a large group, rather than when they are alone (Tanner, 2006). The numerical abilities of invertebrates have been assessed using a variety of methods, including spontaneous preferences and associative learning. For instance, cuttlefish show a spontaneous preference for the larger food amount when presented with 1 *vs.* 2, 2 *vs.* 3, 3 *vs.* 4, and 4 *vs.* 5 shrimps in a two-alternative choice (Yang and Chiao, 2016). The same preference was also observed when cuttlefish are presented with numerical fractions, i.e., non-integer numbers such as 1 *vs.* 1.5, 1.5 *vs.* 2, and 2 *vs.* 2.5 represented by shrimps (Huang et al., 2019).

Among insects, foraging honeybees showed a preference for the visual array containing a larger quantity of items. Free-flying foragers underwent a priming phase where a single yellow disk associated with a drop of sucrose solution was presented. This step allowed the simple association between stimuli (i.e., the yellow disk and food) without providing any numerical information. During the probe test phase, bees were presented with thirteen different novel numerical comparisons ranging from 0.08 to 0.8 ratio difference (i.e., 1 *vs.* 2, 1 *vs.* 3, 1 *vs.* 4, 1 *vs.* 12, 2 *vs.* 3, 2 *vs.* 4, 3 *vs.* 4, 4 *vs.* 5, 4 *vs.* 6, 4 *vs.* 7, 4 *vs.* 8, 4 *vs.* 12, 4 *vs.* 20). Honeybees showed a spontaneous preference for the larger number of elements when the smaller numerosity was “1” and the magnitude difference between quantities was sufficiently high (e.g., 1 *vs.* 12, 1 *vs.* 4 and 1 *vs.* 3; (Howard et al., 2020). The authors further controlled for the role of color preferences and priming effect on the spontaneous preference of quantities of artificial flowers in a subsequent series of control experiments. The results showed the important role of priming (i.e., learning the association between a yellow disk with the reward) to let these numerical preferences emerge (Howard et al., 2020).

Insects, in particular, proved to have impressive learning abilities and to be able to successfully learn to associate numerical quantities to appetitive rewards (Gross et al., 2009; Leppik, 1953). For instance, honeybees are shown to be able to successfully choose the correct numerosity in delayed match-to-sample tasks, even when perceptual features as the overall contour length and area of the stimuli were equated between arrays (Gross et al., 2009). In another study, MaBouDi and colleagues (2020) trained four groups of bumblebees to choose either the smaller or the larger quantity in a 1 *vs.* 3 or 2 *vs.* 4 contrast (i.e., yellow dots or stars). Bees underwent unrewarded (extinction) test trials to assess their ability to transfer the numerical learning to novel shapes (e.g., cross), colours (e.g., purple), and numerical contrasts (i.e., bees trained to discriminate 1 *vs.* 3 elements were tested with 2 *vs.* 4 numerical contrast and vice versa). In order to succeed in the test phase, bees would have needed to apply a relative rule during the training phase (i.e., choose the smaller/larger numerosity based on the relationship with the counterpart and irrespective of the distinct number of elements in the array). The results showed that bees were selectively able to rely on the relative numerical information to solve the discrimination when the elements had different visual properties (shape and colour). Moreover, bees transferred the numerical relations “smaller than” and “larger than” learned during the training to novel numerical comparisons (e.g., bees trained to choose 1 over 3 items, were more likely to select 2 over 4 items in the test phase; MaBouDi et al., 2020), again suggesting the use of a general relative numerical rule. Interestingly, a subsequent analysis of their choice and scanning behavior suggested that bees were not relying on low-level visual cues (e.g., overall area, illusory contours, spatial frequency, convex hull, perimeter) to solve the task. Conversely, in another experiment honeybees trained with small numerical contrasts controlled for visual perceptual variables (i.e., up to 4 items) appeared to spontaneously rely on an absolute numerical value (i.e., selecting the array containing a specific number of items) over a more general relative numerical value (i.e., selecting the smaller/larger array regardless of the number of items contained; Bortot et al., 2019).

Insects are also able to deal with ordinal (other than cardinal) aspect of numerosity. Chittka and collaborators (1995) trained honeybee foragers to collect food from a feeder placed between the third and the fourth of a line of identical tents. To test their ability to “count” the number of landmarks *en route*, at test the distance of the tents was manipulated, creating a contrast between number of landmarks and flying distance. A significant portion of honeybees (22%) landed on the feeder that was placed between the third and the fourth tent, showing to be able to take into account the ordered number of landmarks to locate the food source (Chittka and Geiger, 1995). Similar results were obtained in experiments in which bees were trained to fly into a tunnel and pass a fixed number of landmarks (i.e., stripes, yellow stars, or baffles spaced at regular interval) to find a feeder. Irrespective of the shape, color, and absolute position of the landmarks, honeybees searched for food at the correct position, between the third and fourth landmarks (Dacke and Srinivasan, 2008). Ants (*Camponotus aethiopis*) tested with a similar paradigm, kept track of the relative position of encountered landmarks to find a food source. By randomly changing the spatial disposition of five landmarks the authors showed that ants did not rely on spatial cues (e.g., distance travelled from the starting point) or memory trace to search near the correct trained landmark (d'Ettorre et al., 2021).

Using an appetitive-aversive conditioning paradigm (i.e., correct stimulus associated with positive reward and incorrect stimulus associated with punishment), honeybees were able to correctly perform simple arithmetic operations in a delayed match-to-sample task (i.e., add/subtract one element from a numerical sample). Bees were presented in a Y-maze with a coloured sample number. If the sample was blue (i.e., showing either 1, 2, or 4 blue elements), bees had to choose the one-element larger array in the subsequent numerical comparison in order to be rewarded (i.e., to choose 2, 3 or 5 blue elements as correct). Conversely, if the sample was yellow (i.e., showing either 2, 4 or 5 elements), the bees had to choose the one-element smaller array in the subsequent comparison (i.e., choose 1, 3 or 4 yellow elements as correct). At test, bees were presented with a novel sample number (i.e., 3) whose colour determined the correct choice of the smaller (yellow) or larger number (blue) in the next comparison. To exclude the use of alternative strategies not implicating the formation of arithmetic rules (i.e., choose the smaller/larger option in the comparison, irrespective of the value of the sample), bees were subjected to four different transfer tests. Two of these tests presented the correct option as following the correct numerical direction (e.g., subtraction: sample is 3, the correct option is 2 compared to 4; addition: sample is 3, the correct option is 4 compared to 2), while the other two presented the correct option as following the incorrect numerical direction (e.g., subtraction: sample is 3, the correct option is 2 compared to 1; addition: sample is 3, the correct option is 4 compared to 5). Analyses revealed that bees were able to transfer the arithmetic rule (blue = add, yellow = subtract) to novel numbers without solving the task by simply using a relative rule "choose the smaller/larger option in the comparison", with a percentage of correct choices ranging from 66.4% to 72.1% in addition test, and from 63.6% to 67.9% in subtraction test. These results suggest an ability to use symbolic cues to solve basic arithmetic operations with small numbers in honeybees (up to 5 units; Howard et al., 2019a).

Another important building block of numerical cognition is the ability to understand the concept of zero as a numerosity (i.e., as an "empty" set). Until recently, this ability was thought to be a prerogative of primates such as rhesus monkeys (Merritt et al., 2009; Ramirez-Cardenas et al., 2016), vervet monkeys (Tsutsumi et al., 2011), chimpanzees (Biro and Matsuzawa, 2001) or of bird species such as the African grey parrot (Pepperberg and Gordon, 2005). Honeybees and ants also showed the capacity to process a non-symbolic "concept" of zero numerosity by correctly placing a blank stimulus at the beginning of the numerical continuum (Cammaerts and Cammaerts, 2019; Howard et al., 2018). Bees were divided into two groups and trained to choose either the smaller or the larger of two numerosities (i.e., from 1 to 4 units) across trials. At test, they were presented with a novel comparison between an empty set (i.e., containing no elements) and a novel array containing some items (i.e., from 1 to 4 items). Honeybees previously trained to apply the "smaller than" rule, chose the empty set (64% of correct choices), considered as lower than the other numerosity, while the "larger than" group chose the novel set containing the other elements (74.5% of correct choices). Bees trained to choose the *less* *than* numerical value, preferred the empty set even when compared with the one element with 63% of accuracy. Besides, the zero appeared as placed along a positive numerical continuum because the discrimination accuracy of bees was higher for 0 *vs.* 5 (71.5%) and 0 *vs.* 6 (75.5%) discriminations and lower for 0 *vs.* 1 (58%) or 0 *vs.* 2 (61.5%) discriminations, showing the typical *numerical distance* effect (Howard et al., 2018). Despite the interesting results, it might be argued that the authors lacked in proving a clear evidence of the formation of a true mental concept of zero as numerosity (i.e., zero defined as a numerical quantity, having a null value). As different perceptual variables were still available during the training procedure (e.g., spatial frequency, convex hull), bees could have solved the task by relying on a more general *sense of magnitude* instead of a more precised *sense of number* (MaBouDi et al., 2021). Moreover, a simple neural network with seven sensory neurons encoding spatial frequency projecting to a single decision neuron proved to be able to successfully mimic bee choice in Howard et al. 2018, successfully categorizing a blank stimulus as occupying the lower position in the positive numerical continuum (MaBouDi et al., 2021).

Bees are not the only insects showing numerical abilities (Pahl et al., 2013). Desert ants (*Cataglyphis fortis*) might use a piece of relatively precise numerical information to keep track of the distance covered during navigation activity. Wittlinger and collaborators (2006) hypothesized the existence of a "*step counter*" that allows the measurement of the distance intercourse from the nest to a food site. To test this hypothesis, the leg's length of the ants was manipulated by either increasing or decreasing it (i.e., adding stilts or creating stumps), resulting in longer or shorter steps made by walking ants. When released from the food site, ants with longer legs searched for the nest after having walked a longer distance, while shortened-leg ants searched for the nest at a shorter distance. The authors suggested the existence of a pedometer for distance computation, cautionary hypothesizing it to be some sort of "*step counter*". However, the data presented did not fully support the idea of ants truly counting the exact number of steps (see also Franks et al., 2006) but rather of them being equipped with a proprioreceptor encoding the number of strides made to calculate the itinerary distance (Wittlinger et al., 2006). Scout ants (*Formica polyctena*) seem to communicate the numerical information in order to find food placed in a series of identical sites. Forager ants were randomly placed in one of 25-60 equally spaced branches where they could feed. The ants would then return to the nest and make antennal contact with the nestmates. Such contact lasted longer according to the number of branches the ants had passed on the way back to the nest (Reznikova & Ryabko, 2011). However, it is not clearly reported if the inter-branches distance was carefully controlled to rule out the possibility that ants could have communicated the covered distance (expressed either in time spent or number of steps made) between the rewarded branch and the nest, instead of the numerical order of the food source. As explicated by Czaczkes (2022), the above-mentioned claims regarding the possibility that ants are equipped with the ability to communicate the exact number of branches are not fully supported by the data, as ants could have communicated some magnitude information instead.

Insects are not the only arthropods being able to use numbers. Spiders (*Portia africana*) change their predation strategy according to the number of conspecifics at a prey's nest, preferring to settle when the number is one instead of zero, two or three in order to maximize their probability of hunting success (Nelson & Jackson, 2012). The relationship between numerical cues and searching behavior of another species of spiders (*Nephila clavipes*) was also investigated. These spiders were given the possibility to choose between 1, 2 or 4 small preys, or one single large prey. The total area was equated between groups with different numerosity, in order to minimize the influence of confounding variables. Later, the prey items were removed and the searching time of spiders was recorded. The results showed that spiders spent more time searching for food when they had lost the greater number of prey, highlighting the importance of computing numerosity in their food choice (Rodríguez et al., 2015).

While all these examples cautiously suggest the presence of a *sense of number*, they might fall short in providing direct evidence of the capacity of invertebrates to form the concept of a number. This is defined as a mental construct that represent the exact value of a numerosity and its relationship with other numerosities (Dehaene, 2011). Notably, the *number concept* also entails the ability to generalize a response based on the object's numerical property across different perception or action modalities (Dehaene, 2011). Hence, some of the studies examining numerical abilities in invertebrates within this thesis do not clearly establish the presence of a true identification and representation of numerical values in these species. Rather, they suggest the presence of a *number perception* and *elementary mental computation* allowing to order numerical quantities in ascending/descending order (Butterworth et al., 2018). Since the majority of those studies have focused on investigating numerical discrimination abilities within the small number domain (i.e., up to 4 units; Bortot et al., 2019 for an example) or lacked proper controls over other perceptual features possibly guiding the animal's behavior (e.g., odor bouquet variability in Carazo et al., 2012), assertions regarding the presence of a *true number concept* in invertebrate species might be somewhat overstated. Claims about the ability of a species to form an abstract concept of numbers should be supported by careful methodological controls, aimed at rigorously exclude alternative hypotheses. Therefore, the objective of this review introduction was to present the most recent research on numerical abilities in invertebrates with a critical perspective, while still acknowledging the scientific attempts to provide a clearer and more solid framework about numerical abilities in invertebrates.

1.2.2 Magnitude processing in honeybees: number, size, and time

As previously reported, the non-symbolic and non-verbal numerical abilities of animals are thought to be part of a more general cognitive mechanism that allow the process of different magnitudes in a similar fashion (Gallistel, 1989; Walsh, 2003). This mechanism would have an important adaptive value, allowing the animal to perform simultaneous computations of different dimensions to make relevant decisions (Gallistel, 1989; Walsh, 2003).

As Gallistel suggested:

> *"Given the evidence that animal brains perform operations isomorphic to addition, subtraction, and division with the representatives of temporal intervals and the evidence that they have a closely related system for representing numerosity, it may be reasonably be conjectured that their well-documented ability to match the allocation of their foraging behavior to the relative rates of prey occurrence depends upon a representation of rate obtained by dividing representatives of numerosities by representatives of temporal intervals."* (Gallistel, 1989)

Humans and other vertebrate species process different dimensions in a similar manner (e.g., space, time, length, number) at both behavioral (De Corte et al., 2017; Lourenco and Longo, 2010; Meck and Church, 1983; Merritt et al., 2010; Miletto Petrazzini and Brennan, 2020; Roberts and Mitchell, 1994) and neural level (Walsh, 2003) providing evidence of this magnitude mechanism being widespread among the vertebrates subphylum. In invertebrate species, evidence about the presence of the capacity to represent different dimensions in a single currency has not been provided. Yet, such indication would be essential to give new insights into the universality of this mechanism, as well as to allow speculation on the necessary and sufficient neural substrates to support it.

Honeybees offer a unique opportunity to try to fill this gap, as they are capable of processing different continuous and discrete dimensions separately (Bortot et al., 2021). As briefly described above, bees encode and use the numerical information of visual arrays to solve a delayed-matching-to-sample task (i.e., bees matched a sample stimulus with a subsequent stimulus presented in a comparison, based on its numerousness; DMTS, Gross et al., 2009). They spontaneously rely on the absolute numerical value of visual arrays (Bortot et al., 2019) and can encode the number of landmarks to find a food source (Chittka and Geiger, 1995) employing this strategy even if the perceptual features of markers change (e.g., shape; Dacke & Srinivasan, 2008). They can place a blank stimulus at the beginning of the numerical continuum (i.e., zero numerosity) and show the distinctive distance effect (Howard et al., 2018), perform addition and subtraction operations (Howard et al., 2019a), and categorize non-symbolic numerousness as odd or even (Howard et al., 2022). In a recent study, Howard and colleagues investigated the ability of bees to build a symbolic numerical representation. Free-flying foragers were trained to associate a specific symbol to a specific numerousness with a unidirectional DMTS: one group of bees saw the symbol sample and had to choose the correct numerosity in a subsequent comparison; the other group of subjects saw the numerosity sample and had to choose the corresponding symbol in a subsequent comparison. Despite being able to learn the symbol-numerousness association even if the perceptual features of the latter (e.g., elements' color, shape) varied, bees failed when their ability to reverse the task was tested (e.g., bees previously presented with a symbol and trained to select the corresponding numerosity, they were presented with the familiar numerosity as a sample and asked to choose the corresponding symbol) suggesting an inability to form a true symbolic representation of numerosity (Howard et al., 2019b).

Investigation of the cognitive abilities of honeybees led to the implementation of experiments to study their processing of continuous dimensions of objects, such as size or duration. Avarguès-Weber et al. (2014) investigated the ability of foragers to solve visual discrimination between objects having different relative sizes (i.e., 6 stimuli ranging from 1x1 cm to 6x6 cm side length). To get a food reward, two groups of subjects were independently trained to approach the relatively smaller or larger element (i.e., square or diamond shape), respectively. During the training, each stimulus size could be either reinforced or punished, depending on the alternative option presented (except 1x1 cm size and 6x6 cm size stimuli that were always reinforced for the group trained to the smaller and larger elements, respectively). To solve this task bees could have implemented two different strategies: either categorize the stimuli based on a "*larger than*" or "*smaller than*" conceptual strategy or learn all the specific size comparisons. To test this, bees were presented with a comparison between familiar shapes having novel sizes (i.e., 2.5 x 2.5 *vs.* 4.5 x 4.5) and novel elements with unfamiliar shapes (i.e., 5 points star), colour (i.e., either blue or violet), and size (i.e., 2.5 x 2.5 *vs.* 4.5 x 4.5 cm; Avarguès-Weber et al., 2014). Results supported the use of the cognitively less expensive strategy (Avarguès Weber et al., 2012) based on the abstract categorization of "*larger*" and "*smaller*" elements (Avarguès-Weber et al., 2014).

In the reported study, bees were asked to make a comparison between simultaneously presented sizes, thus not overloading their working memory. However, in an ecological foraging situation, the probability of encountering flowers in a sequential fashion is higher (Howard et al., 2017; but see also Dyer and Chittka, 2004; Dyer and Neumeyer, 2005). In a subsequent experiment, Howard and colleagues examined the capacity of free-flying foragers to make size comparisons of visual stimuli presented in sequence. To this purpose, they trained bees to reach a cube-shaped apparatus that could display 4 elements located in the four facets, thus forcing bees to sequentially explore spatially separated stimuli before making the selection. During training, honeybees were presented with a comparison between square or diamond shape elements having from 3 to 6 cm side length (i.e., 3 x 3 cm, 4 x 4 cm, 5 x 5 cm, 6 x 6 cm); half of the subjects had to choose the relatively smaller size, whereas the other half had to select the relatively larger size to access a food reward. Bees were then tested for their ability to transfer the acquired abstract rule (i.e., choose the larger or smaller element), to two novel comparisons involving novel element dimensions (i.e., 1 x 1 cm *vs.* 2 x 2 cm; 7 x 7 cm *vs.* 8 x 8 cm). Interestingly, bees succeed in the task by correctly transferring the acquired rule to novel stimuli dimensions, presented in a mimicking foraging situation (Howard et al., 2017).

Time, together with size and number among others, is another *prothetic* dimension likely supported by a common metric (Walsh, 2003). In vertebrate species, an interaction between temporal and other continuous and discrete dimensions has been demonstrated (De Corte et al., 2017; Lourenco and Longo, 2010; Meck and Church, 1983; Merritt et al., 2010; Roberts and Mitchell, 1994). Previous studies have tried to establish whether honeybees compute temporal cues without however reaching a unanimous consensus (Ng et al., 2021). Honeybees behave under the influence of circadian rhythms, although age-dependent differences are present (Eban-Rothschild and Bloch, 2008). They learn to visit different food sources depending on the time of the day, irrespective of their location, suggesting an encoding of the temporal feature according to an episodic-like memory (renamed *circadian timed episodic-like memory*; Pahl et al., 2007). In foraging situations, encoding temporal properties might ensure a more efficient behavior (Ng et al., 2020) as the nectar rate of flowers might vary according to a specific moment of the day (Pahl et al., 2007). However, attempts to highlight the capacity to encode fixed temporal duration in laboratory conditions were unsuccessful. In 2020, Ng and colleagues investigated the ability of honeybees to use either the temporal duration or colour of light to correctly solve subsequent shape discrimination. Specifically, bees could experience two different context-setting cues – blue/yellow light, or short/long white light duration (i.e., 1 second or 3 seconds) – anticipating the correct visual stimulus to choose. Results provided evidence of the ability of bees to use the colour cue to subsequently select the correct shape. In contrast, honeybees exposed to light duration did not learn the time-shape association, confirming the apparent inability of bees to use temporal timing information as an effective cue in decision-making (Ng et al., 2020). Despite those results, the importance of temporal information in bees' foraging (Ng et al., 2021) but also communication contexts (von Frisch, 1967) requires further controlled investigation of the presence of the ability to process temporal duration in this species.

In the first part of this thesis, I will present evidence for the existence of a bidirectional cross-transfer between a continuous (size) and a discrete (number) dimension. In Chapter 2, I will present the results of an investigation of the ability of bees to transfer a rule acquired in the numerical domain to the size domain. Free-flying honeybees had to solve a numerical discrimination task by either selecting the smaller or the larger numerical quantity. Subsequently, they were tested for spontaneous generalization over a different size dimension. The results revealed a transfer across dimensions, as bees spontaneously selected the congruent size with respect to their training (i.e., bees previously trained to choose the larger numerousness, selected the larger size, and vice versa).

In Chapter 3, I will present an investigation of bees' ability to make a similar transfer from the size to the numerical dimensions. Free-flying foragers were trained to discriminate between three relatively larger and smaller elements in terms of size. Bees then spontaneously selected the congruent numerosity based on their previous training, when faced with a 4 *vs.* 8 element discrimination (i.e., bees previously trained to select the larger size, they chose the larger numerosity, and vice versa). The role of several perceptual variables, such as area, contour length, and spatial frequency in bees' decision-making, is considered and discussed.

Overall, these results suggest the presence of a general cognitive mechanism for magnitude processing – thought to be a prerogative of vertebrates – also in an insect species, giving new insights into its convergent adaptation and universal value.

1.3 Categorization: the ability to extrapolate regularities

Animals live in a highly complex environment that demands rapid and efficient learning to respond to different stimuli. A strategy where all possible stimulus occurrences are stored in the animal's memory would be quite inefficient (Pahl et al., 2010). Categorization, namely the creation of classes of objects based on perceptual or abstract invariants, offers a more effective approach to deal with external stimuli, requiring less memory capacity, and minimizing learning costs (Zayan and Vauclair, 1998). Thus, the hypothesis that small-brained animals, such as honeybees, possess categorization abilities seems highly plausible (Avarguès-Weber and Giurfa, 2013).

Starting from the late 1990s, a growing amount of evidence focused on the mechanism underlying the ability of bees to discriminate visual objects, reporting categorization strategies in this insect species (Avarguès Weber et al., 2012). Giurfa et al. (1996) trained honeybees to discriminate perceptually distinct visual stimuli based on bilateral symmetry/asymmetry. These properties were the sole consistent invariants that could allow bees to classify various stimuli as symmetrical or asymmetrical. When presented with novel visual configurations, honeybees correctly generalize their response, as those previously trained to select the symmetrical/asymmetrical objects, chose the novel symmetrical/asymmetrical ones. Notably, other perceptual cues, such as area, contour length, density of contours (i.e., area to perimeter ratio), visual angle, spatial frequency, and orientation power, did not influence bees' choice (Giurfa et al., 1996). Furthermore, bees demonstrated the ability to successfully allocate novel and complex visual stimuli to the correct perceptual category (i.e., plant stem, landscape, star-shaped and circular flowers; Zhang et al., 2004). Bees are also able to form a category based on the global configuration of an array of elements. This means that they can perceive and categorize visual stimuli not just based on individual features but by considering the relationship between them, forming a whole object (Avarguès-Weber et al., 2010). Specifically, honeybees were presented with a comparison between visual configurations resembling faces (i.e., comprising two circular top elements, one vertical middle element, and one horizontal bottom element) and non-face-like configurations (i.e., array containing the same elements – two circular, one vertical, one horizontal – but randomly arranged). Bees were able to categorize novel stimuli as belonging to one of the two categories (i.e., face-like or non-face-like), indicating the capacity to recognize complex visual configurations, form the relative category, and correctly generalize their response towards unfamiliar stimuli. Importantly, authors excluded the possibility that these results were influenced by low-level visual features (e.g., spatial frequency, symmetry, center of gravity, visual angle; Avarguès-Weber et al., 2010). These studies provided interesting evidence about bees' capacity to categorize both single visual objects and the configuration of elements based on their physical features. The decision to classify a particular stimulus as part of a category is based on the perceptual similarity between this and other objects forming such a category (Avarguès-Weber et al., 2010). In simpler terms, if a category is defined by a certain attribute (e.g., bilateral symmetry), the novel object encountered is defined as belonging to that category only if it possesses such an attribute, regardless of other features (e.g., colour) (Avarguès-Weber and Giurfa, 2013).

A more sophisticated categorization involves inferring the abstract relationship relating different objects. Such a category would thus be based on abstract rules connecting such items having no perceptual similarity (Avarguès-Weber et al., 2010; Avarguès-Weber and Giurfa, 2013). The ability to form *relational* (or *abstract*) concepts allowing a similar behavioral response towards different stimuli has been precisely investigated in honeybees (see Avarguès-Weber and Giurfa, 2013 for a review). In 2001, a first study by Giurfa et al. presented evidence of the capacity of bees to correctly respond to different objects based on an abstract rule of *sameness* and *difference*. In a series of experiments, the authors trained honeybees to select the stimulus with the same perceptual features (i.e., colour, orientation, odour) as a previously presented sample (delay matching to sample task, DMTS). Irrespective of the perceptual characteristic of the visual sample, bees chose the subsequent identical visual stimulus with an accuracy ranging from 65.3% to 76.3% (e.g., bees presented with a blue sample, chose a blue target over a yellow one). However, bees could have solved this task via simpler associative learning and memorization of the correct outcome depending on the sample (e.g., choose a blue target if the sample is blue), without implying a higher level of abstraction (i.e., choose the stimulus that is the same as the sample, irrespective of the appearances of the sample). To test for that eventuality, bees were subsequently presented with a novel discrimination of stimuli having a different appearance (e.g., bees that were trained to follow the *same* rule with colors, were presented with horizontal and vertical gratings, and vice versa). Here bees showed the capacity to select the subsequent stimulus corresponding to the novel sample based on an abstract *sameness* rule. Interestingly, the formation of this abstract concept was evident irrespective of the perceptual features or sensory modality of the stimuli presented (i.e., bees trained to select the correct odor based on this principle, also selected the correct color when presented with coloured visual targets). Interestingly, honeybees trained in a *delay non-matching to sample* paradigm (i.e., required to choose the stimulus different from the sample; e.g. if a blue target was presented as the sample, bees had to choose a yellow target over a blue one), successfully solved and generalized the task by spontaneously applying a relational abstract rule of *difference*, without being explicitly trained to do so (Giurfa et al., 2001). Similarly, bees presented with three objects (two identical and one different), could be trained to follow an oddity (i.e., select the different stimulus) and non-oddity (i.e., select one of the two similar stimuli) abstract rule, regardless of the physical features of stimuli (i.e., pattern and modality presentation: three-dimensional or two-dimensional objects presented via computer monitor) (Muszynski and Couvillon, 2015; but see also Giurfa, 2021).

Further studies confirmed the capacity of the bee's brain to extrapolate abstract invariant rules to categorize different objects. Bees were presented with a visual stimulus having a strict spatial relationship with a referent object (e.g., a horizontal line). Following an appetitive-aversive conditioning procedure, half of the subjects had to select the *above* relation (i.e., target above the referent line), whereas the other half had to select the stimulus-referent configuration following a *below* rule (i.e., target below the referent line). Bees successfully categorize subsequent novel perceptual stimuli, depending on their abstract spatial relationship with the target line. Careful control experiments demonstrated no role of other simpler perceptual invariants as the center of gravity or the probability of visual stimulation in the upper (for the *above* condition) or lower (for the *below* condition) part of the visual field. Moreover, when bees were presented with a familiar object without the reference target, bees' performance dropped at the chance level, confirming the formation of the abstract spatial *above*/*below* rule (Avarguès-Weber et al., 2011). Similarly, in a subsequent study, bees showed the ability to spontaneously categorize visual stimuli based also on their *left/right* relationship, irrespective of their physical appearance (Avargues̀-Weber et al., 2012). Notably, a recent study unveiled the strategy used by bees to solve the *above/below* conceptual task, implying simpler and more basic perceptual mechanisms (Guiraud et al., 2018; see *General conclusion* section for further comments).

Honeybees forage in an environment where the perceptual characteristics of encountered objects may vary throughout their life. It is thus essential to have the ability to extrapolate physical or abstract regularities to generalize the correct response learned with some configurations, in novel circumstances. Identifying relation and spatial invariants might be one of the core mechanisms at the disposal of such reduced brains to better navigate in the surroundings and deal with changes in foraging conditions (Avargueѐs-Weber et al., 2012; Avarguès-Weber et al., 2011; Avarguès-Weber and Giurfa, 2013; Giurfa et al., 2001).

In the fourth chapter of this thesis, I will present an investigation of the ability of bees to extrapolate the structure of a temporally determined odor sequence. In a series of six different experiments where different conditioning paradigms have been used (i.e., absolute, differential, generalization), the presence of this ability at a spontaneous or acquired level has been investigated. Specifically, bees' ability to learn and generalize a sequence structure over different composing elements (i.e., single odors composing the sequence) might appear only if training explicitly exploit a generalization strategy. Alternative shortcuts used by bees to solve the task became evident when absolute and differential paradigms were used. Overall, these results provide a first attempt to study the ability of bees to acquire and categorize olfactory stimuli based on their temporal structure.

1.4 Neural basis of honeybees' cognitive abilities

The growing fascination and interest in honeybee cognition are also due to the presence of a minuscule - but very efficient - brain composed of 960 000 neurons tightly packed in 1 mm^3, supporting a collection of both fixed and adaptable behaviors (Giurfa, 2003a). Despite its dimensions, the bee brain is a highly specialized organ, subdivided into specific regions (i.e., neuropiles) dedicated to the processing of visual, olfactory, mechanosensory stimuli (e.g., optic and antennal lobes) and their integration (i.e., mushroom bodies; Giurfa, 2003a)

Among the different neuropiles, the antennal lobes (AL) are the first center for the encoding of olfactory information. Each antennal lobe is composed of ≈ 160 000 glomeruli (i.e., clusters of neurons) whose activation is highly dependent on odor chemical structure (Joerges et al., 1997). A specific odor activates a specific glomerular spatial pattern, without any differences between individuals, indicating a species-specific neural specialization in odor encoding (Giurfa, 2003b). Interestingly, an associative learning experience modifies the strength of the activation pattern for the rewarded odor, without altering its spatial feature (Faber et al., 1999). Honeybees' antennal lobes provide the first neural substrate for the formation of olfactory elementary association (Giurfa, 2003b; Menzel and Giurfa, 2001).

However, bees not only form simpler associations between a given odor and a reward, but they are capable of more complex configural and contextual learning (Avarguès Weber et al., 2012; Avarguès-Weber and Giurfa, 2013; Giurfa, 2013, 2003b). This type of learning acquisition implies the integration of multiple sensory inputs to a unique multimodal output regulating the behavior (Giurfa, 2013). In the bee's brain, the regions thought to support such capacity are the mushroom bodies (MBs) (Menzel, 2012). These structures are defined as high-order integration centers, composed of ≈ 170 000 Kenyon cells (Giurfa, 2003b). The MBs receive separated sensory information from the first sensory centers (e.g., antennal, optic lobes), integrate them, and form a non-segregated output (Giurfa, 2013, 2003a). The development of these structures seems to go *pari passu* with the acquired life experience (Farris et al., 2001; and see Menzel, 2014) but also to have an innate component as an increase in their volume has been measured in non-stimulated (i.e., reared in darkness, social isolated) 7-days-old bees (Fahrbach et al., 1998). Interestingly, despite receiving input from the olfactory centers (AL) and the reward pathway (i.e., ventral unpaired median neuron of the maxillary neuromere 1, VUMmx1; Hammer, 1993) mushroom bodies are not necessary for elementary learning to be established (Giurfa, 2003b). Using hydroxyurea (an inhibitor of DNA synthesis), a partial or total unilateral or bilateral lesion of the mushroom bodies can be obtained, without damaging other neuropiles (Malun et al., 2002b). Ablated foragers presented with a differential PER conditioning requiring learning an elemental discrimination between a rewarded odor over a non-rewarded one, showed a similar learning rate as non-ablated animals. Interestingly, ablated bees also showed a similar performance when their retention ability was tested one hour and one day after training, suggesting that the absence of functionally intact mushroom bodies does not prevent the acquisition and formation of an elemental olfactory memory in honeybees (Malun et al., 2002a). Such evidence highlights the peculiar characterization of the bee brain, with a multiple-level specialization to adaptively respond to environmental stimulation. Bees are endowed with neural circuits allowing both elementary but also more complex learning, where integration of information coming from different sensory modalities allows them to solve harder tasks, such as abstract visual regularities and computing the numerical property of an array (see Avarguès-Weber and Giurfa, 2013 for a review).

1.5 Aim of the thesis

The present work aimed to investigate the numerical and abstraction abilities of honeybees. In the second and third chapters, I will detail the investigation of the ability of bees to equally process different magnitudes (i.e., number and size), by using operant conditioning towards visual stimuli. In these two independent studies, I explored the possibility that bees could make a cross-dimensional transfer of a specific rule (i.e., choose the smaller, choose the larger stimulus) between these dimensions. The overarching aim was to provide evidence of the existence, or lack thereof, of a general cognitive mechanism – only described for vertebrates until then - allowing animals to process different continuous and discrete quantities in a similar fashion, in an invertebrate species. In the fourth chapter, I present a study on the investigation of the ability of bees to extrapolate an odor sequence structure, temporally determined. This study further examines the abstraction and generalization abilities of honeybees. Overall, the present work aspires to significantly impact our understanding of the presence of general cognitive mechanisms in an invertebrate species. This will allow a better understanding of their evolution, confirm their high adaptive value, and open new frontiers in their investigation from a neurobiological standpoint. Ultimately, studying small-brained creatures would enhance our knowledge about their capacities and strategies for thriving in their environment.

CHAPTER 2

TRANSFER FROM NUMBER TO SIZE REVEALS ABSTRACT CODING OF MAGNITUDE IN HONEYBEES

This chapter contains a modified version of the following scientific article: *Bortot, M., Stancher, G., & Vallortigara, G. (2020). Transfer from number to size reveals abstract coding of magnitude in honeybees. Iscience, 23(5).* The Journal Author Rights are reported in Appendix A.2. The following materials were used: introduction, methods, results, discussion, figures, and supplementary materials.

2.1 Introduction

Honeybees (*Apis mellifera*) have been shown to be able to process the numerical attributes of visual stimuli (Bortot et al., 2019; Chittka and Geiger, 1995; Dacke and Srinivasan, 2008; Howard et al., 2019a), including the zero as a quantity (Howard et al., 2018). Given their distant phylogenetic origins, it is unclear, however, to what extent bees (invertebrates) and vertebrates share similarities in number cognition.

Humans and others non-human vertebrates make use of a nonverbal, nonsymbolic representation of number, the so-called Approximate Number System (ANS). The ANS obeys Weber’s law – it is thus mainly limited by the ratio between the numerical values being compared - and is thought to be supported by an evolutionarily ancient mechanism for representing quantity in an analog fashion. Gallistel (1989) first argued that discrete countable quantity (i.e., number) and continuous quantity (e.g., space and time) must be represented by a common mental currency to enable animals to perform arithmetic operations across domains (as in the case of the rate of return to a food patch, that can be computed only if organisms represent time and number in a single currency). According to this hypothesis, quantity representations in the various domains, (i.e., number, space, and time), would be processed by a «common magnitude system», which represents these dimensions via the same unit of magnitude (Gallistel, 1989). Evidence that the temporal, spatial, and numerical features of a stimulus can interact with one another has been provided for vertebrates such as monkeys (Merritt et al., 2010) and birds (De Corte et al., 2017), and for prelinguistic human babies (Di Giorgio et al., 2019).

Interestingly, honeybees have been shown to exhibit the numerical distance effect (i.e., the fact that the ability to discriminate between numbers improves as the numerical distance increases (e.g., zero *vs.* four is easier than zero *vs.* one; Howard et al., 2018). The numerical distance effect is one of the signatures of the ANS and suggests the existence of an analog magnitude system in honeybees that would allow the processing of different numbers. Moreover, honeybees have been shown to be able to process the relative size of visual stimuli (Avarguès-Weber et al., 2014), but it is not known whether in bees a common set of coding mechanisms underlies quantity estimation in different domains.

Here we investigated whether honeybees could make a transfer from discrete (number) to continuous (size) magnitudes. Bees were trained to discriminate between different numerical comparisons having either a 0.5 ratio (2 *vs.* 4 and 4 *vs.* 8) or 0.67 ratio (2 *vs.* 3 and 4 *vs.* 6). Half of the subjects learnt to choose the smaller quantity, and the other half the larger quantity. Then at test, bees were presented with stimuli of different size but identical numerosity under extinction condition (i.e., in the absence of reward). If bees possess a common mechanism to process different magnitudes, then animals trained to choose the smaller/larger quantity in the number comparisons were expected to choose the congruent smaller/larger size in the size comparison. Moreover, choice of the congruent size would not be affected by the ratio of the stimuli (i.e., ratios that proved to be discriminable for numbers should prove discriminable for sizes as well).

2.2 General Methods

Experiments were performed during the Summer 2019 at SperimentArea, a field station run by the local Natural History Museum, in Rovereto (North of Italy).

2.2.1 Subjects

Thirty-two free-flying honeybees (*Apis mellifera*) were trained singly to fly into a wooden Y-maze (Fig. 4c). This sample size is common in experiments on free-flying honeybees' visual learning abilities (Bortot et al., 2019; Howard et al., 2018; Howard et al., 2019a), due to the nature of the free-flight condition that does not allow control by the experimenter on the decision of the single bee to come back freely to the apparatus. One half of the bees were trained with a 0.5 ratio (N=16) and the other half with a 0.67 ratio (N=16). In the 0.5 ratio, one half of the subjects were tested with a 2 *vs.* 4 comparison and the other half with a 4 *vs.* 8 comparison; in the 0.67 ratio half of the subjects was tested with a 2 *vs.* 3 comparison, and the other half with a 4 *vs.* 6 comparison.

2.2.2 Apparatus and stimuli

The stimuli consisted of black elements, either squares, diamonds, or dots on a white squared-shape background (8 cm x 8 cm) located at 15 cm distance from the decision chamber (Fig. 4c). The stimuli size ranged from 1.12 cm to 3.56 cm (diameter of dots) and from 1 cm to 2.5 cm (side of squares and diamonds). A total of 30 couples of stimuli were used for each shape (i.e., squares, diamonds, and dots). The spatial disposition and the size of the elements were varied among trials to prevent the use of non-numerical cues. In order to control for the continuous variables that covary with numerosity (e.g., area, contour length, density), we adopted a procedure previously used in other studies on numerical abilities of bees (Bortot et al., 2019). Within each shape, in one quarter of the stimuli (N=7) the cumulative surface area was matched to 100%, whereas in the second quarter (N=8) was not controlled (i.e., the ratio between the cumulative surface area within each pair was congruent with the numerical ratio: 0.5 in 2 *vs.* 4 and 4 *vs.* 8; 0.67 in 2 *vs.* 3 and 4 *vs.* 6). In addition, half of the stimuli was controlled for the convex hull and the other half for the density of the elements. Furthermore, in the third and fourth quarter of the stimuli, the cumulative contour length was matched to 100% (N=8) and not controlled (N=7), respectively, following the same logic. Again, within each shape, half of the stimuli were controlled for the convex hull and the other half for the density of the elements. The control of these variables was performed for each shape (Fig. S3, S4, S5, S6).

During the training phase, half of the bees (N=16) were presented with squares and diamonds, whereas the other half (N=16) was presented with diamonds and dots. Thus, in the training phase bees were presented with 60 couple of stimuli in random order differing in shape, spatial disposition, size of the element, and combinations of controlled continuous variables, and only the numerical information was kept constant. The stimuli used in the *number learning* test were taken from the training sample of stimuli with the area matched to 100%. In the *size transfer* test, stimuli consisted of two pairs of novel shapes (i.e., the shape that was not presented in the training phase) having sizes that differed by a ratio of either 0.5 or 0.67, depending on the numerical training previously completed by each subject. Within each pair, the two arrays had the same number and disposition of elements. In particular, the number of elements presented was equal to the numerosity reinforced during the training phase (e.g., bees trained to select 2 elements over 4 elements during the training phase, were then presented with a 2 *vs*. 2 comparison where one group of 2 elements had the double size of the other group of 2 elements) (Fig. S7).

2.2.3 Training procedure

The experimental procedure comprised a pre-training phase followed by a training and tests phase. All the phases were completed by all subjects in 1 or 2 consecutive days. During the pre-training phase, each bee was individually habituated to fly inside the apparatus and to collect food by landing on two grey poles placed in both arms, in the absence of visual stimuli. In the training phase, four different numerical comparisons (ratio 0.5: 2 *vs.* 4, 4 *vs.* 8; ratio 0.67: 2 *vs.* 3, 4 *vs.* 6) were presented to each independent group, separately. Within each group, half of the subjects was trained to select the smaller numerosity in the comparisons (either 2 or 4), whereas the other half was trained to choose the larger numerosity in the comparison (either 3, 4, 6, or 8) in order to get the food reward. During this phase, an appetitive-aversive conditioning paradigm was used: the correct numerosity was always associated with the food (0.88 M of sucrose solution) whereas the incorrect numerosity was always associated with a bitter 60 mM quinine solution, used as punishment (Avarguès-Weber et al., 2010). The use of this appetitive-aversive conditioning has been shown to improve the ability of bees to discriminate between numerosities (Howard et al., 2019c). Each subject had to complete 60 consecutive trials of training. The stimuli were presented in a pseudo-random sequence (i.e., the correct/incorrect stimulus was never presented for more than two consecutive times on the same side).

2.2.4 Test procedure

Once completed the training phase, honeybees started the test phase. During this phase, two non-reinforced tests were presented: a *number learning* test and a *size transfer* test. Each test was presented twice to counterbalance the position of the correct array and avoid side preferences. The tests lasted 1 minute during which the number of choices (i.e., direct contact made with a body part, either the antennae or legs, on one of the two grey poles placed in front of each stimulus) made by the subjects were counted online. In the *number learning* test, bees were presented with the same numerical comparisons and shapes used during the training but in the absence of any reward. In the *size transfer* test bees were exposed to the novel stimuli displaying only the size information (even in this case without any reward).

2.2.5 Statistical analyses

In the test phase, the percentage of choices for the larger numerosity and larger size was calculated for each subject and analyzed, giving rise to one single value per bee to exclude pseudo-replication. The data were checked for normality (Shapiro-Wilk normality test: W

= 0.98, $P > 0.05$) and homoscedasticity (Levene's test: $P > 0.05$) and then analyzed with parametric statistical tests. An analysis of variance was performed with ratio (0.5 and 0.67), type of training (smaller *vs.* larger as positive), and type of test (*number learning* test *vs. size generalization* test) as factors. The effect of the numerical comparisons, as factor nested in ratio, was analyzed with a nested factorial Anova. The omega-squared effect size of any significant results in the Anova analysis was reported. The proportion of choices for the correct numerosity during the *number learning* test and the proportion of choices for the congruent size during the *size generalization* test were calculated for each subject and analyzed with a two-tailed one-sample *t*-test. The Cohen's *d* effect size of any significant result was also reported.

2.3 Results

The results are shown in Fig. 4 (a, b) as proportions of choices for the larger magnitude. An analysis of variance revealed a significant main effect of the type of training (smaller *vs.* larger numerosity as positive; $F_{(1, 48)} = 45.8$, $P < 0.001$, $\omega^2 = 0.399$) but not of the type of test (*number learning* test *vs. size generalization* test; $F_{(1, 48)} = 3.5$, $P = 0.066$) and the ratio (0.5 *vs.* 0.67; $F_{(1, 48)} = 0.3$, $P > 0.05$).

No significant interactions were observed (ratio x numerical comparisons: $F_{(2, 48)} = 1.9$, $P > 0.05$; ratio x type of training: $F_{(1, 48)} = 1.7$, $P > 0.05$; ratio x type of test: $F_{(1, 48)} = 0.5$, $P > 0.05$; type of training x type of test: $F_{(1, 48)} = 1.2$, $P > 0.05$; ratio x type of training x type of test: $F_{(1, 48)} = 0.09$, $P > 0.05$; ratio x type of training x numerical comparisons: $F_{(2, 48)} = 1.2$, $P > 0.05$; ratio x type of test x numerical comparisons: $F_{(2, 48)} = 1.9$, $P > 0.05$; ratio x type of training x type of test x numerical comparisons: $F_{(2, 48)} = 0.03$, $P > 0.05$).

As can be seen in Fig. 4, bees trained to select the larger/smaller numerousness (Fig. 4a; choice for correct, either larger or smaller, numerousness: 57.16% ± 1.43, mean% ± SEM%; two-tailed one-sample *t*-test: $t_{(31)} = 5.02$, $P < 0.001$, $d = 0.89$) chose the congruent larger/smaller size (Fig. 4b; choice for congruent, either larger or smaller, size: 55.14% ± 1.21, mean% ± SEM %; two-tailed one-sample *t*-test: $t_{(31)} = 4.26$, $P < 0.001$, $d = 0.75$).

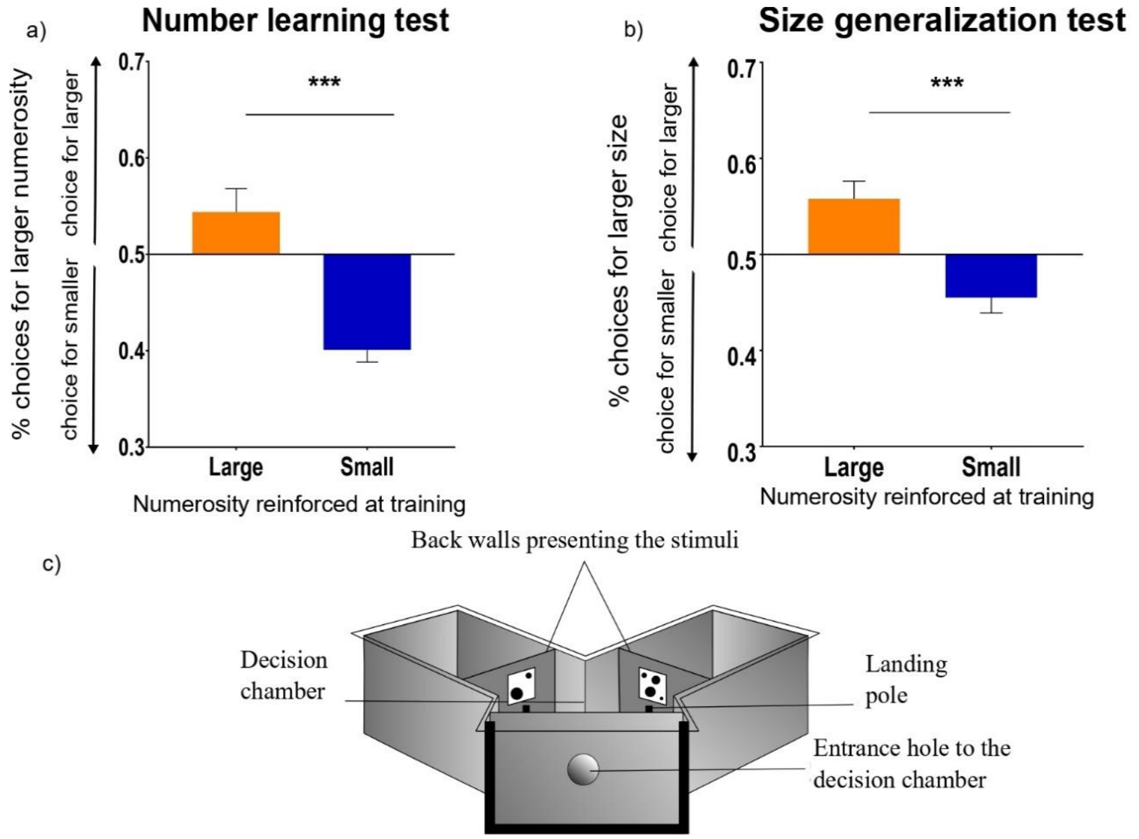


FIGURE 4: a) In the *number learning* test, honeybees trained to discriminate the smaller or the larger numerousness showed correct spontaneous choices in the absence of reward (*** $P < 0.001$, Analysis of variance (ANOVA)). ***b)*** In the *size transfer* test, bees previously trained to select the larger numerosity showed a preference for the larger size, conversely bees previously trained to select the smaller numerosity showed a preference for the smaller size (*** $P < 0.001$, Analysis of variance (ANOVA)). ***c)*** Schematic representation of the Y-maze used to train bees to discriminate numerousness and to test them for transfer from numerical to spatial (size) dimensions.

2.4 Discussion

Results of *number learning* test confirmed previous studies (Bortot et al., 2019; Howard et al., 2018; Howard et al., 2019a) showing that bees can discriminate numerosities with 0.5 and 0.67 ratios when continuous physical variables were controlled for. Moreover, we found that honeybees can make a transfer from discrete (number) to continuous (size) magnitudes. This provides the evidence for a common code for magnitudes in an invertebrate species.

The hypothesis of the existence of a prelinguistic framework to process different prothetic dimensions (i.e., dimensions that can be "more" or "less" than) was first proposed by Gallistel (Gallistel, 1989) and then developed by Walsh (Walsh, 2003). Research in humans and other vertebrates has revealed that the temporal, spatial, and numerical features of a stimulus can interact with one another (Gallistel and Gelman, 2000; Lourenco and Longo, 2010; Merritt et al., 2010; Rugani et al., 2015) and evidence of similar activation in the parietal cortex in humans and non-human primates in quantity discrimination seems to support the hypothesis of an encoding by a common magnitude (Piazza et al., 2007). Our results show that bees generalize from a numerical dimension to a spatial (size) dimension, suggesting that a general magnitude encoding can be shared among vertebrates and invertebrates.

Our experiment demonstrates that, despite their small brains and the absence of a cortex, honeybees generalize from discrete (number) to continuous (size) magnitudes. It could be that there are regions in the honeybee’s brain that play a similar function to the mammalian parietal cortex or to the avian nidopallium caudolaterale (Ditz and Nieder, 2020). The central neuronal structure of the bee’s brain, the mushroom bodies, a very complex high-order integration center, seems to be crucial in mediating the ability of bees to perform conceptual learning and extraction of relational rules (Gallistel, 1989; Giurfa, 2013). Whether mushroom bodies support the cognitive mechanism of processing magnitudes is unknown.

Future studies should investigate whether the ability of insects to generalize between number and space is widespread to other magnitudes such as, for instance, time or brightness.

CHAPTER 3

TRANSFER FROM CONTINUOUS TO DISCRETE QUANTITIES IN HONEYBEES

This chapter contains a modified version of the following scientific article: *Bortot, M. & Vallortigara, G. (2023). Transfer from continuous to discrete quantities in honeybees. Iscience, 26(10).* The Journal Author Rights are reported in Appendix A.3. The following materials were used: introduction, methods, results, discussion, figures, and supplementary materials.

3.1 Introduction

The abilities to process the numerical, spatial, and temporal properties of an object have been the focus of separated research lines for decades. Gallistel (Gallistel, 1989) first proposed the existence of a common mental currency to process different magnitudes (i.e., dimensions that can be perceived as *smaller* or *larger*). These magnitudes are either continuous and uncountable, such as time, space, length, or luminance, or discrete and countable, as number (Tudusciuc and Nieder, 2007). This hypothesis was then expanded into a broader theory (A Theory Of Magnitude, ATOM; (Walsh, 2003) suggesting the presence of a mechanism that would allow a symmetrical interaction between stimulus dimensions (Walsh, 2015). Several studies in both humans and other vertebrate species support this hypothesis (De Corte et al., 2017; Lourenco and Longo, 2010; Meck and Church, 1983; Merritt et al., 2010; Miletto Petrazzini and Brennan, 2020; Roberts and

Mitchell, 1994), providing evidence for this mechanism being widespread in the vertebrate subphylum.

Honeybees (*Apis mellifera*) are an excellent model for studying the presence of a similar magnitude encoding system in invertebrates. Previous research has demonstrated the ability of honeybees to process different dimensions separately. For instance, bees can process the numerical feature of stimuli, and successfully discriminate between sets of numerically different visual elements (Bortot et al., 2019; Gross et al., 2009). Moreover, honeybees perform arithmetic operations (i.e., addition and subtraction; Howard et al., 2019a) and possess a concept of zero numerosity (Howard et al., 2018). Finally, they proved able to discriminate the relative size of visual stimuli and generalize such learning to novel shapes (Avarguès-Weber et al., 2014; Howard et al., 2017).

We recently showed that bees could perform a transfer from the numerical to the size dimension of a stimulus, suggesting the presence of a cross-dimensional transfer in an insect species (Bortot et al., 2020). However, whether the interaction between number and size would be symmetrical was not investigated. We hypothesized that if the coding of different dimensions, such as size and numerousness, originates from a common metric in the honeybee brain, we should observe a cross-dimensional transfer from continuous to discrete magnitudes as well, suggesting a bidirectional relationship between those dimensions.

Here, we aimed to investigate the ability of bees to make a transfer from a continuous (i.e., size) to a discrete (i.e., numerical) dimension. Specifically, bees were trained to associate an array with either three larger or three smaller visual stimuli with a reward, presented in a Y-maze (Fig. 5). The relative size dimension of the stimuli changed during the training, albeit maintaining the same 0.5 ratio difference. At test, bees were presented with numerical comparisons involving small and large numbers of visual stimuli controlled for continuous variables that covary with numerosity. A generalization test for size discrimination ability to transfer over a different shape was also performed.

3.2 General Methods

The experiment was performed from June to October 2023 at SperimentArea, a field station run by the local Natural History Museum, in Rovereto (North of Italy).

3.2.1 Subjects

Free-flying honeybee foragers (*Apis* mellifera; N = 20) were individually trained to enter a wooden Y-maze located in a wooden house in the park. The entrance of the maze was connected to a single window that allowed the control of the entrance of single subjects. The room was illuminated by natural light and by two white light stripes attached to the ceiling (Ledpoint light solution, Honglitronic, 1340 lumen/meter, CCT 2900-3000K white). The artificial lights were positioned above each arm of the maze. Half of the bees (N=10) were trained to associate the larger elements with an appetitive reward, and the other half (N=10) with the smaller elements. Honeybees were marked to allow individual recognition and at the end of the experiment, they were marked with a red dot on the thorax to prevent double testing and then released.

3.2.2 Apparatus and stimuli

The apparatus was a wooden Y-maze (Bortot et al., 2020; Fig. 5). We used landing poles to provide the reinforcement and the punishment to the bees. They consisted of grey poles with an attached open, transparent capsule where a drop of solution was placed. In this way, we prevented bees could discriminate between the positive (i.e., sucrose) and negative (i.e., quinine) solutions by directly touching them with their antennae since bees were forced to land on the pole to be able to taste the solution. Capsules were changed between individuals and cleaned with 30% ethanol at every trial.

All stimuli consisted of yellow elements (RGB: 255, 237, 0; (Avarguès-Weber et al., 2014)) on a grey background (10 x 10 cm; RBG: 144, 150, 146; (Avarguès-Weber et al., 2014)) shown at a 10 cm distance from the decision chamber of the maze. Stimuli could have either a square, diamond, or circular shape (Supplementary Figures S8-10). The first two shapes were used for training purposes, whereas the third one was only presented during the test phase. The training stimuli consisted of comparisons between three larger and three smaller elements having a 0.5 ratio difference. Using GeNEsIS software (Zanon et al., 2022), we created 16 pairs of stimuli (i.e., N=8 squares, N=8 diamond) having four different side dimensions (i.e., 1 *vs.* 2 cm; 1.15 *vs.* 2.3 cm; 1.35 *vs.* 2.7 cm; 1.5 *vs.* 3 cm). Stimuli were controlled for element disposition, that could be arranged in a line (N= 7) or a triangular (N= 9) configuration. The inter-distance between elements (i.e., the distance computed from the center of the elements) was controlled so that larger and smaller elements of specific comparisons had the same position in the array (Supplementary Figures S8-9). During the training, each stimulus was rotated by 90 degrees in the following trials to prevent bees from learning a particular spatial configuration.

The *size generalization* test was composed of a comparison between three large and three small elements having a novel shape (i.e., circular) and a novel relative size (i.e., 1.25 *vs.* 2.5 cm). This test aimed to confirm the learning of the task (i.e., choose the smaller/larger size) irrespective of the element shape and in the absence of any reward (Supplementary Figure S10).

During the *numerical generalization* test, a 4 *vs.* 8 elements comparison was presented under different stimuli control conditions. We maintained the magnitude ratio difference experienced in the training phase (i.e., 0.5). To check for the role of perceptual variables covarying with numerosity, we created a total of three couples of stimuli separately controlled for total perimeter, total area, and element size. In all three pairs of stimuli, the convex hull was controlled for. Moreover, the smaller and larger elements were identically present in both numerical arrays (Supplementary Figure S10). These tests aimed to investigate whether bees were able to make a transfer from the spatial to the numerical dimension irrespective of the availability of perceptual features, such as area, contour length, or element dimensions.

3.2.3 Training procedure

The experimental procedure comprised habituation, training, and test phases. All phases could be completed in 1 or 2 consecutive days. During the habituation phase, each bee was collected from a gravity feeder placed in the apiary and trained to gradually fly inside the apparatus to collect food by landing on the two landing poles placed in both arms, in the absence of visual stimuli. Each subject was individually marked to allow bee recognition. In the training phase, a comparison between relatively larger and smaller elements was presented (Fig. 5). Half of the subjects learned to associate the relatively larger stimuli with positive reinforcement, whereas the other half of the bees learned to choose the relatively smaller stimuli in the comparison. During this phase, an appetitive-aversive conditioning paradigm was used where the correct size was always associated

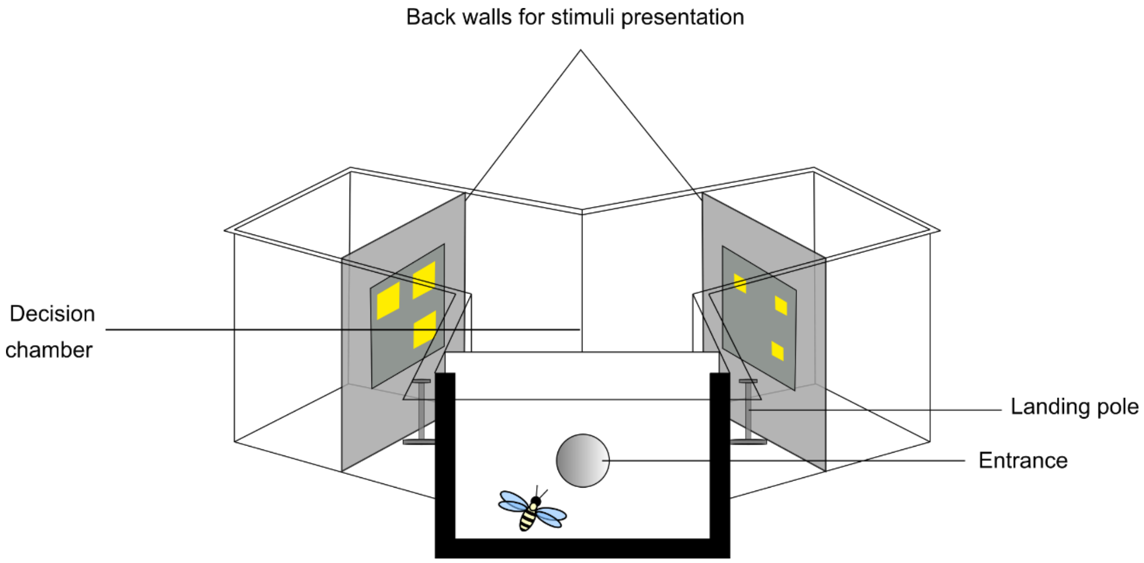


FIGURE 5: Schematic representation of the Y-maze.

with food (i.e., sucrose solution 50% w/w), whereas the incorrect size was always associated with punishment (i.e., 60 mM quinine solution; see Avarguès-Weber et al., 2010). Each bee had to reach a criterion of ≥ 80% accuracy over 10 consecutive trials. The stimuli were presented in a pseudo-random sequence (i.e., the correct stimulus was never presented more than two consecutive times on the same side). For the overall duration of this phase, the position of the experimenter was always at the end of the Y-maze, in a symmetrical position with respect to both arms to avoid any side cue.

3.2.4 Test procedure

Once reached the accuracy criterion, honeybees entered the test phase where four tests were given (i.e., *size generalization* test, *number total area* test, *number total perimeter* test, *number same size* test). Each test was presented twice to counterbalance the position of the correct array and avoid side preferences. The tests lasted one minute during which the behavior of the experimental bee was video recorded (LifeCam Studio, 30 fps). During the test phase, the experimenter moved away from the apparatus and was thus not visible to the bee to avoid any side bias.

The videos were analyzed by the researcher in blind condition (i.e., no information about the specific training undergone by the subject was provided in the videos that were analyzed six months after the conclusion of the experiment; 0.33x velocity). The number of choices computed by the subjects (i.e., landing behavior or direct contact made with antennae or legs, on one of the two capsules on the landing poles placed in front of each stimulus) was scored. All tests were conducted in probe conditions (i.e., in the absence of reward and punishment), and a drop of water was placed in the two capsules positioned in front of each stimulus.

The percentage of choices for the correct size and congruent numerosity (i.e., congruent respect to the specific training: 8 elements for bees trained to choose the larger elements at training, 4 elements for bees previously trained to select the smaller elements at training) was calculated for each subject and analyzed, giving rise one single value per bee to exclude pseudo-replication.

3.2.5 Statistical analyses

The training data were checked for normality (Shapiro-Wilk normality test: $W = 0.97$, $P > 0.05$) and homoscedasticity (Levene's test: $P > 0.05$) and then analyzed with parametric statistical tests. An analysis of variance was performed with group (larger *vs.* smaller size as positive), type of stimulus (square and diamond), and position of the reinforced stimulus (left *vs.* right) as factors.

We performed an offline blind scoring of the videos of the test phase, and we calculated the proportion of choices for the correct stimulus size during the *size generalization* test and the proportion of congruent numerosity during the *number total area* test, the *number total perimeter* test and the *number same size* test. The data were checked for normality (Shapiro-Wilk normality test: $W = 0.97$, $P > 0.05$) and homoscedasticity (Levene's test: $P > 0.05$) and analyzed with parametric statistical tests. An analysis of variance was conducted with group (larger *vs.* smaller size as positive) and type of test (*size generalization*, *number total area*, *number total perimeter*, *number same size*). The proportion of choices for the correct and congruent stimuli was analyzed with a two-tailed one-sample *t*-test.

We calculated the spatial frequency of our images to estimate the contribution of this parameter to the behavioral outcome. Spatial frequency was computed as the fast Fourier transformation of images, followed by the calculation of the radial average of the signal amplitude in the frequency domain and the final sum of the frequency contributions of its power spectrum, as reported in a previous study (Potrich et al., 2022). All the frequency analyses were performed with a custom MATLAB script of the GeNEsIS program (Zanon et al., 2022; https://github.com/MirkoZanon/GeNEsIS). Significant differences between the spatial frequency of training stimuli were analyzed with a non-parametric Wilcoxon rank sum test. In all the analyses, an α-value of 0.05 was specified. All the analyses were conducted with R-Studio (R, 4.1.3 version).

3.3 Results

During the training phase, honeybees trained to select the array containing the relatively larger stimuli reached the 80% accuracy criterion in 25.6 ± 4.18 (mean ± SEM) trials, whereas bees trained to choose the relatively smaller stimuli, reached the 80% accuracy criterion in 38.8 ± 6.40 (mean ± SEM) trials. An analysis of variance revealed no significant heterogeneity associated with the group (larger *vs.* smaller trained: $F_{(1, 72)} = 0.17$, $P = 0.679$), the type of shape presented at training (diamond *vs.* square: $F_{(1, 72)} = 0.27$, $P = 0.605$), the position of the correct stimulus (right *vs.* left: $F_{(1, 72)} = 1.26$, $P = 0.266$), and their interactions (shape x position: $F_{(1, 72)} = 0.19$, $P = 0.666$; shape x group: $F_{(1, 72)} = 2.1$, $P = 0.152$; position x group: $F_{(1, 72)} = 0.21$, $P = 0.648$; shape x position x group: $F_{(1, 72)} = 0.39$, $P = 0.533$).

During the test phase, the proportion of choices for the correct relative size (i.e., larger/smaller elements for bees previously trained to select the larger/smaller elements, respectively) and then the congruent numerical array (i.e., 4 or 8 elements for bees previously trained to choose the smaller/larger elements, respectively) was computed. An analysis of variance revealed no significant differences between groups (larger *vs.* smaller trained: $F_{(1, 72)} = 0.224$, $P = 0.64$), type of test ($F_{(3, 72)} = 2.44$, $P = 0.072$; Fig. 6 for visualization of the performance at *number* tests) and their interaction (group x type of test: $F_{(3, 72)} = 0.52$, $P = 0.67$). We thus merged the three numerical tests (i.e., *number total area*, *number total perimeter*, *number same size*; renamed as *number generalization* test) and the analysis of variance performed on this dataset revealed a significant effect of the type of test (*size generalization* test *vs. number generalization* test: $F_{(1, 36)} = 8.71$, $P = 0.0055$). Again, no significant effect of group (larger *vs.* smaller trained: $F_{(1, 36)} = 0.07$, $P = 0.794$) and interaction between the experimental group and type of test was found ($F_{(1, 36)} = 0.96$, $P = 0.333$).

Honeybees presented with a size comparison of elements having a novel shape (i.e., circle) successfully choose the larger or smaller relative size according to their previous training (*size generalization* test: 59.89% ± 2.01%, mean% ± SEM%; two-tailed one-sample *t*-test: $t_{(19)} = 4.91$, $P < 0.001$; Fig. 7). Similarly, in the *number generalization* test bees selected significantly the congruent stimulus according to their previous training (i.e., 4 elements if they were trained to choose the smaller size stimulus, or 8 elements if they were trained to choose the larger size stimulus during the training; 52.84% ± 1.23%, mean% ± SEM%; two-tailed one-sample *t*-test: $t_{(19)} = 2.31$, $P = 0.032$; Fig. 7).

Spatial frequency (i.e., number of light/dark cycles or oscillations of a pattern occurring per unit of distance in a given space) was proposed to play a role in visual discrimination of honeybees (Hertz, 1933; MaBouDi et al., 2021). Thus, we analyzed whether spatial frequency could have been used as a perceptual feature to solve our cross-transfer task.

In our experiment, spatial frequency covaried with the size of elements (i.e., arrays containing larger stimuli had also the averaged higher spatial frequency) both in stimulus presented during the training (*smaller* size total power = 28878,9 ± 70, mean ± SEM; *larger* size total power = 31594,8 ± 296,8, mean ± SEM; Wilcoxon rank sum test: total power of *smaller vs. larger* size: *W*=256, *p-value* < 0.001; Fig. 8) and the *size generalization* test (*smaller* size total power = 27725,4; *larger* size total power = 29760,3; Fig. 8), and with numerosity only when the stimulus size was controlled (i.e., *number same size* test; 4 elements total power = 27954,02; 8 elements total power = 28851,5; Fig. 8). Conversely, when the total area was equated between arrays (i.e., *number total area* test), spatial frequency was almost identical between arrays (4 elements total power = 29716,9; 8 elements total power = 29715,0; Fig. 8). In the *number total perimeter* test, spatial frequency was inversely related with numerosity, as the higher total power value was associated with the smaller numerosity (4 elements total power = 33944,7; 8 elements total power = 31020,3; Fig. 8). The performance that we observed in our experiment is thus not consistent with a spatial frequency-based strategy, suggesting a true cross-dimensional transfer from the size to the numerical dimension not supported by low-level perceptual cues.

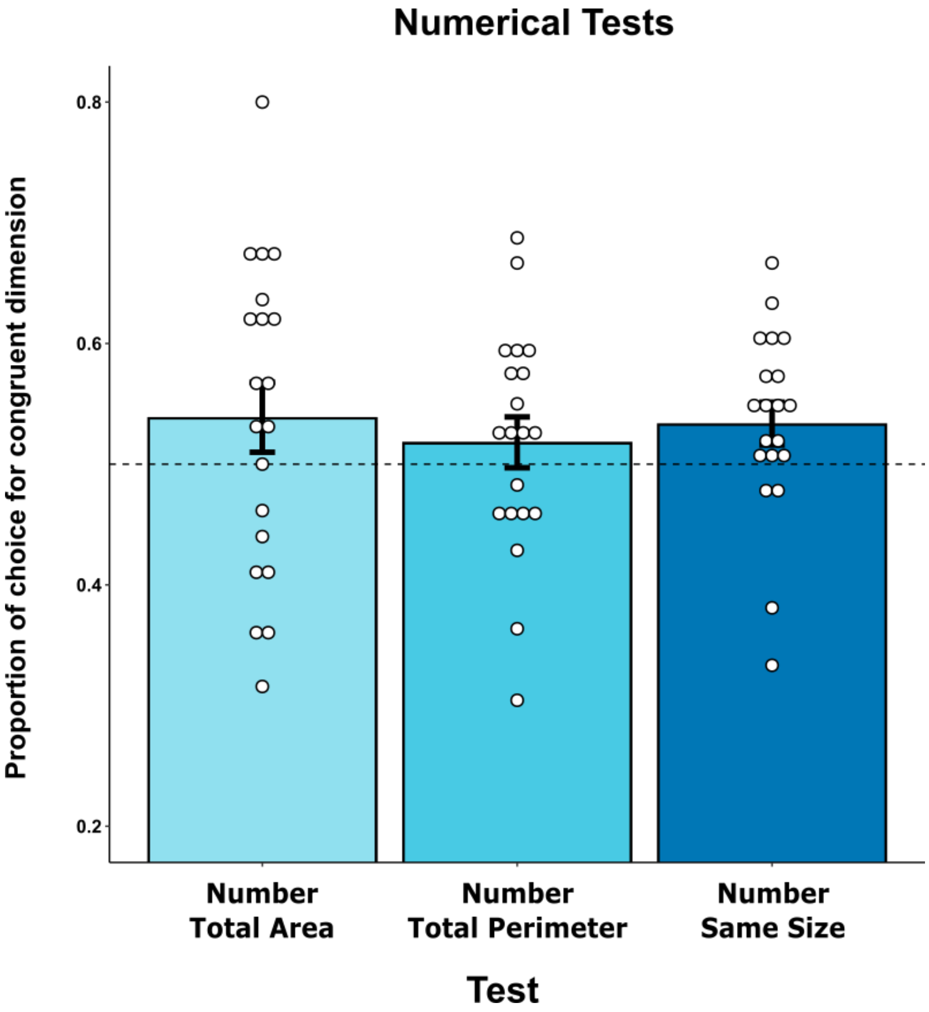


FIGURE 6: Results of the numerical tests (group means with SEM are shown); white dot represents single subject performance. No significant effect of the type of test was found (Analysis of variance (ANOVA): type of test ($F_{(3, 72)} = 2.44$, $P = 0.072$; no asterisks indicate no significance).

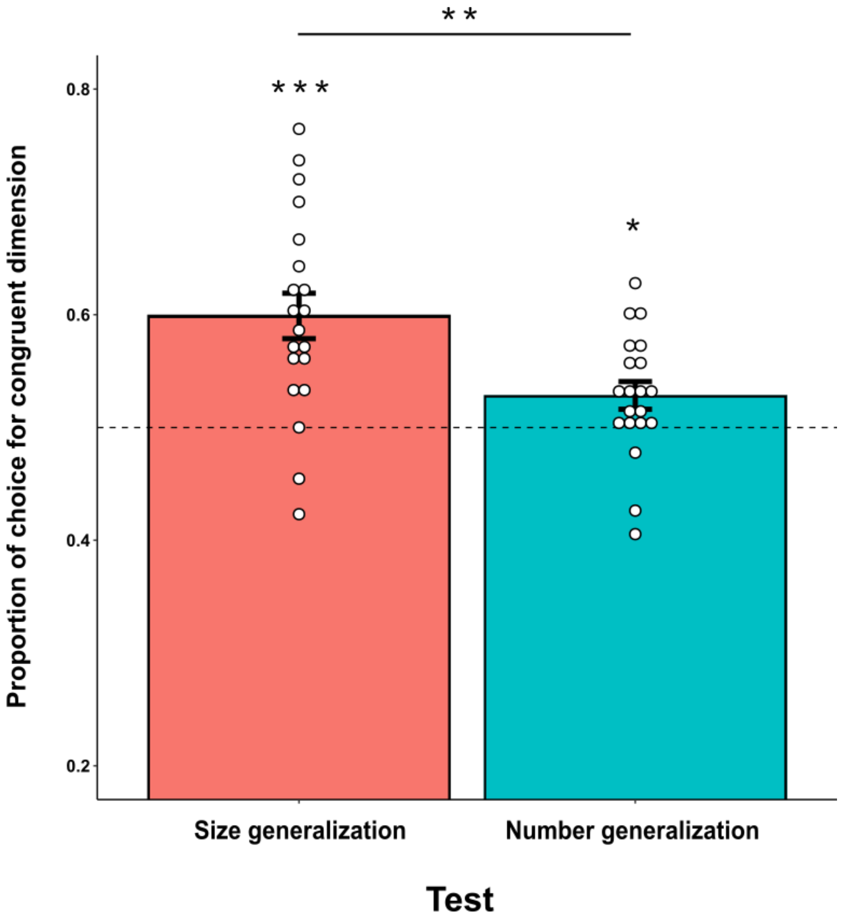


FIGURE 7: Results of the *size* and *number generalization* (overall) tests (groups means with SEM are shown; white dot represents single subject performance). In the *size generalization* test, honeybees chose the larger or smaller relative size according to their previous training (*size generalization* test: 59.89% ± 2.01%, mean% ± SEM%; two-tailed one-sample *t*-test: $t_{(19)} = 4.91$, $P < 0.001$). In the *number generalization* test bees chose the congruent stimulus according to their previous training (i.e., 4 elements if they were trained to choose the smaller size stimulus, and 8 elements if they were trained to choose the larger size stimulus during the training) (*number generalization* test: 52.84% ± 1.23%, mean% ± SEM%; two-tailed one-sample *t*-test: $t_{(19)} = 2.31$, $P = 0.032$). (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

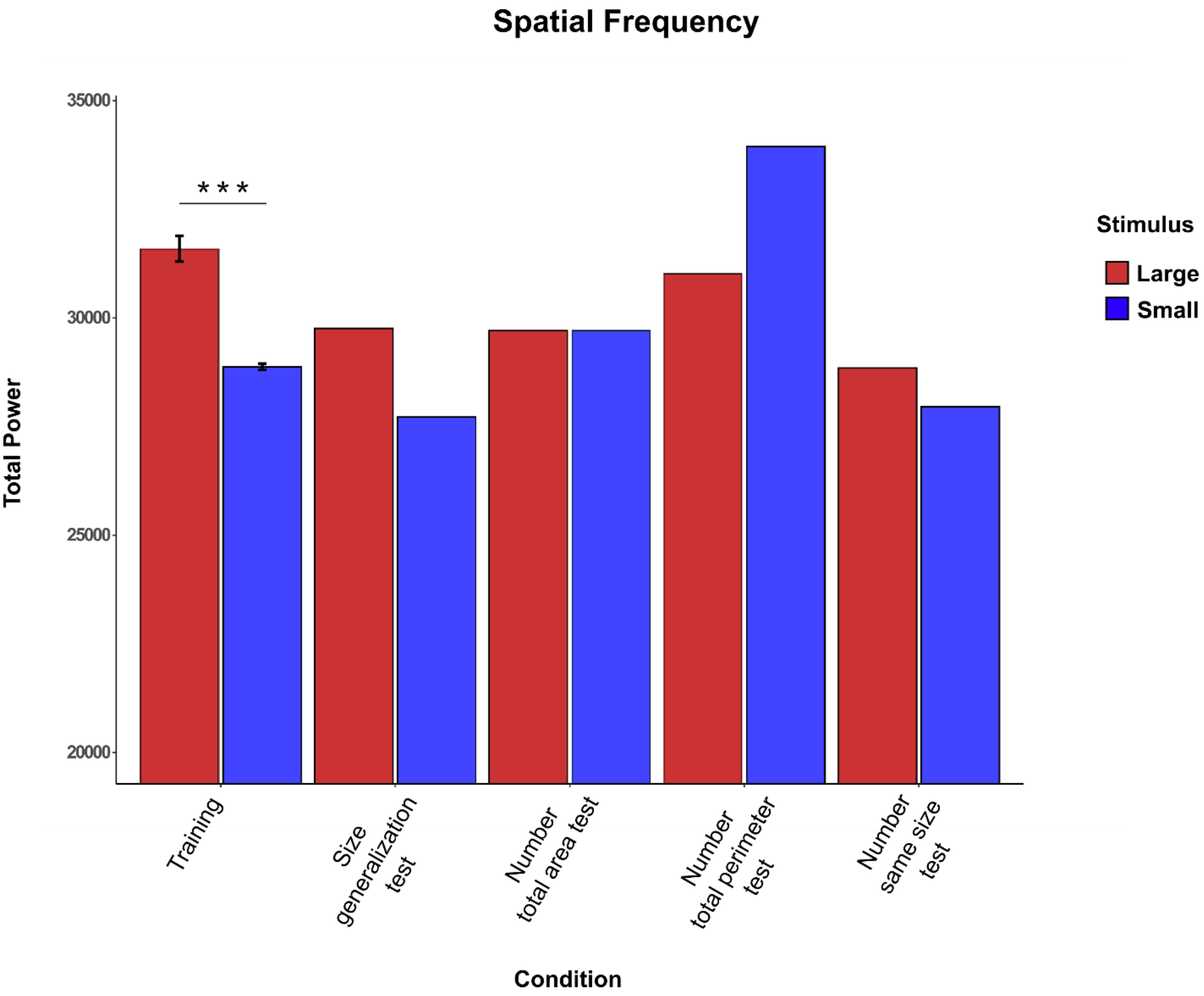


FIGURE 8: Spatial frequency of the stimuli presented during the training and the test phase. Spatial frequency varied directly along the stimulus dimension (i.e., either size or numerousness) during training (*smaller* size total power = 28878,9 ± 70, mean ± SEM; *larger* size total power = 31594,8 ± 296,8, mean ± SEM; Wilcoxon rank sum test: total power of *smaller vs. larger* size: *W*=256, *p-value* < 0.001), the *size generalization* test (*smaller* size total power = 27725,4; *larger* size total power = 29760,3), and when the stimulus size was controlled (i.e., *same size* test; 4 elements total power = 27954,02; 8 elements total power = 28851,5). Spatial frequency was similar when the total area was equated between arrays (i.e., *number total area* test; 4 elements total power = 29716,9; 8 elements total power = 29715,0), while it was inversely related with numerosity when the total contour length was controlled (4 elements total power = 33944,7; 8 elements total power = 31020,3).

3.4 Discussion

The results of the *size generalization* test confirmed that bees can discriminate between different relative sizes (Howard et al., 2017) and can generalize such learning to novel shapes (Avarguès-Weber et al., 2014).

The results of the *number tests* (i.e., *number total area*, *number total perimeter,* and *number same size* test) revealed an overall spontaneous, although weak, preference for congruent numerosity. Bees showed a lower tendency to choose the congruent numerosity in comparison to choosing the correct size, highlighting that transferring the learned "choosing the smaller/larger dimension" rule from a continuous to a discrete dimension could be a more difficult task than transferring within a single dimension. Nonetheless, bees might be equipped with the capacity to make a transfer from a continuous (size) to a discrete (number) dimension.

Despite the lack of any significant difference among the three numerical tests, the graph may suggest that the best performances were observed in the *number total area* and *number same size* tests (Fig. 6). It could be that honeybees mainly used contour length as the primary perceptual feature in the size discrimination during training. This could have favored the choice towards the congruent numerosities when the total area and the stimuli size were controlled since here contour length varied in a congruent way with numerosity (i.e., a larger numerosity also has a larger overall perimeter, and vice versa). The use of contour length as a primary cue could also explain the results of the only study that showed evidence of transfer from length to number in non-human vertebrates (angelfish; (Miletto Petrazzini and Brennan, 2020). Fish trained to select the shorter line (with the smaller perimeter) chose the smaller numerosity in a 10 *vs.* 20 elements comparison whose area was matched between arrays. The smaller quantity contained a smaller amount of contour length: thus, angelfish could have relied on this cue to solve the task.

However, although contour length has been argued to be used during numerosity discrimination in human infants (Clearfield and Mix, 1999), recent studies demonstrated that humans and non-human species, including honeybees, can perform numerosity discrimination when the perimeter is equated between sets of stimuli (human infants: Di Giorgio et al., 2019; Lourenco and Longo, 2010; archerfish: Potrich et al., 2022; chicks: Rugani et al., 2015, 2013, 2008; honeybees: Gross et al., 2009). We believe therefore that the slight tendency for a worse performance in the *number total perimeter* test could be explained in another way. The geometrical constraints associated with this condition led to the creation of the numerically smaller array with three larger elements and one smaller element, and the numerically larger array with only one larger element and seven smaller ones (Supplementary Figure S10). These configurations could have been perceived by the bees as more similar to the size comparison experienced during the training. Thus, a response towards the more perceptually familiar configuration (i.e., larger elements contained in the numerically smaller group for bees previously trained to select the larger size, and smaller elements contained in the numerically larger group for subjects previously trained to choose the smaller size) could explain the tendency.

Our data potentially show the presence of individual differences in solving our visual task. The occurrence of different strategies to solve cognitively demanding tasks could indeed benefit the colony as more diversification in individuals' behavior would allow a more efficient resource acquisition (Burns and Dyer, 2008; Finke et al., 2021). Interestingly, those individual differences are maintained within a sensory modality, despite the changing of the perceptual discrimination or increasing level of abstraction of the task (Finke et al., 2021). Conversely, the observed individual differences between bee foragers are not conserved between different sensory modalities, as a higher performance in the visual domain does not implicate a subsequent higher performance in an olfactory discrimination task (Finke et al., 2021).

Interactions between different magnitudes, such as space, time, and quantity have been reported in different species (time, space, quantity: Lourenco and Longo, 2010; time and quantity: Meck and Church, 1983; Roberts and Mitchell, 1994; space and time: De Corte et al., 2017; Merritt et al., 2010; space and quantity: Miletto Petrazzini and Brennan, 2020); in humans and non-human primates, they seem to be associated with a common activation of the parietal cortex (Walsh, 2003). Recent evidence suggested the columnar neurons of *Drosophila melanogaster* lobula as a probable neural substrate for number perception in this insect species (Bengochea et al., 2023).

There are ecological reasons to posit that time, space, and number must be represented by a common magnitude system in organisms as different as vertebrates and invertebrates. As stressed by Gallistel (Gallistel, 1989) discrete quantity (i.e., numerousness) and continuous quantity (i.e., space or time) must be represented by some sort of common mental currency, which he refers to as ‘magnitude’, to enable biological organisms to perform arithmetic operations across domains. For instance, when a bee calculates the rate of return to a flower it has to compute number and time in a single currency. Our previous work in honeybees demonstrated the existence of a cross-dimensional transfer from the discrete (numerical) to a continuous (size) dimension (Bortot et al., 2020). The present results demonstrated that bees could make a transfer from the size to the numerical dimension of visual stimuli as well, suggesting a symmetrical and bidirectional interaction between those dimensions. This strongly advocates for the presence of a universal magnitude processing mechanism in both vertebrate and invertebrate species.

CHAPTER 4

AN INVESTIGATION OF THE ABILITY OF HONEYBEES TO LEARN ODOR SEQUENCE STRUCTURES IN A PER CLASSICAL CONDITIONING PARADIGM

4.1 Introduction

From an evolutionary perspective, inferring a rule that allows the treatment of different stimuli encountered in the environment as belonging to the same category could be extremely important. That might help an animal in anticipating a particular outcome, as well as to generalize its response, thus maximizing its fitness. This capacity has been thought to be one of the core mechanisms underlying human language acquisition, albeit not limited to our species (Dehaene et al., 2015).

The ability to extrapolate regularities and generalize them to new stimuli has been found in different vertebrate species, such as primates and birds (Endress et al., 2009; Sonnweber et al., 2015; Spierings and Cate, 2016; Versace et al., 2019). Evidence from precocial species suggests that this ability might be present at the onset of life. Exploiting imprinting, an early form of learning by exposure, domestic chicks (*Gallus gallus*) successfully discriminated between XX and XY multimodal patterns (Versace et al., 2017) and between triplets of simultaneous visual elements having an AAB *vs.* ABA structure, without explicit training (Santolin et al., 2016). Newborn ducklings imprinted with visual 3D stimuli being the same or different in shape or color, at test chose novel stimuli having the same relationship as the imprinted ones, even though an alternative explanation involving stimuli visual symmetry might be in place (Martinho and Kacelnik, 2016).

Using combinatory signals having a specific structure to convey a message is observed even in invertebrates. For instance, the treehopper males (*Enchenopa binotata*) combine two different elements to attract females during mating: an initial whine followed by several pulses (Cocroft et al., 2010). Females of this species seem to respond to the typical structure of this stimulus, being guided by a combinatory strategy (Desjonquères et al., 2020; Speck et al., 2020). Likewise, during the final phase of courtship, males of the black widow spider (*Latrodectus hesperus*) display an organized signal whose stereotypical structure is different from that of the signals emitted in other phases of the mating (Sivalinghem and Mason, 2021).

The ability to abstract and generalize structural sequence regularities has been marginally investigated in invertebrates. Macquart et al. (2008) studied the ability of an ant species (*Gigantiops destructor*) to learn simple motor sequences. In order to reach their nest, ants were forced to explore Y-mazes according to rule of turns of increasing difficulty: form constant and repetitive turns (e.g., RRRRRRRR or LLLLLLLL; R = right, L = left) to simple alternating (e.g., RLRLRLRL), double alternating (e.g., RRLLRRLL) and irregular alternating turns (e.g., RLRRL). At test, they were presented with two additional chambers at the end of the maze. Ants correctly extended a constant-turn learning to the novel chambers. However, they failed in consistently choosing the second correct chamber when trained to apply both a simple and double alternating rule, with performance dropping from 88.8%-71.4% to 55.5%-14.3%, respectively (Macquart et al., 2008). A probable explanation implies the memorization of the maze length or number of turns needed to reach the nest, causing ants in deploying a more natural searching behavior after the first novel chamber (Czaczkes, 2022). Nevertheless, these results suggest the possible use of a sensorimotor sequence learning in this species, highlighting its adaptive value respect to a costlier landmark memorization during navigation (Macquart et al., 2008). Honeybees offer a unique opportunity to try to further study the ability to form a sequence learning since they appear to be equipped with impressive cognitive and learning abilities (Bortot et al., 2020). Bees show evidence of generalization capacity, treating equally different visual stimuli based on specific physical characteristics, such as radial, circular, and bilateral symmetry (Lehrer et al., 1995). Bees can also master abstract concepts such as *same/different* (Giurfa et al., 2001), *above/below* (Avarguès-Weber et al., 2011), and *right/left* (Avargues̀-Weber et al., 2012). They have been also tested for their ability to encode and use the temporal information of a visual stimulus to solve a subsequent task, albeit with negative results (Ng et al., 2020).

Here we aimed to investigate whether honeybees could abstract the underlying structure of a temporal sequence of three odor and then generalize their responses to novel stimuli. We performed six experiments using the *proboscis extension response* (PER) conditioning paradigm which allows the formation of an association between an odor stimulus and a sucrose solution that, when provided to the antennae, elicits the spontaneous extroversion of the ligula (Giurfa and Sandoz, 2012).

The first two experiments investigated whether bees could learn an arbitrary odor sequence, spontaneously generalize their response to novel stimuli with a similar structure but composed of novel odors, and reject novel different configurations although composed of familiar odors. We investigated the role of the position of a particular odor in the sequence and its temporal closeness to the reward, studying whether this affected bees' memorization and recall abilities in a third control experiment. We also investigated whether differential conditioning could lead to successful discrimination of sequence structures (Exp. 4, 5). Lastly, we aimed to determine whether, by using a conditioning procedure in which a generalization strategy is favored to solve the task, a spontaneous encoding of the internal structure of odor sequences could be established and used to respond accordingly to novel stimuli.

4.2 General Methods

All experiments were conducted at the Animal Cognition and Comparative Neuroscience Lab (ACN Lab, CIMeC, University of Trento) in Rovereto (Italy). Experiments 1, 2, and 4 were conducted from May to September 2021. Experiments 3 and 5 were conducted from April to May 2022. Experiment 6 was partly conducted in September 2021 and completed in May 2022.

4.2.1 Subjects

Honeybee foragers (*Apis mellifera*) were obtained from colonies located in the apiary of the ACN Lab. Honeybees were anesthetized on ice until they stopped moving. Then, they were individually harnessed on metal supports. A piece of poliplak was placed on the wings in other to prevent their damage. Each fixed bee was fed with 3 µl of 50% sucrose solution and kept in a dark, humid box for about 1 hour before starting training. In general, the day after training, all subjects were checked for their PER response following the antennal stimulation with the sucrose solution. Bees that did not exhibit the ligula extension response, were discarded from the analysis. After the experiments, all subjects were released after marking them with a UNIPOSCA color to avoid cross-testing.

Overall, 324 honeybee foragers were tested (Exp.1: N = 47; Exp. 2: N = 48; Exp. 3: N = 41; Exp. 4: N = 66; Exp.5: N = 38; Exp.6: N = 84). The minimum sample size of each experiment was determined based on previous guidelines, suggesting the use of a large sample size for PER experiments (i.e., 40/50 bees per group; Matsumoto et al., 2012).

4.2.2 Apparatus and stimuli

In all experiments, the setup used to deliver the odor sequences was composed of a computerized olfactometer controlled by a MATLAB program (MATLAB R2019a, The MathWorks Inc., Natick, MA, USA), connected to six valves.

The stimuli consisted of six different odors, used according to the aim of each experiment: 3-hexanol (A), acetophenone (B), 1-nonanol (C), citral (D), benzaldehyde (E) and 2-octanone (F) (SIGMA-ALDRICH®), with a 1:200 concentration (5 µl odor / 1000 µl mineral oil). We selected odors that have not been proven to elicit an innate preference in honeybees (Nouvian et al., 2015). For details on the sequence stimuli used see Table 1 and *Result* section.

Each odor was prepared and changed before every training session. Each odor sequence was composed of three distinct puffs (1 second each), separated by 500 ms of air. A frontal continuous airflow and an aspirator behind the subject were used to clear the environment from the odor flow.

Experiment	**Conditioning**	**Training stimuli**		**Test stimuli**	
				Similar structure	*Similar odor*
Experiment 1	Absolute	ABA		CDC	BAA
Experiment 2	Absolute	CDC		ABA	DCC
				First element	*Second element*
Experiment 3	Absolute	ABA		AAA	BBB
		Reinforced	*Non reinforced*	*Congruent*	*Incongruent*
Experiment 4	Differential	ABA	BAA	CDC	DCC
		BAA	ABA	DCC	CDC
Experiment 5	Differential	ABA	AAB	CDC	CCD
		AAB	ABA	CCD	CDC
				Similar structure	*Different structure*
Experiment 6	Absolute generalization	ABA, CDC		EFE	FEE

TABLE 1: Schematic representation of the experimental design of the six experiments.

4.2.3 Training procedure

All experiments followed a *proboscis extension response* conditioning procedure (Matsumoto et al., 2012) (PER), where harnessed bees were individually conditioned to associate an odor stimulus with positive reinforcement. Each conditioning trial lasted ≈ 1 minute. The bee was placed in front of a continuous airflow for 25 seconds before the odor stimulation (4 seconds). The reinforcement (3 seconds) was delivered with a toothpick soaked in sucrose solution (i.e., ≈ 2μl of 50% sucrose solution w/w), after 3 seconds from the beginning of the odor. This allowed an overlapping of 1 second between odor and sucrose delivery. Then, after the stimulation, the bee was kept in front of the airflow for another 27 seconds and then removed. An inter-trial interval (ITI) of 10 minutes was used. The use of toothpicks does not introduce any odor bias and has been demonstrated not to be a confound for the classical CS-US learning of honeybees (Giurfa and Malun, 2004).

In experiments using the *absolute conditioning* paradigm (Exp. 1, 2, 3), bees completed a total of 10 trials, divided into 5 conditioning trials, pseudo-randomly interposed with 5 blank trials (Matsumoto et al., 2012) (Supplementary Figure S11). In the conditioning trials, each subject was exposed to the training odor sequence, associated with the reinforcement. Conversely, during blank trials, bees were exposed to a sequence of three non-reinforced air puffs, following the identical timing sequence described above. The blank trials were used to avoid the simple learning of the puff occurrence, irrespective of the odors presented.

In experiments using the *differential conditioning* paradigm (Exp. 4, 5), bees underwent a total of 10 trials: 5 reinforced and 5 non-reinforced, pseudo-randomly presented (Supplementary Figure S12). During reinforced trials, the odor sequence was associated with positive reinforcement, while in non-reinforced trials, the other odor sequence was associated with water.

In experiment 6, *absolute conditioning* was used to provide a sort of generalization training. In this case, bees underwent 14 trials divided into 8 conditioned trials and 6 blank trials. During the conditioned trials, one of two odor sequences was presented (4 trials per odor sequence), while in the blank trials, bees were presented with three non-reinforced air puffs (Supplementary Figure S13). The blank trials were used to avoid the simple learning of the puff occurrence, irrespective of the odors presented.

In all experiments, a spontaneous PER response at the first conditioning trial was set as the exclusion criterion.

4.2.4 Test procedure

After training, bees underwent two testing sessions: the *memory* and the *recall* test. The test phase aimed to investigate memory formation at a different post-conditioning time. The *memory* test was conducted one hour after the end of the training, while the *recall* test was performed the morning after the training (≈ 18h delay). Both tests were conducted in probe conditions, thus not providing either reinforcement or punishment associated with the odor stimulation. An ITI of 10 minutes was set between test sequences. As the dependent variable for the analysis, the PER response to odor sequence was considered.

During the testing phase, the order of presentation of the sequences was randomized across subjects. Moreover, the order of sequence presentation was changed between *memory* and *recall* tests within each subject.

In experiments 1 – 3, only subjects showing the PER response to the previously trained sequence (i.e., ABA in Exp. 1 and 3, CDC in Exp. 3) during the *memory* test were considered for the analysis.

In experiments 3 and 5, we encoded also at what stage of the sequence the PER response was observed (see Supplemental Material).

After the presentation of the last testing sequence, the presence of PER response was checked for every subject by gently stimulating its antennae with a toothpick soaked in 30% sucrose solution (w/w). Bees that did not display the response were discarded from the analysis.

4.2.5 Statistical analyses

In the training phase, the percentage of accuracy for each trial was computed as the proportion of PER response observed on the totality of tested subjects. The response was computed as 1 if the response was present after the odor onset, and 0 if it was not present or present before and maintained after the onset of odor.

The training phase was characterized by a situation of complete separation of data, described as a condition of the allocation of all the observations in the same variable (Albert and Anderson, 1984). In particular, because of experiment criteria, the first trial of training was always composed of 0s (i.e., only bees not responding to the first odor presentation were considered for the analysis). Thus, the data were fitted in a *Bayesian Generalized Linear Mixed-Effect Model* (Bayesian GLMM, *bglmer* function of *blme* package) with a binomial distribution (*logit* link). The number of trials was set as a fixed factor in the model, while bee id and day were set as random factors in the models. In experiment 6, the odor type was also inserted as a factor in the model. To determine the significant parameters of the models, the *anova* function (*car* package) was used and the best model was selected based on the *Akaike Information Criterion* (AIC).

In the test phase, the percentage accuracy for each test sequence was computed as the proportion of PER response observed on the totality of tested subjects. The response was computed as 1 if the response was present after the odor onset, and 0 if it was not present or present before and maintained after the onset of odor. The performance during the test phase was analyzed with a *Generalized Linear Mixed Effect Model* (GLMM, *glmer* function of *lme4* package) with a binomial distribution (*logit* link). The type of sequence (i.e., sequences presented during the test phase) and order of presentation of each sequence during the test (i.e., as first, second, third – or fourth presented sequence in the case of Exp. 4, 5, and 6) were set as fixed effects in the model, while bee id and day were set as random factors. The performance during the *memory* test of experiments 1, 2, and 3, was characterized by a situation of complete separation of data (i.e., the response to the trained sequence – ABA for the first and third experiment, CDC for the second experiment – was only composed of 1s). Thus, these data were analyzed with a *Bayesian Generalized Linear Mixed-Effect Model* (Bayesian GLMM, *bglmer* function of *blme* package) with a binomial distribution (*logit* link). The type of sequence and presentation order were set as fixed effects in the model, while bee id and day were set as random factors. When necessary, models were optimized with the iterative algorithm *BOBYQA*. To determine the significant parameters of the models, the *anova* function (*car* package) was used and the best model was selected based on the *Akaike information criterion* (AIC).

In all the analyses, an α-value of 0.05 was specified. All the analyses were conducted with R Studio (R, 4.1.3 version).

4.3 Results

4.3.1 Experiment 1 – ABA absolute conditioning paradigm

The aim of this experiment was to study whether honeybees could learn an odor sequence and then spontaneously generalize their responses to novel odor sequences having a similar or a different structure. Honeybees were individually trained to associate the ABA odor sequences with positive reinforcement. Then, during the test phase, bees were presented with three different sequences: the trained one (i.e., ABA), a sequence having a different structure but composed of the same odorants (i.e., BAA), and a new sequence having the same structure, but composed of new odorants (i.e., CDC).

In the training phase, honeybees ($n = 47$) successfully learned to respond to the ABA sequence. In particular, there was a significant improvement in the PER response between the first and last trial of training (Bayesian GLMM: PER response ~ number of trials + (1|bee) + (1|day); Trial 1 *vs.* Trial 5: *z-value* = 7.06, *p-value* < 0.001, Fig.9a).

At *memory* test (i.e., test to investigate memory formation at 1 hour after training), a significant difference between sequences was found (Bayesian GLMM: PER response ~ type of sequence + (1|bee) + (1|day); ABA *vs.* BAA: *z-value* = - 2.16, *p-value* = 0.03; ABA *vs.* CDC: *z-value* = -3.99, *p-value* < 0.001; BAA *vs.* CDC: *z-value* = -3.29, *p-value* = 0.001; Fig. 9b). Equally, significant differences among the three tested sequences were found at *recall* test (i.e., test to investigate memory formation the morning after training) (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); ABA *vs.* BAA: *z-value* = 4.5, *p-value* < 0.001; ABA *vs.* CDC: *z-value* = -5.48, *p-value* < 0.001; BAA *vs.* CDC: *z-value* = -5.91, *p-value* < 0.001; Fig. 9c).

Bees were thus able to learn and memorize the trained sequence (ABA), albeit without being able to generalize their response to novel odors presented with the same structure (CDC). When presented with the novel BAA sequence, bees showed however an increased response at *recall* test.

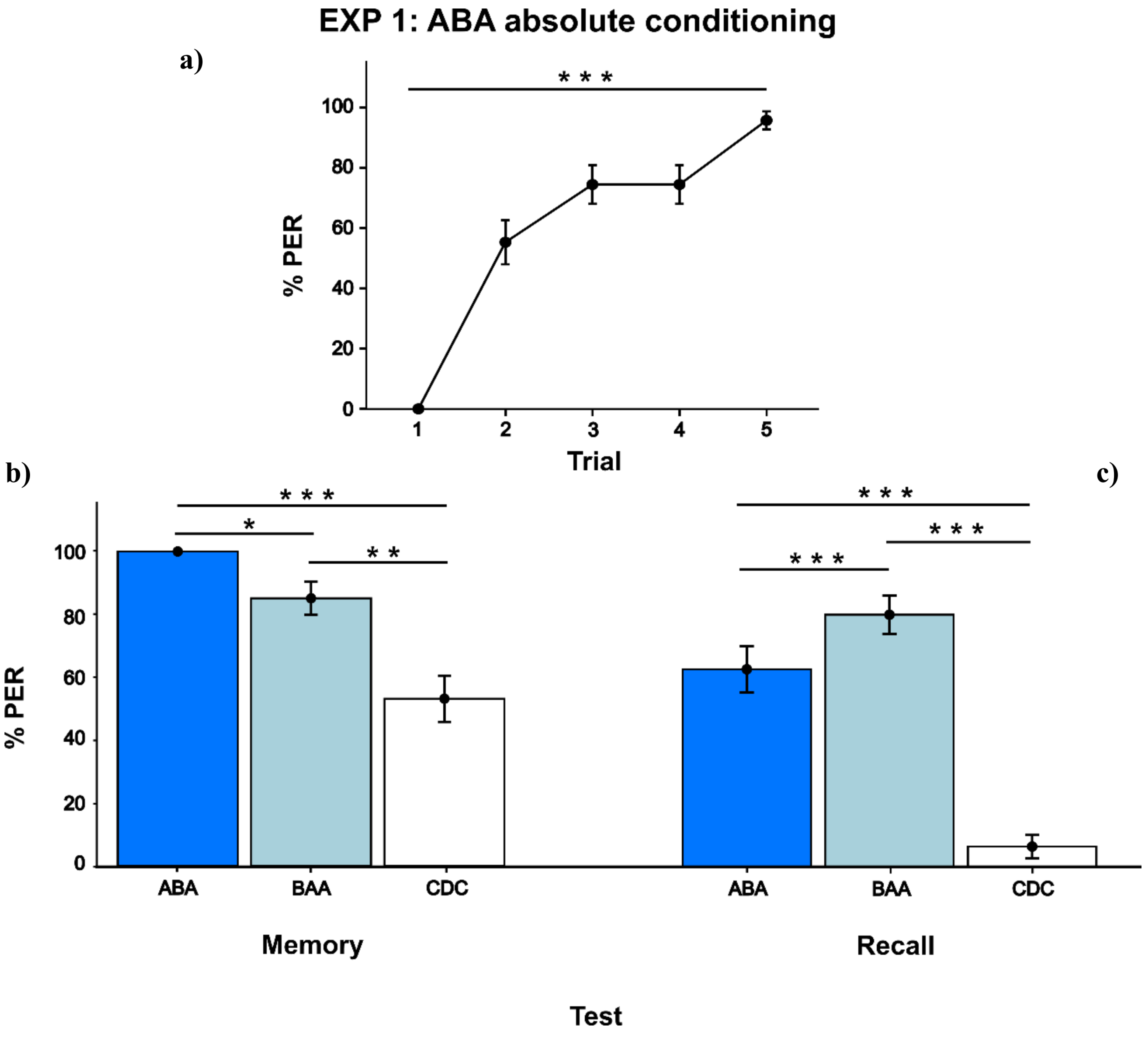


FIGURE 9: Experiment 1 learning and test performance (N=47). ***a)*** Learning curve of bees trained to show the PER response when the ABA sequence (i.e., 3-hexanol – acetophenone – 3-hexanol) was presented. Data shown are means ± SEM. Bees increased their percentage of response from the first to the last trial. ***b)*** Performance during the *Memory* test (1 hour after the ending of training). ***c)*** Performance during the *Recall* test (completed the morning after the training). Data shown are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.3.2 Experiment 2 – CDC absolute conditioning paradigm

This experiment was a control replica of Experiment 1, to confirm the results previously obtained using a different set of odors. Honeybees were individually trained to associate the CDC odor sequences with positive reinforcement. Then, during the test phase, bees were presented with three different sequences: the trained one (i.e., CDC), a sequence having a different structure but composed of the same odorants (i.e., DCC) and a new sequence having the same structure but composed of new odorants (i.e., ABA).

In the training phase, honeybees (n = 48) successfully learned to respond to the CDC sequence with a significant improvement in the PER response between the first and last trial of training (Bayesian GLMM: PER response ~ number of trials + (1|bee) + (1|day); Trial 1 *vs.* Trial 5: *z-value* = 6.98, *p-value* < 0.001, Fig.10a).

At *memory* test, a significant difference between CDC and ABA sequences (Bayesian GLMM: PER response ~ type of sequence + (1|bee) + (1|day); CDC *vs.* ABA: *z-value* = -3.84, *p-value* < 0.001) and between DCC and ABA sequences was found (DCC *vs.* ABA: *z-value* = -3.84, *p-value* < 0.001). Conversely, no differences were found between the response to the CDC and DCC sequences at test (CDC *vs.* DCC: *z-value* = -0.21, *p-value* = 0.83, Fig. 10b).

At *recall* test, significant differences between sequences were found (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); CDC *vs.* DCC: *z-value* = 5.08, *p-value* < 0.001; CDC *vs.* ABA: *z-value* = -3.83, *p-value* < 0.001; DCC *vs.* ABA: *z-value* = -5.19, *p-value* < 0.001; Fig. 10c).

The results confirmed a lack of spontaneous generalization between the trained and novel sequences despite having the same structure (i.e., CDC *vs.* ABA). Again, we observed an enhanced response to the novel DCC sequence at *recall* test.

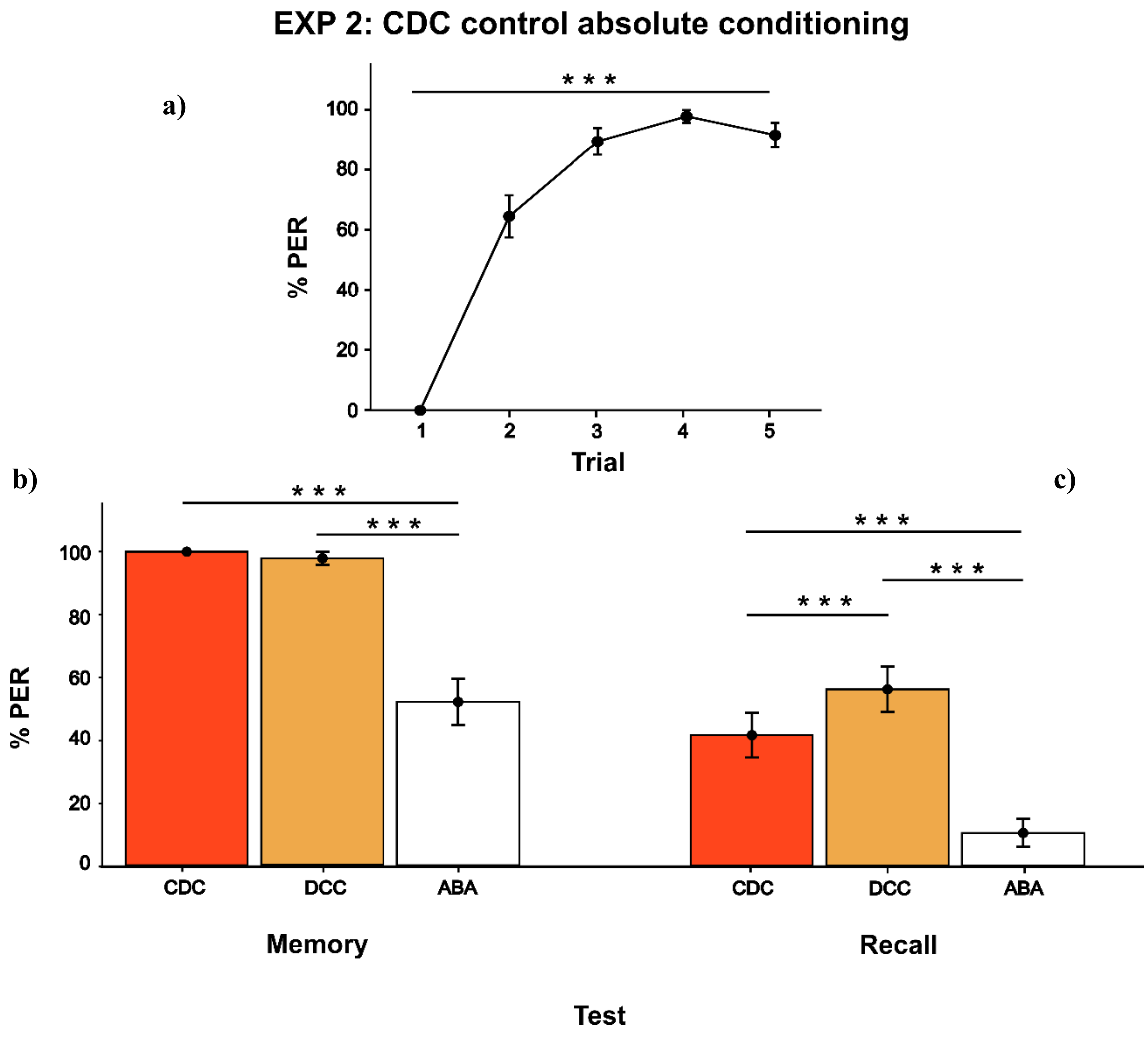


FIGURE 10: Experiment 2 learning and test performance (N=48). ***a)*** Learning curve of bees trained to show the PER response when the CDC sequence (i.e., 1-nonanol – citral – 1-nonanol) was presented. Data shown are means ± SEM. Bees increased their percentage of response from the first to the last trial. ***b)*** Performance during the *Memory* test (1 hour after the ending of training). ***c)*** Performance during the *Recall* test (completed the morning after the training). Data shown are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.3.3 Experiment 3 – ABA control absolute conditioning paradigm

In experiment 3, we investigated whether some elements of the ABA sequence were memorized better thanks to their internal position in the sequence and temporal closeness to the reward. Thus, we trained honeybees to learn the association between the ABA sequence and positive reinforcement. Then, during the test phase, bees were presented with three different sequences: the trained one (i.e., ABA), a sequence composed of the A odor only (i.e., AAA), and a sequence composed of the B odor only (i.e., BBB).

Results showed that bees (n = 41) were successfully able to perform the task, by significantly increasing their response to odors during training (Bayesian GLMM: PER response ~ number of trials + (1|bee) + (1|day); Trial 1 *vs.* Trial 5: *z-value* = 6.17, *p-value* < 0.001, Fig. 11a).

At *memory* test, honeybees responded significantly less to the AAA sequence with respect to the ABA sequence (Bayesian GLMM: PER response ~ type of sequence + (1|bee) + (1|day); ABA *vs.* AAA: *z-value* = -2.59, *p-value* = 0.009) but not to the BBB sequence (Bayesian GLMM: PER response ~ type of sequence + (1|bee) + (1|day); ABA *vs.* BBB: *z-value* = -1.58, *p-value* = 0.11). No significant differences between the novel sequences were found (Bayesian GLMM: PER response ~ type of sequence + (1|bee) + (1|day); AAA *vs.* BBB: *z-value* = 1.26, *p-value* = 0.21, Fig. 11b).

Analysis of the *recall* test showed no significant differences between models, and the simplest model was selected to describe the data (GLMM: PER response ~ 1 + (1|id), Fig. 11c). This highlighted a non-significant effect of the type of sequence as a factor.

We investigated whether the results previously obtained at *recall* test of experiments 1 and 2 (i.e., higher response to novel sequences composed of the same odors with respect to the trained ones) could be explained by a different encoding of one element of the sequence (i.e., odor A or odor B) due to their position in the sequence and closeness to the reward delivery. The results of *recall* test did not support this hypothesis since a similar response to the A and B odors was found.

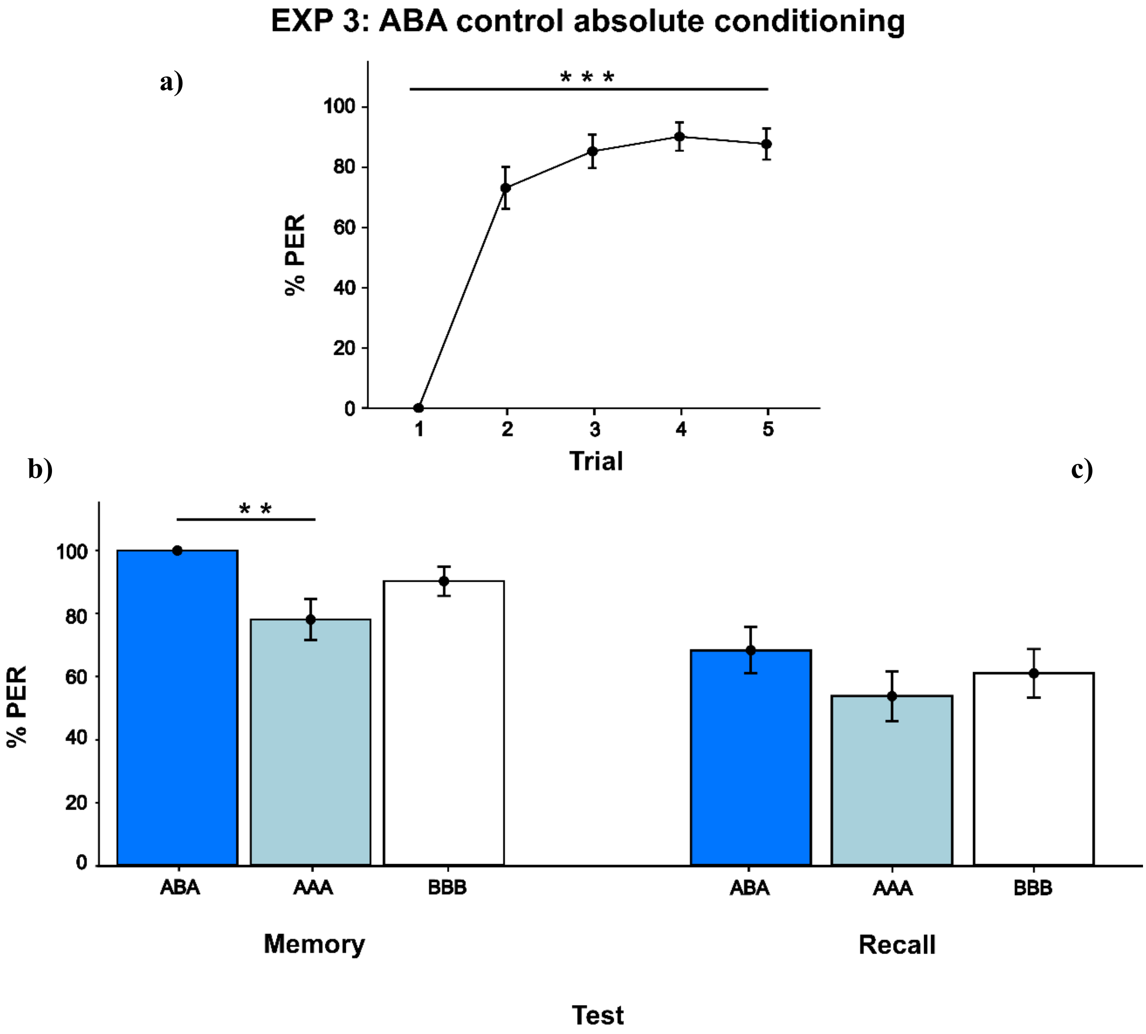


FIGURE 11: Experiment 3 learning and test performance (N=41). ***a)*** Learning curve of bees trained to show the PER response when the ABA sequence (i.e., 3-hexanol – acetophenone – 3-hexanol) was presented. Data shown are means ± SEM. Bees increased their percentage of response from the first to the last trial. ***b)*** Performance during the *Memory* test (1 hour after the ending of training). ***c)*** Performance during the *Recall* test (completed the morning after the training). Data shown are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.3.4 Experiment 4 – ABA *vs.* BAA differential conditioning paradigm

The purpose of Experiment 4 was to determine whether the specific learning of the structure of an odor sequence could be obtained by exploiting a differential conditioning paradigm. Two independent groups of honeybees (group 1: n = 36; group 2: n = 30) were trained to discriminate between the ABA and BAA sequences (first group: ABA reinforced, BAA non-reinforced; second group: BAA reinforced and ABA non-reinforced). Then, at test, their response to the previously reinforced and non-reinforced sequences was scored, together with their spontaneous PER response to congruent (i.e., CDC for the first group; DCC for the second group) and incongruent (i.e., DCC for the first group; CDC for the second group) sequences composed of novel odors.

Results showed a significant effect on the number of trials, with a positive increase from the first to the last trial (Bayesian GLMM: PER response ~ number of trials + type of sequence + number of trials*type of sequence + (1|bee) + (1|day); trial 1 *vs.* trial 5: *z-value* = 7.88, *p-value* < 0.001). However, no differences between sequences were found (Bayesian GLMM: PER response ~ number of trials + type of sequence + number of trials*type of sequence + (1|bee) + (1|day); ABA reinforced *vs.* BAA non-reinforced: *z-value* = -1.58, *p-value* = 0.11; ABA reinforced *vs.* BAA reinforced: *z-value* = 0.64, *p-value* = 0.52; ABA reinforced *vs.* ABA non-reinforced: *z-value* = -1.61, *p-value* = 0.11; ABA non-reinforced *vs.* BAA reinforced: *z-value* = 1.86, *p-value* = 0.063; ABA non-reinforced *vs.* BAA non-reinforced: *z-value* = -0.5, *p-value* = 0.62; BAA reinforced *vs.* BAA non-reinforced: *z-value* = -1.26, *p-value* = 0.21). The interaction term showed a significant impact on data. When we analyzed the crucial comparisons (i.e., between reinforced and non-reinforced sequences presented to the same group), post-hoc analyses revealed only a difference between ABA reinforced and BAA non-reinforced sequences in the second trial (post-hoc: *z-ratio* = -2.13, *p-value* = 0.03) and between BAA reinforced and ABA non-reinforced in the first trial (post-hoc: *z-ratio* = 2.23, *p-value* = 0.03). This was not considered a reliable indicator of the capacity of bees to discriminate between reinforced and non-reinforced sequences, as this difference was not maintained across the subsequent trials.

Thus, we collapsed the two experimental groups, creating two new sequence categories (i.e., reinforced, and non-reinforced sequences), and analyzed the data. Again, the number of trials had a significant impact on the bees' performance, with an improvement from the first to the last trial (Bayesian GLMM: PER response ~ number of trials + (1|bee) + (1|day); Trial 1 *vs.* Trial 5: *z-value* = 10.76, *p-value* < 0.001; Fig. 12a). The best model did not include the sequence factor (i.e., reinforced *vs.* non-reinforced sequences), suggesting the inability of bees to discriminate between them.

During the test phase, four different sequences were presented in probe conditions: previously reinforced (i.e., ABA for group 1, BAA for group 2), previously non-reinforced (i.e., BAA for group 1, ABA for group 2), congruent (i.e., sequence with new odor and same structure of the previously reinforced one: CDC for group 1, DCC for group 2) and incongruent (i.e., sequence with new odor and same structure of the previously non-reinforced one: DCC for group 1 and CDC for group 2).

At *memory* test, no differences were found between the reinforced and non-reinforced sequences, (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); reinforced *vs.* non-reinforced: *z-value* = -1.25, *p-value* = 0.21; Fig. 12b). The response to the congruent and incongruent sequences was not significantly different (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); congruent *vs.* incongruent: *z-value* = -1.19, *p-value* = 0.23). Bees showed a significantly lower PER response to both congruent and incongruent sequences with respect to the reinforced and non-reinforced ones (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); reinforced *vs.* congruent: *z-value* = -5.79, *p-value* < 0.001; reinforced *vs.* incongruent: *z-value* = -5.68, *p-value* < 0.001; non-reinforced *vs.* congruent: *p-value* = -6.1, *p-value* < 0.001; non-reinforced *vs.* incongruent: *z-value* = -5.91, *p-value* < 0.001; Fig. 12b).

Similar results were found at *recall* test, where bees did not differentiate between the reinforced and non-reinforced sequences (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); reinforced *vs.* non-reinforced: *z-value* = 0.59, *p-value* = 0.55; Fig. 12c) and between the congruent and incongruent sequences (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); congruent *vs.* incongruent: *z-value* = -0.47, *p-value* = 0.64). Significant differences were found between novel congruent and incongruent sequences compared to the reinforced and non-reinforced ones (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); reinforced *vs.* congruent: *z-value* = -4.38, *p-value* < 0.001; reinforced *vs.* incongruent: *z-value* = -4.33, *p-value* < 0.001; non-reinforced *vs.* congruent: *p-value* = -4.46, *p-value* < 0.001; non-reinforced *vs.* incongruent: *z-value* = -4.4, *p-value* < 0.001; Fig. 12c).

In summary, in experiment 4 honeybees neither discriminated between ABA and BAA sequences nor generalized their response to novel congruent (i.e., having the same structure as the previously reinforced one) or incongruent (i.e., having the same structure as the previously non-reinforced sequence) sequences.

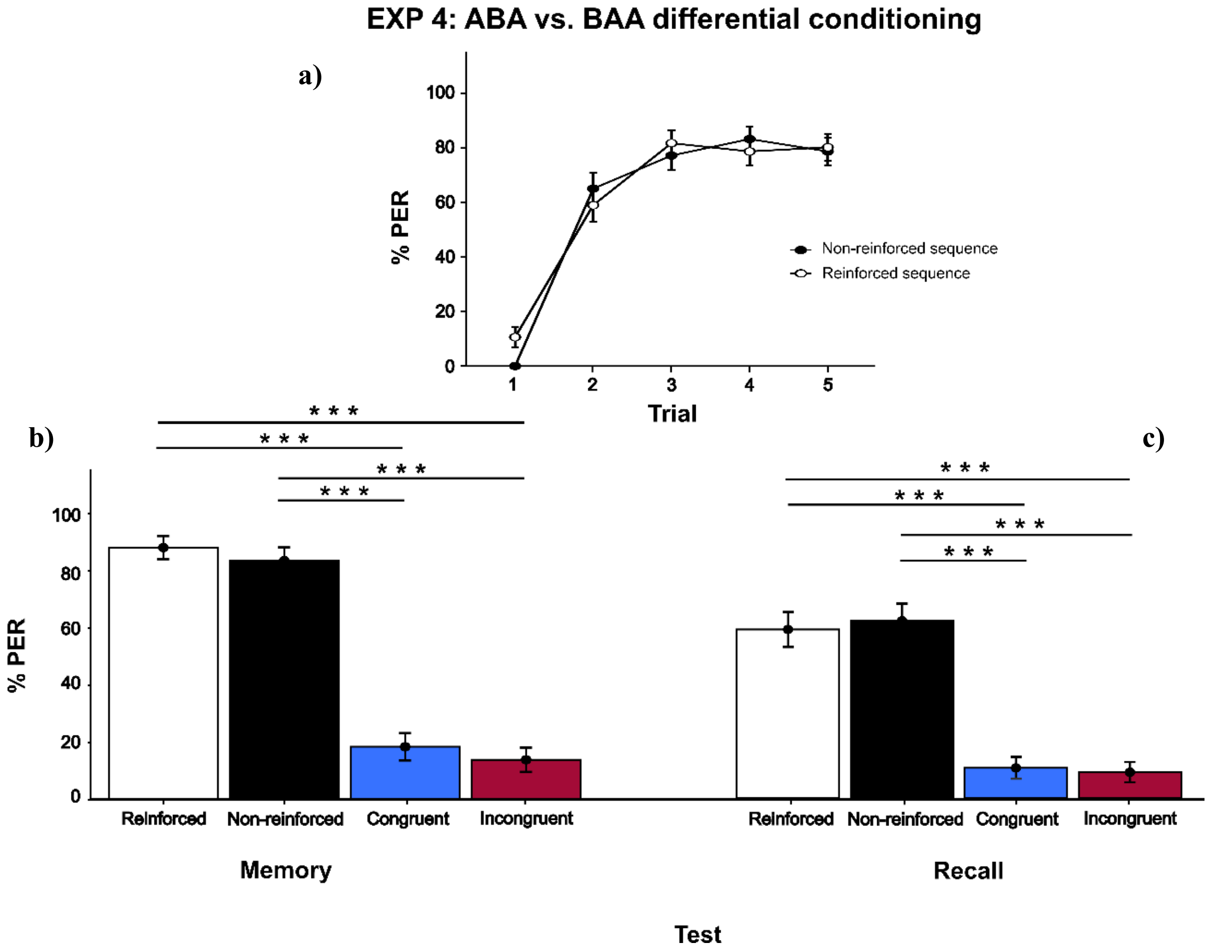


FIGURE 12: Experiment 4 learning and test performance (N=66). ***a)*** Learning curve of bees trained to discriminate between the ABA (i.e., 3-hexanol – acetophenone – 3-hexanol) and BAA (i.e., acetophenone – 3-hexanol – 3-hexanol) sequences. *Reinforced* and *non-reinforced* sequences were defined as sequences associated during the training with sucrose solution and water, respectively. *Congruent* and *incongruent* sequences were defined as sequences composed of novel odors having the same structure as previously reinforced and non-reinforced sequences, respectively. Data shown are means ± SEM. Bees did not show any differences between the two sequences. ***b)*** Performance during the *Memory* test (1 hour after the ending of training). ***c)*** Performance during the *Recall* test (completed the morning after the training). Data shown are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.3.5 Experiment 5 – ABA *vs.* AAB differential conditioning paradigm

In this experiment, we aim to investigate whether the last element of the sequence was more informative and thus used by bees to discriminate between two odor structures. We trained two independent groups of honeybees (group 1: n = 20; group 2: n = 18) to discriminate between the ABA and AAB sequences. The procedure was the same as described for experiment 4. We also encoded the occurrence of the PER response in relation to the presentation of a specific element of the sequence (i.e., first, second, or third element).

At training, results showed a significant effect of the number of trials, with an increase in response from the first to the last trial (Bayesian GLMM: PER response ~ number of trials + type of sequence + (1|bee) + (1|day); Trial 1 *vs.* Trial 5: *z-value* = 8.36, *p-value* < 0.001). The model highlighted a difference between the two reinforced sequences (Bayesian GLMM: PER response ~ number of trials + type of sequence + (1|bee) + (1|day); ABA reinforced *vs.* AAB reinforced: *z-value* = -2.69, *p-value* = 0.007). However, no other differences among sequences were found (Bayesian GLMM: PER response ~ number of trials + type of sequence + (1|bee) + (1|day); ABA reinforced *vs.* ABA non-reinforced: *z-value* = -1.13, *p-value* = 0.26; ABA reinforced *vs.* AAB non-reinforced: *z-value* = -1.91, *p-value* = 0.056; AAB reinforced *vs.* ABA non-reinforced: *z-value* = 1.89, *p-value* = 0.06; AAB reinforced *vs.* AAB non-reinforced: *z-value* = 1.17, *p-value* = 0.24; ABA non-reinforced *vs.* AAB non-reinforced: *z-value* = 0.29, *p-value* = 0.77)

The significant difference between the two rewarded sequences was not considered a reliable indicator of differences in the ability of bees to better learn one of the sequences over the other. Honeybees of both groups could not discriminate between rewarded and non-rewarded sequences during training. Thus, we decided to proceed with the analysis of the totality of data, collapsing the two groups together. The rationale behind that decision was the lack of evidence that one of the two groups was able to discriminate the presented sequences during the training.

Thus, we created two new sequence categories, as experiment 4 (i.e., reinforced, and non-reinforced sequences), and analyzed the data. The number of trials had a significant impact on the bees' performance, with an improvement from the first to the last trial (Bayesian GLMM: PER response ~ number of trials + (1|bee) + (1|day); Trial 1 *vs.* Trial 5: *z-value* = 8.31, *p-value* < 0.001; Fig. 13a). The model did not report any significant impact of the sequence factor (i.e., reinforced *vs.* non-reinforced) on the data.

During the test phase, we presented the four different sequences in probe conditions: previously reinforced (i.e., ABA for group 1, AAB for group 2), previously non-reinforced (i.e., AAB for group 1, ABA for group 2), congruent (i.e., sequence with new odor and same structure of the previously reinforced one: CDC for group 1, CCD for group 2) and incongruent (i.e., sequence with new odor and same structure of the previously non-reinforced one: CCD for group 1 and CDC for group 2).

At *memory* test, no differences were found neither between the reinforced and non-reinforced sequences (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); reinforced *vs.* non-reinforced: *z-value* = 1.6, *p-value* = 0.11) nor between congruent and incongruent sequences (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); congruent *vs.* incongruent: *z-value* = -0.52, *p-value* = 0.6). Conversely, bees responded significantly less to congruent and incongruent sequences with respect to the reinforced and non-reinforced ones (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); reinforced *vs.* congruent: *z-value* = -2.81, *p-value* = 0.005; reinforced *vs.* incongruent: *z-*

value = -3.15, *p-value* = 0.002; non-reinforced *vs.* congruent: *z-value* = -3.25, *p-value* = 0.001; non-reinforced *vs.* incongruent: *z-value* = -3.45, *p-value* < 0.001; Fig. 13b).

At *recall* test, bees successfully discriminate between reinforced and non-reinforced sequences (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); reinforced *vs.* non-reinforced: *z-value* = -2.13, *p-value* = 0.03), and between reinforced, non-reinforced, and congruent, incongruent sequences (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); reinforced *vs.* congruent: *z-value* = -4.31, *p-value* < 0.001; reinforced *vs.* incongruent: *z-value* = -4.49, *p-value* < 0.001; non-reinforced *vs.* congruent: *z-value* = -3.6, *p-value* < 0.001; non-reinforced *vs.* incongruent: *z-value* = -3.88, *p-value* < 0.001; Fig. 13c). Again, no differences in the PER response to congruent and incongruent sequences emerged (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); congruent *vs.* incongruent: *z-value* = -0.74, *p-value* = 0.46).

As in experiment 4, honeybees trained to discriminate between ABA and AAB sequences did not show a spontaneous generalization of response to novel congruent and incongruent sequences (i.e., having the same structure of previously reinforced and non-reinforced sequences, respectively). Bees confirmed the inability to discriminate between ABA and AAB sequences during the training and *memory* test phase. However, this ability emerged during the *recall* test, suggesting the use of different strategies based on particular elements of the sequence in long-term memory formation (see Discussion).

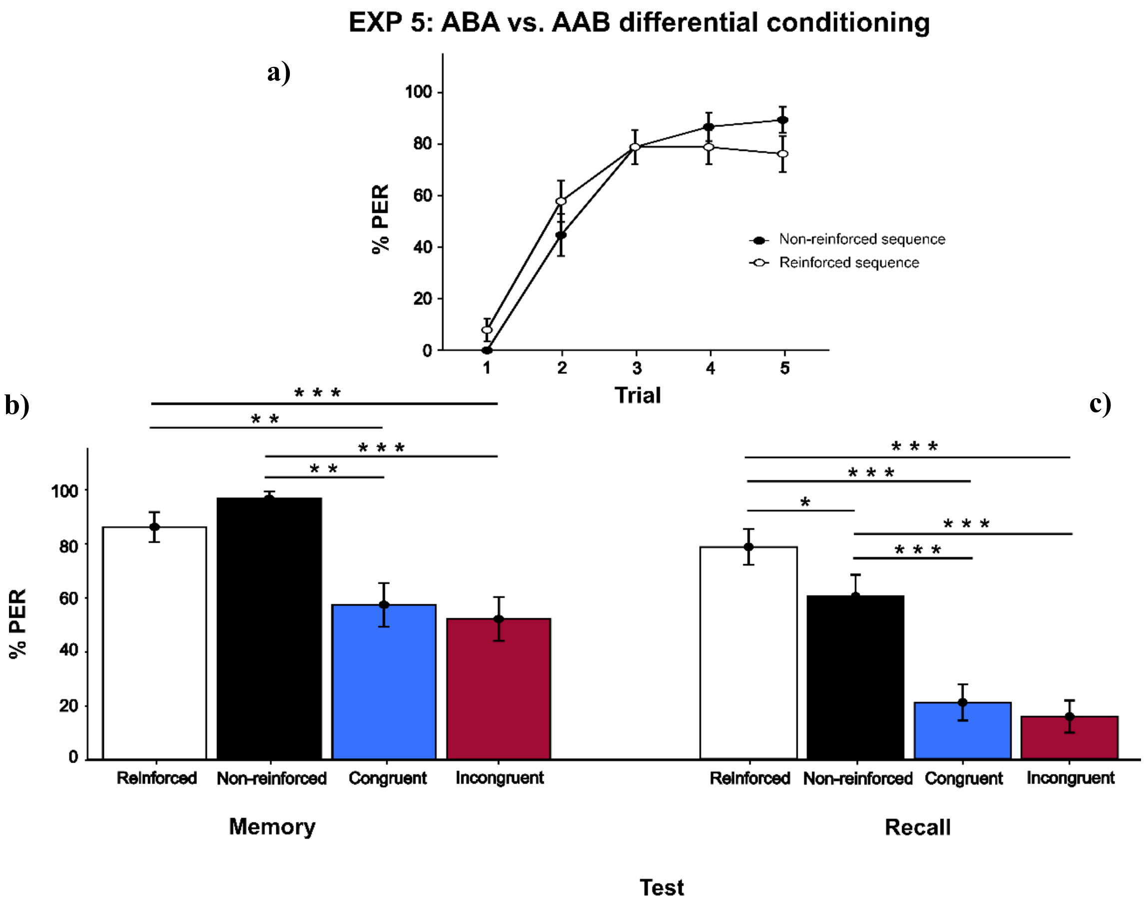


FIGURE 13: Experiment 5 learning and test performance (N=38). ***a)*** Learning curve of bees trained to discriminate between the ABA (i.e., 3-hexanol – acetophenone – 3-hexanol) and AAB (i.e., 3-hexanol – 3-hexanol - acetophenone) sequences. *Reinforced* and *non-reinforced* sequences were defined as sequences associated during the training with sucrose solution and water, respectively. *Congruent* and *incongruent* sequences were defined as sequences composed of novel odors having the same structure as previously reinforced and non-reinforced sequences, respectively. Data shown are means ± SEM. Bees did not show any differences between the two sequences. ***b)*** Performance during the *Memory* test (1 hour after the ending of training). ***c)*** Performance during the *Recall* test (completed the morning after the training). Data shown are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.3.6 Experiment 6 – Generalization conditioning paradigm

In this experiment, a group of honeybees (n = 84) was trained to learn to respond to two sequences having the same structure but different odors (i.e., ABA and CDC). Then, the ability of bees to generalize to a new odor sequence having the same internal structure (i.e., EFE) and to differentiate it from a sequence having a different structure (i.e., FEE) was tested.

During the training phase, the best model highlighted a significant effect of the number of trials on the percentage of response, with a response accuracy increasing from the first to the last trial (Bayesian GLMM: PER response ~ number of trials + (1|bee) + (1|day); Trial 1 *vs.* Trial 8: *z-value* = 9.15, *p-value* < 0.001; Fig. 14a). The type of odor presented (i.e., ABA - 3-hexanol/acetophenone/3-hexanol - and CDC - 1-nonanol/citral/1-nonanol) was not highlighted as a significant factor by models, suggesting that during training, honeybees responded equally to the two sequences.

At *memory* test, we investigated the proportion of bees responding to the trained sequences (i.e., ABA, CDC) and novel ones, having either new odors with the same (i.e., EFE) or different structures (i.e., FEE). Honeybees responded significantly less to the latter sequence with respect to the trained ones (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); ABA *vs.* FEE: *z-value* = -2.46, *p-value* = 0.014; CDC *vs.* FEE: *z-value* = -2.46, *p-value* = 0.014), but not to the new sequence having the same structure (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); ABA *vs.* EFE: *z-value* = -0.87, *p-value* = 0.38; CDC *vs.* EFE: *z-value* = -0.87, *p-value* = 0.38). No differences were found between the EFE and FEE sequences during the test (GLMM: PER response ~ type of sequence + (1|bee) + (1|day); EFE *vs.* FEE: *z-value* = -1.65, *p-value* = 0.09; Fig. 14b).

Results of *recall* test showed a significant effect of type of sequence (GLMM: PER response ~ type of sequence + order of presentation + (1|bee) + (1|day); ABA *vs.* CDC: *z-value* = -2.22, *p-value* = 0.03; ABA *vs.* EFE: *z-value* = -4.11, *p-value* < 0.001; ABA *vs.* FEE: *z-value* = -4.69, *p-value* < 0.001; CDC *vs.* EFE: *z-value* = -2.12, *p-value* = 0.03; CDC *vs.* FEE: *z-value* = -2.96, *p-value* = 0.003; EFE *vs.* FEE: *z-value* = -1.01, *p-value* = 0.31; Fig. 14c) and order of presentation (GLMM: PER response ~ type of sequence + order of presentation + (1|bee) + (1|day); first *vs.* second: *z-value* = 0.97, *p-value* = 0.33; first *vs.* third: *z-value* = 2.07, *p-value* = 0.04; first *vs.* fourth: *z-value* = 2.52, *p-value* = 0.01; second *vs.* third: *z-value* = 1.27, *p-value* = 0.20; second *vs.* fourth: *z-value* = 1.67, *p-value* = 0.09; third *vs.* fourth: *z-value* = 0.37, *p-value* = 0.71; Supplementary Figure S16).

Results of *memory* test showed a decreased response for the sequence composed of novel odors presented with a novel sequence (i.e., FEE), compared to sequences having a familiar structure irrespective of the constituent odors (i.e., ABA, CDC, EFE). At *recall* test, this result was not replicated since a generally higher response to ABA and CDC sequence with respect to the novel ones (i.e., EFE, FEE) was found.

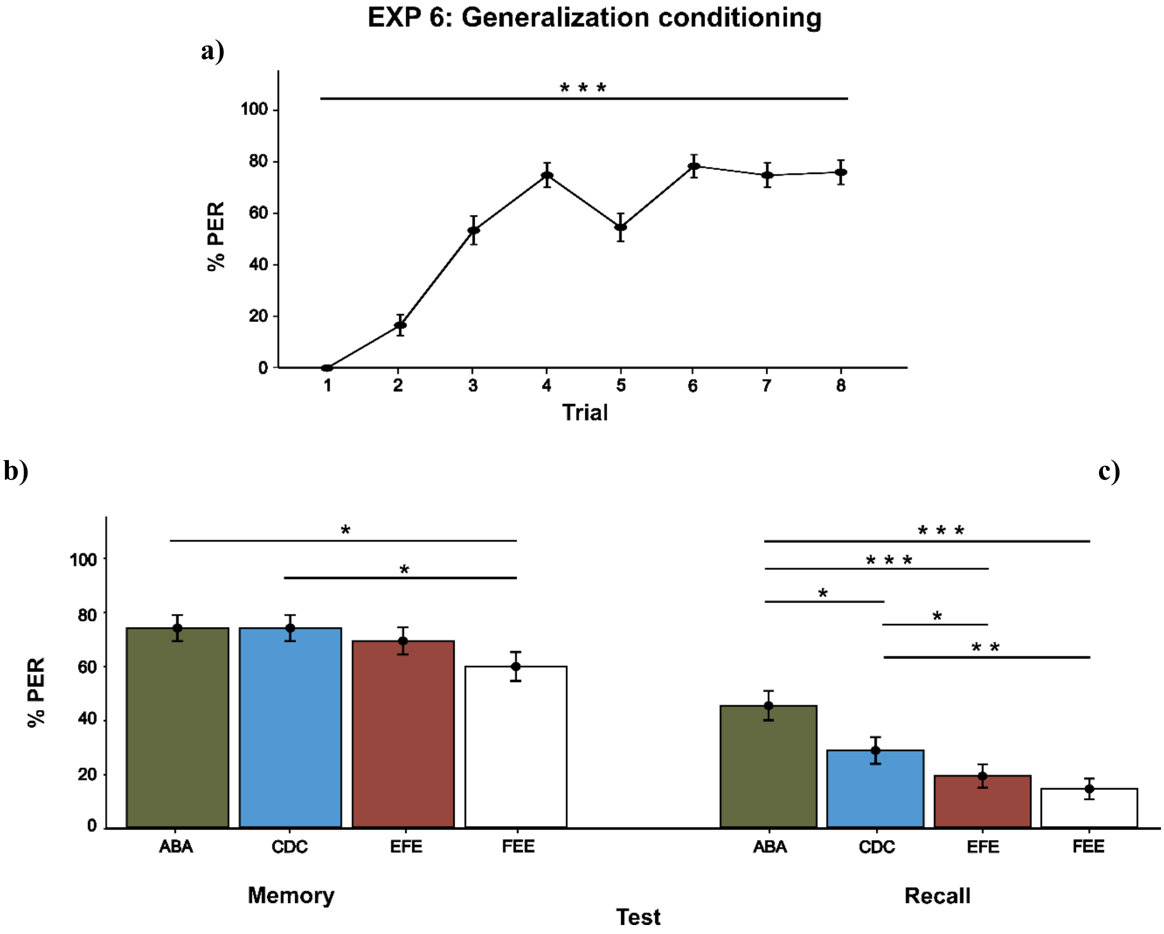


FIGURE 14: Experiment 6 learning and test performance (N=84). ***a)*** Learning curve of bees trained to show the PER response when the ABA and CDC sequences (i.e., ABA: 3-hexanol – acetophenone – 3-hexanol; CDC: 1-nonanol – citral – 1-nonanol) were presented. Data shown are means ± SEM. Bees increased their percentage of response from the first to the last trial, without any differences between the proportion of response to the two sequences. ***b)*** Performance during the *Memory* test (1 hour after the ending of training). ***c)*** Performance during the *Recall* test (completed the morning after the training). Data shown are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.4 Discussion

We investigated whether honeybees could extract an underlying temporal odor regularity from the environment. To this aim, we adapted the classical PER paradigm and observed bees' ability to extrapolate an underlying structure of consecutively presented odors forming a sequence. Our results do not strongly support the hypothesis about the existence of this ability in honeybees, as they rather favoured the use of alternative strategies when available.

Experiments 1 and 2 revealed that bees associated the trained sequences to positive reinforcement (Fig. 9a, 10a) without, however, being spontaneously able to generalize this response to novel odors presented with the same structure (i.e., CDC, Exp. 1, Fig. 9b, 9c; ABA, Exp. 2, Fig. 10b, 10c). In these conditions, honeybees' behavior suggested a primary encoding of the individual odor properties instead of the entire sequence order presentation. Interestingly, bees also showed a significant increase in response to novel-structured sequences at *recall* test (i.e., BAA, Exp. 1, Fig. 9c; DCC, Exp. 2, Fig. 10c). This outcome was not driven by a different retrieval of one of the two odors constituting the sequence stimulus (see Results of Exp.3, *recall* test). It might be argued that bees could have learned a A+B odor mixture compound, instead of a sequential ABA odor presentation where each elemental component has a unique position in the sequence. First and foremost, we temporally separate the three sequential odor puffs, providing a continuous air flow to clean the environment and minimize any possible odor overlapping. Furthermore, results from the first two experiments might partially answer this concern. If bees were mainly learning to respond to a compound formed by mixed odors (e.g., A+B rather than ABA in Exp. 1; C+D rather that CDC in Exp. 2), we would not have expected to observe the significantly different percentage of response between trained sequences (i.e., ABA, Exp. 1; CDC, Exp.2) and novel structure sequences composed of familiar odors in the *recall* test of Experiment 1 and 2 (i.e., BAA, Exp. 1; DCC, Exp. 2) as they equally carried the ‘mixture’ cue, despite the temporal order of their composing elements.

When we analyzed the exact moment of PER display, bees preferentially responded to both the A and B odors presented in the first position, suggesting again a similar encoding of the two odors irrespective of their ordering structure and temporal closeness to the reward (Supplementary Figure 14). The existence of better memorization of the peculiar dyad composed of second and third elements (e.g., BA, DC) could be another possible explanation for this result. This option might have been favored by the stricter temporal contingency of the second and third elements with the sucrose provided as reinforcement (i.e., just before and during the sucrose delivery, respectively). From this perspective, the results of the fourth and fifth experiments can further support the idea of the importance of this dyad (i.e., second and third elements), as honeybees were only able to discriminate between ABA and AAB sequences (*recall* test Exp. 5, Fig. 13c), where this dyad was not present in one of the sequences.

Observation of the exact moment of PER display (Exp. 5) suggested the presence of an additional *response to the odor change* strategy. In fact, when novel odors were presented (i.e., CDC, CCD; *recall* test of Exp. 5), a considerable number of bees responded to the D odor, namely the odor different from the first one presented (C; Supplementary Figure S15). Similarly, when the trained ABA and AAB stimuli were presented, bees were more likely to respond not only to the first odor (A) but also to the subsequent different odor (B) irrespective of the position of the latter (either second or third in the sequence; Supplementary Figure S15), supporting a possible spontaneous use of this strategy. Arguably, a generalization response among citral (D) and 3-hexanol (A) or between citral (D) and acetophenone (B) could explain these results. However, past research demonstrated bees' discrimination ability between citral and 1-hexanol compounds (Laska et al., 1999). Since also a strong generalization at both behavioral (Guerrieri et al., 2005) and neuronal levels between different hexanols (i.e., 1-hexanol, 2-hexanol, 3-hexanol; Paoli et al., 2018) has been suggested, we could acknowledge a low probability for bees to generalize their response between citral and 3-hexanol in our study. Likewise, citral and acetophenone have different neuronal activity, sustained by specific glomeruli activation in the bee brain (Joerges et al., 1997; Paoli et al., 2018). The low generalization response between sequences in Exp. 1 and 2 (i.e., ABA *vs.* CDC) also supports the idea that an overlapping among odors was unlikely to be present in our research.

Honeybees showed a weak response congruent with structure similarity (i.e., ABA, CDC, and EFE) and dissimilarity strategy (i.e., FEE) in experiment 6 (*memory* test, Fig. 14b). The capacity to generalize the structure across different odors was present only one hour after training. When we tested the long-term memory capacity, a higher response for the two trained sequences (i.e., ABA, CDC) was found, together with a significant difference between them (*recall* test, Fig. 14c). This latter difference might be caused by a more robust memorization of the 3-hexanol and acetophenone odors (composing the ABA stimulus) compared to 1-nonanol and citral odors (composing the CDC stimulus). Results of the *recall* test of experiments 1 and 2 support this hypothesis, with the proportion of bees responding to the ABA-trained sequence (61,7%, Exp. 1; Fig. 9c) being higher than the proportion of subjects responding to the CDC-trained sequence (41,7%, Exp. 2; Fig. 10c). We did not find a significant difference between novel stimuli with either a familiar or a non-familiar structure (i.e., EFE, FEE). This might be an effect of the relatively short training (i.e., 8-trials training providing only two odor sequences with the same structure). Future research should investigate whether a more extended and diverse training procedure could enhance the ability of bees to generalize their response and differentiate between novel odors with familiar and unfamiliar structures. In visual discrimination task, the use of appetitive-aversive conditioning has been shown to enhance honeybees' solving performance (Avarguès-Weber et al., 2010). Unfortunately, the use of quinine solution as aversive stimulus is not fully supported during PER conditioning paradigm due to its toxicity rate (just under 60% of mortality for 100mM quinine concentration; Wright et al., 2010) .

The experimental design of this study involved a presentation of odor stimuli that might appear to be as not ecologically relevant, for it could be argued that foragers might not encounter temporally separated odor bouquets during their flight, so that any direct comparison between our experimental conditions and natural behavior may appear weak. However, to investigate the ability of bees to learn and generalize among sequences, it is mandatory to use a design that allows for rigorous control over independent variables (e.g., the timing of stimuli occurrence) and presentation of ecologically salient sequences with which honeybees could create an association with positive reinforcement (i.e., odor sequences). Thus, the main goal of our experimental design was not to mimic natural behavior and study the underlying cognitive process but to provide an effective experimental method to investigate the presence of a specific cognitive ability in an insect species, shedding light on our understanding of bees' cognition.

Our results do not provide conclusive evidence on the ability of bees to extrapolate regularities from olfactory temporal sequences and generalize these structures to novel stimuli. Indeed, the type of experimental paradigm of conditioning used was crucial to highlight the use of different strategies when bees were presented with our sequence discrimination task. This should not be surprising as it has been demonstrated that the use of different conditioning procedures (i.e., absolute or differential) could determine variations in stimuli discrimination, with higher discrimination performance with appetitive-aversive differential conditioning (Avarguès-Weber and Giurfa, 2014).

The ability to process, learn, and generalize an abstract rule to novel stimuli has been mainly investigated in vertebrate species (Chen et al., 2015; Stobbe et al., 2012; Versace et al., 2017). Previous studies suggested the existence of abstraction abilities of bees (Avargues̀-Weber et al., 2012; Avarguès-Weber et al., 2011; Giurfa et al., 2001). Our results do not provide further and clear demonstration of the ability of bees to extrapolate temporal contingencies and successfully generalize that knowledge to novel stimuli. Rather, our findings support the implementation of simpler rules to address an odor sequence discrimination task. Still, we do not exclude the possibility that the ability to extrapolate a structure regularity might be present or better developed in other sensory modalities (i.e., visual, tactile) in honeybees.

CHAPTER 5

GENERAL CONCLUSION

Everything started with our eyes. In fact, the first evidence claiming bees are complex living creatures with intricate life came from ethological studies where their behavior was observed and then precisely described (Turner, 1910; von Frisch, 1914). Over the decades, the adaptation and standardization of behavioral, neurobiological, neuroimaging, and genetic techniques allowed a deeper understanding of honeybees' cognitive capacities and their evolution. I studied such abilities by using two different, well-known paradigms: the training of flight towards visual targets and the classical conditioning of the proboscis extension response to olfactory stimulation (Giurfa, 2003a).

The aim of this thesis was to investigate the presence of two cornerstones of cognition in honeybees: quantity processing and extrapolation of regularities from the environment. As to the first topic, I gained evidence for a similar, abstract encoding of continuous and discrete quantities (i.e., magnitudes) in honeybees. For the first time, we could speculate on the existence of a general mechanism allowing magnitude processing in an insect species. This result is crucial to set a step forward to the understanding of quantity processing, with a focus on *number sense*. The second part of this thesis reported the results of an investigation of temporal odor sequence processing in honeybees. Albeit bees already proved to the capable of deep levels of abstraction and categorization, their ability to extrapolate the sequential structure of odor stimuli was never assessed. The results reported here failed in reporting the existence of an ability to extrapolate temporal odor regularities in honeybees, suggesting that this level of abstract might not be supported by such miniature brains, at least in the olfactory domain.

Specifically, in the first two studies I provided new data about bees' ability to similarly process different dimensions (i.e., size and numerousness). The hypothesis of the existence of a general cognitive mechanism that supports magnitude processing (i.e., dimensions that can be “more or less than” in time, space, or number) was proposed by Gallistel (1989) and later included in a more general framework by Walsh (2003). Such a mechanism would have a high adaptive value for vertebrates and invertebrates, as it would enable animals to make arithmetic operations across domains (Gallistel, 1989; Walsh, 2003). This theory has been supported by several pieces of evidence from both humans and other vertebrate species demonstrating the presence of similar magnitude processing at both behavioral (De Corte et al., 2017; Lourenco and Longo, 2010; Meck and Church, 1983; Merritt et al., 2010; Miletto Petrazzini and Brennan, 2020; Roberts and Mitchell, 1994) and neural levels (see Walsh, 2003 for a review). In the first study, I investigated whether bees could transfer a particular rule (i.e., choose the larger/smaller item) from a discrete (number) to a continuous (size) dimension. Independent groups of free-flying bee foragers were presented with four different numerical contrasts having either a 0.67 or 0.5 ratio difference and were trained to select either the smaller or larger numerosity to get the food reward. Their ability to transfer such knowledge to the size dimension was then tested by presenting bees with a comparison between relatively larger and smaller elements having identical numerical properties (e.g., 4 relatively larger *vs.* 4 small elements). Bees spontaneously selected the congruent novel size dimension (i.e., bees trained to select the smaller/larger numerosity, also selected the smaller/larger size). Interestingly, bees were able to make that transfer regardless of their training (i.e., select the larger or smaller numerousness), numerical contrasts and ratio difference experienced. The results of this study provided evidence that this magnitude processing mechanism might be a property in common between vertebrates and invertebrates (Bortot et al., 2020).

The ATOM theory suggests similar processing of dimensions such as size, time, and number. However, whether their interactions are always symmetrical is still debated (Walsh, 2015). The first study provided data suggesting a unilateral relationship from the numerical to the size dimension in honeybees (Bortot et al., 2020). However, whether this interaction is bidirectional, as already demonstrated in other vertebrate species (De Corte et al., 2017; Lourenco and Longo, 2010; Merritt et al., 2010; Miletto Petrazzini and Brennan, 2020), was not known. In the third chapter of this thesis, I presented the results describing bees' ability to make a transfer from a continuous (size) to a discrete (number) dimension. A two-independent group design was used where free-flying foragers were trained to discriminate between three relatively larger and three smaller elements (i.e., squares, diamonds). Their rule generalization ability over novel shapes (i.e., three larger *vs.* three smaller circles) and novel dimensions (i.e., numerosity) was confirmed by the test phase. This provides the first evidence of an insect’s ability to make a transfer from a continuous to a discrete dimension (Bortot and Vallortigara, 2023).

In both studies, it might be argued that these results could have been affected by the use of other perceptual variables by the bees, instead of categorizing our stimuli only on the basis of an abstract relationship (i.e., larger and smaller dimension). Indeed, continuous features such as total area, contour length, convex hull (i.e., the area occupied by the elements), density, and inter-stimulus distance have a direct relationship with numerosity and size: the larger the latter, the greater the formers (Zanon et al., 2020). In the first study, those variables were carefully manipulated during the numerical training, to prevent bees from relying on them instead of extrapolating the numerical value of the arrays (Bortot et al., 2020). Our results are in line with previous studies reporting bees' discrimination ability based on arrays' numerousness despite the type of perceptual variables controlled (Bortot et al., 2019; Gross et al., 2009; Howard et al., 2018; Howard et al., 2019a). Thus, we can claim that bees discriminated our training stimuli based on their numerosity and then made a transfer to size without using other perceptual variables. In the second study, bees were first trained to discriminate between three relatively larger and smaller elements. In this context, as only inter-item distance was equated, a bee trained to select the dimensionally larger set could have achieved this task by using different strategies involving either the processing of perceptual features (area, perimeter, convex hull, single element dimensions, spatial frequency) or abstract the dimensional relationship between them. Unfortunately, it is not possible to vary such continuous variables when presenting discrimination between sizes, as those are truly embedded in this dimension. When bees were tested for their ability to transfer the acquired rule to the numerical dimension, we created different sets of numerical tests. Using the GeNEsIS software developed in our lab (Zanon et al., 2022), we presented three 4 *vs.* 8 elements comparisons where we controlled different perceptual variables separately: total area, total perimeter, convex hull, and element size. The GeNEsIS software permits careful simultaneous control over several variables at the pixel level (Zanon et al., 2022). The aim was to check whether bees were possibly relying on perceptual features – and if so, which ones - to solve both the size discrimination and the subsequent numerical one. If on the contrary, we would have observed a general congruent resolution of the numerical contrast, we could have hypothesized a spontaneous use of a more abstract rule (i.e., choose the smaller/larger quantity, irrespective of its dimension – either size or number). The results showed an overall significant choice towards the congruent numerosity irrespective of the perceptual variables available, suggesting the ability of bees to make a cross-dimensional transfer from continuous to discrete dimension. However, a closer look revealed a slight decrease in performance when the total contour length was equated between numerical arrays (Bortot and Vallortigara, 2023). Despite this being a non-significant observation, we could speculate on the role of perimeter as a primary visual cue used by honeybees. Indeed, honeybees prefer shapes bearing larger contour lengths (Hertz, 1929a) and their discrimination ability decreases as the contour length similarity between two element shapes increases (Hertz, 1929b). However, the perimeter was demonstrated not to be the only feature accountable for pattern discrimination ability of bees (Hertz, 1935) as it was further postulated that the perimeter-to-area ratio (i.e., contour density; Anderson, 1977) might account for shape similarity perception ($\frac{P}{A} < 1$ indicates dissimilarity; $\frac{P}{A} > 1$ indicates similarity) (Anderson, 1977). The *contour density* hypothesis has been proposed to account for shape discrimination ability in honeybees (Anderson, 1977), but not for other visual pattern discrimination. Indeed, when the perimeter feature was carefully equated between arrays bearing different numerical (Gross et al., 2009) or shape information (Hertz, 1935) bees were able to solve the task. An alternative explanation for the slightly lower performance at the *numerical perimeter* test could better account for the observed performance. The geometrical constraints set for this stimulus (i.e., perimeter and convex hull equated between arrays and the presence of both the larger and smaller elements in both sets) led to the creation of a conflict between the dimensional property of the stimuli (i.e., their numerical value) and their visual configuration. The numerically smaller set, composed of three large and one small element, and the numerically larger one, composed of only one large and seven small elements, could have been perceived as more similar to the size sets with the larger and smaller elements experienced during the training (Bortot and Vallortigara, 2023).

Bees can discriminate between stimuli based on their differences in spatial frequency (i.e., the number of dark/light cycles per spatial unit (Hertz, 1933; MaBouDi et al., 2021). We assessed whether this parameter could explain the results observed in our second study. The spatial frequency directly correlated with the elements' size during training (i.e., the larger elements had the larger spatial frequency) but could not account for bees' performance at the numerical tests. The spatial frequency was directly related to the numerousness of the array only when the elements' size was equated (*number same size* test), while it indirectly covaried with numerosity when contour length was controlled (*number total perimeter* test). Spatial frequency was almost identical when the total area was equated between arrays (*number total area* test) (Bortot and Vallortigara, 2023).

In the presented experiments, a crucial aspect involves the reinforcement and punishment disposal. In fact, a recent study suggested that bumblebees seem to visually differentiate sucrose solution from water (Kienitz et al., 2022), even though whether this might be applicable also to honeybees is still unknown. Regardless the interesting point raised by Kienitz et al. (2022), their results are not fully transferable to the studies presented here and previous literature on bee visual abilities. One notable difference is the use of appetitive-aversive conditioning procedures in the studies presented in this thesis, involving sucrose solution and quinine solution. While there is no indication yet about bees' ability to discriminate between these two substances, the second study of this thesis (Chapter 3), implemented further measures to address this possibility. Precautions were taken by placing the substance drops in capsules, limiting the possible visual interaction of the subjects with the substances. In the study by Kienitz et al. (2022) the substance drops were placed at 15 mm distance, thus allowing the bees to simultaneously inspecting them before the choice. In both studies presented in this thesis, the substances were presented in association with a visual stimulus positioned at 10 cm distance from the decision chamber. This arrangement prevented simultaneous access to the visual stimulation (either stimulus or substance drop). Moreover, the eventuality of bees applying a discrimination rule based on potential visual differences between substances (e.g., spectral difference, light scattering; Kienitz et al., 2022) is controlled by the subsequent test phase. In this phase only water is provided, ensuring that any potential learning of visual physical characteristics of substances should result in chance-level performance. Hence, it is reasonable to exclude the possibility that bees solved our tasks by relying on a "simpler" discrimination between substances.

Overall, our results and careful controls are inclined to suggest the existence of a "*common mental currency*" (Gallistel, 1989) allowing a simultaneous processing of continuous and discrete dimensions (i.e., size and number) in an insect species. Nevertheless, the existence of such a mechanism supporting the encoding of different quantities was mainly proposed for vertebrate species (Walsh, 2015, 2003). Its universality and evolution in phylogenetically more distant organisms, such as insects, were only broadly hypothesized in the absence of direct scientific evidence (Gallistel, 1989; Walsh, 2003). The behavioral suggestion of such ability and the existence of neurobiological and imaging tools in honeybees can open new frontiers in the understanding of the neural mechanisms involved, their characterization, and their function. However, it is crucial to point out that we are basing our claims on relatively low percentages of correct choices (ranging from 55.14% to 57.16% in the first study and

52.84% in the second study). Further experiments and additional replicates are profoundly needed to strongly supporting our claims that, up to now, are limited to the studies and data reported here.

In the third chapter of this thesis, I presented an investigation of the ability of bees to extrapolate an odor sequence structure and abstract that knowledge to correctly generalize their response towards familiar structures composed of unfamiliar odors. In the past decades, the existence of the ability to extrapolate and generalize visual and auditory regularities was mainly studied in several vertebrate species (Endress et al., 2009; Santolin et al., 2016; Sonnweber et al., 2015; Spierings and Cate, 2016; Versace et al., 2019, 2017). Our results present an investigation of this topic where honeybees, an insect species, is the main character. We used three versions of the *proboscis extension response* (PER) classical conditioning paradigm to investigate the effect of the type of training on bees' capacities, i.e., absolute, differential, and generalization paradigms. The obtained results do not firmly suggest a primary encoding of the sequence structure. Interestingly, we pointed out the adoption of relatively simpler strategies to solve this task, such as specific odor learning irrespective of order presentation, a response to odor change, and partial encoding of the structure, depending on the implementation of different conditioning paradigms (see Chapter 4, *Discussion* section). Only the findings from the *memory* test of Experiment 6 might provide some support for the hypothesis that bees could possess the ability to encode the structure of a rewarded sequence regardless of its specific odor components when employing a generalization strategy. However, this conclusion is mainly based on the significant difference between the two previously rewarded sequences (i.e., ABA, CDC) and the novel sequence having an unfamiliar structure and novel composing odors (i.e., FEE). Unfortunately, this result was not maintained across time, disappearing ≈ 18 hours after the training. The missing significant difference between the EFE and the FEE sequences at *memory* test might again cast doubt on bees' true encoding of the sequence structure (see Chapter 4, *Discussion* section). From an experimental point of view, our investigation confirmed the crucial influence of the conditioning procedure used, that can shape the animal behavior and strategic choice to succeed in the selected task. The use of different conditioning methods has already been proven to affect bees' decision-making, with higher correct choices obtained after an appetitive-aversive differential conditioning procedure in visual task discrimination (Avarguès-Weber and Giurfa, 2014).

The discovery of alternative strategy implementation is pivotal for understanding honeybees learning processes, the construction of abstract knowledge, and which experimental tools are necessary to let it occur. Avarguès-Weber et al. (2011) initially proposed the existence of an abstract *above/below* categorization rule in honeybees during a visual discrimination task based on element's spatial relationship. However, a more recent study highlighted that honeybees might have solved the above-mentioned task by using different strategies based on the perceptual properties of the stimuli, instead of relying on a contextual relationship categorization (Guiraud et al., 2018). In both studies, bees learned to discriminate visual stimuli based on the spatial relationship between a shape and a referent line (i.e., shape above the referent for the "above" condition; shape below the referent for the "below" condition). High-speed videography analysis in the later study (Guiraud et al., 2018) suggested that bees might have solved the task by employing a scanning behavior of the lower visual item before making a decision, without necessarily relying on a more abstract conceptual rule as proposed by Avarguès-Weber et al. (2011). The results obtained by Guiraud et al. (2018), did not necessarily discredit the findings of Avarguès-Weber et al. (2011), as they observed one subject that appeared to solve the task according to the proposed *above/below* abstract generalization strategy, indicating potential individual differences in strategy utilization. The variation in experimental protocols between these studies might have also influenced their different outcomes and data interpretation (Guiraud et al., 2018).

The implementation of behavioral strategies implying “simpler” mechanism do not necessary rule out the possibility that bees have access to more cognitively demanding abilities. In fact, the development of different mechanisms supporting low and high-cognitively demanding behaviors could be adaptive in ecological situations (MaBouDi et al., 2020b). After extensive training, bumblebees trained in a *small* vs. *large* visual shape discrimination, opted for a simpler win-stay/lose-switch strategy, albeit still retaining the learning of a relative size rule (MaBouDi et al., 2020b). The implementation of the “simpler” strategy would have allowed bees to cope with more cognitively demanding task by engaging in less-cognitively approaches and, at the same time, speeding up the foraging processes, enhancing individual’s food gain (MaBouDi et al., 2020b). Nevertheless, the learning of a relative size rule (i.e., discriminate between relatively smaller and larger items) would have been useful in the first stages of learning to successfully avoid incorrect items associated with quinine solution (MaBouDi et al., 2020b).

The discovery and exploration of alternative behaviors is crucial in expanding our knowledge in the field of animal behavior. This further step is already feasible thanks to the application of advanced techniques (e.g., tracking software, closed-loop virtual environment). The implementation of rigorous observations of animal behavior will enhance our capacity for objective investigations, and further scientific accuracy in describing natural occurrences, transcending prior limitations and broadening our understanding of the ecology and evolution of different species.

While the studies presented in this thesis aimed at investigating honeybees cognitive abilities, there are essential considerations often overlooked, such as species ecology, how the data obtained would be comprehensive of the ability of the species, and the practical implications of the results. The focus of the described investigations was to understand specific cognitive abilities in bees involving an equal magnitude processing and abstraction of external structures, which are potentially vital for their environmental adaptation. In fact, the existence of a common mental currency allowing animals to perform arithmetic operation across domains (Gallistel, 1989) could be of high ecological impact for honeybees as pollinators. As briefly discussed in the third chapter, bees could have developed this ability (likely supported by a defined neural circuit) to assess different dimension simultaneously during ecologically relevant situations, as foraging. For instance, a bee forager would need to assess the number of flowers on a site or landmarks encountered to reach that position, as well as the time spent visiting flower sources and their size (Gallistel, 1989). All of this processing would be done at the same time to have a broader knowledge of the external environment. Also, in the fourth chapter of this thesis, I investigated the existence of another ecologically relevant capacity, namely the extrapolation of regularities from the environment. This capacity would be of high adaptive value by allowing animals to conform their behavior in response to stimuli that share common regular structures despite being composed by different elements, ultimately helping to discover the underlying environmental structure (Santolin et al., 2016). Bees are equipped with a minimal circuit favouring the construction of categorical and generalization processes rather than overloading the memory capacity (Avarguès

Weber et al., 2012). Moreover, bees communicate through a sensorimotor display known as the waggle dance, which comprises elements presented according to a sequential structure (von Frisch, 1967). Therefore, the existence of an abstraction capacity related to sequence structures in bees could be hypothesised.

When considering studies of bees visual cognitive abilities (see for instance: Avarguès-Weber et al., 2011; Giurfa et al., 2001; Howard et al., 2018), there exist a tendency to generalize findings to the entire *Apis mellifera* genus, without considering potential individual cognitive and experience differences. The sample sizes used in these investigations and in the ones reported here (i.e., between 20 and 32 honeybee foragers in the first and second study), seems poor if compared to the hundreds of thousands of individuals composing a typical bee colony. The constrains of the training procedure and the narrow temporal window available for experiment completion (i.e., summer period) often limits the number of individuals that can be tested, restricting the variation in sample size. Consequently, this limitation may confine the conclusions and subsequent hypotheses to the specific sample size tested, preventing a comprehensive view of the ecological variations and individual adaptations. While the results presented in the second and third chapter imply the existence of important and compelling abilities in bees that were only previously observed in vertebrates, there is a need to carefully consider the possibility that such capacities could be an attribute showed by only a minority of subjects. The majority of bees may display alternative responses that do not involve any complex cognitive process. Indeed, the use of different strategies and the existence of individual differences have been suggested in previous studies (Burns and Dyer, 2008; Finke et al., 2021; Guiraud et al., 2018).

The studies reported in this thesis were primarily intended to contribute new and compelling data on bees cognition, offering new support for evolutionary theories and expanding the knowledge about this species' biology. They were not planned to have a direct application in the agricultural and beekeeping industry. Nevertheless, the never-ending expansion of bee research contributes to increasing awareness of the complexity of this species. New discoveries about their biology, ecology and evolution will emphasize their importance and the crucial need of their protection on a global scale.

Altogether, the present thesis adds two pieces of evidence to the hallmark of bees' cognition. Honeybees were demonstrated to be an effective animal model to fill missing gaps in support of scientific theories and expand our understanding of the evolution of abilities in the absence of – what was thought – essential brain structures (i.e., cortex). Further exploration of the brain configuration and networks involved will give us a larger and clearer picture of the sufficient but essential mechanisms of cognition.

SUPPLEMENTARY MATERIALS

SM.1

TRANSFER FROM NUMBER TO SIZE REVEALS ABSTRACT CODING OF MAGNITUDE IN HONEYBEES

Supplemental figures and analyses

This chapter contains a modified version of the Supplemental Materials of the following scientific article: *Bortot, M., Stancher, G., & Vallortigara, G. (2020). Transfer from number to size reveals abstract coding of magnitude in honeybees. Iscience, 23(5).* The Journal Author Rights are reported in Appendix A.2.

Analyses Blind Video Coding

We performed an offline scoring in blind condition for the videos of the test phase and compared them with the original analysis (due to the corruption of two videos, 30 subjects were used in both conditions). The results are shown the Supplementary Figure S1 (offline blind scorings) and Figure S2 (original scoring) below together with statistical analyses and confirmed the original analyses.

For each group – *offline blind scoring* and *original* scoring - we calculate the percentage of choices for the larger numerosity during the *number learning* test and for the larger size during the *size generalization* test for each subject and analyzed. The data were checked for normality (*offline blind scoring*: Shapiro-Wilk normality test: W: 0.99, $P > 0.05$; *original scoring*: Shapiro-Wilk normality test: W: 0.98, $P > 0.05$) and homoscedasticity (*offline blind scoring*: Levene's test: $P > 0.05$; *original scoring*:

Levene's test: $P > 0.05$) and then analyzed with parametric statistical tests. An analysis of variance was performed with ratio (0.5 and 0.67), type of training (smaller *vs.* larger as positive), and type of test (*number learning* test *vs. size generalization* test) as factors. The effect of the numerical comparisons, as factors nested in ratio, was analyzed with a nested factorial Anova. The omega-squared effect size of any significant results was reported.

Results

Offline blind scoring

An analysis of variance revealed a significant main effect of the type of training (smaller *vs.* larger numerosity as positive; $F_{(1, 44)} = 35.9$, $P < 0.001$, $\omega^2 = 0.371$; Fig. S1) but not of the type of test (*number learning* test *vs. size generalization* test; $F_{(1, 44)} = 3.5$, $P = 0.069$) and of the ratio (0.5 *vs.* 0.67; $F_{(1, 44)} = 0.19$, $P > 0.05$). No significant interactions were observed (ratio x numerical comparisons: $F_{(2, 44)} = 1.7$, $P > 0.05$; ratio x type of training: $F_{(1, 44)} = 2.2$, $P > 0.05$; ratio x type of test: $F_{(1, 44)} = 1.06$, $P > 0.05$; type of training x type of test: $F_{(1, 44)} = 0.09$, $P > 0.05$; ratio x type of training x type of test: $F_{(1, 44)} = 1.2$, $P > 0.05$; ratio x type of training x numerical comparisons: $F_{(2, 44)} = 0.04$, $P > 0.05$; ratio x type of test x numerical comparisons: $F_{(2, 44)} = 0.1$, $P > 0.05$; ratio x type of training x type of test x numerical comparisons: $F_{(2, 44)} = 0.5$, $P > 0.05$).

Original scoring

An analysis of variance revealed a significant main effect of the type of training (smaller *vs.* larger numerosity as positive; $F_{(1, 44)} = 41.6$, $P < 0.001$, $\omega^2 = 0.389$; Fig.S2) and of the type of test (*number learning* test *vs. size generalization* test; $F_{(1, 44)} = 5.1$, $P = 0.029$, ω^2

= 0.039) but not of the ratio (0.5 *vs.* 0.67; $F_{(1, 44)} < 0.0005$, $P > 0.05$). No significant interactions were observed (ratio x numerical comparisons: $F_{(2, 44)} = 2.2$, $P > 0.05$; ratio x type of training: $F_{(1, 44)} = 2.3$, $P > 0.05$; ratio x type of test: $F_{(1, 44)} = 0.4$, $P > 0.05$; type of training x type of test: $F_{(1, 44)} = 1.1$, $P > 0.05$; ratio x type of training x type of test: $F_{(1, 44)} = 0.6$, $P > 0.05$; ratio x type of training x numerical comparisons: $F_{(2, 44)} = 0.8$, $P > 0.05$; ratio x type of test x numerical comparisons: $F_{(2, 44)} = 1.09$, $P > 0.05$; ratio x type of training x type of test x numerical comparisons: $F_{(2, 44)} = 0.06$, $P > 0.05$).

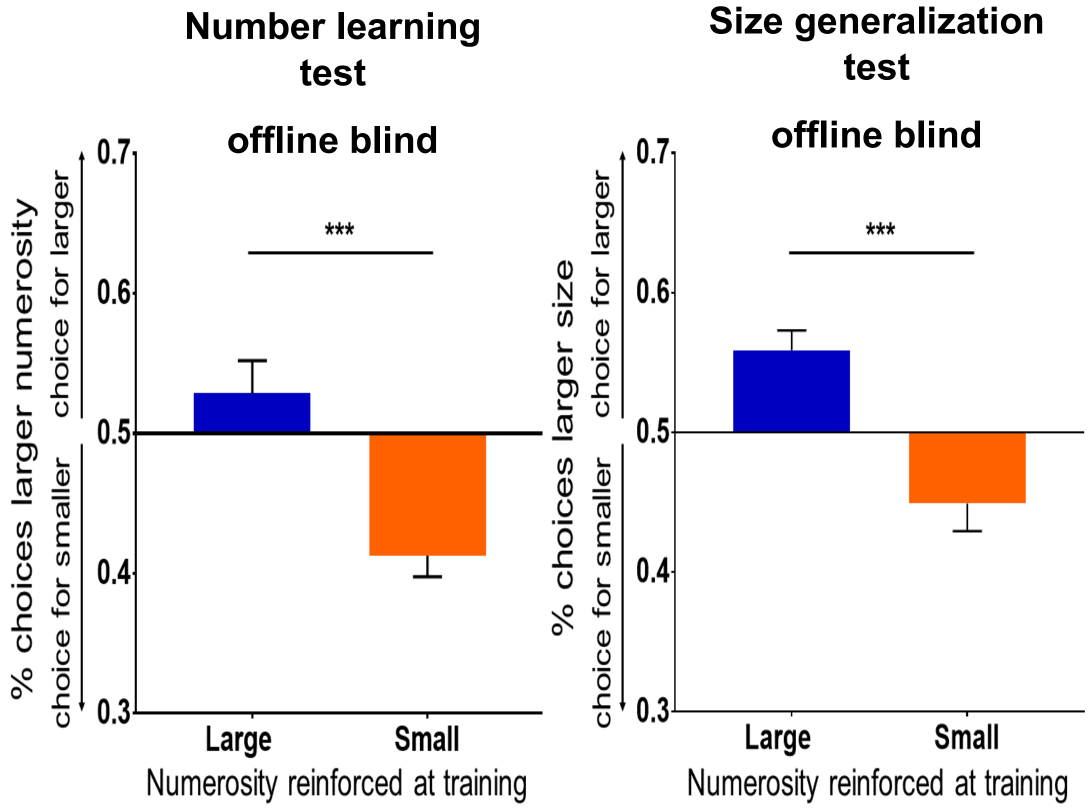


FIGURE S1: The offline scoring of the test phase of 30 subjects (*** $P < 0.001$, Analysis of variance (ANOVA))

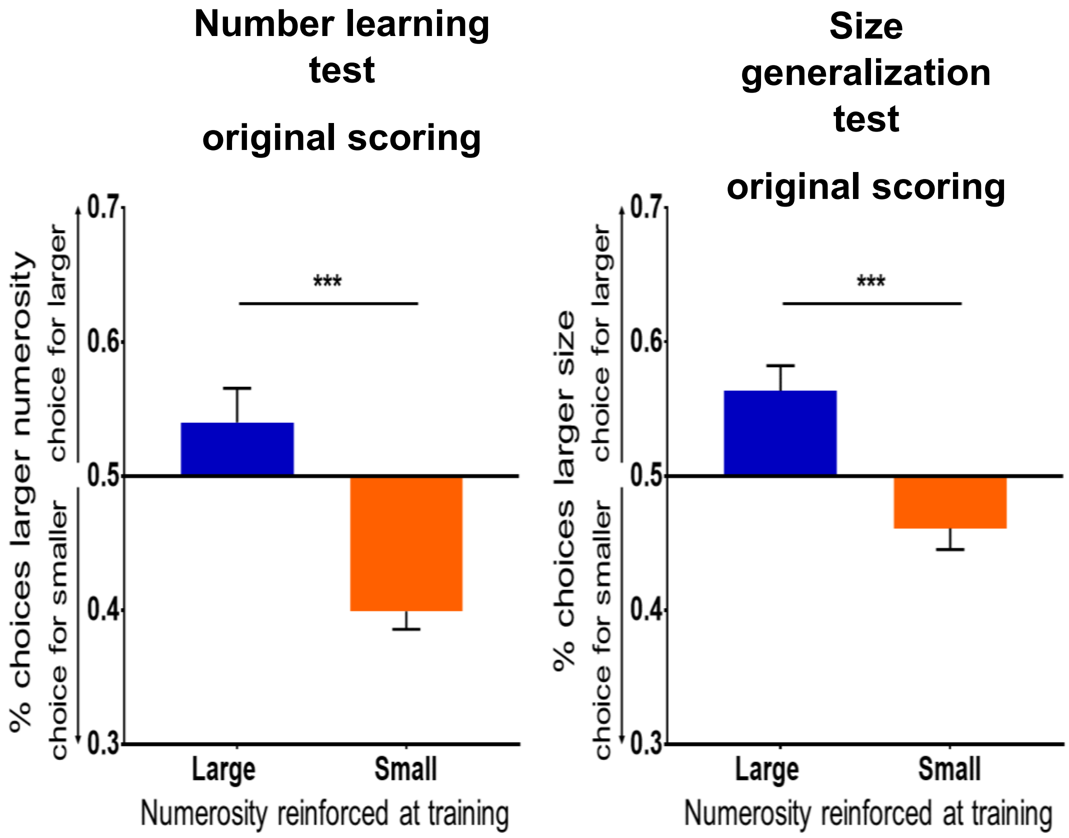


FIGURE S2: The original scoring of the test phase of 30 subjects (*** $P < 0.001$, Analysis of variance (ANOVA))

Figure S3. Numerical contrast 2 *vs.* 3, Related to Figure 4a.

The 90 stimuli pairs displaying the 2 *vs.* 3 numerical contrast. Stimuli are divided by shape categories: circles, diamonds, and squares. Within each shape, in one quarter of the pairs the cumulative surface area was matched to 100% whereas in the second quarter it was not controlled. In the third and the fourth quarter the contour length was matched to 100% and not controlled, respectively. Moreover, within each shape, half of the stimuli were controlled for the convex hull and the other half for the density of the elements.

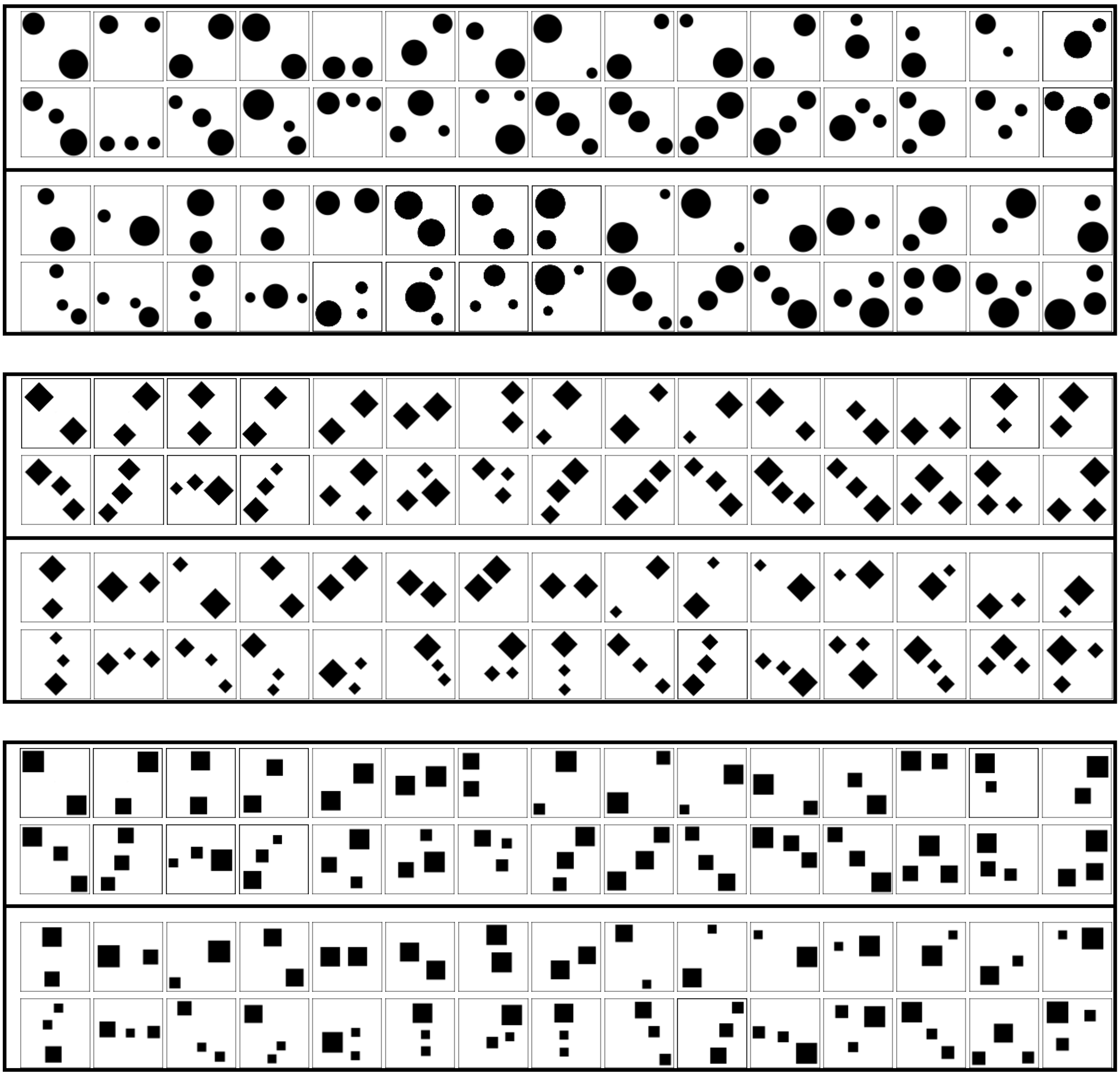


Figure S4. Numerical contrast 2 *vs.* 4, Related to Figure 4a.

The 90 stimuli pairs displaying the 2 *vs.* 4 numerical contrast. Stimuli are divided by shape categories: circles, diamonds, and squares. Within each shape, in one quarter of the pairs the cumulative surface area was matched to 100% whereas in the second quarter it was not controlled. In the third and the fourth quarter the contour length was matched to 100% and not controlled, respectively. Moreover, within each shape, half of the stimuli were controlled for the convex hull and the other half for the density of the elements.

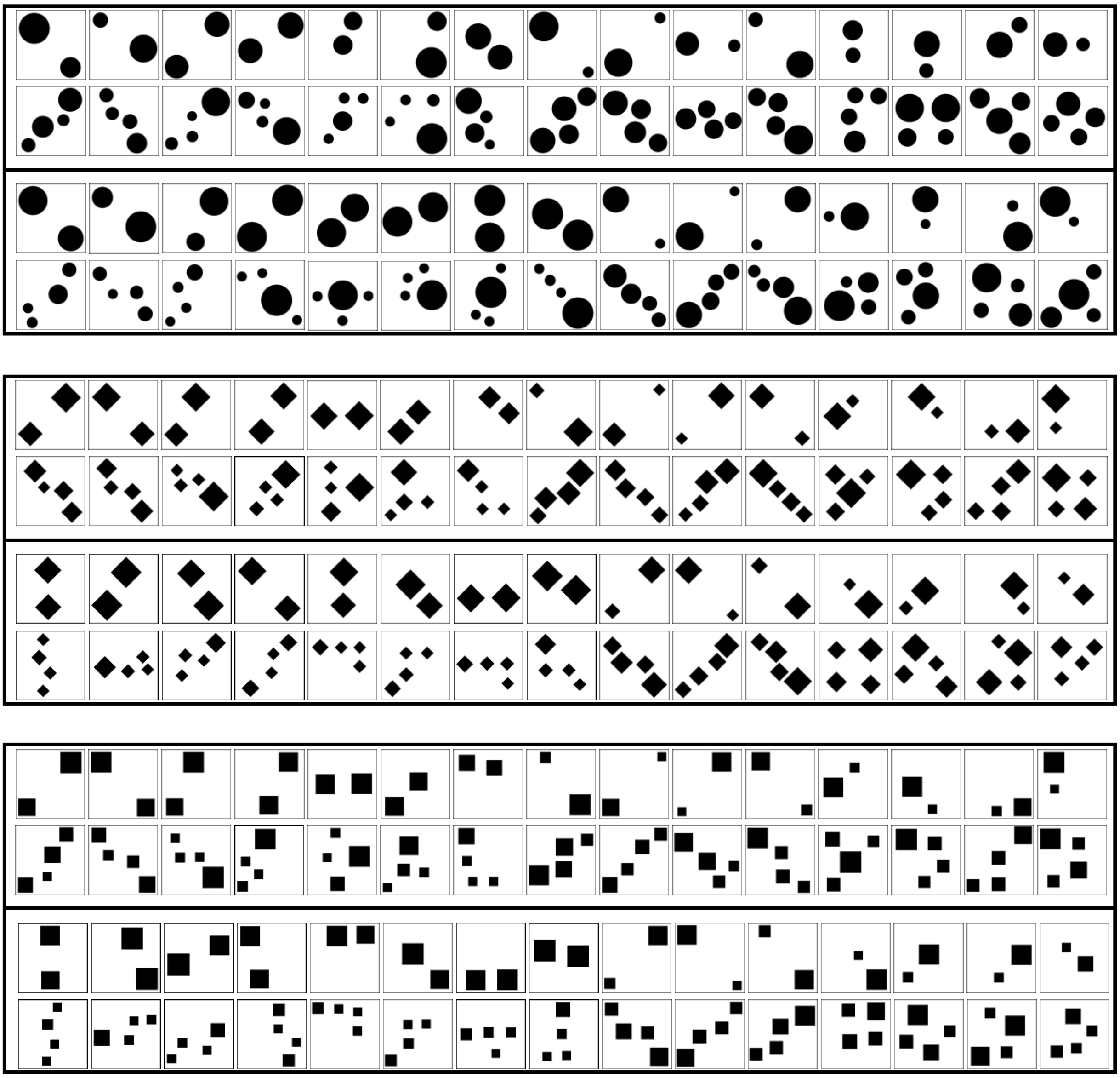


Figure S5. Numerical contrast 4 *vs.* 6, Related to Figure 4a.

The 90 stimuli pairs displaying the 4 *vs.* 6 numerical contrast. Stimuli are divided by shape categories: circles, diamonds, and squares. Within each shape, in one quarter of the stimuli the cumulative surface area was matched to 100% whereas in the second quarter it was not controlled. In the third and the fourth quarter the contour length was matched to 100% and not controlled, respectively. Moreover, within each shape, half of the stimuli were controlled for the convex hull and the other half for the density of the elements.

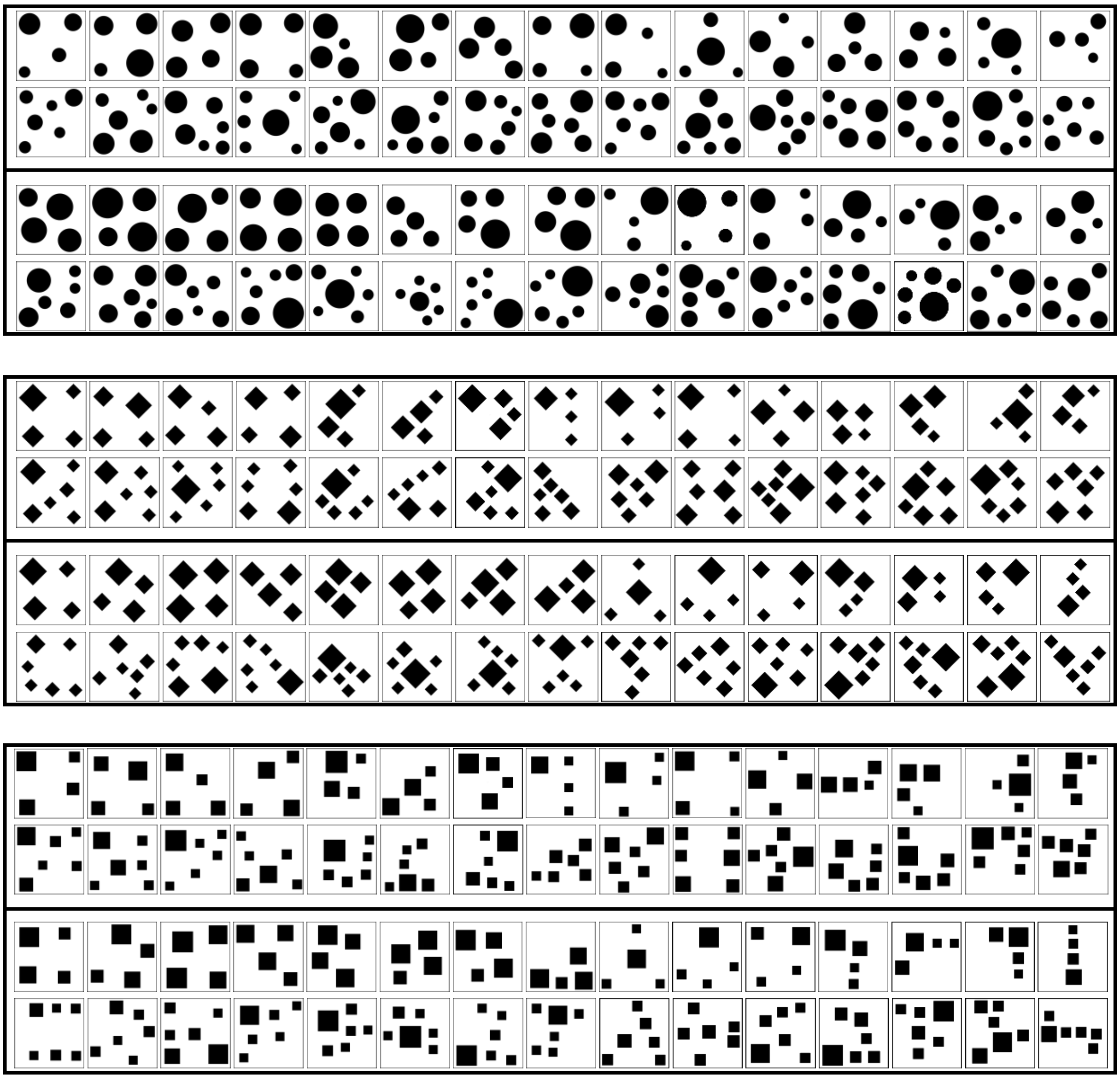


Figure S6. Numerical contrast 4 *vs.* 8, Related to Figure 4a.

The 90 stimuli pairs displaying the 4 *vs.* 8 numerical contrast. Stimuli are divided by shape categories: circles, diamonds, and squares. Within each shape, in one quarter of the pairs the cumulative surface area was matched to 100% whereas in the second quarter it was not controlled. In the third and the fourth quarter the contour length was matched to 100% and not controlled, respectively. Moreover, within each shape, half of the stimuli were controlled for the convex hull and the other half for the density of the elements.

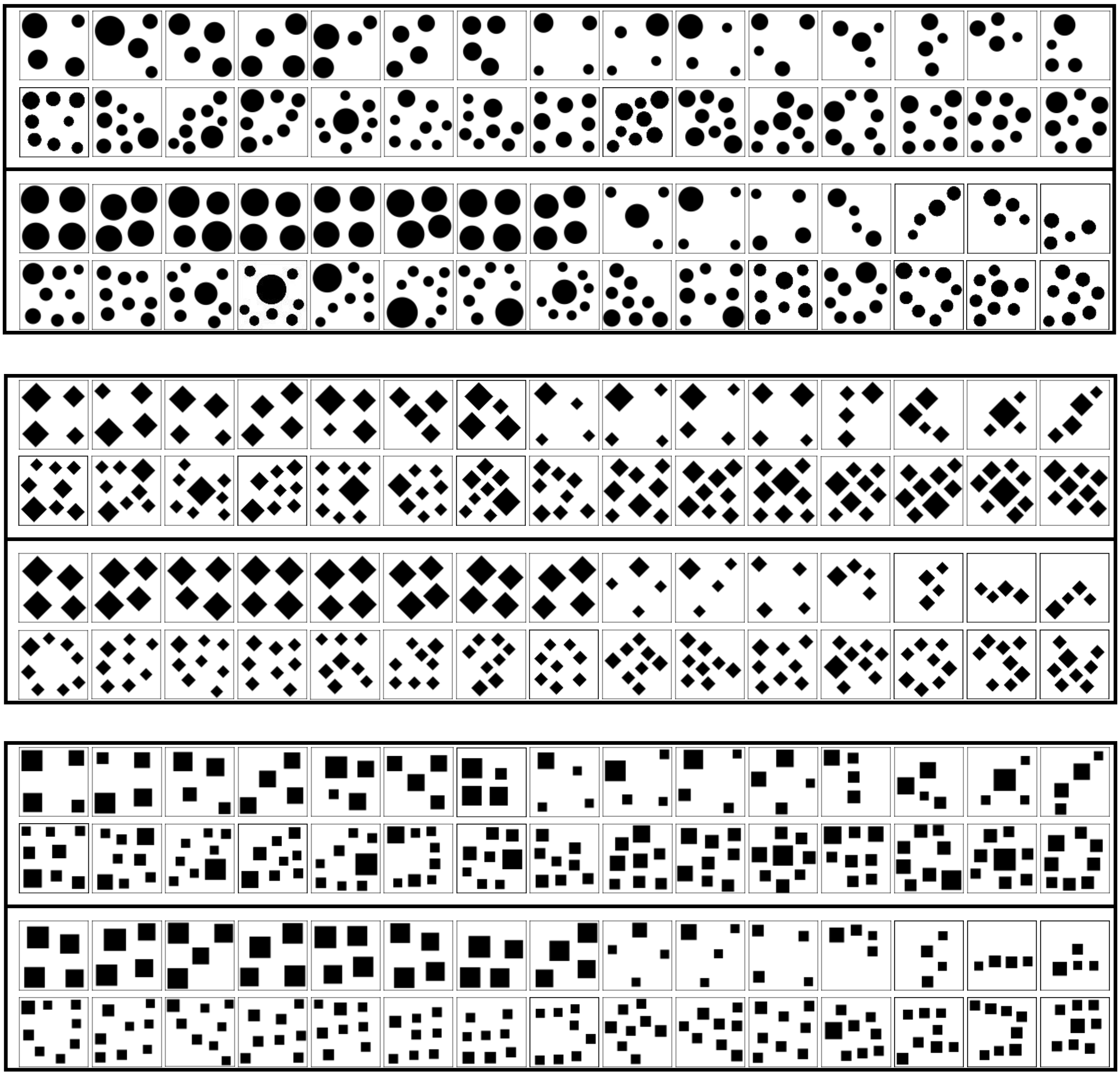
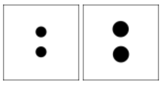
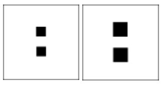
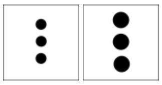
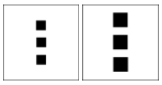
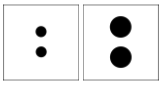
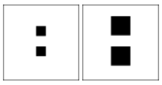
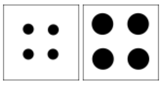
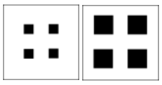
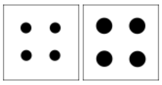
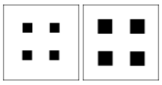
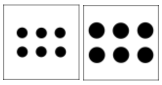
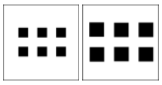
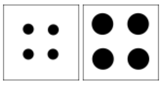
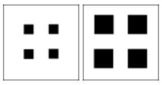
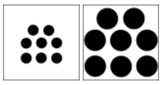
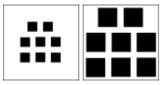


Figure S7. Size contrast, Related to Figure 4b.

The stimuli pairs used during the *size transfer* test. The stimuli consisted of two pairs of novel shapes (i.e., the shape that was not presented in the training phase) with sizes that differed by a ratio of either 0.5 or 0.67, depending on the numerical training previously completed by each subject. Within each pair, the two arrays had the same number and disposition of elements.

Numerical contrast	Correct numerosity during training	Stimuli *size transfer* test	
		Circles	Squares
2 *vs.* 3	2 elements		
2 *vs.* 3	3 elements		
2 *vs.* 4	2 elements		
2 *vs.* 4	4 elements		
4 *vs.* 6	4 elements		
4 *vs.* 6	6 elements		
4 *vs.* 8	4 elements		
4 *vs.* 8	8 elements		

SM.2

TRANSFER FROM CONTINUOUS TO DISCRETE QUANTITIES IN HONEYBEES

Supplemental figures

Figure S8: Training stimuli with square shape.

The 8 stimulus pairs display a comparison between three relatively larger and three relatively smaller squared elements. The ratio difference between sizes was kept at 0.5. We created four different size contrasts: 1 *vs.* 2 cm; 1.15 *vs.* 2.3 cm; 1.35 *vs.* 2.7 cm; 1.5 *vs.* 3 cm. The inter-distance between elements (i.e., the distance computed from the center of the elements) was controlled so that larger and smaller elements of specific comparisons had the same position in the array. All stimuli were created with the GeNEsIS software (Arena radius of 13; Zanon et al., 2021). All values are reported in pixel units.

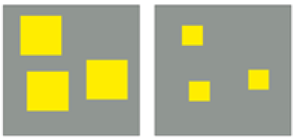
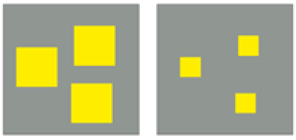
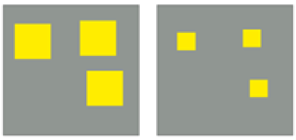
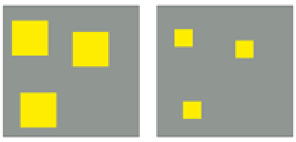
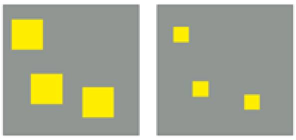
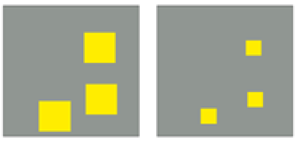
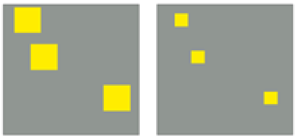
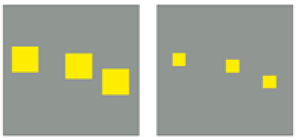
Training stimuli comparison	Area	Perimeter	Inter distance Convex hull	Element size
	Large: 138.7 Small: 34.7	Large: 81.6 Small: 40.8	Large: 233.3 CH 10.96 ID Small: 129.03 CH 10.96 ID	Large: 6.8 Small: 3.4
	Large: 138.7 Small: 34.7	Large: 81.6 Small: 40.8	Large: 225.3 CH 10.4 ID Small: 124.4 CH 10.4 ID	Large: 6.8 Small: 3.4
	Large: 108 Small: 27	Large: 72 Small: 36	Large: 206.6 CH 11.3 ID Small: 117.8 CH 11.3 ID	Large: 6 Small: 3
	Large: 108 Small: 27	Large: 72 Small: 36	Large: 231.7 CH 12.1 ID Small: 137.4 CH 12.1 ID	Large: 6 Small: 3
	Large: 81.1 Small: 20.28	Large: 62.4 Small: 31.2	Large: 184.8 CH 11.8 ID Small: 103.7 CH 11.8 ID	Large: 5.2 Small: 2.6
	Large: 81.1 Small: 20.28	Large: 62.4 Small: 31.2	Large: 162.6 CH 10.3 ID Small: 91.8 CH 10.3 ID	Large: 5.2 Small: 2.6
	Large: 58.1 Small: 14.52	Large: 52.8 Small: 26.4	Large: 172.4 CH 13.6 ID Small: 95.7 CH 13.6 ID	Large: 4.4 Small: 2.2
	Large: 58.1 Small: 14.52	Large: 52.8 Small: 26.4	Large: 110.9 CH 10.4 IT Small: 54.8 CH 10.4 ID	Large: 4.4 Small: 2.2

Figure S9: Training stimuli with diamond shape.

The 8 stimulus pairs display a comparison between three relatively larger and three relatively smaller diamond elements. The ratio difference between sizes was kept at 0.5. We created four different size contrasts: 1 *vs.* 2 cm; 1.15 *vs.* 2.3 cm; 1.35 *vs.* 2.7 cm; 1.5 *vs*. 3 cm. The inter-distance between elements (i.e., the distance computed from the center of the elements) was controlled so that larger and smaller elements of specific comparisons had the same position in the array. All stimuli were created with the GeNEsIS software (Arena radius of 13; Zanon et al., 2021). All values are reported in pixel units.

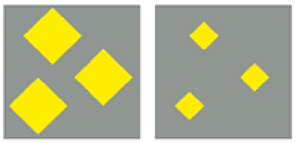
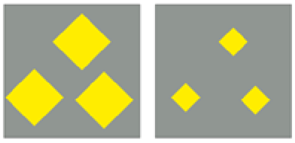
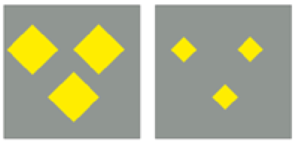
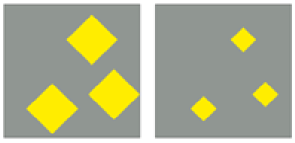
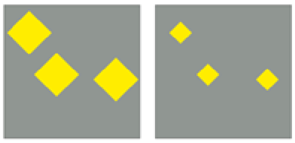
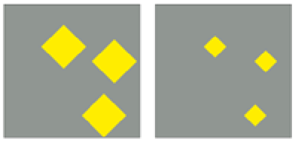
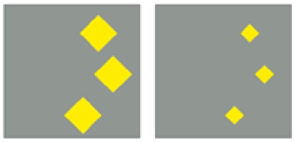
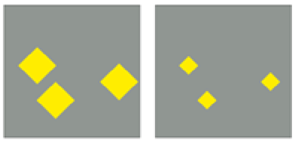
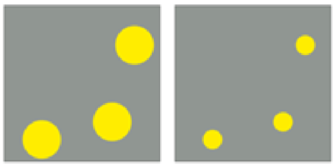
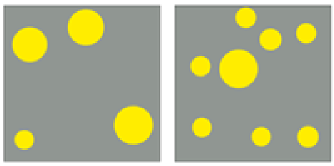
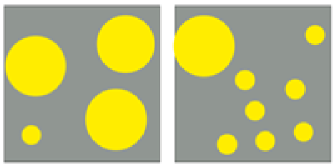
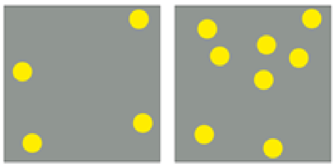
Training stimuli comparison	Area	Perimeter	Inter distance Convex hull	Element size
	Large: 138.7 Small: 34.7	Large: 81.6 Small: 40.8	Large: 201.6 CH 11.6 ID Small: 124 CH 11.6 ID	Large: 6.8 Small: 3.4
	Large: 138.7 Small: 34.7	Large: 81.6 Small: 40.8	Large: 173.8 CH 11.4 ID Small: 109.1 CH 11.4 ID	Large: 6.8 Small: 3.4
	Large: 108 Small: 27	Large: 72 Small: 36	Large: 176.7 CH 10.2 ID Small: 105.8 CH 10.2 ID	Large: 6 Small: 3
	Large: 108 Small: 27	Large: 72 Small: 36	Large: 168.5 CH 11.3 ID Small: 105.6 CH 11.3 ID	Large: 6 Small: 3
	Large: 81.1 Small: 20.28	Large: 62.4 Small: 31.2	Large: 128.2 CH 11.5 ID Small: 77.2 CH 11.5 ID	Large: 5.2 Small: 2.6
	Large: 81.1 Small: 20.28	Large: 62.4 Small: 31.2	Large: 161.3 CH 10.5 ID Small: 97.6 CH 10.5 ID	Large: 5.2 Small: 2.6
	Large: 58.1 Small: 14.52	Large: 52.8 Small: 26.4	Large: 121.7 CH 10 ID Small: 71.3 CH 10 ID	Large: 4.4 Small: 2.2
	Large: 58.1 Small: 14.52	Large: 52.8 Small: 26.4	Large: 115.3 CH 10.5 IT Small: 73.05 CH 10.5 ID	Large: 4.4 Small: 2.2

Figure S10: Test stimuli, related to Figure 6-8.

Stimuli used during the test phase. The *size generalization* test was composed of a comparison between three large and three small elements having a novel shape (i.e., circular) and a novel relative size (i.e., 1.25 *vs.* 2.5 cm). During the *numerical* test phase, a 4 *vs.* 8 elements comparison was presented under different stimuli control conditions. We maintained the magnitude ratio difference experienced in the training phase (i.e., 0.5). To check for the role of perceptual variables covarying with numerosity, we created a total of three couples of stimuli separately controlled for total perimeter (i.e., *number total perimeter* test), total area (i.e., *number total area* test), and element size (*number same size* test). In all three couples of stimuli, the convex hull was controlled. Moreover, the smaller and larger elements were present in both numerical arrays All stimuli were created with the GeNEsIS software (Arena radius of 33.75, 0.4 radius variability, 0.1 accepted error; Zanon et al., 2021). All values are reported in pixel units. The value of the convex hull was approximated to the first decimal point in centimeters.

	Test stimuli comparison	Area	Perimeter	Inter distance Convex hull	Element size
Size generalization test		Large: 500.9 Small: 125.2	Large: 137.4 Small: 68.7	Large: 1321.8 CH 36.2 ID Small: 801.1 CH 36.2 ID	Large: 7.29 Small: 3.4
Number total area test		Large: 492.5 Small: 492.5	Large: 214.9 Small: 153.1	Large: 2187.1 CH 32.2 ID Small: 2187.2 CH 40.1 ID	Larger element: 7.3 Smaller element: 3.65
Number total perimeter test		Large: 733.2 Small: 1260.1	Large: 237.3 Small: 237.1	Large: 2383.7 CH 28.5 ID Small: 2383.5 CH 35 ID	Larger element: 11.7 Smaller element: 3.65
Number same size test		Large: 333.9 Small: 166.9	Large: 183.9 Small: 91.6	Large: 2100.2 CH 31.2 ID Small: 2100.1 CH 45.1 ID	Element: 3.65

SM.3

AN INVESTIGATION OF THE ODOR SEQUENCE LEARNING IN HONEYBEES USING A PER CLASSICAL CONDITIONING PARADIGM

Supplemental figures

Figure S11: Schematic representation of the absolute conditioning training paradigm, Related to Figures 9a, 10a, and 11a.

During the training of Exp. 1, Exp. 2, and 3 honeybees were presented with a total of 10 trials, divided into 5 experimental trials and 5 blank trials. During the blank trials (represented as white bars) a sequence of three puffs of air was provided without any positive reinforcement. Conversely, in the experimental trials, the odor sequence (represented as blue bars) was provided to the bee in association with a drop of sucrose solution (represented as light green bar).

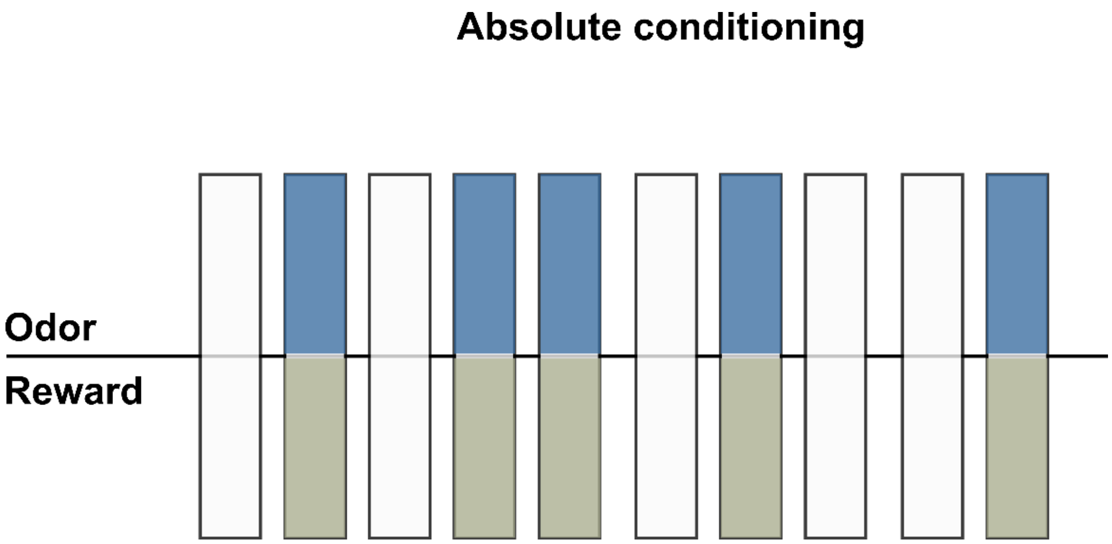


Figure S12: Schematic representation of the differential conditioning training paradigm, Related to Figures 12a and 13a.

During the training of Exp. 4 and 5 honeybees were presented with a total of 10 trials, divided into 5 correct trials and 5 incorrect trials. Depending on the experimental group, during the correct trials, bees were presented with a certain odor sequence (either ABA, BAA, or AAB; represented by blue bars) in association with the sucrose solution (represented as light green bars). Conversely, during the incorrect trials, a different sequence was presented (either ABA, BAA, or AAB; represented by red bars) in association with a neutral stimulus (water; represented as white bars).

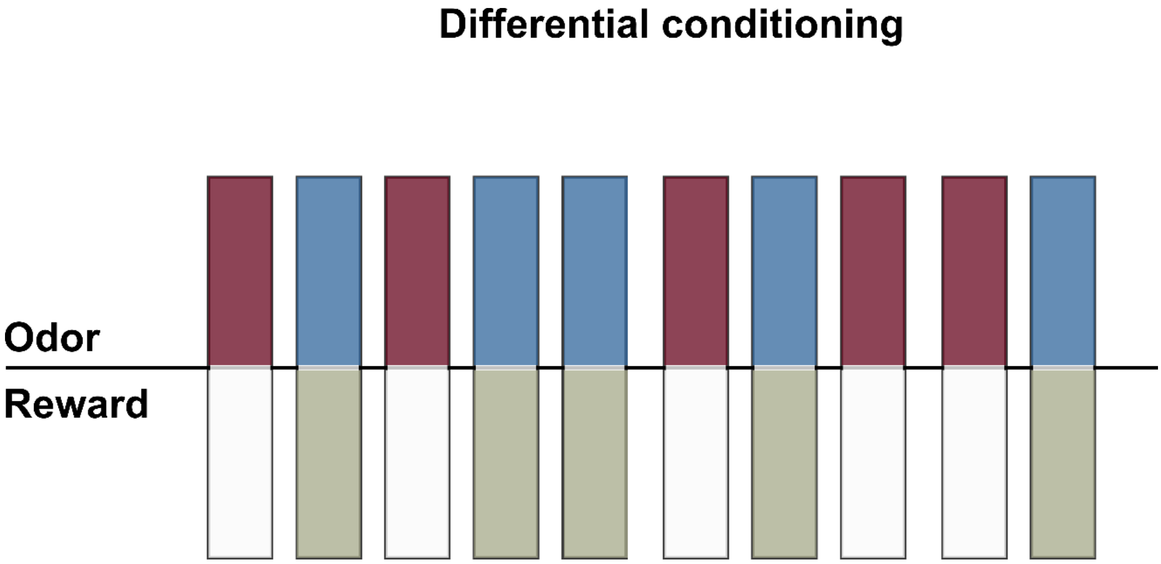


Figure S13: Schematic representation of the generalization conditioning training paradigm, Related to Figure 14a.

During the training of Exp. 6, honeybees were presented with a total of 14 trials, divided into 8 experimental trials and 6 blank trials. During the blank trials (represented as white bars) a sequence of three puffs of air was provided without any positive reinforcement. In the experimental trials, two odor sequences (ABA or CDC) were presented (first sequence represented as blue bars; second sequence represented as green bars) to the bee in association with a drop of sucrose solution.

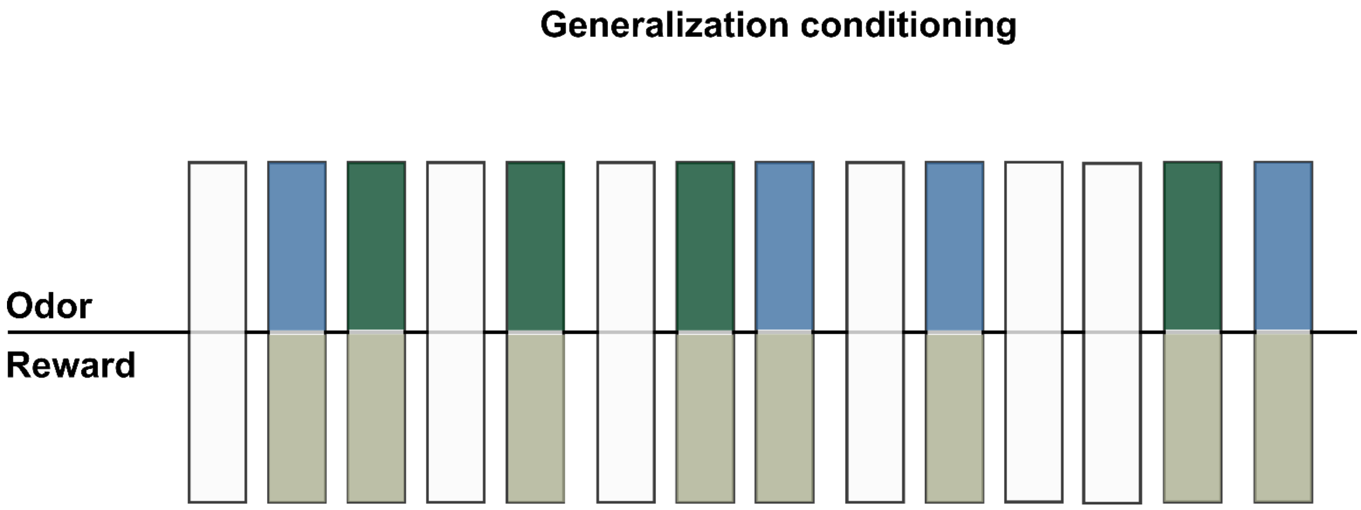
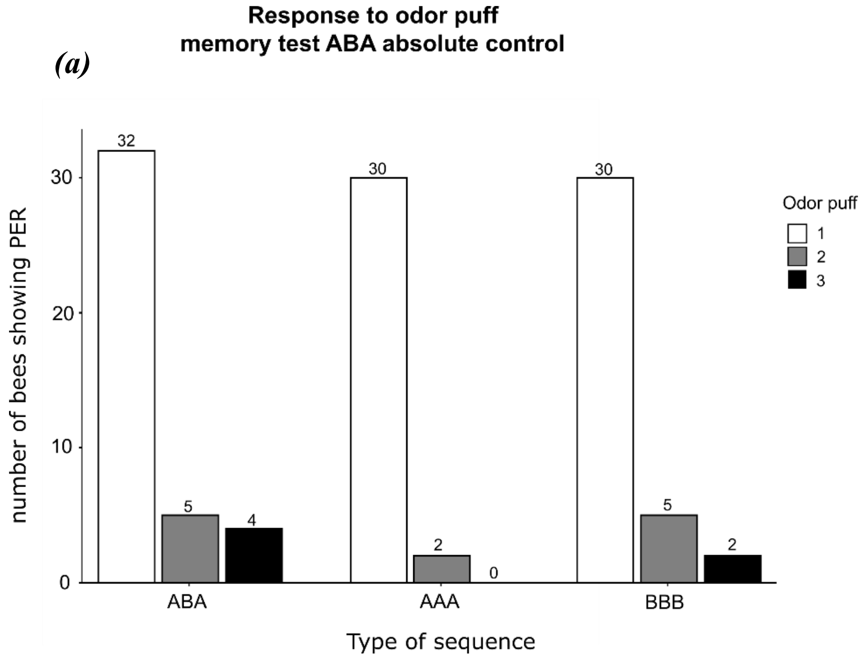


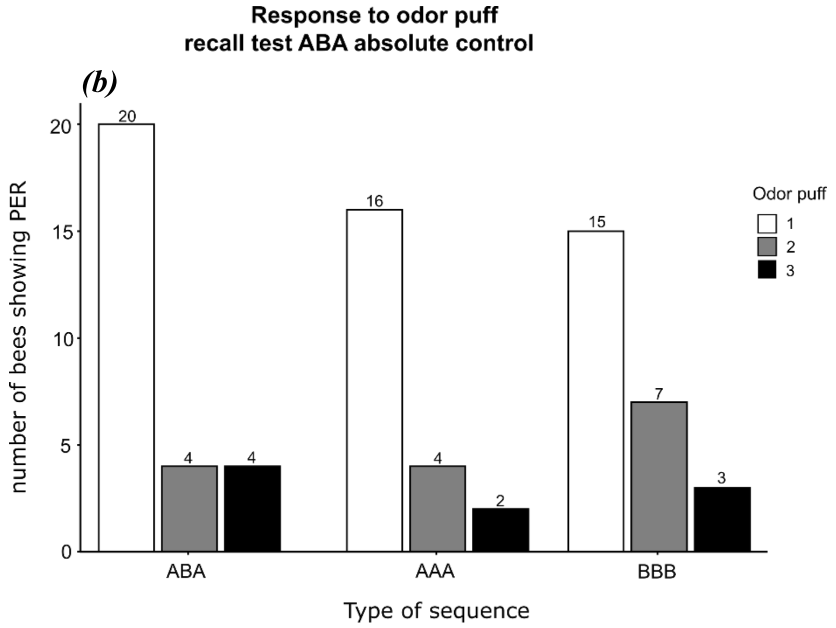
Figure S14: Representation of PER occurrences to the elements of the sequences, Related to Figures 11b and 11c.

In experiment 3, we encoded the occurrence of the PER response in relation to the presentation of a specific element of the sequence (i.e., first, second, or third element). A similar tendency was observed in both tests (i.e., *memory* test (a) and *recall* test (b)) in which honeybees preferentially responded to the first odor provided, regardless of whether it was the first (A, 3-hexanol) or second (B, acetophenone) element of the previously trained sequence (i.e., ABA).



Response to odor puff memory test ABA absolute control

(a)



Response to odor puff recall test ABA absolute control

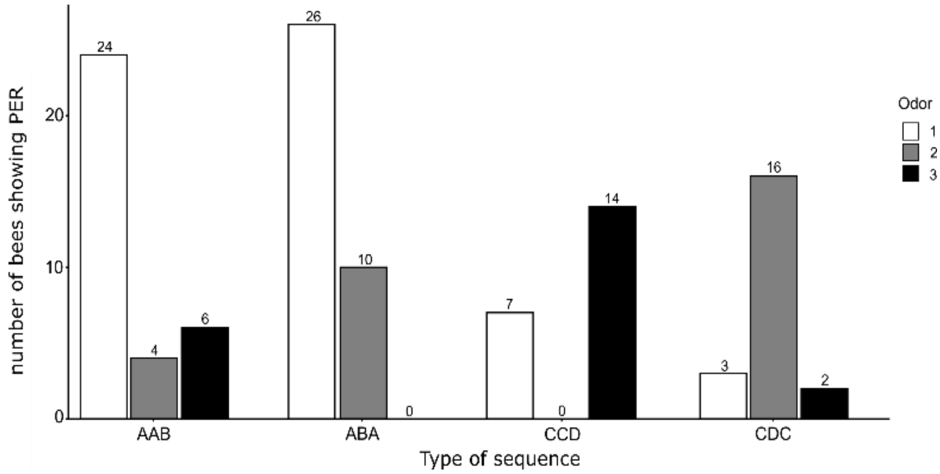
(b)

Figure S15: Representation of PER occurrences to the elements of the sequences, Related to Figures 13b and 13c.

In experiment 5, we encoded the occurrence of the PER response in relation to the presentation of a specific element of the sequence (i.e., first, second, or third element). When either an ABA or AAB sequence was presented (i.e., those were either reinforced or non-reinforced during the training phase), a higher number of bees responded to the odor A (i.e., 3-hexanol) when it occupied the first position in the sequence, compared to when the same odor occupied the second or third position in the sequence. Similarly, when the odor B (i.e., acetophenone) was presented in the second or third position, bees most likely responded to this event. Interestingly, when the response time to novel sequences was scored (i.e., CDC and CCD), the majority of bees responded to the D odor (i.e., citral), irrespective of its position in the sequence. These results were observed both at *memory* test (a) and *recall* test (b).

Response to odor puff
memory test ABA vs. AAB

(a)



Response to odor puff
recall test ABA vs. AAB

(b)

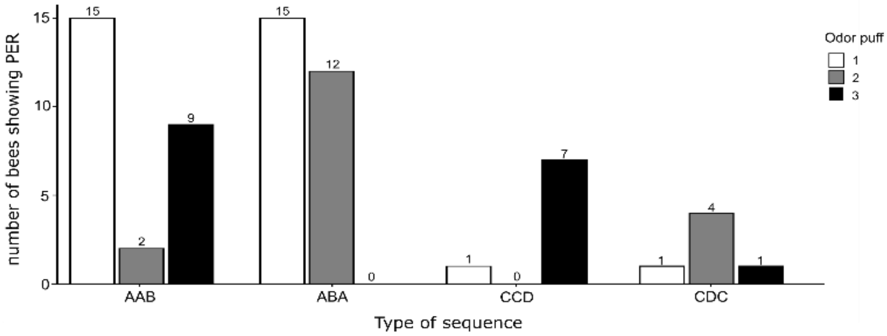
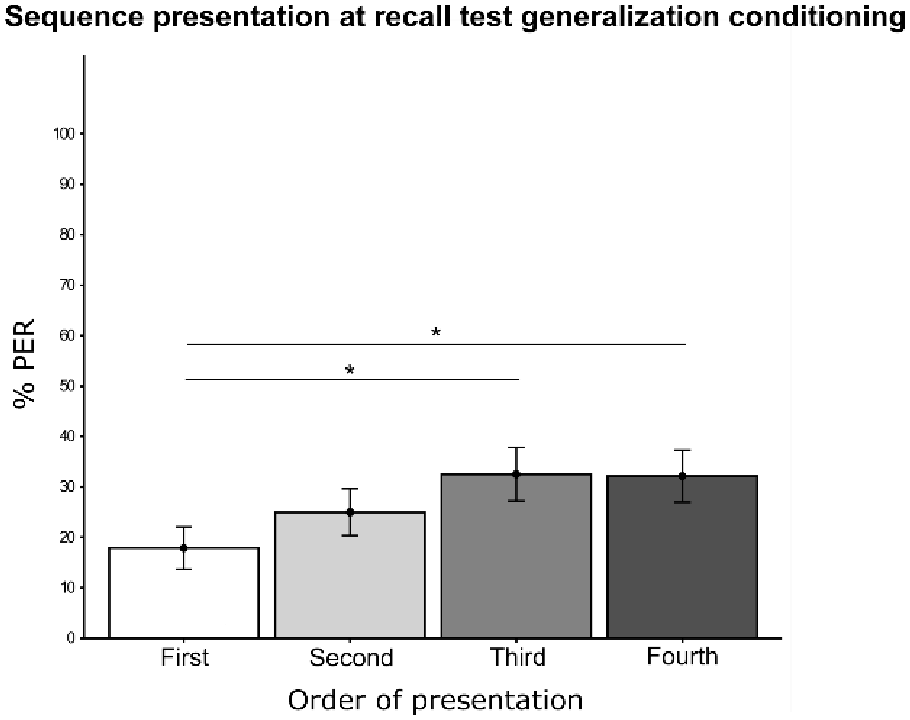


Figure S16: Sequence presentation during *Recall* test of experiment 6, Related to Figure 14c.

Analysis of PER response during the *recall* test highlighted a significant effect of the order of presentation (i.e., as first, second, third, or fourth presented sequence). In particular, the GLMM model showed a significant difference in the probability of response between first and third presentation (GLMM: PER response ~ type of sequence + order of presentation + (1|bee) + (1|day); first *vs.* third: *z-value* = 2.07, *p-value* < 0.05) and between first and last presentation (first *vs.* fourth: *z-value* = 2.52, *p-value* < 0.05).



SM.4

DATA AND CODE REPOSITORY

Chapter 2: Transfer from number to size reveals abstract coding of magnitude in honeybees

Original data have been deposited to Mendeley Data: https://doi.org/10.17632/dgwbncbs58.1.

Chapter 3: Transfer from continuous to discrete quantities in honeybees

Original data have been deposited to Mendeley Data Repository: https://doi.org/10.17632/zyjjngtdwv.1

Original code has been deposited to Mendeley Data Repository: https://doi.org/10.17632/zyjjngtdwv.1.

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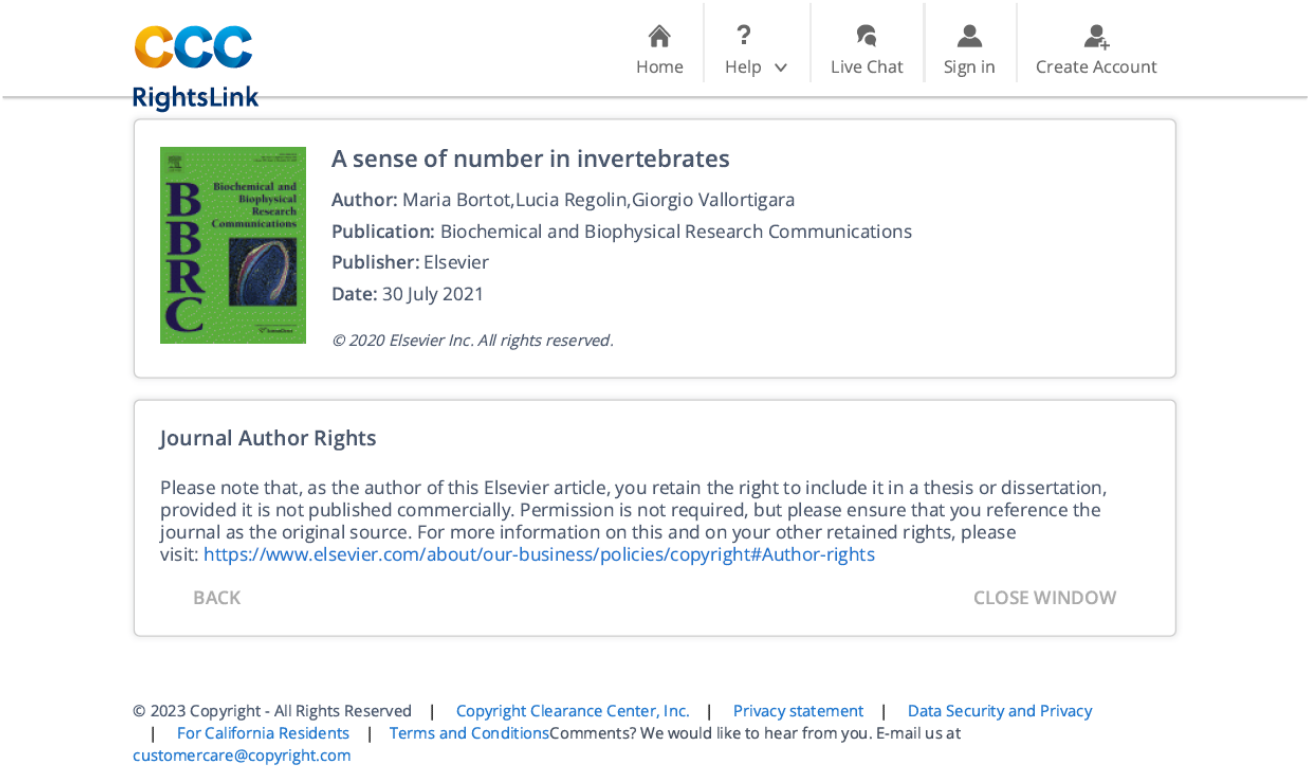
A.1 Introduction: A sense of number in invertebrates



A sense of number in invertebrates

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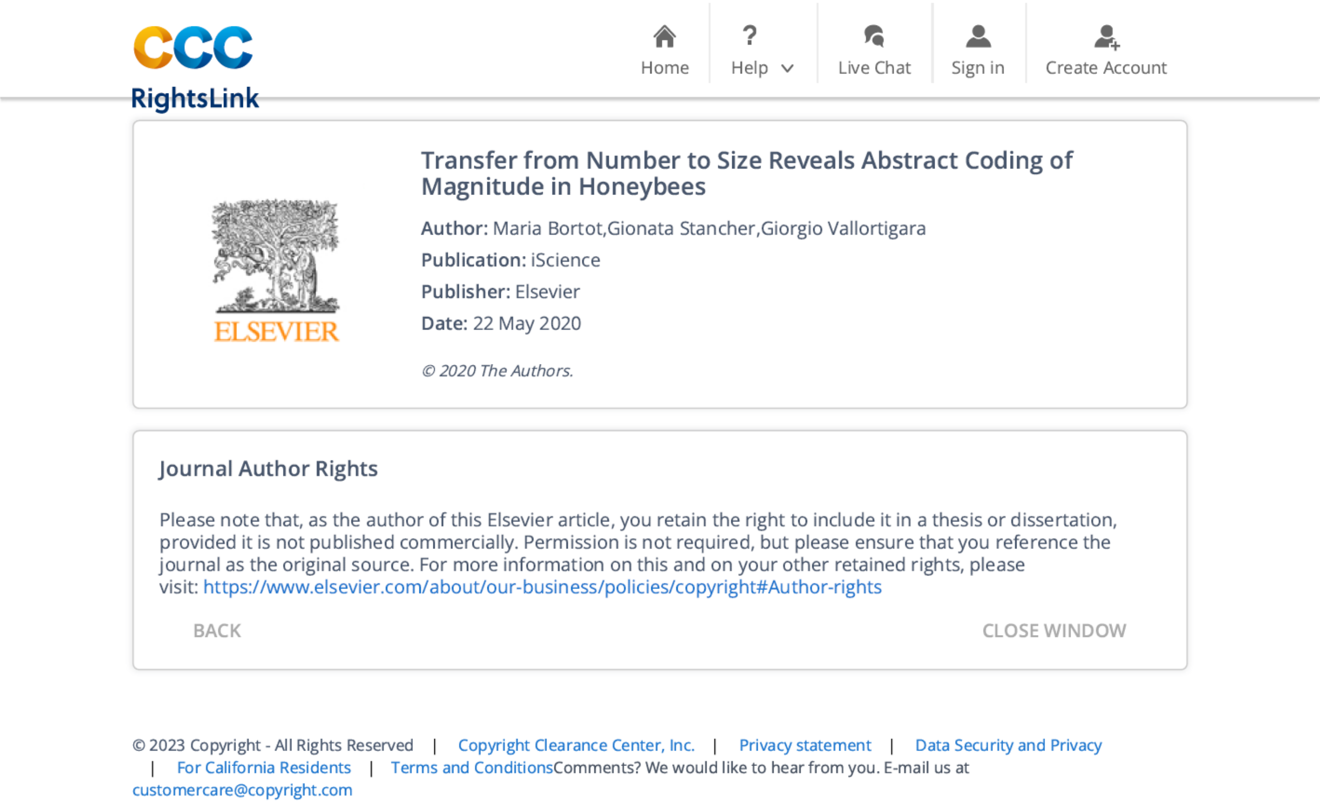
A.2 Chapter 2: Transfer from number to size reveals abstract coding of magnitude in honeybees



Transfer from Number to Size Reveals Abstract Coding of Magnitude in Honeybees

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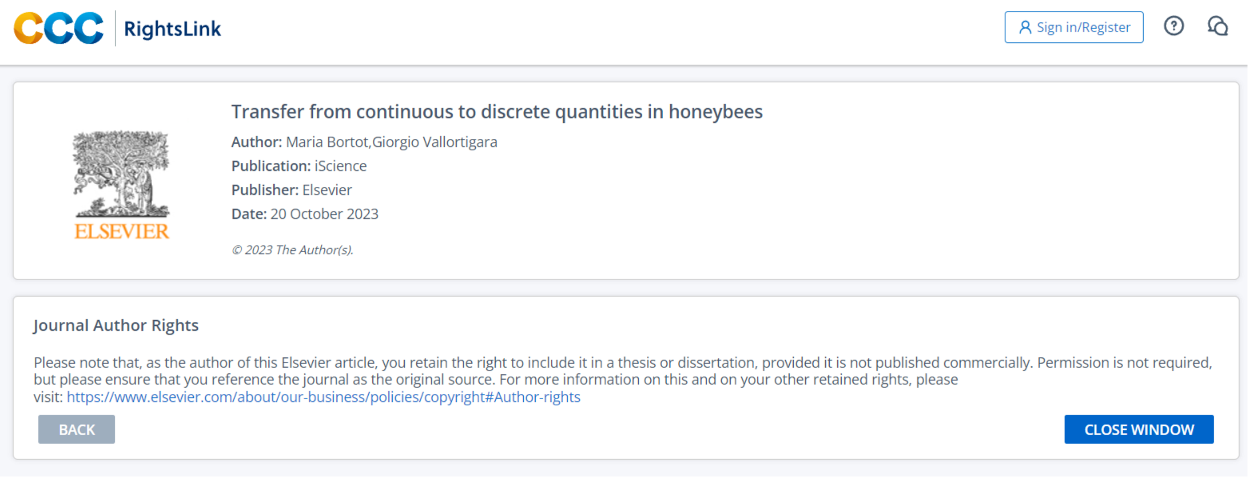
A.3 Chapter 3: Transfer from continuous to discrete quantities in honeybees



Transfer from continuous to discrete quantities in honeybees

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