



Numerical cognition in honeybees: rule learning enables quantity discrimination, zero comprehension, simple arithmetic, and symbol use by an insect

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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Preface

The work presented here was completed for this thesis and is predominantly my own. It was conducted under the supervision of Associate Professor Adrian Dyer (School Media and Communication, RMIT University), Professor Andrew Greentree (School of Science, RMIT University), Dr Jair Garcia (School Media and Communication, RMIT University), and Dr Aurore Avarguès-Weber (Centre de Biologie Intégrative (CBI), Université de Toulouse). Some of the work in this thesis has been published and some of it is in review, thus the journal formatting is retained throughout the thesis and figure titles are specific to the chapters they appear in. Each chapter only refers to figures within the same chapter. Publications and contributions from others are detailed below.

Chapter Two has been submitted: Howard, S.R., Avarguès-Weber, A., Garcia, J.E., Greentree, A.D. and Dyer, A.G., (Submitted). Surpassing the subitizing threshold: Appetitive-aversive conditioning improves quantity discrimination in honeybees. All authors contributed to experimental design, interpretation of the data, and editing of the manuscript. SRH predominantly designed the experiments, collected and analysed the data, and wrote the manuscript. All authors gave final approval for submission. Chapter Two has also been presented at the international conference International Congress of Neuroethology 2018.

Chapter Three is a published paper: Howard SR, Avarguès-Weber A, Garcia JE, Greentree AD, Dyer AG (2018) Numerical ordering of zero in honey bees. *Science*. 360: 1124-1126. SRH, AAW, JEG, ADG, and AGD were involved in the design of the experiment, data interpretation, and drafting of the manuscript. SRH, JEG, AGD, ADG, and AGD analysed data. SRH collected data and wrote the manuscript. All authors gave final approval for

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Chapter Four has been accepted in Scientific Advances and will be published on February 8th 2019: Howard SR, Avarguès-Weber A, Garcia JE, Greentree AD, Dyer AG (2019) Numerical cognition in honeybees enables addition and subtraction. *Scientific Advances*. SRH, AAW, JEG, and AGD designed the experiment. SRH performed data collection and wrote the manuscript. SRH, JEG, and ADG analysed data. All authors were involved in the interpretation of results and drafting of the manuscript.

Chapter Five is a draft paper: Howard SR, Avarguès-Weber A, Garcia JE, Greentree AD, Dyer AG (Submitted) Symbolic representation of number in the honeybee (*Apis Mellifera*). SRH, AAW and AGD designed the experiment. SRH collected and analysed the data, and wrote the manuscript.

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Guntarik O, Garcia JE, **Howard SR**, Dyer AG. (2018). TRACES: Mobile Eye Tracking Captures User Sensory Experience in an Outdoor Walking Tour Environment, *Leonardo* 51: 163-164.

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Shrestha M, Garcia JE, Chua JHJ, **Howard SR**, Tscheulin T, Dorin A, Nielsen A, Dyer AG. (2019). Fluorescent pan traps affect the capture rate of insect orders in different ways, *Insects*.

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

Karl Von Frisch (1886 – 1982)

Thesis abstract

Many non-human animals demonstrate some level of numerical ability which includes an understanding of complex numerical concepts such as arithmetic, sequential ordering of numbers, or an understanding the concept of zero. Although very little research has been done on numerical ability in invertebrates, honeybees and several other insects have been shown to possess some numerical capabilities. In my thesis, I have assessed the capacity of bees to exhibit complex numeric skills such as number categorisation, extrapolation, and simple arithmetic. I show that the use of appetitive-aversive differential conditioning improves the honeybees' ability to discriminate between quantities above the subitizing range compared to appetitive-differential conditioning. Honeybees have demonstrated an understanding of the quantitative value of nothing and placed an empty set in the correct position along a line of sequential numbers, learnt to categorise numbers as greater or lesser in context, acquired abstract colour-based rules to solve elementary incremental and decremental problems, and demonstrated an ability to match symbols with specific quantities. In some cases, honeybees have mastered numerical concepts at a level that parallels abilities demonstrated by primates, mammals, birds, and other vertebrates. I discuss these findings in relation to how number processing developed in human culture, and how subsequent work can search for number processing regions in animal brains.

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Chapter 1

1. Introduction

In this introduction, I will explore the literature on numerical ability in humans, non-human vertebrates, and invertebrates with a final focus on the honeybee, as cognitive studies on this species are numerous and my later experimental chapters present data demonstrating the numerical capacity of honeybees. As each experimental chapter is written in manuscript form and thus contains a subject-specific introduction, the Chapter 1 Introduction is general to the field and our current understanding of numerical abilities in humans and non-human animals.

It is hypothesised by some authors that relatively complex numerical competency (e.g. numerical cognition such as arithmetic; Figure 1) is not an evolved capacity and is in fact an artefact of the development of human language and culture (Núñez, 2017a; Núñez, 2017b). Other authors argue that non-human animals are both capable of performing complex numerical tasks and that some of these tasks may be evolved capacities in humans and non-human animals (Nieder, 2005; Nieder, 2017). The experimental work I will present in Chapters 2 – 5 will add valuable data and insight into this debate. I examined whether an invertebrate could learn and perform complex numerical tasks. The model species I have tested is the honeybee, as invertebrates are relatively understudied for their numerical capacity compared to vertebrates (Figure 1; Carazo et al., 2009; Gross et al., 2009), and bees demonstrate both complex natural (Biesmeijer and Seeley, 2005; Gallo and Chittka, 2018; Grüter and Farina, 2009; Riley et al., 2005; Srinivasan, 2014; Srinivasan et al., 2000; Von Frisch, 1967) and learnt behaviours (Avergues-Weber and Giurfa, 2013; Dyer, 2012; Srinivasan, 2010; Zhang, 2006). Additionally, the honeybee is separated from humans by 600 million years of evolution (Consortium, 2006; Grimaldi et al., 2005), thus experimental data

on the numeric capacity of honeybees could inform the evolutionary processes by which numerical capacity may, or may not, have evolved.

In this review chapter, I will discuss the current and emerging literature regarding definitions and examples of numerical categories including basic vs. complex numerical abilities, the mechanisms of counting, and numerosity discrimination. I will also discuss and evaluate the role of human language and culture in number faculty with input from studies of human adults, infants, and pre-verbal children across cultures. In this chapter, there will be a focus on numerical abilities in non-human animals, which will include a discussion of the different levels of numerical abilities which non-human animals exhibit, neurobiological evidence of numerical ability, limitations of animal number skills, and the understanding of complex numerical concepts across species. Finally, I will introduce the study species used in my empirical research, *Apis Mellifera*, and discuss the current literature which identifies the honeybee as a model species for the study of cognition and numerical competency in invertebrates.

1.1. Part I: Numerical and quantal concepts and tasks

Numerical abilities differ by their complexity. For example, the ability of a species to choose a greater quantity of food could be influenced by non-numerical cues that correlate with increasing numerosity, such as surface area, weight, or perimeter, and thus this would be considered a more basic use of numerical cues than performing arithmetic operations such as addition, subtraction, multiplication, or division. In the following sections I will discuss the specific tasks and behaviours required for demonstrating numerical competency, use of the approximate number system (ANS) or object file system (OFS), quantal cognition, numerical cognition, arithmetic, and an understanding of zero numerosity, which have requirements varying from basic numerosity tasks to the use of symbolic number representation (Figure 1). It is important to note that often numerical tasks are required for

demonstrating more than one numerical concept. For example, the demonstration of true counting *and* numerical cognition requires an individual, or species, to symbolically represent numerosities and quantitatively value those symbols (Figure 1; see definitions below).

1.1.1. Numerical competency

Numerical competency can be classified into three categories: cardinal number (cardinality or numerosity), ordinal number, and nominal number (Nieder, 2005). **Cardinality** is *the ability to quantitatively value sets discretely and continuously*. **Ordinality** is *the ranking of individual elements in a sequence*. **Nominal number** assignment can be considered non-numerical *and uses number to classify individual objects within a set*. Nominal number is believed to only be used by linguistic humans (Nieder, 2005).

1.1.1.1. Cardinality:

Cardinality involves the ability to value and quantitatively order sets of items (Nieder, 2005). The valuation of sets of objects differing in their numerosity is observed in chimpanzees (Beran et al., 1998; Biro and Matsuzawa, 1999; Biro and Matsuzawa, 2001; Boysen and Berntson, 1989; Boysen et al., 1995; Boysen et al., 1993; Murofushi, 1997) and rhesus monkeys (Brannon and Terrace, 2000). Additionally, both rhesus monkeys (Brannon and Terrace, 2000; Merritt et al., 2009) and chimpanzees (Beran and Rumbaugh, 2001; Beran et al., 1998; Biro and Matsuzawa, 1999; Boysen and Berntson, 1989), are able to correctly place sets of objects in the correct order following the quantitative number line.

1.1.1.2. Ordinality:

Ordinality is the ranking of different elements in a set, for example, a runner coming 5th in a race (Nieder, 2005). Animals demonstrate an ability to rank non-numerical items with training, but also in natural environments. For example, baboons demonstrate a tendency to rank conspecifics in their group by dominance (Bergman et al., 2003), thus establishing an

ordering system of individual rank. Both rhesus monkeys (Swartz et al., 1991; Terrace et al., 2003) and pigeons (Straub et al., 1979) were able to learn to order different lists of items, such as abstract photographs of objects, humans, and animals, in an exact rank position for each item with training on the tasks.

1.1.1.3. Nominal number:

Nominal number is used to label objects, for example, in a laboratory a scientist may label samples 1, 2, 3, 4... etc. to differentiate them from each other. Nominal number is not considered a numerical skill and is believed to only be used by humans (Figure 1; Nieder, 2005).

1.1.2. Object File System (OFS) vs. Approximate Number System (ANS)/ Analog Magnitude System (AMS)

There are two well accepted mechanisms for numerical discrimination involved for different number ranges (Agrillo et al., 2008; Kaufman et al., 1949; Piazza et al., 2002; Tomonaga and Matsuzawa, 2002; Trick and Pylyshyn, 1994). The first is known as **subitizing** or **the object file system**, *the quick and accurate estimation of four and fewer objects* (Figure 2a). The second mechanism is the approximate number system (ANS), also known as the analog magnitude system (AMS) for quantifying numerosities above four objects.

These different mechanisms may have led to a phenomenon observed across species, where the threshold of accurately estimating objects, ‘counting’, and discriminating between numerosities is set at about four objects. This threshold is reported in humans (Carazo et al., 2009; Dehaene and Cohen, 1994; Jevons, 1871; Piazza et al., 2002; Riggs et al., 2006; Starkey and Cooper Jr, 1995), fish (Agrillo et al., 2008; Gómez-Laplaza and Gerlai, 2011; Seguin and Gerlai, 2017), and bees (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008; Gross et al., 2009). Some species can surpass this threshold through the use of the AMS,

however this results in more errors, or the need for a larger magnitude to separate two quantities for discrimination (see definitions and examples below).

1.1.2.1. Subitizing/ Object File System (OFS)

Subitizing, also called the **object file system** (OFS) or the **parallel individuation system**, is considered to be a type of counting mechanism with low quantities. The object-file system represents each individual object symbolically, rather than representing the set symbolically. There is a limit on the number of object files available so that this system can only represent up to three or four individual elements (Brannon, 2006; Feigenson et al., 2004; Hyde, 2011). However, it can also be used to explain thresholds of quantity discrimination and estimation in non-human animals. The threshold of subitizing, the ability to accurately identify a small numerosity ‘at a glance’ without sequential counting, is considered to be at about four or sometimes five (Agrillo et al., 2008; Kaufman et al., 1949; Piazza et al., 2002; Tomonaga and Matsuzawa, 2002; Trick and Pylyshyn, 1994). Other literature suggests the threshold could be in the range of four to seven (Cowan, 2010; Miller, 1956; Saaty and Ozdemir, 2003; Simons and Langheinrich, 1982). Different species have been tested for their upper numerosity limits in the form of matching-to-sample tasks, quantity discrimination, spontaneous choice, among other protocols. The German zoologist, Otto Koehler (Hassenstein, 1974), determined the upper numerosity limit of different species, showing a limit of five for pigeons, six for budgerigars and jackdaws, and seven for ravens, African grey parrots, Amazones, magpies, and squirrels (Davis and Pérusse, 1988; Hassmann, 1952; Nieder, 2005). More recent studies on non-human animals show that the discrimination between two quantities of objects which differ by one element is four. For example, honeybees tested on the ability to discriminate between 2 vs. 3 were successful at these quantities as well as 3 vs. 4, but not 4 vs. 5 or 5 vs. 6 (Gross et al., 2009). A similar result is found in angelfish, mosquitofish, and zebrafish, where the ability to choose the larger shoal

Figure 1: A schematic describing the relative complexities of different **numerical concepts** (overall titles) and **numerical tasks** (smaller titles), how the numerical concepts relate, and which species have demonstrated each task and concept. The diagram shows which tasks belong at varied levels of complexity ranging from non-numerical through to basic, mid-range, and high complexity tasks. The tasks are grouped into overall concepts which are defined across the literature. In some cases all tasks need to be demonstrated for a concept to be shown (e.g. true counting required all criteria to be met), in other cases a single task needs to be shown to demonstrate the concept (e.g. any task in quantical cognition is evidence of using quantical cognition). The interaction between concepts is also shown by the dark areas of the diagram. Some concepts have overlapping tasks needed to demonstrate more than one concept. Beside or below each task I have shown the animal species which have successfully performed the task. The star in the key classifies the task as having been demonstrated by all species shown on the schematic. The **non-numerical level** includes representations of number which do not require numerical capacity. At the non-numerical level, there is the lowest level of *understanding zero numerosity* which is a sensory representation of ‘nothing’, such as neurons not firing in relation to the absence of a stimulus (Nieder, 2016b). There is also *nominal number* representation, which is the use of number to label elements of a set which does not correlate with numerosity or rank of that element (Nieder, 2005). Between the non-numerical and basic numerical sections, there is an overlap. This overlap includes the use of *non-numerical cues* to judge numerosity or discriminate between numerosities. These cues can include parameters such as size, weight, or surface area. The **basic level** of numerical capacity includes *quantity discrimination of numerosities below five elements*. At the overlap between the basic and mid-range complexity, there is the task of *subitizing*, the ability to quickly and accurately enumerate four or less elements (Agrillo et al., 2008; Kaufman et al., 1949; Piazza et al., 2002; Tomonaga and Matsuzawa, 2002; Trick and Pylyshyn, 1994). This task is within the overlap as it is defined as a mechanism of counting and is a basis for *true counting*. Within the **mid-range numerical complexity**, two of the levels of understanding *zero numerosity* are based. The *categorical understanding of ‘nothing’* and the *quantitative understanding of zero numerosity* are fairly complex tasks which lead to the final fourth stage of understanding zero (Nieder, 2016b). The lowest level of complexity of *arithmetic* is also included in this section where species can *use arithmetic-like reasoning*, without training, to solve problems. In this section is also the most complex type of quantity discrimination which I have identified, *quantity discrimination which obeys Weber’s Law*. The reason this is considered of higher complexity than quantity discrimination is due to the research demonstrating that Weber’s Law mechanisms allow species to discriminate between numerosities well-above the subitizing range of four elements (AMS). This mid-range section also includes the *translation of number tasks to novel representations of numerosities* and *novel numerosities*, both aspects of true counting. *Ordinality*, the ranking of elements in a set is also included in the mid-range under the concept of *numerical competency*. In the overlap between the mid-range and high-level complexity of numerical tasks is the ability to *discriminate between numbers about four* as this surpasses the common threshold of enumerating, or discriminating between numerosities, however is not yet showing the most advanced level of complexity in numerical tasks. Finally, the **most complex numerical tasks** include the *final understanding of zero numerosity*, the use of *symbolic representation of numbers in arithmetic*, all stages of *numerical cognition (symbol and number matching; exact number use, arithmetic using abstract elements, and the symbolic representation and quantitative valuing of symbols)*. This final section also includes many of the tasks needed to demonstrate *true counting (serially counting past four elements, translation of number tasks to a different modality, symbol and number matching, cardinality, the translation of number tasks between modalities, and the symbolic valuation of numerosities)*.

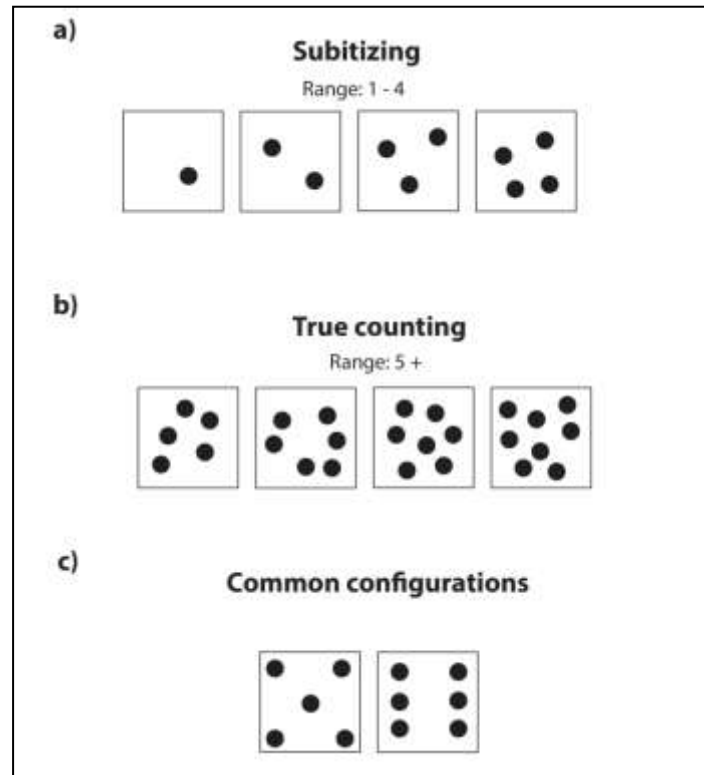


Figure 2: Subitizing is defined as the quick and accurate estimation of positive integer quantities one to four (a). True counting is defined as the slow and sometimes error-prone process of serially counting more than four items. It is also known as the AMS/ANS (b). Accurate quantity estimation (subitizing) can be extended when the quantity pattern is ordered in a common configuration, such as dots on dice (c) (Krajcsi et al., 2013).

(group of conspecifics) option when the shoals differ by one fish is successful in 1 vs. 2, 2 vs. 3, and 3 vs. 4, but not 4 vs. 5. A classic study by Jevons (1871) also demonstrated that human estimation of items is 100 % accurate when there are four or less objects, but when there are five or more, the estimation of the numerosities of objects results in errors. These studies show that a threshold limit of about four objects exists for species as evolutionarily distant as honeybees and humans which have been separated for 600 million years (Consortium, 2006; Grimaldi et al., 2005).

1.1.2.2. Approximate Number System (ANS)

The approximate number system (ANS), also known as the Analog Magnitude System (AMS), is a non-symbolic representational system of processing of quantities above the subitizing range (Núñez 2017b). Animals which are able to process quantities above four are considered to be using the ANS/AMS mechanism of numerosity judgement. The ANS is thought to be an ancient evolutionary foundation, shared with non-human animals, for our ability to perform mathematics and use symbolic numerosity mechanisms (Brannon and Merritt, 2011; DeWind et al., 2015; Feigenson et al., 2004). While symbolic number systems, such as the use of Arabic numerals to discriminate between numerosities, allows us to easily discriminate between two numbers, non-symbolic number systems, such as ANS, show that ratio-dependent number discrimination exists. This means that two numbers close together in magnitude are harder to discriminate between than two numbers which are distant in magnitude (Brannon and Merritt, 2011; Nieder, 2016). This phenomenon is also known as Weber's Law which describes how well two stimuli are able to be differentiated based on their proportional difference, such as time, sound, numerosity, and touch, among other examples (Akre and Johnsen, 2014; Fechner, 1965; Weber, 1978). Thus, humans and non-human animals share the evolutionary ancient quantification system known as the approximate number system.

1.1.3. True counting

True counting is considered to only be accessible to humans as it is considered to use symbolic numerosity mechanisms (Núñez 2017b). True counting is the ability to enumerate and discriminate between numbers above four but requires a set number of criteria to be met before a species or individual can be identified as performing true counting (Figure 1; Table 1). The literature (Agrillo et al., 2008; Davis and Pérusse, 1988; Kaufman et al., 1949;

Lazareva and Wasserman, 2017; Piazza et al., 2002; Tomonaga and Matsuzawa, 2002; Trick and Pylyshyn, 1994) identifies these criteria as:

1. **Serially counting** numerosities of five and higher.
2. Extrapolation to **novel representations** of numerosities.
3. Extrapolation to **novel numerosities**.
4. **Symbolic representation** of number.
5. Quantitative valuing of sets (**cardinality**).
6. Translation of number tasks to **different modalities**.
7. Translation of number tasks to **different procedures**.

Table 1: The criteria for demonstration of ‘true counting’ as defined across the literature.

Criteria	Description	Example
Serially counting more than four elements	The error-prone and slow process of counting elements one-by-one above four	Being able to count the number of apples you put in a basket as 1, 2, 3, 4, 5, 6, 7, and so on.
Extrapolation to novel representation of numerosity	The ability to transfer numerical knowledge to novel stimuli.	Three blue circles is the same number of items as three birds.
Extrapolation to novel numerosities	The ability to transfer numerical knowledge to unfamiliar numerosities	A child learning to count from one to four and then understanding that five is higher than four even though they have not been taught the quantitative value of five.
Symbolic representation of number	Being able to match symbols to specific numerosities for representation and manipulation of number	Symbols such as Arabic numerals (1, 2, 3) or Roman numerals (I, II, III).
Quantitatively valuing sets of objects	Assigning quantitative value to different sets of objects and being able to order them by this value	Understanding that a set of six circles is greater in number than a set of five circles.
Translation of numerosity between modalities	Number skills should be transferable between modalities (e.g. visual to tactile)	Being able to understand that three flashes of light is the numerical equivalent to three bell rings.
Translation of numerosity between procedures	Number skills should be transferable between procedures (e.g. sequential to simultaneous)	Seeing a set of items and being able to sequentially count to that numerosity

1.1.3.1. Serial counting:

Serially counting above four elements is a necessary aspect of true counting (Agrillo et al., 2008; Davis and Pérusse, 1988; Kaufman et al., 1949; Lazareva and Wasserman, 2017; Piazza et al., 2002; Tomonaga and Matsuzawa, 2002; Trick and Pylyshyn, 1994). The ability to serially count more than four objects is demonstrated by chimpanzees and rhesus monkeys. Chimpanzees have demonstrated serial counting of up to seven elements (Beran and Rumbaugh, 2001). When shown an Arabic numeral of 1 – 7, chimpanzees could serially select elements until they reached the quantitative value of the Arabic numeral as shown by the number of selected elements. For example, if shown a ‘7’, the chimpanzee could serially select seven dots from a pool of ten dots to match the numeral. When rhesus monkeys were presented with a number of items on a screen, the monkeys had to respond by touching the sets of objects in either an ascending or descending order. Rhesus monkeys could order numerosities one – nine by quantitative value when presented with a number of abstract elements (Brannon and Terrace, 2000).

1.1.3.2. Extrapolation to novel representation of numerosities:

The demonstration of true counting and numerical ability should be object-independent (Dacke and Srinivasan, 2008; Davis and Pérusse, 1988). Thus if a species, or individual, learns to count using sets of circles, they should be able to count sets of apples, lemons, or buildings. The requirement of transferring counting to novel items is known as the ‘abstraction principle’. Without this transfer to novel elements the behaviour is not classified as true counting but ‘protocounting’ (Davis and Pérusse, 1988). The extrapolation of counting to novel representations is demonstrated by dolphins, which can transfer to novel representations of stimuli, such as transferring number tasks from three-dimensional objects to two dimensional objects (Kilian et al., 2003).

1.1.3.3. Extrapolation to novel numerosities:

The extrapolation of counting to novel numerosities is also required for the demonstration of true counting (Davis and Pérusse, 1988; Lazareva and Wasserman, 2017). For example, rhesus monkeys were trained to count from one to four items. The monkeys were then able to extrapolate this counting task to the novel numerosities of five to nine (Brannon and Terrace, 1998; Brannon and Terrace, 2000), thereby demonstrating extrapolation of numerical ordering and counting to new and unfamiliar numerosities (Lazareva and Wasserman, 2017).

1.1.3.4. Symbolic representation of number:

The symbolic representation of number and ordering of those symbolic representations is considered a necessary aspect of true counting (Lazareva and Wasserman, 2017). Alex, the African grey parrot, was able to learn the English names of numbers and subsequently use those labels to count and add, thereby demonstrating numerical skills required for numerical cognition and true counting (Pepperberg, 1987; Pepperberg, 1994; Pepperberg, 2006a; Pepperberg, 2006b; Pepperberg, 2012; Pepperberg and Carey, 2012; Pepperberg and Gordon, 2005). Chimpanzees which have learnt Arabic numerals can then order them quantitatively (Beran, 2004b; Beran and Rumbaugh, 2001; Beran et al., 1998; Biro and Matsuzawa, 1999; Biro and Matsuzawa, 2001; Boysen and Berntson, 1989; Boysen et al., 1995; Boysen et al., 1993; Murofushi, 1997) as well as sum the numerals (Boysen et al., 1996). Rhesus monkeys have also learned to choose the larger of two Arabic numerals to receive a corresponding number of food pellets (Washburn and Rumbaugh, 1991).

1.1.3.5. Translation of number tasks to different modalities:

Numerical ability should not be restricted to a certain modality (Davis and Pérusse, 1988). There should be an ability to transfer numerical tasks, such as counting, across modalities (e.g. visual to auditory or tactile to visual). The ability to transfer between modalities was demonstrated in rats (Church, 1984), which were able to learn to press a right lever when two

sounds were presented and a left lever when four sounds were presented. This is potentially the only study which successfully demonstrates cross-modal transfer of a numerical task in a non-human species, according to a recent review (Lazareva and Wasserman, 2017), which more likely indicates the difficulty of testing the transfer of numerical tasks between modalities than the inability of a non-human animal to demonstrate it under the appropriate conditions.

1.1.3.6. Translation of number tasks to different procedures:

Numerical ability should not be limited to a certain procedure. Numerical tasks should be transferable across different procedures (e.g. simultaneous to successive presentation; Lazareva and Wasserman, 2017). Chimpanzees, as described in section 1.1.3.1, could view an Arabic numeral (e.g. seven) and sequentially count objects to reach this numerosity (Beran et al., 1998).

1.1.3.7. True counting criteria:

It appears that true counting should consist of an ability to serially count objects above four, extrapolate counting to novel objects and numerosities, demonstrate quantitative ordering of sets of objects, demonstrate symbolic representation of number, and transfer numerical ability to different modalities and procedures. This is a substantial list of criteria to fulfil in order to demonstrate true counting, all of which have not been demonstrated in a single non-human animal species (Figure 1). However, true counting is considered to be a level of numerical ability not accessible to nonhuman animals as it requires symbolic numerosity, which nonhuman animals are not thought to be capable of performing.

In Chapters 2 – 5 of my thesis, I examine the capacity of honeybees to demonstrate some of these true counting characteristics in numerical tasks. I specifically test the ability to discrimination between numerosities greater than four elements, the extrapolation of number

tasks to novel representations and numerosities, the ability to make symbol and numerosity associations, and the ability to demonstrate cardinality.

1.1.4. Quantical cognition

Quantical cognition includes *the biologically evolved preconditions for numerical cognition which are non-symbolic and imprecise*. Importantly, quantical cognition does not scale up to number and arithmetic, which require cultural mediation (Núñez, 2017a). Quantical cognition encompasses the following capacities (Figure 1):

1. The use of **non-numerical cues** which correlate with numerosity to solve numerical problems.
2. The ability to **discriminate between low numerosities** (four or less) based on numerical cues.
3. The ability to discriminate between high quantities (five and greater) through mechanisms which are consistent with **Weber's Law** – performance increases as magnitude between numerosities increases.

1.1.4.1. Non-numerical cues:

Quantical cognition includes the ability to judge quantity using non-numerical cues such as surface area, weight, perimeter, or movement. For example, golden orb-web spiders (*Nephila clavipes*) can keep track of the number of prey in their webs using numerical information combined with non-numerical cues, such as the weight of prey (Rodríguez et al., 2015).

1.1.4.2. Quantity discrimination:

Quantical cognition also encompasses low-level quantity discrimination for numerosities below five elements. Many animals demonstrate quantical cognition in the form of natural activities such as resource management, predator avoidance, and navigation. For example, fish, such as mosquitofish and angelfish, can discriminate between two shoals of different

quantities when they differ by just one element at low numerosities (e.g. 1 vs. 2, 2 vs. 3, 3 vs. 4) but fail at 4 vs. 5 in some studies (Agrillo et al., 2008; Gómez-Laplaza and Gerlai, 2011).

1.1.4.3. Quantity discrimination and Weber's Law:

An aspect of quantal cognition, which is observed in multiple species, is the improved ability to differentiate between two quantities as the magnitude between two quantities increases. This is observed when comparing numerosities of four or more elements. For example, the ability of fish to discriminate between two shoals (groups of conspecifics) consisting of four or more individual fish appears to be related to mechanisms consistent with Weber's Law (Agrillo et al., 2008; Gómez-Laplaza and Gerlai, 2011). Weber's Law describes how sensory systems distinguish between two stimulus magnitudes based on their proportional difference (Akre and Johnsen, 2014; Fechner, 1965; Weber, 1978). Also known as proportional processing, Weber's Law is used by a number of animals to detect changes in sound, light, odours, electrical fields, pressure, number, and time (Akre and Johnsen, 2014). When animals adhere to Weber's law, they compare stimuli on the basis of proportional differences. In quantity discrimination, Weber's Law allows fish to surpass the threshold limit of four by comparing quantities based on their proportional differences, this is also evident when animals use AMS/ANS. Thus, the ability of mosquitofish and angelfish to discriminate between two shoals (as described above in section 1.1.2.2.) improved as the numerical distance between the shoals increased (Agrillo et al., 2008; Gómez-Laplaza and Gerlai, 2011). Mosquitofish are successfully able to compare the quantities of 1 vs. 2, 2 vs. 3 and 3 vs. 4. When the limit of discriminating between numbers in the subitizing range is reached, mosquitofish are unable to successfully discriminate between quantities of 4 vs. 5, 4 vs. 6, 4 vs. 7, 5 vs. 6, 6 vs. 7, 6 vs. 8, 7 vs. 8, or 8 vs. 12. However, when the magnitude of difference between the two quantities at and above the subitizing threshold is increased to a

ratio of 1 : 2, fish are successfully able to discriminate between quantities of 4 vs. 8, 8 vs. 16, and 4 vs. 10 (Agrillo et al., 2008).

In Chapter 2, I examine the quantity discrimination ability of honeybees above the subitizing range and demonstrate that different conditioning procedures will yield different discrimination limits in this invertebrate species.

1.1.5. Numerical cognition

Numerical cognition is *the exact and symbolic use of number including arithmetic operations and symbolic representation of numerosities* (Núñez, 2017a). Unlike quantal cognition, numerical cognition tasks cannot rely on non-numerical traits to make discriminations between numerosities. Numerical cognition encompasses tasks including:

1. **Exact number use** (including arithmetic and symbolic representation).
2. **Arithmetic** (e.g. operations such as addition, subtraction, multiplication, and division)
3. **Symbolic representation and quantitative valuation of symbols**, including symbol and number matching.

1.1.5.1. Arithmetic:

Arithmetic operations, such as addition and subtraction, are demonstrated widely throughout the animal kingdom. In addition to adult humans (Pica et al., 2004), they are exhibited by human infants (McCrink and Wynn, 2004; Wynn, 1992), chimpanzees (Beran, 2004a), orangutans (10), rhesus monkeys (Sulkowski and Hauser, 2001), vervet monkeys (Tsutsumi et al., 2011), an African grey parrot (Pepperberg, 2006a; Pepperberg, 2012), and pigeons (Brannon et al., 2001). Some invertebrates are able to utilize arithmetic-type reasoning under natural conditions (Figure 1). For example, jumping spiders (*Portia Africana*) are able to use numerical information, independent from non-numerical cues, to keep track of prey counts in

their webs (Cross and Jackson, 2017). The numerical concept of arithmetic is discussed in more detail below (Section 1.1.6).

1.1.5.2. Symbolic representation of numerosity and valuation of symbols:

A few vertebrates have demonstrated the ability to match specific symbols with quantities. For example, pigeons (Xia et al., 2001; Xia et al., 2000) can learn to match different symbols with a certain number of pecks. Three non-human species have demonstrated symbolic number use in arithmetic operations and through quantitative valuation, including an African grey parrot (Pepperberg, 1987; Pepperberg, 1994; Pepperberg, 2006a; Pepperberg, 2006b; Pepperberg, 2012; Pepperberg and Carey, 2012; Pepperberg and Gordon, 2005), chimpanzees (Beran, 2004b; Beran and Rumbaugh, 2001; Beran et al., 1998; Biro and Matsuzawa, 1999; Biro and Matsuzawa, 2001; Boysen and Berntson, 1989; Boysen et al., 1995; Boysen et al., 1993; Murofushi, 1997), and rhesus monkeys (Washburn and Rumbaugh, 1991). With the exception of spiders (Cross and Jackson, 2017; Nelson and Jackson, 2012; Rodríguez et al., 2015), evidence of numerical cognition in invertebrates is uncommon, thus in the following experimental chapters (Chapters 2 – 5) I explore numerical cognition abilities in an invertebrate species, the honeybee.

In Chapter 4, I determine the ability of bees to learn and perform an arithmetic-type task consisting of learning to add or subtract one element from a sample stimulus based on colour cues. In Chapter 5, I examine the capacity of honeybees to acquire symbol and numerosity associations to determine if an invertebrate could learn a symbolic number language created by humans at a basic level.

1.1.6. Arithmetic

Arithmetic can be identified as *the operational use of numbers* such as in addition, subtraction, multiplication and division. Arithmetic, such as addition and subtraction problem

solving, requires complex management of quantities in both working memory and longer term rule based memory (Tsutsumi et al., 2011). Arithmetic is thus a complex concept, cognitively challenging, and is considered an aspect of numerical cognition (Figure 1; Núñez, 2017a; Núñez, 2017b). Arithmetic can be demonstrated at varying levels of complexity (Figure 1):

1. Spontaneous arithmetic-type reasoning.
2. Arithmetic problems using abstract objects.
3. Symbolic representation of numerosity for use in arithmetic (including the symbolic representation of zero numerosity).

Human infants (McCrink and Wynn, 2004; Wynn, 1992), vervet monkeys (Tsutsumi et al., 2011), chimpanzees (Beran, 2004a; Beran and Beran, 2004; Boysen and Berntson, 1989), orang-utans (Call, 2000), rhesus monkeys (Sulkowski and Hauser, 2001), Alex the parrot (Pepperberg, 2006a; Pepperberg, 2012), pigeons (Brannon et al., 2001), and spiders (Nelson and Jackson, 2012; Rodríguez et al., 2015) demonstrate the ability to add and/or subtract.

1.1.6.1. Spontaneous arithmetic-type reasoning:

As shown in Figure 1, arithmetic can vary in complexity with the lowest level of arithmetic classified as the spontaneous use of arithmetic-like reasoning which is shown in wild vervet monkeys. Vervet monkeys observing an experimental apparatus in their environment understood subtraction-like problems with no training on the tasks. When shown food pieces being placed in an opaque cup and then observing when none or some were removed, the monkeys preferred to approach the cup when the subtraction operation should result in food being present (e.g. $2 - 1$) rather than absent (e.g. $1 - 1$) resulting in a spontaneous understanding of a subtraction problem (Tsutsumi et al., 2011).

1.1.6.2. Arithmetic problems using abstract objects:

The next level of complexity in arithmetic is the addition and subtraction of abstract elements (Figure 1). This is where a species, or individual, is able to add two quantities of objects to get a correct answer. For example, when human infants observe a number of objects become covered by an opaque screen and then see either more objects added (addition) or some objects removed (subtraction), they will stare longer when the screen is removed if the resulting number of objects is not consistent with the addition or subtraction problem they had witnessed. If infants observed five objects become occluded, then saw five more objects added behind the screen, once the screen is removed they will stare longer if the result is five objects rather than ten objects (McCrink and Wynn, 2004).

1.1.6.3. Symbolic representation of numerosity for use in arithmetic:

Arithmetic using symbolic representations of numerosities is a complex problem (Núñez, 2017a). Some primates have been trained to use symbolic representations of number such as Arabic numerals. For example, a chimpanzee was able to sum Arabic numerals ranging from 0 – 4 (Boysen and Berntson, 1989).

There is currently a paucity of research on the ability of invertebrates to perform arithmetic-like problem solving. Thus, in Chapter 4, I examine the capacity of honeybees to simultaneously learn simple addition and subtraction tasks and apply these rules to unfamiliar stimuli and novel numerosities.

1.1.7. Zero numerosity

The concept of zero is processed at a number of levels ranging from the sensory representation of zero up to the symbolic use of zero in mathematics. Zero numerosity is the *understanding that the null amount of zero has a quantitative value and belongs at the low end of the positive integer number line.* There are considered to be four stages of

understanding zero numerosity across human culture, ontogeny, phylogeny, neurophysiology, learning, and non-human animal understanding (Figure 1; Nieder, 2016b):

1. The **first stage** is defined as a '*sensory representation*' understanding of zero, the absence of stimulation.
2. The **second stage** is a *categorical understanding* of zero (e.g. nothing vs. something).
3. The **third stage** is the understanding of zero numerosity as having a *quantitative value* and belonging at the low end of the positive number line.
4. The final **fourth stage** is the *symbolic representation of zero for the use in mathematics*.

A categorical understanding of zero (stage 2) is evident by Alex the parrot who could answer with the word “none” spontaneously when asked how many items of a particular object there were when the object in question was not present (Pepperberg, 2006a; Pepperberg and Gordon, 2005). A quantitative understanding of zero numerosity (stage 3) is demonstrated by rhesus monkeys, which were able to order sets of objects including an empty set containing no objects (Merritt et al., 2009). The fourth stage of understanding zero numerosity, the use of a symbolic representation of zero in mathematics, may be accessible to a chimpanzee which was able to sum Arabic numerals ranging from 0 – 4 (Boysen and Berntson, 1989), however it is not yet confirmed that this constitutes the fourth level of understanding zero numerosity.

Additionally, monkeys have demonstrated a neural representation of zero numerosity (Ramirez-Cardenas et al., 2016). When monkeys were performing a number matching task consisting of matching numerosities including an empty set stimulus, the ventral intraparietal area (VIP) encoded the empty set as a separate category to other numerosities, however neurons in the prefrontal cortex (PFC) represented the empty set stimulus as similar to numerosity one, and less similar to higher numerosities. These results demonstrate numerical

distance effects of zero numerosity in the brain, suggesting a neural understanding of zero at the third stage, a quantitative representation.

Early, under-developed concepts of zero are evidenced in Chinese, Babylonian, and Mayan mathematics (Boyer, 1944; Houston et al., 2001; Joseph, 2008; Nieder, 2016b). The understanding of zero in early human culture ranged from defining it as a placeholder for a lack of a numerical value in the Babylonian number system to Egyptian mathematics having a magnitude or direction-separator concept of zero (i.e. separating the numbers above zero from those below zero) over four thousand years ago (Joseph, 2008; Joseph, 2011). However, these representations of ‘zero’ did not have a quantitative value associated with them (Boyer, 1944; Nieder, 2016b). It was not until 628AD that zero had a written record which noted it as a number in its own right, thus giving it a quantitative value and rules for its use in arithmetic (Boyer, 1944; Nieder, 2016b).

Considering advanced ancient human civilizations did not fully understand the importance and necessity of zero in their own numeric system, it is of particular interest that non-human animal species are able to learn the quantitative value of zero (Biro and Matsuzawa, 2001; Merritt et al., 2009; Ramirez-Cardenas et al., 2016) and represent an empty set as a quantitative value in the brain (Ramirez-Cardenas et al., 2016).

The question of whether animals such as fish or insects are “able to represent empty sets as a quantitative category”, as posed by Nieder (2016b), is addressed in Chapter 3. I examine the capacity of honeybees to apply previously learnt numerical rules to an empty set to determine if, and to what level, bees understand zero numerosity.

1.2. Part II: Numerical ability as an evolved capacity

1.2.1. Evolution of numerical capacity

Language has provided humans with the ability to develop advanced mathematical skills and a high level of numerical ability (Gelman and Gallistel, 2004; Nieder, 2005), and research in humans has established counting and numerical competency as an important ability evident across cultures of verbal and written systems (Brannon and Van de Walle, 2001). Quantical cognition, such as quantity discrimination, is widely accepted as a part of ‘number sense’ which has evolved in humans and non-human human animals (Núñez, 2017a). More advanced numerical abilities involving complex concepts, such as arithmetic, are thought to be an artefact of the evolution of human culture and language (Núñez, 2017a; Núñez, 2017b) and accordingly some authors believe non-human animals do not possess the general “intelligence” to solve these problems (Lenneberg, 1971). Conversely, other authors argue that numerical capacity has in fact developed separately to language (Nieder, 2005; Nieder, 2017). The argument that numerical ability is linked to language (Brannon and Van de Walle, 2001; Lenneberg, 1971), is disputed by the evidence of numerical ability in pre-verbal children (McCrink and Wynn, 2004; Wynn, 1992; Wynn, 1998), humans who speak languages lacking number nomenclature (Pica et al., 2004), and non-human animals (Nieder, 2005; Nieder, 2017). In this section I will examine the evidence for the evolution of complex numerical skills in humans and non-human animals.

Human infants have demonstrated the ability to discriminate between small numbers (four and less) of objects (Antell and Keating, 1983; Starkey and Cooper, 1980; Strauss and Curtis, 1981; Wynn, 1998) and the capacity to understand addition and subtraction of small (Wynn, 1992) or large (greater than four) numbers of items (McCrink and Wynn, 2004). Arithmetic such as addition and subtraction is considered an aspect of numerical cognition (Figure 1; Núñez, 2017a). Thus, the demonstration of numerical cognition skills in human infants

supports the argument that even complex numerical skills cannot be exclusively linked to the development of human language and culture.

In addition to pre-verbal children and infants, some languages have limited or no words to describe number. For example, those who speak Mundurukú, an Amazonian language, do not have words to describe numbers above five, however can still add large groups of elements (Pica et al., 2004). In studies where verbal counting was difficult or not possible, humans were able to accurately compare sets of elements (evidence of cardinality; Figure 1; Cordes et al., 2001; Whalen et al., 1999). These studies support the argument that verbal language is not a necessary prerequisite for basic or even advanced numerical skills (Butterworth et al., 2008; Frank et al., 2008), however other authors argue that the spoken or written systems of number that humans use will impact the level of numerical cognition they can reach (Gordon, 2004). Nieder (2005) suggests that humans adults, infants, and non-human animals demonstrate an evolutionarily ancient quantification system that operates independently of language, as supported by studies of non-verbal number tasks in humans (Butterworth et al., 2008; Frank et al., 2008; Pica et al., 2004).

It is interesting to attempt to quantify what skills and behaviours constitute the use of language in numerical tasks. Chimpanzees and rhesus monkeys can be taught to use and give quantitative value to Arabic numerals (Beran, 2004b; Biro and Matsuzawa, 2001; Harris et al., 2007), Alex the parrot could count, sum numbers of items, respond to questions with the correct English label for numerals (Pepperberg, 2006b). Furthermore, training on the Arabic numeral symbolic number system in monkeys resulted in the analogous populations of neurons activating in monkeys as in human children (Diester and Nieder, 2007), demonstrating a precursor of human symbolic language in monkeys (see section 1.2.2. below).

Thus, the evidence that learning a symbolic language for number tasks activates analogous neurons in a monkey's brain as in humans (Diester and Nieder, 2007) suggests a similar evolution of numerical processing in the brain.

1.2.2. Number neurons

Evidence of the evolution of numerical capacity in non-human animals is strengthened by studies on the neurobiology of number skills (Dehaene, 2003; Nieder, 2016a; Nieder et al., 2002; Nieder and Miller, 2003). In some non-human animals there is evidence of neurons which only activate with specific numbers, known as 'number neurons' (Nieder, 2016a). Number-encoding neurons (Dehaene, 2002) are observed in different species of monkeys, domestic cats, and crows. Number-encoding neurons are activated in the parietal cortex of the monkey (*Macaca fuscata*) brain (predominantly the superior parietal lobule; Sawamura et al., 2002). These neurons are also observed in rhesus monkeys (*Macaca mulatta*) within the prefrontal cortex (Nieder et al., 2002; Nieder and Merten, 2007; Nieder and Miller, 2003; Nieder and Miller, 2004; Viswanathan and Nieder, 2013), posterior parietal cortex (Nieder and Miller, 2004; Tudusciuc and Nieder, 2007), parietal lobe (Nieder et al., 2006; Tudusciuc and Nieder, 2007; Viswanathan and Nieder, 2013), and the lateral and ventral intraparietal areas of the intraparietal sulcus (IPS) (Nieder, 2012; Nieder and Miller, 2004; Viswanathan and Nieder, 2013). The cortex of the domestic cat (*Felis catus*; Thompson et al., 1970), and the crow (*Corvus corone corone*) endbrain (nidopallium caudolaterale (NCL); Ditz and Nieder, 2015) also have similar responses of neurons to number stimuli. The evidence of number-encoding neurons in species as evolutionary separate as monkeys, domestic cats, and crows suggests that numerical processing and abilities are a result of convergent evolution (Nieder, 2016a).

One of the arguments supporting the view that numerical competency has not evolved and is an artefact of the development of human language and culture (Núñez, 2017a; Núñez,

2017b), is that animals have not demonstrated numerical cognition, including symbolic representation of number. However, monkeys demonstrate the activation of similar neuron populations as children after having been taught the quantitative values of Arabic numerals (Diester and Nieder, 2007).

Further research on more evolutionary distant animals will inform whether numerical competency is a conserved or convergent trait. For example honeybees and humans have been separated for 600 million years (Consortium, 2006; Grimaldi et al., 2005), thus the demonstration of similar numerical skills and limits of such evolutionary distant species would suggest convergent evolution is a likely explanation (see section 1.2.3), as the common ancestor for both species must have been less neurologically complex than either.

1.2.3. Outstanding questions

I aim to give insight into the question of whether basic and advanced numerical abilities could have evolved in an invertebrate species evolutionarily separated from humans for 600 million years (Consortium, 2006; Grimaldi et al., 2005). I examine the numerical competency of the honeybee to determine what level of numeric understanding and performance this species could reach to provide an insight into whether high-level numeric skills are restricted to humans with culture and language, and to determine whether number sense in other non-human animals has evolved via convergent evolution or conserved evolution.

If the honeybee is able to perform numerical tasks of a high-complexity, this would suggest the evolution of numerical capacity in humans and non-human animals. If numerical capacity is a conserved trait, it would be expected that the last common ancestor of honeybees and humans had the capacity to perform analogous tasks common to both honeybees and humans. If numerical capacity is a result of convergent evolution, then this suggests that over time different species from humans to invertebrates have separately developed an ability to

perform numerical tasks due to the demands in their own environments. If the honeybee is not able to perform numerical tasks, this suggests that numerical capacity may not be an evolved trait (Núñez, 2017a; Núñez, 2017b), or conserved evolution of numerical abilities of ancestors common to humans, non-human primates, and perhaps other vertebrate species occurred, or that convergent evolution of numerical abilities in vertebrate species demonstrating numerical capacities has occurred (Figure 1).

Therefore, in my experimental chapters (Chapters 2 – 5), I explore the ability of honeybees to learn, apply, and solve numerical problems to determine what level of numeric ability they can reach and how this may compare to humans and other vertebrates. The ability to compare numeric abilities in an invertebrate species with our current knowledge of animal numeric ability will give insights into the possible evolutionary pathways of numeric ability.

1.3. Part III: A model invertebrate species for the study of animal numerical ability

1.3.1. Cognition in bees

Bees have long been used as a classic research model for understanding insect sensory perception (Dyer and Arikawa, 2014; Von Frisch, 1914; Von Frisch, 1967). Honeybees, *Apis mellifera*, demonstrate very complex, naturally evolved behaviours such as comb-building (Gallo and Chittka, 2018) and dance communication of the location of food sources to nest mates (Biesmeijer and Seeley, 2005; Grüter and Farina, 2009; Riley et al., 2005; Srinivasan, 2014; Srinivasan et al., 2000; Von Frisch, 1967). In addition to these observed behaviours, honeybees can learn a number of complex tasks with extended visual experience that were previously assumed to require a large mammalian brain (Chittka and Niven, 2009; Dyer, 2012; Srinivasan, 2010).

Honeybees can be trained to perform simple and conceptual tasks with the use of classical conditioning, providing a food source of sucrose (sugar water) for a correct choice

(Avarguès-Weber and Giurfa, 2013; Dyer, 2012; Srinivasan, 2010; Zhang, 2006). For example, they can solve problems using rules such as ‘left vs. right’ and maze navigation (Collett et al., 1993; Zhang et al., 1996; Zhang et al., 2000), ‘above vs. below’ (Avarguès-Weber et al., 2011), ‘same vs. different’ (Giurfa et al., 2001), ‘larger vs. smaller’ (Avarguès-Weber et al., 2014; Howard et al., 2017a; Howard et al., 2017b), and they can combine learnt rules, known as dual concept use (Avarguès-Weber et al., 2012). Honeybees have also demonstrated counting (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008; Menzel et al., 2010) and number discrimination (Gross et al., 2009) up to four objects. The capacity of bees to learn and apply rules to solve problems creates many new questions in invertebrate cognition, learning, and goal-orientated tasks.

1.3.2. Numerical limits in bees

In previous studies on honeybee numerical ability, bees reached a number competency threshold at four (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008; Gross et al., 2009; Skorupski et al., 2018). As discussed above, this phenomenon is also seen across other animal species (Agrillo et al., 2008; Cowan, 2010; Gómez-Laplaza and Gerlai, 2011; Jevons, 1871; Seguin and Gerlai, 2017; Simons and Langheinrich, 1982).

Honeybees have demonstrated an ability to count landmarks to find the correct position of food sources (Figure 3; Chittka and Geiger, 1995; Dacke and Srinivasan, 2008; Menzel et al., 2010). A study conducted in a field showed that honeybees were able to learn the correct position of a food source after one, two, three, or four landmarks, large yellow tents (Chittka and Geiger, 1995), although some bees preferred to use distance rather than landmark number (Figure 3a; Chittka and Geiger, 1995; Menzel et al., 2010). In another study, honeybees were trained to collect sucrose solution at positions of up to four objects in a flight tunnel that allowed the exclusion of other cues that might occur in natural environments (Figure 3b; Dacke and Srinivasan, 2008). In both studies bees could not count beyond four landmarks.

Honeybees have also shown the capacity to match patterns containing up to four elements by number alone (Gross et al., 2009). When viewing a pattern of up to four elements bees were able to view the pattern, fly into a tunnel that presented multiple options, and choose the correct option, the stimulus that contained the same number of elements as the sample, independent of the shape, colour, or pattern of the individual elements. For example, bees could match three blue circles with three yellow stars (Figure 3c). Honeybees were unable to discriminate between the quantities of 4 vs. 5 or 5 vs. 6 in this experiment.

1.3.3. Conditioning procedures in bees

More recently, a study explored the effect of using either classical appetitive conditioning, or appetitive-aversive conditioning on honeybee performance in cognitive and visual tasks (Avarguès-Weber et al., 2010). Appetitive and absolute conditioning rewards bees for a correct choice while appetitive-aversive conditioning results in a rewarding or aversive outcome for correct and incorrect choices respectively. Avarguès-Weber et al. (2010) showed that appetitive-aversive conditioning significantly increased bee performance in perceptually difficult goal-orientated tasks. As previous studies on honeybee numerical competency were performed using classical appetitive conditioning, further research should aim to determine if the numerical competency of honeybees could be extended using the appetitive-aversive framework that has proven effective in many other cognitive and visual tasks (Avarguès-Weber et al., 2014; Avarguès-Weber et al., 2012; Avarguès-Weber et al., 2011; Howard et al., 2017a; Howard et al., 2017b; Ravi et al., 2016). Thus, perhaps the use of appetitive-aversive differential conditioning could improve the numerical ability of honeybees.

In Chapter 2, I test the impact of using appetitive-aversive conditioning on quantity discrimination in the honeybee to answer this question.

1.3.4. Conclusions

Due to the impressive but limited amount of research into invertebrate numerical competency (Carazo et al., 2009; Dacke and Srinivasan, 2008), further studies should be carried out testing numerical capacity in these species. The honeybee is an ideal model species to test how numerical skills may be modulated or improved with appetitive-aversive differential conditioning as they have previously demonstrated learning and application of concepts and rules, and have shown some numerical competency in previous studies (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008; Gross et al., 2009). In addition, the comparison of the capacity for an invertebrate, separated from humans for 600 million years (Consortium, 2006; Grimaldi et al., 2005), to perform numeric tasks will greatly inform the current debate on numerical ability in non-human animals and the evolutionary pathway which may have resulted in ‘number sense’.

This introduction has explored the current ideas and research surrounding numerical capacities of vertebrate and invertebrate species, and has outlined some of the on-going discussions in this area. I suggest that basic and more advanced numerical skills such as addition, subtraction, and understanding the concept of zero may not have developed alongside verbal language as human infants, non-human primates, and birds have demonstrated some level of understanding in these areas (Biro and Matsuzawa, 2001; McCrink and Wynn, 2004; Merritt et al., 2009; Pepperberg, 2006a; Pepperberg and Gordon, 2005; Wynn, 1992).

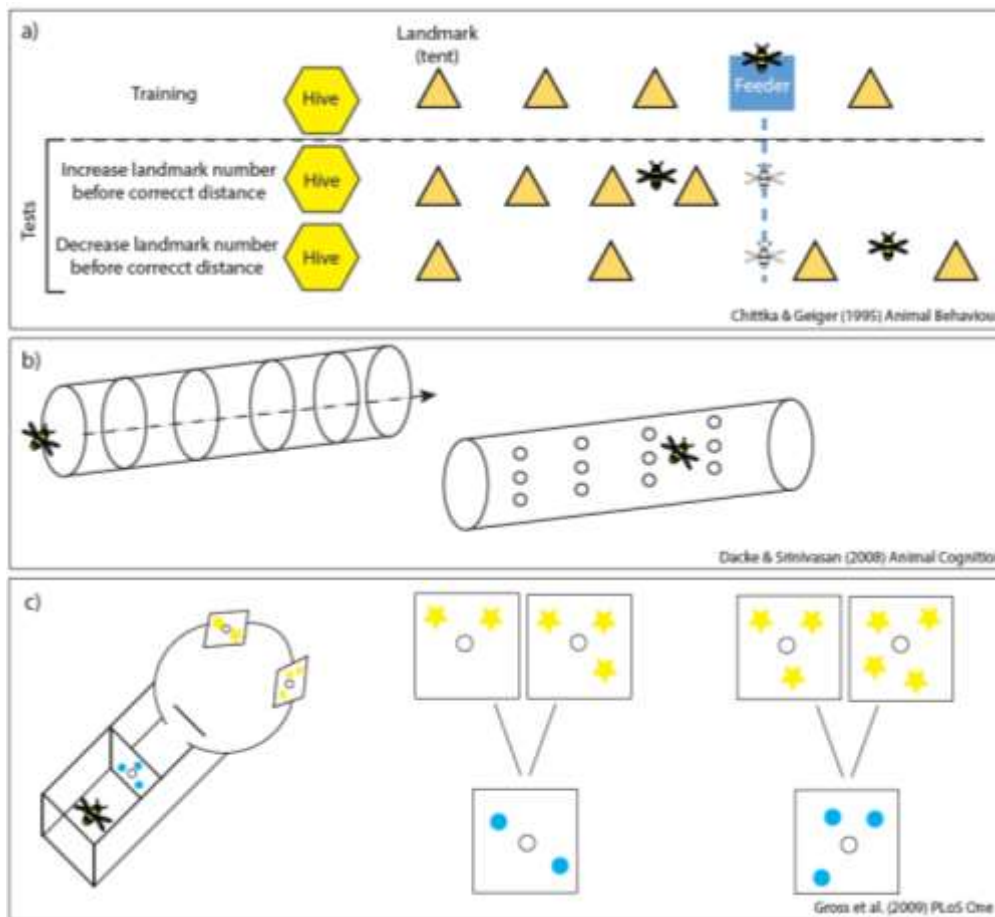


Figure 3: The representation of three studies on honeybee counting and number matching which have informed this research. a) A representation of the methodological set-up of Chittka & Geiger (1995). A number of yellow tents were placed 200 meters apart. Bees were trained to visit a feeder dispensing sucrose between the 3rd and 4th tent. When the number of tents before the distance of the feeder was increased, some bees (yellow and black icon) chose to visit the location of the 3rd and 4th tent in the new location suggesting they used landmark number as a cue while other bees (white and black icon) chose the return to the distance the original feeder was at suggesting they preferred to use distance rather than landmark number as a cue. This was also found when the number of tents before the original feeder was decreased. While some bees preferred to visit at the correct landmark number (yellow and black icon), between the 3rd and 4th tents, other bees preferred to use distance cues (white and black icon), visiting the original distance of the feeder. b) A representation of the methodological set-up of Dacke & Srinivasan (2008). Bees were trained to enter a flight tunnel to find sucrose solution at a certain number of landmarks. Non-numerical cues such as distance and the use of other landmarks were controlled for using the flight tunnel. Bees demonstrated that they could count to four landmarks of different representations in the flight tunnel tests to find sucrose when distance and other cues were controlled. c) A representation of the methodological set-up of Gross et al. (2009). Bees were trained to use a Y-maze apparatus which presented a sample stimulus (e.g. three blue dots) in a tunnel, and then two options one meter along the tunnel. Bees learnt to match the sample number of two or three elements to novel representations of elements containing two, three, but not four (e.g. yellow stars).

1.4. Part IV: Outline and summary of thesis

Number sense is a vital aspect of survival for foraging, predator avoidance, and navigation in non-human animals. Both spontaneous numerical abilities and learnt numerical tasks are demonstrated across species, which can extend to very high-level number skills (Figure 1). Despite the arguments that complex number skills and concepts are only accessible to humans with developed culture and language (Lenneberg, 1971; Núñez, 2017a; Núñez, 2017b), pre-verbal human infants, non-verbal adult humans, and non-human animals have displayed evidence of numerical cognition. In my thesis, I use the honeybee as a model species to formally test the numerical ability of an invertebrate, separated from humans by 600 million years.

Chapter Two presents experimental evidence that honeybees are able to surpass their previous number discrimination limit of four items through changes to training and conditioning. I demonstrate that with appetitive-aversive differential conditioning, the honeybee can be trained to discriminate between numerosities at and above the subitizing range. This is in comparison to honeybees trained with classical appetitive-conditioning which do not clearly demonstrate quantity discrimination above the subitizing range. These results are important precursors to the following chapters where I extend the limits of honeybee numeric ability to more advanced concepts and tasks.

Chapter Three presents my research on the ability of honeybees to learn the relational rules of ‘greater than’ vs. ‘less than’. Honeybees were able to learn these numeric rules and apply them to novel numerosities, representations of number, and even use the rules to understand the quantitative value of an empty set.

Chapter Four presents experimental results demonstrating that honeybees are able to learn simple arithmetic. Bees were able to learn a symbolic representation (colour) of addition and

subtraction and subsequently perform the operations $+ 1$ or $- 1$ using novel numerosities and representation of numerosities.

Chapter Five presents my research on the ability of bees to match symbols and numerosities. Honeybees were able to learn to match two numerosities to two symbols in either a symbol-to-number-matching task or a number-to-symbol-matching task. Bees could also transfer the task to novel representations of the quantities but were unable to reverse the association.

Chapter Six provides a discussion on the addition of my research to the existing literature. I compare the ability of bees to perform numerical tasks in my experimental work to other species and past research on honeybees. I discuss the implications of my experimental research on the debate regarding evolved numeric ability and numerical competency in non-human animals.

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Chapter 2

Title: Surpassing the subitizing threshold: Appetitive-aversive conditioning improves discrimination of numerosities in honeybees

Running title: Surpassing the subitizing threshold

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Summary statement

We show that appetitive-aversive differential conditioning enables bees to learn to discriminate quantities of 1- 10 elements, whereas classical appetitive conditioning does not allow bees to learn the same task.

Abstract

Animals including humans, fish, and honeybees have demonstrated a quantity discrimination threshold at four objects, often known as subitizing elements. Discrimination between numerosities at or above the subitizing range is considered a complex capacity. In the current study, we trained and tested two groups of bees on their ability to differentiate between quantities (4 vs. 5 through to 4 vs. 8) when trained with different conditioning procedures. Bees trained with appetitive (reward) differential conditioning demonstrated no significant learning of this task, and limited discrimination above the subitizing range. In contrast, bees trained using appetitive-aversive (reward-aversion) differential conditioning demonstrated significant learning and subsequent discrimination of all tested comparisons from 4 vs. 5 to 4 vs. 8. Our results show conditioning procedure is vital to performance on numerically challenging tasks, and may inform future research on numerical abilities in other animals.

Introduction

Quantity discrimination has been explored in a number of species through different methods (e.g. spontaneous choice or training on quantities; limited vs. extensive training), which sometimes yield different results (Agrillo and Bisazza, 2014; DeWind and Brannon, 2012; Gatto et al., 2017; Gazes et al., 2018; Miletto Petrazzini et al., 2018). The ability to discriminate quantities varies across different species, with insects such as mealworm beetles able to discriminate ratios (calculated by dividing the lower number by the higher number) of 0.25 – 0.33 (Carazo et al., 2009). Species which can discriminate the more difficult ratio of

0.80 include monkeys (Addessi et al., 2008; Gazes et al., 2018), jungle crows (Bogale et al., 2011), ponies (Gabor and Gerken, 2018), Clark's nutcrackers (Tornick et al., 2015), and dolphins (Jaakkola et al., 2005). Some animals including elephants (Irie-Sugimoto et al., 2009), Great apes (Hanus and Call, 2007), Mexican jays (Kelly, 2016), Western scrub jays (Kelly, 2016), and guppies (Bisazza et al., 2014; Lucon-Xiccato et al., 2017) can discriminate ratios over 0.80, an impressive feat. Previous studies on quantity discrimination suggest some variation between low quantities (four and less) and high quantities (four and greater). For example, frogs (*Bombina orientalis*) are able to discriminate ratios of 0.67 with four items or less but only ratios of 0.5 with four or more items (Stancher et al., 2015). Discrimination ability can also vary with different number comparisons of the same ratio, for example North Island robins can discriminate a ratio of 0.875 when comparing the quantities of seven vs. eight, but not with the quantities of 14 vs. 16, despite both comparisons being at the same ratio (Garland et al., 2012).

Several species, including humans, exhibit a numerical competency threshold at four (Agrillo et al., 2008; Cowan, 2010; Gómez-Laplaza and Gerlai, 2011a; Jevons, 1871; Kaufman et al., 1949; Simons and Langheinrich, 1982; Trick and Pylyshyn, 1994). This threshold is observed in visual object and tactile stimulation estimation in humans (Dehaene and Cohen, 1994; Jevons, 1871; Piazza et al., 2002; Riggs et al., 2006; Starkey and Cooper Jr, 1995), quantity discrimination in fish (Agrillo et al., 2008; Gómez-Laplaza and Gerlai, 2011a; Seguin and Gerlai, 2017), as well as counting and number-generalisation in honeybees (Boysen, 1988; Dacke and Srinivasan, 2008; Gross et al., 2009). The threshold phenomenon is observed across a diverse range of species and may be due to the hypothesis that 'counting' has two mechanisms: 'subitizing' (accurately and quickly recognising four or less objects) and 'true counting' (the process of sequentially incrementing the number of identified elements, theoretically without bound; Agrillo et al., 2008; Kaufman et al., 1949; Piazza et al., 2002;

Tomonaga and Matsuzawa, 2002; Trick and Pylyshyn, 1994). Some non-human animal species have been shown to surpass this threshold limit of four. For example, the ability of mosquitofish to discriminate between two shoals (groups of conspecifics) consisting of more than four individuals was shown to improve as the numerical distance between them increased. The ability to discriminate between two shoals consisting of four or more fish was found only with ratios of 0.50 or smaller (4 vs. 8, 8 vs. 16 and 4 vs. 10; Agrillo et al., 2008).

It appears that Weber's Law may have an impact on counting and estimation. Weber's Law describes how sensory systems distinguish between two stimulus magnitudes based on their proportional difference (Akre and Johnsen, 2014; Fechner, 1965; Weber, 1978). Also known as proportional processing, Weber's Law is used by a number of animals to detect changes in sound, light, odours, electrical fields, pressure, number, and time (Akre and Johnsen, 2014). When animals adhere to Weber's law, they compare stimuli on the basis of proportional differences. This is also the case in angelfish, where Weber's Law plays a role in their ability to discriminate between numbers four and above. Angelfish can discriminate between 'higher-level' numbers (four and higher) at a ratio of 0.50 (Gómez-Laplaza and Gerlai, 2011a). Recent studies suggest that numerical processing may be flexible in animals when using different training and/or testing procedures (Bisazza et al., 2014; Lucon-Xiccato et al., 2017); thus comparative studies of different animals can provide important insights into general principles of numeric processing.

Honeybees have exhibited a numerical threshold at four. Studies testing the ability of bees to count landmarks in either open natural environments or controlled laboratory conditions both found that bees were able to reliably count up to four landmarks, but failed at counting more than four landmarks, in order to find a source of food (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008). In a study using a delayed-matching-to-sample (DMTS) procedure in a Y-maze, bees were able to accurately match specific quantities of up to three elements

irrespective of shape, colour, and pattern of the objects to receive a reward of sucrose, but were unable to do this with higher numbers (Gross et al., 2009). Gross et al. (2009) showed a discrimination ability of 2 vs. 3 and 3 vs. 4 but not 4 vs. 5 or 4 vs. 6. These studies were important steps in determining that bees could both count and match numbers, and that numerical ability was a biologically meaningful skill for either navigation (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008; Menzel et al., 2010), or counting the numbers of flowers in a flower patch during foraging (Gross et al., 2009).

While many studies have explored the numerical abilities of vertebrates, relatively little research has been done on the numerical competency of invertebrates (Carazo et al., 2009; Dacke and Srinivasan, 2008), even in well-studied model species, such as the honeybee, which has demonstrated a limited numerical ability when using classical conditioning. The previous studies on number matching and counting in honeybees used classic appetitive or appetitive-differential conditioning frameworks, where bees received a reward of sucrose for a correct choice and no outcome for an incorrect choice (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008; Gross et al., 2009). Relatively recent advancements in training protocol have shown that when bees are trained on a perceptually difficult colour task, performance significantly increases when appetitive-aversive differential conditioning is used in training compared to appetitive-differential conditioning (Avarguès-Weber et al., 2010; Dyer, 2012). Appetitive-aversive differential conditioning is where a bee receives a reward of sucrose for a correct choice and an aversive outcome of quinine for an incorrect choice, whereas appetitive-differential conditioning rewards a bee for a correct choice and gives no outcome for an incorrect choice. When a colour discrimination task was easy to learn, the conditioning procedure had no significant effect on performance during training or in unconditioned tests, however, when the task was perceptually challenging, only bees which were trained with appetitive-aversive conditioning demonstrated learning in tests (Avarguès-Weber et al., 2010;

Dyer, 2012). There is evidence that including a distractor associated with an aversive outcome also improves the strength of the association between correct option and the reward in vertebrates (Rescorla and Wagner, 1972), which is potentially linked to attentional resources (Avarguès-Weber et al., 2010; Dyer, 2012; Giurfa, 2004; Rescorla and Wagner, 1972). Recent work on spatial visual processing in honeybees shows that learning and behavioural outcomes can be significantly influenced by the conditioning procedure employed during training (Dyer and Griffiths, 2012; Dyer et al., 2005; Giurfa et al., 1999; Howard et al., 2017; Stach and Giurfa, 2005), and so bees have become an important model species for understanding comparative visual perception and cognition.

Recently honeybees were shown to learn the rules of ‘less than’ and ‘greater than’ and apply these rules to the novel numerosities of zero and five using appetitive-aversive differential conditioning (Howard et al., 2018). Bees demonstrated an ability to learn to discriminate between all combinations of the numbers zero up to six during training in one of the experiments, a discrimination ability which has not been previously shown in honeybees (Howard et al., 2018). This result is likely a due to the use of appetitive-aversive differential conditioning during training and thus in this study we formally test and compare the numerical discrimination ability of bees when trained with appetitive-differential conditioning or appetitive-aversive differential conditioning. We will determine whether numerical discrimination is improved with different conditioning procedures, and thus provide some insights into why different quantity discrimination results are observed across a range of animal taxa.

Materials and Methods

Study design:

We aimed to determine if there was a difference in quantity discrimination performance between bees which were trained using appetitive-differential conditioning (Group 1) compared to bees trained with appetitive-aversive differential conditioning (Group 2). To do this, we trained bees to choose four elements when presented against the quantities one to ten. We then tested bees on their ability to discriminate between quantities of four vs. five, six, seven, and eight.

Study species:

We used 22 free-flying honeybees (*Apis mellifera*) foragers for this experiment. All bees were marked with a coloured dot on the thorax to identify individuals. A gravity feeder was set up within 20 meters of the experiment to provide 10 – 30 % sucrose which attracted a regular number of bees for use in experiments. Bees were randomly assigned to test groups.

Apparatus:

Individual honeybees were trained to enter a Y-maze (Fig. 1; as described in Avarguès-Weber et al., 2011). Each bee had to fly through an entrance hole to enter an initial chamber and then fly through another hole into the decision chamber where the bee would be presented with two different options in each arm of the chamber, one correct and one incorrect (Fig. 1).

Stimuli were presented on grey backgrounds located 5 cm away from the decision lines to ensure each element was above the minimum resolution threshold for free-flying honeybees (Srinivasan and Lehrer, 1988). During training in Group 1, a 10 μ L drop of 50 % sucrose solution (appetitive/rewarding outcome) was paired with a correct choice and a 10 μ L drop of

water (neutral unreinforced outcome) was paired with the incorrect choice (appetitive-differential conditioning). During training in Group 2, a 10 μ L drop of 50 % sucrose solution (appetitive/rewarding outcome) was paired with a correct choice and a 60 mM quinine solution (aversive outcome) was paired with an incorrect choice (appetitive-aversive differential conditioning). Each stimulus had a transparent landing pole located below it which held either the drop of sucrose, quinine, or water. Poles were replaced when touched by a bee and cleaned with 20 % ethanol then water and dried to exclude olfactory cues. The side of correct and incorrect stimuli were randomly changed between choices. If a bee made an incorrect choice and started to imbibe the quinine; it was allowed to fly to the pole in front of the correct stimulus to collect sucrose to maintain motivation; but only the first choice was recorded for statistical analysis following standard procedures (Avarguès-Weber et al., 2015). Once the bee was finished imbibing the sucrose, it was allowed to fly back to the hive if satiated or make another decision by re-entering the maze from behind an opaque screen. During the non-reinforced tests, a drop of water was placed on each of the poles placed in front of the stimuli. Ten choices (touches of the poles) were recorded for each of the four tests to enable statistical comparisons, consistent with standard testing procedures for honeybees (Avarguès-Weber et al., 2015; Howard et al., 2018; Howard et al., 2017).

Stimuli:

Each stimulus was a 6 x 6 cm white square card containing a number of black elements (Fig. 2), and was covered with 80 μ m Lowell laminate. Elements in the learning phase could be one of five shapes: square, diamond, circle, triangle, or star. Randomised shapes were used for the four tests to ensure patterns and shapes were unfamiliar to bees during tests (Fig. 3A). Stimuli ranged from having one to ten elements, and no stimulus was shown in more than a single bout (return to hive to offload sucrose; approximately 2 – 5 choices).

There were 3 sets of stimuli consisting of (i) equal overall surface area (set 1; $n = 164$; surface area = $10 \pm 0.3 \text{ cm}^2$), (ii) consistently equal element size (set 2; $n = 160$), or (iii) novel randomised shapes (set 3; $n = 20$; Fig. 2). There were 344 stimuli in total; and furthermore stimuli were rotated to one of four orientation positions determined randomly by dice rolls to provide training and testing sets with over 1000 options.

Training procedure:

Bees were incrementally trained to enter the Y-maze and both arms of the apparatus over 30 – 60 minute periods. Once each bee was able to fly into the entrance hole and the hole that led to the decision chamber and could find the poles in both Y-maze arms, the experiment began.

Each bee completed either 50 appetitive-differential conditioning choices (Group 1) or 50 appetitive-aversive differential conditioning choices (Group 2). Bees in group 1 were rewarded with sucrose for a choice of four elements and received no outcome (a drop of water on pole) for an incorrect choice of any other number. Bees in group 2 were rewarded with sucrose for a choice of four elements and received an aversive outcome, quinine, for a choice of any other number of elements ranging from one to ten (excluding four; Fig. 3A).

Testing procedure:

Once bees had completed the training, there were four tests of 10 unreinforced choices each for bees in either group. Between each of the four tests there were 10 refresher reinforced choices to maintain bee motivation (same procedure as the learning phase). The sequence of these tests was randomised. The tests were non-reinforced (no reward or punishment) and used a 10 μL drop of water (neutral outcome) instead of quinine or sucrose to motivate bees to land. Bees were shown comparisons of four vs. five, four vs. six, four vs. seven and four

vs. eight elements (Fig. 3A), these tests were comparing quantity ratio discriminations of 0.80, 0.67, 0.57 and 0.50, respectively.

Statistical analysis:

To test for the effect of training on bee performance (number of correct choices) in both groups, data from the learning phase of 50 choices were analysed with a generalized linear mixed-effect model (GLMM) with a binomial distribution using the ‘glmer’ package within the R environment for statistical analysis. We fitted a full model with trial number as a continuous predictor, and subject as a random factor to account for repeated choices of individual bees.

To determine whether bees were able to learn to choose four elements in tests, we analysed the test data by employing a GLMM including only the intercept term as fixed factor and subject as a random term. The proportion of ‘correct’ choices (MPCC) recorded from the tests were used as response variable in the model. The Wald statistic (z) tested if the mean proportion of correct choices recorded from the learning test, represented by the coefficient of the intercept term, was significantly different from chance expectation, i.e. $H_0: MPCC = 0.5$.

All analyses were performed within the R environment for statistical analysis.

Results

Training phase:

In this experiment, bees were trained to choose four elements, when presented against quantities ranging from one to ten elements. Two groups of bees were trained for 50 trials of either appetitive-differential conditioning (Group 1), or appetitive-aversive differential conditioning (Group 2) using sets of stimuli with either equal overall surface area (set 1) or consistently equal element size (set 2; Fig. 3A). Only bees trained using appetitive-aversive

differential conditioning demonstrated significant learning over the period of 50 trials (statistical test: generalized linear mixed-effect model (GLMM) with a binomial distribution with trial number as a continuous predictor, and subject as a random factor to account for repeated choices of individual bees; Group 1: $z = 0.102$; $P = 0.918$; $n = 10$; Fig. 3B; Group 2: $z = 5.48$; $P < 0.001$; $n = 12$; Fig. 3C).

Testing phase

After the acquisition phase, honeybees were evaluated on their discrimination ability between four elements and higher numbers in four tests (4 vs. 5, 4 vs. 6, 4 vs. 7 and 4 vs. 8), for ten unreinforced choices per comparison using randomly-shaped novel elements (set 3), presented in a random order. Bees trained using an appetitive only procedure were unable to differentiate between 4 vs. 5 (statistical test: logistic regression with individual as random term tested differences between observed proportion of bee choices and chance level, $y = 0.5$; 51.0 ± 3.8 % (mean \pm s.e.m.); $z = 0.200$; $P = 0.841$), 4 vs. 6 (50.0 ± 4.90 %; $z = 0.000$; $P = 1.000$) or 4 vs. 8 (54.0 ± 5.00 %; $z = 0.600$; $P = 0.549$) at a level significantly different from chance, except during the test of 4 vs. 7 (62.0 ± 3.60 %; $z = 2.18$; $P = 0.029$; Fig. 3D). Because the bees were unable to discriminate 4 vs. 8, it is likely that the 4 vs. 7 discrimination is a statistical anomaly, although more studies are required to confirm this hypothesis. In contrast, bees trained using appetitive-aversive differential conditioning were able to discriminate between 4 vs. 5 (mean = 59.2 ± 3.10 %; $z = 2.00$; $P = 0.046$), 4 vs. 6 (mean = 60.8 ± 3.80 %; $z = 2.35$; $P = 0.019$), 4 vs. 7 (mean = 63.3 ± 3.30 %; $z = 2.89$; $P = 0.004$), and 4 vs. 8 (mean = 64.2 ± 3.40 %; $z = 3.06$; $P = 0.002$; Fig. 3E), all at a level significantly different to chance expectation. Thus our results show that bees perform quantity discrimination at a level significantly different to chance expectation when trained using appetitive-aversive differential conditioning, but not with appetitive-differential conditioning.

Discussion

Our results show that numerical discrimination above the subitising range is possible with appetitive-aversive differential conditioning. Bees trained using this method were able to discriminate quantities over four at a ratio of 0.80 (4 vs. 5), a finer discrimination than what was previously observed in honeybees considering appetitive conditioning (Gross et al., 2009). There is some evidence that bees trained with appetitive-differential conditioning may be able to discriminate ratios of 0.57 (4 vs. 7) above the threshold of four objects, however this is still an open question as these bees failed at discriminating the less challenging ratio of 0.50 (4 vs. 8). Honeybees are currently at a level of quantity discrimination observed in species such as African grey parrots (Al Aïn et al., 2009), capuchin monkeys (Addessi et al., 2008; Gazes et al., 2018), squirrel monkeys (Gazes et al., 2018), dolphins (Jaakkola et al., 2005), ponies (Gabor and Gerken, 2018), jungle crows (Bogale et al., 2011), and guppies (Bisazza et al., 2014). The question of whether the introduction of an aversive outcome for an incorrect choice improves performance has been asked for colour discrimination tasks in bees (Avarguès-Weber et al., 2010; Chittka et al., 2003), but this is the first time appetitive and appetitive-aversive differential conditioning have been directly compared for a numerical visual task.

A potential explanation for why bees perform better at numerosity discrimination with appetitive-aversive differential conditioning may be that the attentional processes are improved due to the presence of an aversive outcome for an incorrect choice (Avarguès-Weber et al., 2010). When the penalty for making an incorrect decision is low there is less motivation to ensure performance is as accurate as possible, however, when the penalty is increased, there is an increase in motivation to be more accurate and thus attention may be modulated by conditioning procedure (Avarguès-Weber et al., 2010). By pairing the incorrect option with an aversive outcome, the conditioning procedure is also improving the strength of

the association between the rewarding outcome and the correct option, as demonstrated in vertebrates (Rescorla and Wagner, 1972).

Through the possible modulation of attention, we show that previous studies on animal numerical ability, specifically quantity discrimination, may be underestimating the potential numeric ability of non-human animals. In the current study we found a difference in the results considering both the training and test phases of honeybee quantity discrimination when bees were trained with the respective procedures. Our result is supported by previous quantity discrimination studies that show varied results within the same species when different methods are employed. For example, guppies are a well-studied species for their quantity discrimination abilities (Agrillo et al., 2012; Bisazza et al., 2014; Gatto et al., 2017; Lucon-Xiccato and Dadda, 2017; Lucon-Xiccato et al., 2017; Lucon-Xiccato et al., 2015; Piffer et al., 2012; Piffer et al., 2013), but their discrimination ability appears dependent on the procedure by which they are tested (Agrillo and Bisazza, 2014). Guppies tested on quantity discrimination using spontaneous choice, training, extensive training, or new methods exhibit different limits of discrimination. Recording the spontaneous choices of guppies has resulted in a discrimination ability at the ratio of 0.75 for low (four and less) number comparisons (Piffer et al., 2012) and 0.67 for high (four and higher) number comparisons (Miletto Petrazzini and Agrillo, 2016), however with extensive training, this has been extended to 0.80 with higher numbers (Bisazza et al., 2014), and even 0.83 using a recently developed method (Lucon-Xiccato et al., 2017). In the current study, we also show that different methods yield different results and quantity discrimination abilities, thus perhaps non-human animals in other studies which are not as motivated to avoid incorrect choices may not have been pushed to their cognitive limits. Numerical processing is likely to be a valuable skill for animals operating successfully in complex environments (Gómez-Laplaza and Gerlai, 2011a; Gómez-Laplaza and Gerlai, 2011b; Lucon-Xiccato et al., 2017;

Miletto Petrazzini and Agrillo, 2016; Nieder, 2017; Seguin and Gerlai, 2017), and we show that motivation, as modulated through conditioning, is critical to understanding what level of numerical abilities an animal demonstrates.

Author contribution

All authors contributed to experimental design, interpretation of the data, and editing of the manuscript. SRH collected and analysed the data. All authors gave final approval for submission.

Competing interests

The authors declare no competing interests.

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Data availability

The raw choice data from tests of individual bees that support the findings of this study will be made available on Dryad Data Repository.

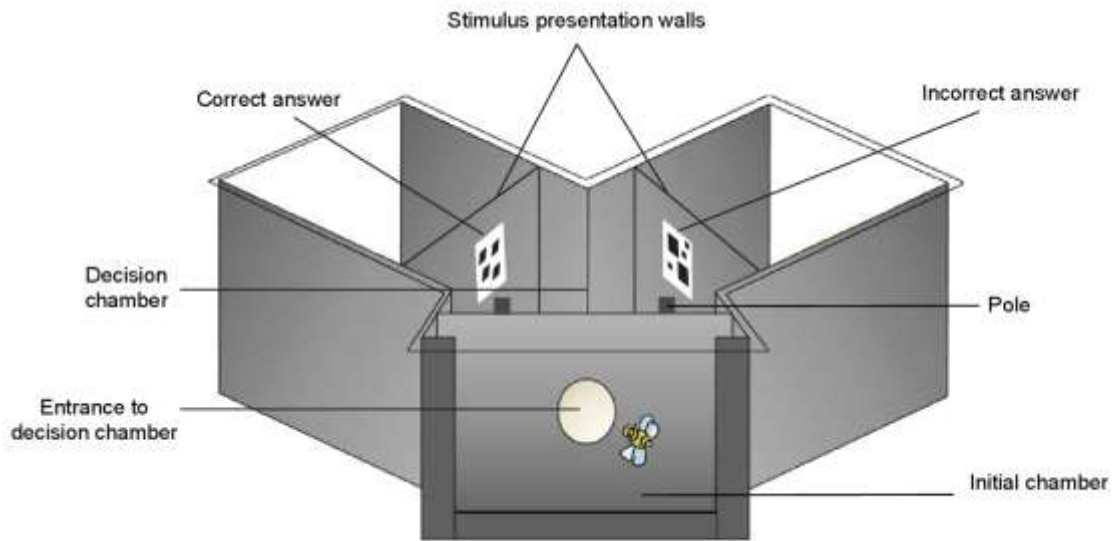


Figure 1: Y-maze apparatus set-up for the experiments. The diagram shows parts of the Y-maze and the stimuli positions. Bees were trained to choose four elements when presented against the incorrect options of one – ten (excluding four).

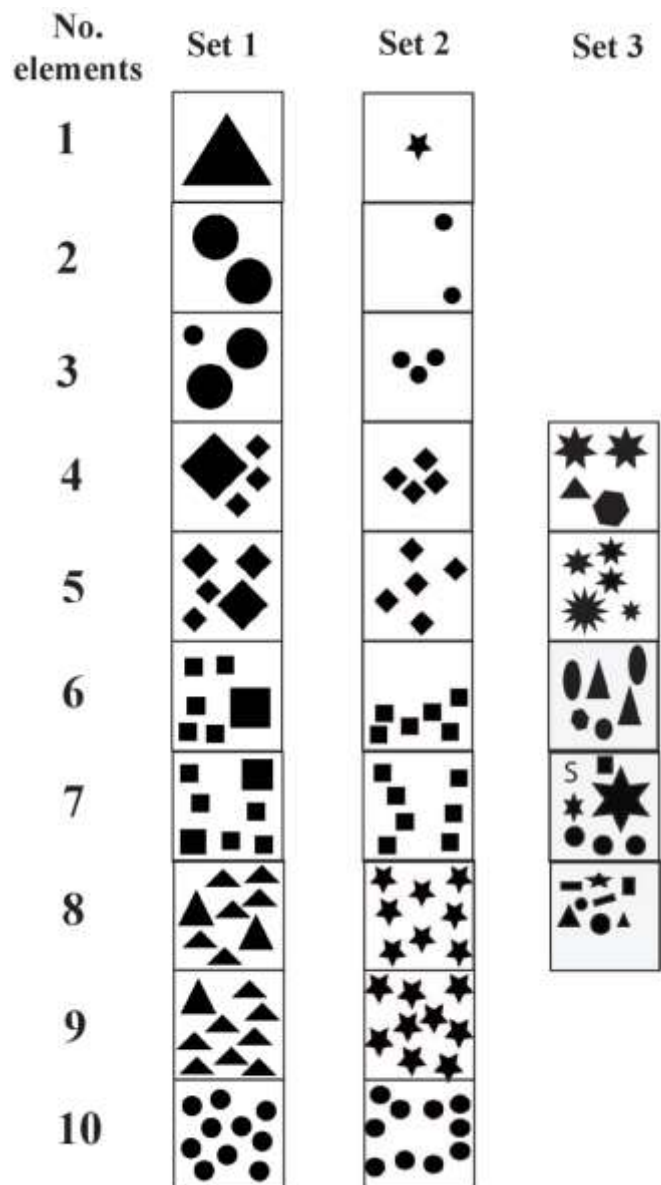


Figure 2: An example subset of the stimuli used in the training (set 1; set 2) and the testing (set 3) phases. We provide an example of one stimulus per quantity per set. Stimuli rotational axis was randomized to one of four positions to further exclude low-level cues.

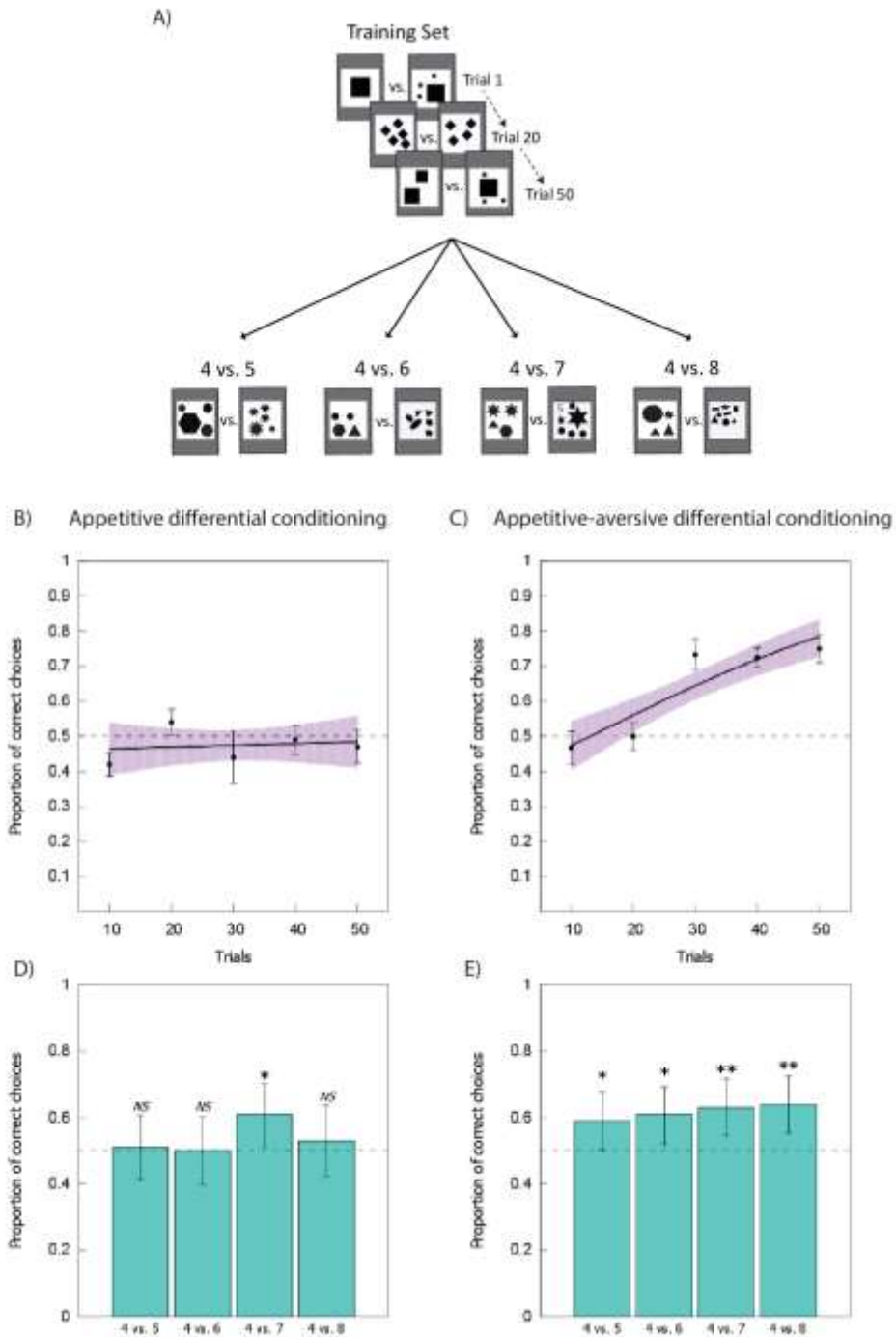


Figure 3: Graphic representation of the method and results of Group 1 and 2 training and testing. A) Examples of possible stimuli combinations during trials and tests. B-C) Performance during the training phase of 50 trials of either appetitive-differential conditioning (B) or appetitive-aversive differential conditioning (C). D-E) Performance during the unreinforced testing phases of Group 1 (D) and 2 (E). Data shown are means \pm

s.e.m. for both groups. Broken black line at 50 % indicates chance level performance.

Significance from chance level performance is indicated by * ≥ 0.05 , ** ≥ 0.01 , *** ≥ 0.001 ,

NS indicates performance which was not significant from chance.

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Chapter 3

Numerical ordering of zero in honey bees

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Abstract:

Some vertebrates demonstrate complex numerosity concepts including addition, sequential ordering of numbers, or even the concept of zero, but whether an insect can develop an understanding for such concepts remains unknown. We trained individual bees to the numerical concepts of ‘greater than’ or ‘less than’ using stimuli containing 1 – 6 elemental features. Bees could subsequently extrapolate the concept of ‘less than’ to order zero numerosity at the lower end of the numerical continuum. Bees demonstrated an understanding that parallels animals such as an African grey parrot, non-human primates, and even pre-school children.

One Sentence Summary: Honeybees use the acquired number rule of ‘less than’ to place an empty set at the low end of the numerical continuum.

Main Text:

Four stages are used to describe the acquisition of understanding zero in human history, psychology, animal cognition, and neurophysiology (*I*). First is the ability to define zero as nothing – the absence of a stimulus. Second is the categorical classification of zero as “nothing” versus “something”. The third stage is understanding zero as a quantity at the low end of the positive integer numerical continuum. The fourth, and currently designated as the most advanced stage of understanding zero, is the symbolic representation of zero, as with an Arabic number, as employed in modern mathematics and calculations (*I*).

Several ancient human civilizations lacked the full understanding and importance of zero, leading to constraints in their numeric systems (*I*). Interestingly, some vertebrate animals have recently demonstrated a capacity to acquire and understand this numerical concept. Rhesus monkeys learnt that empty sets of objects occupy a position on a numerical

continuum (2, 3), vervet monkeys used subtraction-like reasoning to determine if food was present or absent (4), a chimpanzee reached near-perfect performance on zero-concept tasks with training (5), and an African grey parrot spontaneously labeled absent objects as ‘none’(6).

Honeybees have previously demonstrated the capacity to count and discriminate up to four objects (7-10) using classic conditioning techniques. Recent advancements in conditioning protocols (11) reveal that bees can acquire rule-based relational concepts (12, 13), thus enabling remarkable plasticity to acquire and apply seemingly advanced concepts such as size ordering (14). In this study, we test the capacity of honeybees to extrapolate the acquired concepts of ‘greater than’ and ‘less than’ as shown in primates (15, 16), and thus formally demonstrate that an invertebrate can understand the concept of zero numerosity.

We designed a set of experiments to test the extent to which honeybees may understand the concept of zero numerosity (17). In Experiment 1, we trained bees to understand the concepts of ‘less than’ and ‘greater than’ using appetitive-aversive differential conditioning (11). Bees were trained to the respective concepts using white square stimuli containing 1 – 4 black elements (Figure 1A; S1; Table S1). After reaching a criterion of $\geq 80\%$ accuracy, bees demonstrated in non-reinforced tests they had learnt the concept of ‘numerically less’ ($75.0 \pm 4.1\%$ (mean \pm standard error of the mean (s.e.m.)); $H_0 = 50\%$, $z = 5.08$, $P < 0.001$, $n = 10$) and ‘numerically greater’ ($75.5 \pm 3.3\%$; $z = 6.556$, $P < 0.001$, $n = 10$) when presented with novel stimuli of 1 – 4 elements. Furthermore, bees were able to apply these concepts to determine that five elements were greater than two or three elements (‘less than’: $68.0 \pm 5.0\%$; $z = 3.411$, $P < 0.001$, $n = 10$; ‘greater than’: $75.0 \pm 3.9\%$; $z = 5.333$, $P < 0.001$; $n = 10$). Interestingly, bees demonstrated an understanding that zero numerosity lies at the lower end of the numerical continuum by choosing an ‘empty set’ stimulus containing no elements if trained to ‘less than’ ($64.0 \pm 5.4\%$; $z = 2.795$, $P = 0.005$, $n = 10$; Figure 1C), or choosing

stimuli containing elements if trained to ‘greater than’ ($74.5 \pm 2.6 \%$; $z = 6.609$, $P < 0.001$; $n = 10$; Figure 1C).

In Experiment 2, we tested the extent to which bees may understand the quantitative concept of zero in comparison with other animals. As some animals find it challenging to differentiate between the numbers zero and one (5, 6, 18), we trained bees to ‘less than’ using stimuli containing 2 – 5 elements and then tested their ability to differentiate between the novel numerosities of one and zero (Figure 1B). After reaching a criterion of $\geq 80 \%$ accuracy, bees demonstrated the learnt concept of ‘numerically less’ when presented with numbers 2 – 5 ($73.8 \pm 1.9 \%$; $z = 10.18$, $P < 0.001$). When presented with the novel numbers of 1 vs. 0, bees chose the lower number of zero ($63.0 \pm 2.9 \%$; $z = 4.23$, $P < 0.001$; Figure 1D) showing an understanding that an empty set is lower than one, which is challenging for some other animals (5, 6, 18).

When bees were presented with two conflicting pieces of information, 2 vs. 0, where the two element stimuli had always been rewarding in training, and zero was the correct lower number, bees chose the empty set at a level which was not significantly different from chance ($56.2 \pm 3.4 \%$; $z = 1.64$, $P = 0.101$; Figure 1D); thus, bees perceived both plausible alternatives as consistent with their conditioning experience. These results demonstrate that bees were using both an associative mechanism for choosing two elements, and a concept-based mechanism for choosing zero numerosity. This phenomenon was also observed in a dolphin trained to choose the numerically less option using white dots on a black background. This result is explained in terms of an artifact of training set conditioning causing a bias towards consistently rewarding stimuli (19).

To test if bees understood an empty set quantitatively along the numerical continuum, we evaluated numerical distance effects, where accuracy of performance potentially improves as

the difference in magnitude between two respective numbers increases (*1*). In Experiment 3, we trained and tested bees on the ‘less than’ concept using numbers 0 – 6. If bees considered zero numerosity as a number along the numerical continuum, we would expect accuracy of decisions to be the greatest with 0 vs. 6 and poorer for lower numbers vs. zero numerosity. Figure 2A photographically shows that bee vision, as described in (22), is able to easily view our stimuli. After reaching a criterion of ≥ 80 % accuracy during training, bees demonstrated in tests that they could discriminate an empty set from numbers 1 – 6 accurately (ESM 1; Figure 2B). While bees could accurately discriminate all numbers from zero numerosity, there was a significant effect of numerical distance on accuracy (Figure 2B). Bees were more accurate when numbers were numerically more distant (0 vs. 5 and 0 vs. 6) than when numerically closer (0 vs. 1), showing bees are impacted by number magnitude and thus exhibit numerical distance effects.

An alternative explanation for our results could be that bees have a preference for the novel presentation of an empty set stimulus. However, control experiments showed that the bees’ understanding of zero belonging at the lower end of the numerical continuum was rule-based, and not driven by a novel preference (ESM 2; Figure S2). The spatial frequencies of stimuli are also ruled out as a potential explanation for results (ESM 1; Table S1). We additionally conducted further control experiments to exclude bees learning to match pairs of numbers during training (ESM 3; Figure S2).

Our findings show that honeybees can learn and apply the concepts of ‘greater than’ and ‘less than’ to interpret a blank stimulus as representing the conceptual number of zero and place zero in relation to other numerical values. Bees thus perform at a consistent level to that of non-human primates by understanding that zero is lower than one (*5*).

An open question remains as to whether such advanced numerical understandings may be widespread across many animals that deal with complexity in their environments, or if our findings are the result of independent evolution in honeybees. Recent comparative studies of primate and crow brains found that similar levels of numeric processing are facilitated by very different brain structures, suggesting independent evolution of numeric processing (20, 21). By demonstrating that an insect, with a different brain structure from primates and birds, can understand the concept of zero, it would be of high value to consider such capacities in other animals.

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Data and materials availability: Additional data, including the individual choices of all bees, are available in the Supplementary Materials and raw data is available in the Dryad Data Repository: doi:10.5061/dryad.7187rf5.

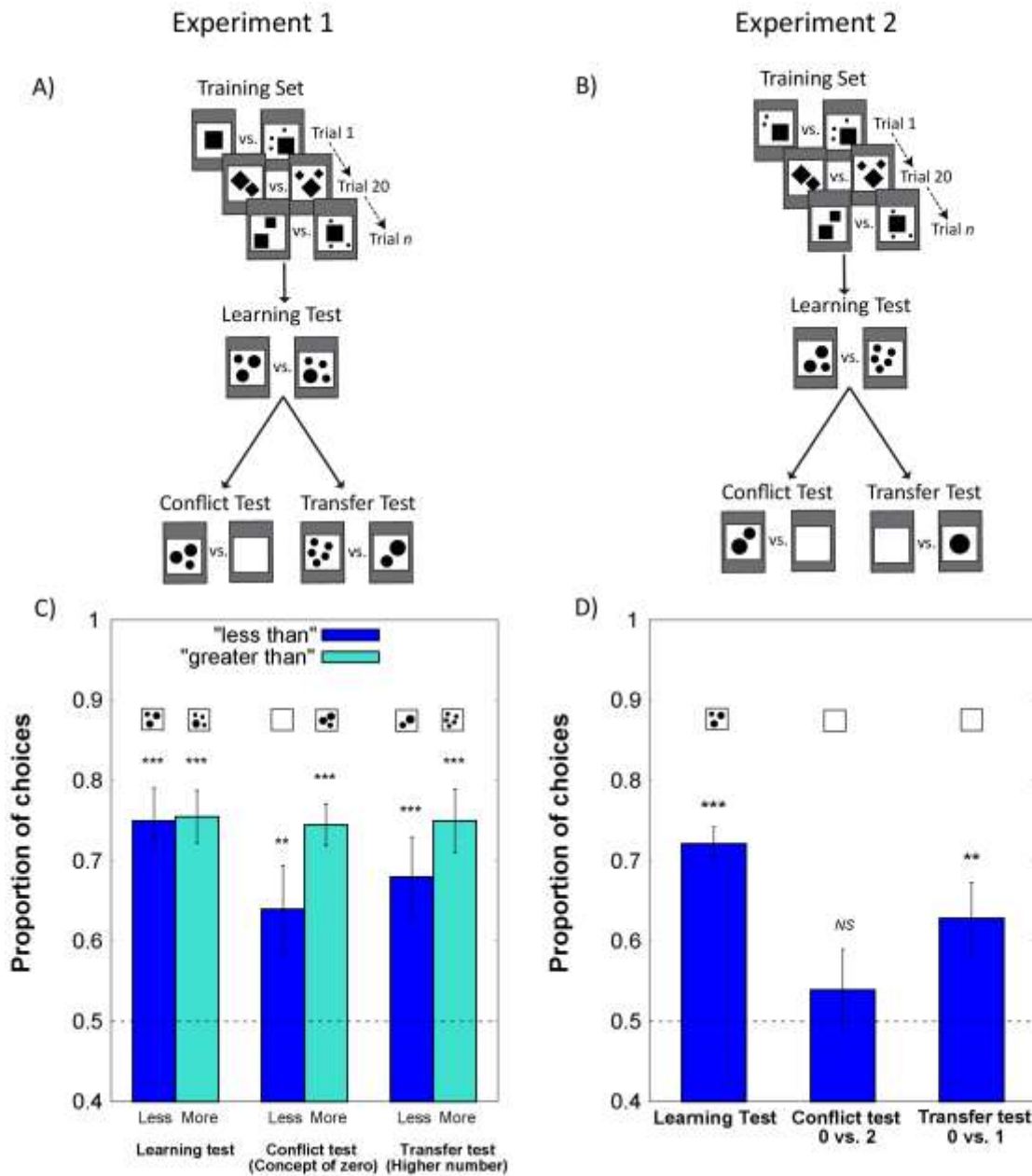


Fig. 1. Graphic representation of the method and results of Experiment 1 and 2. A-B) Examples of possible stimuli combinations during trials and tests in Experiments 1 and 2. C-D) Performance during the unreinforced testing phases during Experiment 1 and Experiment 2. Data shown are means \pm s.e.m. for both treatment groups (Bees trained to ‘less than’ are shown in dark blue, bees trained to ‘greater than’ are shown in turquoise). Stimuli above the columns represent the choices for those stimuli in the data. In Experiment 1 the conflict test evaluating the bee’s concept of zero, data shown for the ‘less than’ group ($n = 10$) are choices

for zero, and data shown for the 'greater than' ($n = 10$) group are choices for stimuli containing elements. In the transfer test to a higher number, data shown for bees trained to 'less than' are choices for a lower number, and for bees trained to 'greater than', data shown are choices for the higher number of five. In Experiment 2 ($n = 25$) the conflict and transfer tests show the bee's choices for zero. Broken black line at 50 % indicates chance level performance. Significance from chance level performance is indicated by * ≥ 0.05 , ** ≥ 0.01 , *** ≥ 0.001 .

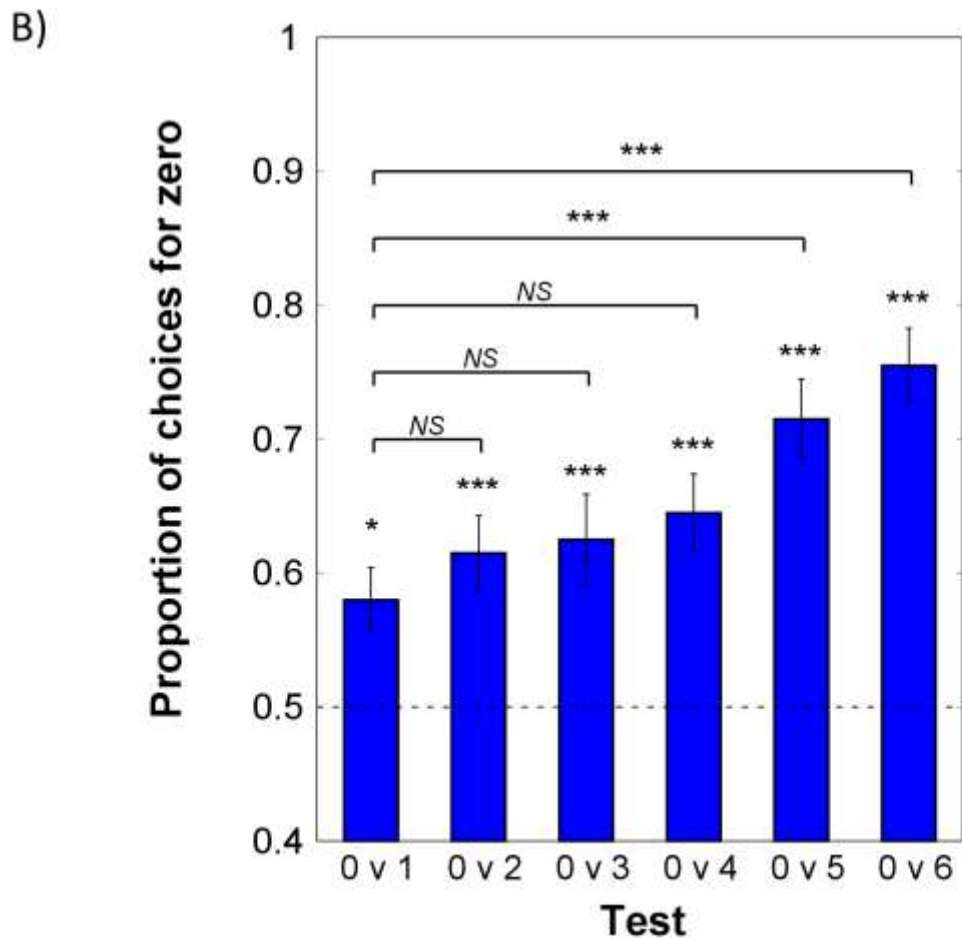
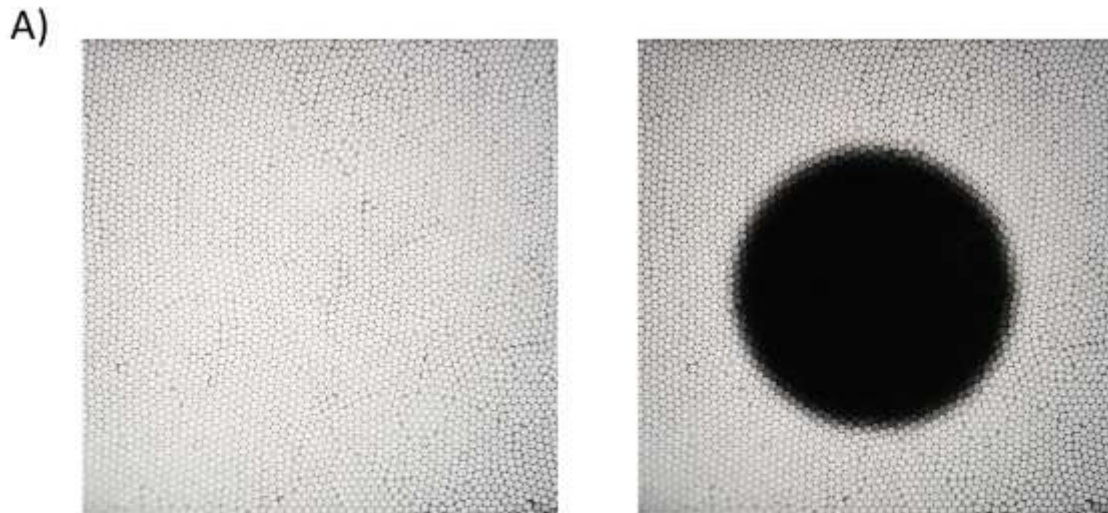


Fig. 2. Photographic representation of stimuli and results from Experiment 3. A) Representation of honeybee spatial vision when viewing stimuli of either zero or one (22). **B)** Honeybee performance during Experiment 3 testing the behavioural effects of numerical

distance of numerosity zero. Data shown are means \pm s.e.m. for the choice of the zero stimuli. Broken black line at 50 % indicates chance level performance. Significance from chance level performance and from other tests is indicated by * \geq 0.05, ** \geq 0.01, *** \geq 0.001, non-significance is indicated by *NS*.

Supplementary Materials for

Bees extrapolate ordered relations to place numerosity zero on a numerical continuum

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This includes:

Materials and Methods
Electronic Supplementary Material (ESM) 1
ESM 2
ESM 3
Figure S1
Figure S2
Figure S3
Figure S4
Table S1

Materials and Methods

Experimental Design

The objective of this study was to determine if bees could learn the concepts of ‘greater than’ and ‘less than’ and then extrapolate these acquired rules to the novel number of zero. This enabled us to determine if, and to what extent, bees may understand the concept of zero. A set of five experiments were designed to test: i) whether bees could extrapolate the learnt rules of ‘greater than’ and ‘less than’ to zero numerosity and five elemental features (Experiment 1); (ii) if bees understood that zero elements was less than one elemental feature (Experiment 2); (iii) if bees displayed number magnitude effects with zero numerosity (Experiment 3); (iv) whether bees had a preference for choosing the zero stimulus which lacked any elements (Experiment 4); and (v) whether bees could replicate the results of Experiment 1 using a completely novel pair of numbers during the testing phase.

General Procedure

Study site and species:

Experiments were conducted with free-flying honeybees (*Apis mellifera*) from maintained hives. Foragers were marked on the thorax with a coloured dot to identify individuals used in the experiments. An ad-lib von Frisch type gravity feeder providing ca. 10 - 30 % sucrose was set-up to maintain a regular number of bees available for testing (13).

Apparatuses:

Honeybees were trained to visit a rotating screen for Experiments 1, 2, 4 and 5 (ESM 2; ESM 3; Figure S1a) which was 50 cm in diameter (23). By using this screen, the spatial arrangement of stimulus choices could be randomly changed, thus excluding position orientation cues. Stimuli were presented vertically on 6 x 8 cm hangers with a landing platform attached below the presentation area. The screen and landing platforms were made

of a colour which is perceived by humans as being 'grey'. Hangers and surrounding screen areas were washed with 30 % ethanol between foraging bouts and before each test was conducted to exclude the use of olfactory cues.

In Experiment 3, a y-maze was used (Figure S1b). One bee was tested at a time during training and testing phases, typically taking 2 – 4 hours/bee to complete the protocol for each individual bee. Landing poles and surrounding areas were washed with 30 % ethanol between foraging bouts and before each test was conducted to exclude the use of olfactory cues.

Stimuli:

Each stimulus was a 6 x 6 cm white square with black elements presented on it, and was covered with 80 μm Lowell laminate. Elements could be one of three shapes: square, diamond, or circle. Two of these shapes were used in training, and the other novel shape was used for testing to ensure patterns and shapes were novel to bees (Figure S2). To control for surface area, each stimulus presented a pattern of elements culminating to a surface area of $10 \pm 0.3 \text{ cm}^2$ regardless of shape, pattern, or number of elements; and each element was above the minimum resolution threshold for honeybee vision as based on previous psychophysics findings (24).

There were a total of 82 different patterns, comprising of 0 - 5 elements of the three different shapes (square; diamond; circle) which could be presented throughout Experiments 1, 2 and 5. In Experiment 3, there were a total of 97 different stimuli patterns also comprising of three different shapes ranging from 0 – 6 elements. This large diversity of stimuli with closely matched spatial parameters controlled for the potential use of an associative mechanism by bees to learn the outcomes of each stimulus (Figure S2). This was especially the case with the requirement to solve non-reinforced tests with novel stimuli. In addition to this array, stimuli were randomly rotated to one of four possible positions (numbers 2 – 6) to further exclude low-level cues resulting in 376 possibilities, however bees were not exposed at all possibilities during training.

General Training Procedure

A 10 μL drop of either a 50 % sucrose solution (CS+) or a 60 mM quinine solution (CS-) were used as rewarding and punishing outcomes for correct or incorrect choices respectively during the learning phase, as this conditioning method promotes visual attention (9).

In all four experiments, the choices of individual bees were recorded until a criterion of $\geq 80 \%$ for any 10 consecutive choices was reached (after a minimum of 20 conditioned choices). A choice was defined as an individual landing on a platform or pole and touching the solution (sucrose or quinine) with the proboscis, leg, or antenna. Stimuli were randomly allocated for each bee and changed between bouts (returns to hive to offload sucrose). After a bee had made a correct choice, new stimuli were presented and the previous stimuli were cleaned with 30 % ethanol solution. When the bee completed imbibing the sucrose, it could choose to make additional choices or return to the hive if satiated. If a bee made an incorrect choice, it would taste the bitter quinine solution and then was allowed to continue making choices until a correct choice was made, at which point the same procedure for a correct choice would be followed.

General Testing Procedure

To collect data for analysis of concept learning and extrapolation, each bee underwent non-reinforced tests. For all non-reinforced tests, we put a 10 μL drop of water on platforms to motivate bees to land. A random number of refreshing conditioned choices (range 2 – 6 choices) were presented between each non-reinforced test to maintain bee motivation.

(a) Experiment 1: Can bees apply acquired numerical information to understand a novel zero-concept problem?

Training and testing phases:

A counter-balanced design was used for this experiment, where one group of bees was trained to associate stimuli consisting of more elements with a reward ($n = 10$), while a second group of bees was trained to associate stimuli consisting of less elements with a reward ($n = 10$).

On the rotating screen, four stimuli (two identical correct stimuli; two identical incorrect stimuli) were presented simultaneously above landing platforms on the hangers which could be positioned in different random spatial positions which were changed following a decision.

Learning phase:

During the learning phase, bees were trained to either associate a reward of sucrose with the numerical concepts of ‘less than’ or ‘greater than’ using different shapes and patterns consisting of 1 – 4 elements. As stated above, bees were trained using appetitive-aversive conditioning until they reached a criterion of $\geq 80\%$ after a minimum of 20 choices (Figure 1A).

Learning test:

After a bee had reached criterion ($\geq 80\%$) in the learning phase, we presented a learning test using novel shapes and patterns consisting of 1 – 4 elements to determine if bees had learnt the ‘less than’ and ‘greater than’ concepts. If bees were able to learn ‘greater than vs. less than’, then they would choose the correct stimulus during the learning test at a level significantly higher than what is expected by chance. The null hypothesis was that choices would not be significantly different to chance expectation.

Conflict test:

The conflict and transfer tests were conducted in pseudo-random order for each bee (Figure 1A). The conflict test was done to determine a bee’s concept of zero. The two stimuli (elements two or three) were presented as novel shapes and patterns against novel plain stimuli of white (zero numerosity). These stimuli had previously resulted in ambiguous outcomes during training as whether the numbers 2 or 3 were correct or incorrect was dependent on the number that they were compared with. In this conflict test we hypothesized that if bees trained to ‘less than’ chose the stimuli which were of a novel square stimulus of white, then they understood that zero numerosity was less than any stimuli with elements on it. The null hypothesis however was that bees have either no preference or even a preference for the numbered elements. However, the experiment was designed to test for potential concept of zero, and thus did not seek to resolve statistically if there might be a preference for stimuli containing elements.

Transfer test:

The transfer test to a higher number consisted of presenting bees with ambiguous stimuli (two or three elements) of novel shape and pattern against a novel higher number, consisting of five elements which bees had not previously seen. This test was done as a counter-balance for the concept of zero numerosity test to determine if bees could extrapolate the ‘greater than’ and ‘less than’ concepts in both directions. In this test, we hypothesised that bees trained to ‘less than’ would choose the lower number of elements if they understood the task,

while bees trained to ‘greater than’ would choose the stimuli consisting of five elements as it was a higher number.

(b) Experiment 2: Can bees demonstrate an extended understanding of the concept of zero?

In this experiment, we trained bees ($n = 25$) to a ‘less than’ concept using elements: [2, 3, 4, 5]. Bees were then tested on 1 vs. 0 and 2 vs. 0. This would determine to what level bees understood the concept of zero. As in Experiment 1, the element shape and pattern changed between bouts. Two shapes were used in training and the novel shape was used in the testing phase (Figure 1B; Figure S2).

Learning phase:

During the learning phase, bees were trained to associate a reward of sucrose with the numerical concept of ‘less than’ using different shapes and patterns consisting of 2 – 5 elements. As stated above, bees were trained using appetitive-aversive conditioning until they reached a criterion of $\geq 80\%$ after a minimum of 20 choices (Figure 1B).

Learning test:

After a bee had reached criterion ($\geq 80\%$) in the learning phase, we presented a learning test using novel shapes and patterns consisting of 2 – 5 elements to determine if bees had learnt the ‘less than’ concept (Figure 1B). If bees were able to learn this concept, then they would choose the correct lower numbered stimulus during the learning test at a level significantly higher than what is expected by chance.

Conflict test:

The conflict and transfer tests were conducted in pseudo-random order for each bee. This conflict test presented 2 vs. 0 to bees. This information was in conflict as both stimuli were potentially correct options based on the training received. Two elements had always been rewarding during training (lowest number), while the empty set stimulus was the correct lower number when compared with two elements. This experiment enabled determining whether bees were employing an associative mechanism if they preferred to choose two in this test. If bees preferred to choose zero numerosity, then we could determine that they were using the rule-based learning and additionally understood that zero numerosity was the lower number. Bees could also choose at chance level as potentially both stimuli are potentially correct.

Transfer Test:

Some animals confuse the numerical values of one and zero, understanding that they are at the lower end of the number scale, but often mistaking zero numerosity for one (4,5,13).

In this transfer test, we presented bees with novel stimuli of zero and one element simultaneously on the rotating screen (Figure S2) to determine if, when trained to the concept of ‘less than’, they would understand that zero elements was less than one. We expected that if bees had an adequate understanding of the concept of zero and one, then they would choose zero numerosity stimuli in this test. If bees could not understand this concept and like other animals, could not differentiate between which was the lower number, they may choose at chance level. If bees were to mistake one for the lower number then bees would more frequently choose stimuli with one element displayed in this transfer test.

(c) Experiment 3: Distance effects

We tested whether bees would display numerical distance effects with zero numerosity, an empty set, and positive integers. We used a y-maze for this experiment (as described (12,

13)) to control for viewing distance and visual angle. Stimuli were set at a distance of 15 cm from the decision chamber. In this experiment we trained bees ($n = 20$) to a ‘less than’ concept using elements including: [0, 1, 2, 3, 4, 5, 6]. Bees would then be tested 0 vs. 1; 0 vs. 2; 0 vs. 3; 0 vs. 4; 0 vs. 5; 0 vs. 6. This would determine if bees were more accurate at performing with a lower number than a higher number. As in Experiment 1, the element shape and pattern changed between bouts. Two of the stimuli shapes were used in training, and the novel alternative shape was used in the testing phase.

Learning phase:

During the learning phase, bees were trained to associate a reward of sucrose with the numerical concept of ‘less than’ using different shapes and patterns consisting of 0 – 6 elements. As stated above, bees were trained using appetitive-aversive conditioning until they reached a criterion of $\geq 80\%$ after a minimum of 20 choices.

Learning test:

After a bee had reached criteria ($\geq 80\%$) in the learning phase, we presented 6 learning tests (10 choices each) using novel shapes and patterns consisting of 0 – 6 elements to determine if bees had learnt the ‘less than’ concept. If bees were able to learn this concept, then they would choose the correct lower number of zero numerosity during the learning test at a level significantly higher than what is expected by chance. If there were also numerical distance effects of zero numerosity, we would see a difference in accuracy between the number which is closest to zero elements, one, compared to numbers which are quantitatively further away from zero (e.g. 6).

(d) Experiment 4: Will bees choose to opt-out of a difficult and potentially punishing numerical task by choosing a novel ‘zero’ stimulus?

In this experiment we trained one group of bees ($n = 10$) to associate two elements with a reward of sucrose and three elements with the aversive substance of quinine. A second group of bees ($n = 10$) were trained to associate two elements with quinine and three elements with sucrose. The element shape and pattern changed between bouts as in Experiment 1 using the same protocol (Figure S2). Two shapes were used in training and the novel shape was used in the testing phase. The same apparatus and similar training procedure was used as in Experiment 1 (ESM 2).

Training and testing phases:

Learning phase:

The learning phase target stimuli (2 vs. 3 elements) were displayed and choices of individuals were recorded until bees reached criterion of $\geq 80\%$ for any ten choices after 20 initial choices has been made. Stimuli were randomly allocated for each bee and changed between bouts (ESM 2; Figure S2).

Learning test:

After a bee had reached criterion ($\geq 80\%$) in the learning phase, we presented a learning test using a novel shape and pattern of 2 vs. 3 elements to determine if bees had learnt the conditioned stimuli (ESM 1). If bees had learnt to solve the problem, then they should choose stimuli of either two or three elements depending on the group they were in at a level significantly higher than what is expected by chance.

Conflict test:

The conflict and transfer tests were conducted in pseudo-random order for each bee. In this test, bees were presented with the stimulus which was punishing during training vs. a novel empty set stimulus. We aimed to determine if bees would choose the punishing stimulus as it was more visually similar to the rewarding one, or if they would ‘opt out’ of this potentially punishing option and choose the empty set stimulus as it was novel and had not previously been punishing. This test would determine if results in Experiment 1 of bees choosing zero numerosity in the ‘less than’ group during the conflict test may be due to an ‘opt out’ mechanism rather than understanding the concept of zero. If bees chose stimuli containing elements, then we could conclude they did not use an ‘opt out’ mechanism. If bees were to choose the empty set stimulus, this would suggest that they prefer a novel stimulus to something that is punishing and may provide an alternative explanation for the results in the conflict test in Experiment 1.

Transfer test:

The transfer test was done to determine whether bees would prefer the empty set over the rewarding number they had been trained to (either two or three elements depending on their group). Bees should prefer the stimulus of a previously rewarding element number when compared to a novel empty set stimulus. If bees chose the novel empty set stimulus, it may indicate that they have a preference for a blank white stimulus.

(e) Experiment 5: Do bees transfer learnt number rules to novel pairs of numbers?

Training and testing phases:

A counter-balanced design was used for this experiment, where one group of bees was trained to associate stimuli consisting of more elements with a reward ($n = 6$), while a second group of bees was trained to associate stimuli consisting of less elements with a reward ($n = 6$).

On the rotating screen, four stimuli (two identical correct stimuli; two identical incorrect stimuli) were presented simultaneously above landing platforms on the hangers which could be positioned in different random spatial positions which were randomly changed following a decision.

Learning phase:

During the learning phase, bees were trained to either associate a reward of sucrose with the numerical concepts of ‘less than’ or ‘greater than’ using different shapes and patterns consisting of 1 – 5 elements. As stated above, bees were trained using appetitive-aversive conditioning until they reached a criterion of $\geq 80\%$ after a minimum of 20 choices. During training, we did not present the bees with the pair of numbers 2 vs. 3. Thus we presented this novel pair of numbers to bees during the learning test. The ratio of three or two elements being correct during training was the same whether bees were trained to a ‘greater than’ or ‘less than’ rule.

Learning test:

After a bee had reached criterion ($\geq 80\%$) in the learning phase, we presented a learning test using novel shapes and patterns consisting of 2 vs. 3 elements to determine if bees had learnt the ‘less than’ and ‘greater than’ concepts. If bees were able to learn ‘greater vs. less’, then they would choose the correct stimulus during the learning test at a level significantly higher than what is expected by chance.

Conflict test:

The conflict test was done to determine a bee's concept of zero. The two novel paired stimuli (elements two or three) were presented with novel shapes and patterns against an empty set stimulus (zero). These stimuli had previously resulted in either reward or punishment dependent upon which number the stimuli had been previously paired with. In this conflict test, we hypothesized that if bees trained to 'less than' chose the stimuli which were of a novel empty set, then they understood that zero numerosity was less than any stimuli with elements on it. The null hypothesis however was that bees have either no preference or even a preference for the numbered elements. However, the experiment was designed to test for potential concept of zero, and did not thus seek to resolve if there might be a preference for stimuli containing elements.

(f) Statistical analysis:

Did bees understand the numerical concepts?

To determine how bees performed on the tests in all experiments, we estimated the mean of the "correct" choices, the intercept of a generalised linear mixed model with a binomial response, using individual bees as a random, categorical variable to account for the repeated measurements. Choice (correct or incorrect choice) was used as a binary response as a fixed effect. These statistical tests and models were performed on the R environment for statistical analysis.

Experiment 1: In the concept of zero conflict test, we considered the empty set stimulus to be correct for bees trained to 'less than' and incorrect for bees trained to 'greater than' (thus stimuli containing two or three elements were correct). For the transfer test to the higher number of five, we considered the stimuli with five elements to be incorrect for bees trained to 'less than' and correct for bees trained to 'greater than'.

Experiment 2: The control test to determine the bee's level of understanding of zero, we considered the empty set stimuli to be correct in both the transfer and conflict tests.

Experiment 3: In all 6 tests, the empty set (zero), was the correct option if bees understood the concept of zero.

Experiment 4: In the control experiment which involved testing the bee's preference to opt-out of choices, we considered the stimuli consisting of numerical elements in both the conflict and transfer tests to be correct.

Experiment 5: In the learning test we considered two elements to be correct if bees were trained to 'less than' and three elements to be correct if bees were trained to 'greater than'. In the concept of zero conflict test, we considered zero numerosity to be correct for bees trained to 'less than' and incorrect for bees trained to 'greater than' (thus stimuli containing two or three elements were correct).

Were results consistent across groups and numerical concept tests?

Experiment 1 and 4:

Separate analyses were performed to determine if there were any asymmetries within tests and between the two groups in Experiment 1 and (trained to 'less than' or 'greater than'), and Experiment 4 (trained to two or three elements). For this analysis, a mixed between-within subjects ANOVA (Split-Plot) experimental design was implemented. The design consisted of two factors: test and experimental group with three and two levels, respectively. The three levels of the test factor were: learning; conflict; transfer. The two levels of the experimental group factor were the two groups in each separate experiment. For both experiments, a total of 20 bees were divided across the two levels of the experimental group factor ($n = 10$ in each group for Experiment 1 and 4), and each bee was then tested

under all levels of the test factor. For this analysis, we used proportion data converted into degrees by means of an arc-sine angular transformation (25). This test was conducted to determine whether the three tests differed in the number of correct choices across both groups. Normality across tests was determined using a Shapiro-Wilk test in SPSS. For this analysis, we used proportion data converted into degrees by means of an arc-sine angular transformation (25).

Experiment 2:

To determine if there were any asymmetries within the three tests in Experiment 2, a one-way repeated measures ANOVA was conducted. The design consisted of an independent categorical variable (test type) which had three levels (learning; conflict; transfer) and one dependent continuous variable (correct choices in tests). For this experiment, a total of 25 bees were tested. Each bee participated in all three tests. For this analysis, we used proportion data converted into degrees by means of an arc-sine angular transformation (25). These statistical tests and models were performed in SPSS Statistics.

Experiment 3:

Normality across tests was determined using a Shapiro-Wilk test in SPSS. For this analysis, we used proportion data converted into degrees by means of an arc-sine angular transformation (25).

To determine if the tests differed in terms of accuracy, we estimated the mean of the “correct” choices, the intercept of a generalised linear mixed model with a binomial response, with numerical distance as a predictor using individual bees as a random, categorical variable to account for the repeated measurements. Choice (correct or incorrect choice) was used as a binary response as a fixed effect. These statistical tests and models were performed on the R environment for statistical analysis.

To determine if the tests were significantly different from each other, we ran a GLMM with a logit link with choice as a binary response and numerical distance as a categorical predictor with six levels: 0 vs. 1, 0 vs. 2, 0 vs. 3, 0 vs. 4, 0 vs. 5, and 0 vs. 6; selecting the first category as the baseline.

Experiment 5:

Normality across tests was determined using a Shapiro-Wilk test in SPSS. For this analysis, we used proportion data converted into degrees by means of an arc-sine angular transformation (25).

(g) Spatial frequency of stimuli:

For all 97 stimuli, we produced a spatial frequency image, a power spatial graph, and a transect graph using the program MATLAB to determine if spatial frequency could potentially explain results from all experiments (see Table S1).

Supplementary Results:

ESM 1: Results

(a) Experiment 1: Bees learn and can extrapolate the concepts of ‘greater than’ and ‘less than’ to a novel higher number and the concept of zero

Learning Phase:

Bees trained to ‘less than’ ($n = 10$) took an average of 42 ± 4 choices and bees trained to ‘greater than’ ($n = 10$) took an average of 49 ± 7 choices. On average, all bees in Experiment 1 took 45 ± 4 choices.

Learning test:

In the learning test, bees trained to ‘less elements’ chose the correct stimulus in 75.0 ± 4.1 % (mean \pm standard error of the mean (s.e.m)) of choices. The number of correct choices made during the non-reinforced learning test were significantly higher than the chance expectation equivalent to 50 % ($Z = 5.08$, $P < 0.001$). Bees trained to ‘more elements’ chose the correct stimulus in 75.5 ± 3.3 % of choices ($Z = 6.556$, $P < 0.001$; Figure 1c).

Conflict test:

In the conflict test of zero elements vs. two or three elements, bees trained to ‘less elements’ chose the stimulus representing zero in 64.0 ± 5.4 % of choices ($Z = 2.795$, $P < 0.005$). Bees trained to ‘more elements’ chose against zero numerosity, thus choosing the stimulus with elements on it, in 74.5 ± 2.6 % of choices ($Z = 6.609$, $P < 0.001$; Figure 1c).

Transfer test:

In the transfer test where either two or three elements were shown against the higher, previously unseen, amount of five elements, bees trained to ‘less than’ chose the correct stimuli of two or three elements in 68.0 ± 5.0 % of choices ($Z = 3.411$, $P < 0.001$). Bees trained to ‘more elements’ extrapolated their learning to choose the stimuli with five elements in 75.0 ± 3.9 % of choices ($Z = 5.333$, $P < 0.001$; Figure 1c).

Consistency and normality of tests:

There was no significant main effect between the proportion of correct choices between bees trained to ‘less than’ and ‘greater than’ in the split-plot ANOVA analysis ($F_{1,2} = 1.859$, $P = 0.190$). Considering the within-subjects factor, we found no significant difference between the different tests (Wilks Lambda (Λ) = 0.871, $F_{17,2} = 1.254$, $P = 0.310$, multivariate partial eta squared = 0.129), nor a significant interaction between tests and subjects ($\Lambda = 0.914$, $F_{17,2} = 0.802$, $P = 0.465$, multivariate partial eta squared = 0.086).

However, data for the bees trained to ‘less than’ was not normally distributed (Shapiro Wilcoxon (W) = 0.815, $df = 10$, $P = 0.022$), while data for bees trained to ‘greater than’ was normally distributed ($W = 0.918$, $df = 10$, $P = 0.341$). We subsequently removed an outlier and found that the data for bees trained to ‘less than’ become normalized ($W = 0.892$, $df = 9$, $P = 0.207$). Graphical data summary by means of boxplot, revealed that one of the bees (individual 9) in the ‘less than’ group demonstrated high performance (100 % of correct choices) relative to the median performance of all the bees in the treatment group. Consequently, subsequent GLMM analyses were performed on data sets containing and excluding individual number 9. Results for the analyses evidenced a significant effect of treatment for the two data sets. The fit of the model, measured by overdispersion, including individual 9 was better (1.07) than the model excluding the outlier (1.19); and in both cases,

analysis indicated that bees chose the correct target more frequently than expected by chance alone (including outlier: $z = 5.08$, $P < 0.001$; excluding outlier: $z = 5.742$, $P < 0.001$). After the removal of the outlier, there was still no significant main effect between the proportion of correct choices between bees trained to ‘less than’ and ‘greater than’ ($F_1 = 2.674$, $P = 0.120$, multivariate partial eta squared = 0.136), or between the different tests ($\Lambda = 0.905$ $F_{16,2} = 0.840$, $P = 0.450$, multivariate partial eta squared = 0.095), nor a significant interaction between tests and subjects ($\Lambda = 0.956$ $F_{16,2} = 0.372$, $P = 0.695$, multivariate partial eta squared = 0.044). This shows bees reliably transferred the acquired concepts from the learning set of 1 – 4 elements to the novel concepts of zero, and a higher number outside of their current numerosity range. This performance is consistent with the application of rule-based problem solving, but would not be expected if bees were using an associative mechanism to solve the different visual problems since to choose a zero numerosity stimulus involved not choosing stimuli elements to which a bee had actually been trained.

(b) Experiment 2: Bees can extend their understanding of the concept of zero

Learning Phase:

Bees ($n = 25$) took an average of 34 ± 2 choices to learn a ‘less than’ rule.

Learning test:

In the learning test, bees were trained to the concept of ‘less than’ and tested on two random element numbers from the training set of 2 – 5 elements. Bees chose the lower number in 73.8 ± 1.9 % of choices ($Z = 10.180$, $P < 0.001$; Figure 1d).

Conflict test:

In the conflict test where bees were shown 2 vs. 0, bees chose the lower number of zero elements in 56.2 ± 3.4 % of choices, which was not significantly different from chance expectation ($Z = 1.639$, $P = 0.101$; Figure 1d). This demonstrated that when both options were potentially correct, bees did not demonstrate a preference for either option.

Transfer test:

In the transfer test where bees were presented with 1 vs. 0, bees chose the lower number of zero elements in 63.0 ± 2.9 % of choices ($Z = 4.233$, $P < 0.001$; Figure 1d). This showed that bees understood that zero elements was less than one on the number scale.

Consistency and normality of tests:

There was a significant main effect across the proportion of correct choices between the three tests ($\Lambda = 0.454$ $F_{2,23} = 13.811$, $P < 0.001$). This means there were differences between the number of correct choices across the learning, conflict, and transfer tests. The main effect was caused by a significant difference between the learning test and the transfer test when we look at the pairwise comparisons ($P = 0.020$) and a significant difference between the learning and conflict test ($P < 0.001$). There was no significant difference between the transfer and conflict tests ($P = 0.200$).

However, data for the learning test was not normally distributed ($W = 0.917$, $df = 25$, $P = 0.044$) while data for bees in the other tests was normally distributed ($P > 0.05$). There were not outliers to remove in order to normalize the data.

(c) Experiment 3: Distance effects

Learning Phase:

Bees ($n = 20$) took an average of 39 ± 3 choices to learn a ‘less than’ rule.

Learning tests:

In all 6 tests bees performed significantly higher than chance expectation when choosing zero elements as the lowest number: 0 vs. 1 (58.0 ± 2.4 %; $Z = 2.253$, $P = 0.024$); 0 vs. 2 (61.5 ± 2.8 %; $Z = 3.497$, $P < 0.001$); 0 vs. 3 (62.5 ± 3.4 %; $Z = 3.634$, $P < 0.001$); 0 vs. 4 (64.5 ± 2.9 %; $Z = 3.906$, $P < 0.001$); 0 vs. 5 (71.5 ± 3.0 %; $Z = 5.872$, $P < 0.001$); 0 vs. 6 (75.5 ± 2.8 %; $Z = 6.728$, $P < 0.001$; Figure 2b).

Based on a GLMM using binary choice as a response and numerical distance as a categorical fixed effect, we found significant differences in response between the baseline category 0 vs. 1 and the category 0 vs. 5 ($z = 2.812$, $P = 0.005$). We also found a significant difference between the baseline and the 0 vs. 6 category ($z = 3.571$, $P < 0.001$). All other comparisons against the baseline were non-significant ($P > 0.219$).

Consistency of tests:

There was a significant effect of numerical distance on accuracy ($Z = 4.004$, $P < 0.001$; Figure 2b). Bees were more accurate when discriminating zero numerosity from higher numbers (e.g. 6) than lower numbers (e.g. 1).

Normality of tests:

The Shapiro-Wilk test showed that data from the tests of 0 vs. 2 ($W = 0.916$, $df = 20$, $P = 0.083$) and 0 vs. 5 ($W = 0.909$, $df = 20$, $P = 0.062$) were normally distributed while data from the tests of 0 vs. 1 ($W = 0.892$, $df = 20$, $P = 0.029$), 0 v. 3 ($W = 0.851$, $df = 20$, $P = 0.006$), 0 vs. 4 ($W = 0.899$, $df = 20$, $P = 0.040$) and 0 vs. 6 ($W = 0.664$, $df = 20$, $P < 0.001$) were not normally distributed.

(d) Experiment 4: Bees do not choose zero to opt-out of a difficult or potentially punishing problem-solving task

Learning Phase:

Bees trained to two elements reached the criterion in 36 ± 3 trials and bees trained to three elements reached the criterion in 32 ± 2 trials.

Learning test:

Bees trained to two elements chose the correct number in the learning test at a level of 77.0 ± 4.7 % which was significantly higher than chance level ($H_0 = 50$ %, $Z = 4.240$, $P < 0.001$). Bees trained to three elements chose the correct element number during the learning test in 68.5 ± 5.8 % of choices ($Z = 4.244$, $P < 0.001$; Figure S3).

Conflict test:

In the conflict test, bees trained to two elements chose the stimuli with three elements when compared to the zero numerosity stimuli in 78.0 ± 4.7 % of choices ($Z = 5.407$, $P < 0.001$). Similarly, bees trained to three elements chose stimuli with two elements in the conflict test when compared to zero numerosity in 79.0 ± 3.4 % of choices ($Z = 4.353$, $P < 0.001$; Figure S3).

Transfer test:

In the transfer test, bees trained to two elements chose the stimuli consisting of two elements in 84.0 ± 4.1 % of choices when presented against the empty set stimuli ($Z = 4.938$, $P < 0.001$). Bees trained to three elements chose stimuli with three elements in the transfer test in 79.0 ± 3.4 % of choices when compared to the empty set stimuli ($Z = 7.104$, $P < 0.001$; Figure S3).

Consistency and normality of tests:

There was no significant main effect between the proportion of correct choices between bees trained to two and three elements in the split-plot ANOVA analysis ($F_1 = 0.395$, $P = 0.538$). Considering the within-subjects factor, we found no significant difference between the different tests ($\Lambda = 0.800$ $F_{17,2} = 2.131$, $P = 0.149$, multivariate partial eta squared = 0.200), nor a significant interaction between tests and groups ($\Lambda = 0.922$ $F_{17,2} = 0.720$, $P = 0.501$, multivariate partial eta squared = 0.078).

However, data for the bees trained to two elements in the learning test was not normally distributed ($W = 0.816$, $df = 10$, $P = 0.023$) while data for bees in all other tests was normally distributed ($P > 0.050$). We subsequently removed two outliers and found that the data for bees trained to two elements in the learning test become normalized ($W = 0.872$, $df = 8$, $P = 0.157$). After the removal of the outliers, there was still no significant main effect between the proportion of correct choices between bees trained to two or three elements ($F_1 = 0.287$, $P = 0.599$, multivariate partial eta squared = 0.018), or between the different tests ($\Lambda = 0.754$, $F_{15,2} = 2.444$, $P = 0.121$, multivariate partial eta squared = 0.246), nor a significant interaction between test and group ($\Lambda = 0.983$, $F_{15,2} = 0.132$, $P = 0.877$, multivariate partial eta squared = 0.017). This shows bees reliably transferred the acquired concepts from the learning phase to the learning set, and preferred the stimuli with elements on them rather than an empty set stimulus representing zero.

(e) Experiment 5: Do bees transfer learnt number rules to novel pairs of numbers?

Learning Phase:

Bees trained to ‘less than’ reached the criterion in 45 ± 9 trials and bees trained to ‘greater than’ reached the criterion in 42 ± 7 trials.

Learning test:

In the non-reinforced learning test of 2 vs. 3, bees in the group trained to ‘less than’ chose the correct number stimulus containing two elements in 75 ± 4.83 % of choices ($z = 4.776$, $P < 0.001$, $n = 6$). Bees trained to the rule of ‘greater than’ chose the correct stimulus containing three elements in 72.5 ± 6.68 % of choices ($z = 3.082$, $P = 0.002$, $n = 6$).

Conflict test:

In a conflict test where bees were presented with novel stimuli containing two or three elements against an empty set stimulus (zero), bees trained to ‘less than’ chose the correct stimulus of zero numerosity in 65 ± 4.83 % of choices ($z = 3.179$, $P = 0.001$, $n = 6$). Bees trained to ‘greater than’ chose the correct stimulus of two or three elements in 70.83 ± 4.55 % of choices ($z = 4.368$, $P < 0.001$, $n = 6$).

Normality of tests:

The data for both non-reinforced tests was normally distributed: Learning test for bees trained to ‘less than’: ($W = 0.950$, $df = 6$, $P = 0.739$). Learning test for bees trained to ‘greater than’:

($W = 0.890$, $df = 6$, $P = 0.319$). Transfer test for bees trained to ‘less than’: ($W = 0.947$, $df = 6$, $P = 0.719$). Transfer test for bees trained to ‘greater than’: ($W = 0.958$, $df = 6$, $P = 0.801$).

(f) Spatial frequency of stimuli:

For all 97 stimuli, we produced a spatial frequency image, a power spatial graph, and a transect graph (see Table S1). When comparing these outputs between stimuli, it is clear that the use of spatial frequency could not allow bees to transfer a learnt spatial frequency rule (rather than a number rule) to an empty set stimulus. The spatial properties of the zero stimulus are too different to the properties of the other ‘numbered’ stimuli for bees to use these properties to solve the tasks and choose zero numerosity as the lowest number, thus bees must have been using a learnt number rule to order zero elements as less than the other numbers. In addition, there is little difference between stimuli containing different numbers of elements in terms of spatial frequency, thus it appears that bees are using number of elements rather than spatial frequency to solve the task and perform the tests.

ESM 2: Experiment 4: Do bees prefer a novel ‘zero’ stimulus?

We conducted a control experiment to test whether bees were choosing zero elements in Experiments 1 and 2 due to the stimulus being novel rather than accurately understanding that zero numerosity was the correct solution. Bees were trained to stimuli consisting of two and three elements of different shape and pattern (Supplementary Figure S2; Figure S3a). One group was trained to associate two elements with an appetitive reward and three elements with an aversive substance, while a second group was trained to associate three elements with a reward and two elements with aversion. In non-reinforced tests, bees trained to two elements chose the correct number in the learning test at a level of $77.0 \pm 4.7\%$ ($z = 4.240$, $P < 0.001$, $n = 10$) and bees trained to three elements chose the correct number in $68.5 \pm 5.8\%$ of choices ($z = 4.244$, $P < 0.001$, $n = 10$, Figure S3b). In a transfer test, the group trained to two elements chose the number of elements that had been associated with sucrose (two) when compared to the empty set in $84.0 \pm 4.1\%$ of choices ($z = 4.938$, $P < 0.001$, $n = 10$) and bees in the group trained to three elements correctly chose the novel stimuli consisting of three shapes in $79.0 \pm 3.4\%$ of choices ($z = 7.104$, $P < 0.001$, $n = 10$; Figure S3b). When bees were shown the stimuli associated with aversion, they chose against zero elements and for the stimuli containing elements in $78.0 \pm 4.7\%$ of choices ($z = 5.407$, $P < 0.001$, $n = 10$) for those trained to two elements, and in $79.0 \pm 3.4\%$ of choices ($z = 4.353$, $P < 0.001$, $n = 10$; Figure S3b) for those trained to three elements. This showed that regardless of whether bees had the option of choosing a number which was always rewarding or always punishing, they did not, in this numeric task, prefer to choose the novel empty set stimulus, but instead chose the stimuli which they had previously been exposed to, even if that stimulus was associated with an aversive substance. Interestingly, this is consistent to observed behaviour in honeybees choosing between perceptually similar colours (26).

ESM 3: Experiment 5: Do bees transfer learnt number rules to novel pairs of numbers?

We conducted a control experiment to test whether bees were able to demonstrate performance significantly better than chance on the learning test in experiment 1 due to having seen all paired numbers (i.e. 1 vs. 2, 1 vs. 3, 1 vs. 4, 2 vs. 3, 2 vs. 4, 3 vs. 4) during the training phase and thus may have remembered the outcomes of all the paired numbers. To control for this potential confound, we conducted an additional experiment where bees were

trained to stimuli containing elements 1 – 5, where the pairing of 2 vs. 3 elements was excluded from the training phase. One group of six bees was trained to the rule of ‘less than’ and a second group six of bees was trained to the rule of ‘greater than’. Bees were trained using differential appetitive-aversive conditioning until they reached a criterion of $\geq 80\%$ (Figure S4A). Bees trained to ‘less than’ reached the criterion in 45 ± 9 trials and bees trained to ‘greater than’ reached the criterion in 42 ± 7 trials. In the non-reinforced learning test of 2 vs. 3, bees in the group trained to ‘less than’ chose the correct number stimulus containing two elements in $75.00 \pm 4.83\%$ of choices ($z = 4.776$, $P < 0.001$, $n = 6$). Bees trained to the rule of ‘greater than’ chose the correct stimulus containing three elements in $72.50 \pm 6.68\%$ of choices ($z = 3.082$, $P = 0.002$, $n = 6$). In a conflict test showing bees novel stimuli containing two or three elements against an empty set stimulus (zero), bees trained to ‘less than’ chose the correct stimulus of zero numerosity in $65.00 \pm 4.83\%$ of choices ($z = 3.179$, $P = 0.001$, $n = 6$). Bees trained to ‘greater than’ chose the correct stimulus of two or three elements in $70.83 \pm 4.55\%$ of choices ($z = 4.368$, $P < 0.001$, $n = 6$; Figure S4B). Bees demonstrated that even when they had not previously been presented with a specific pairing of numbers during training, they could still extrapolate their rule learning to a novel pair of stimuli.

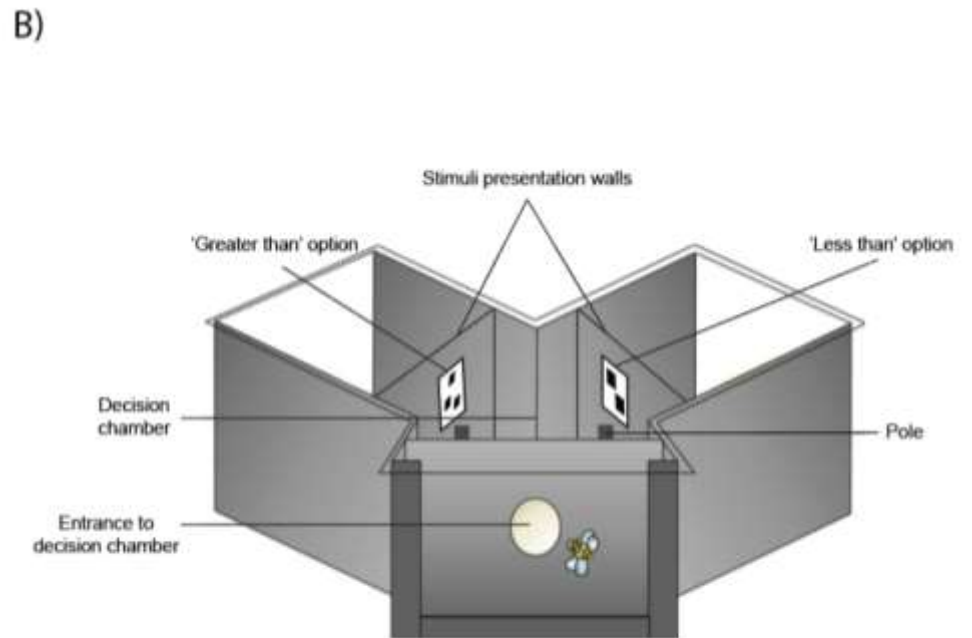
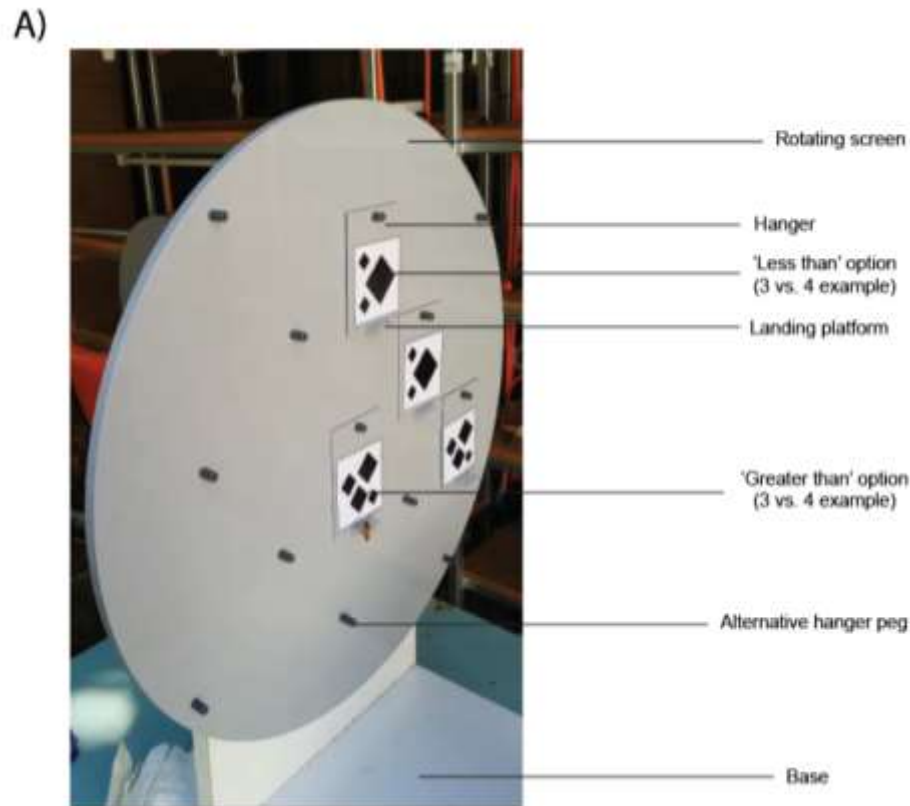


Fig. S1:

Apparatuses used for the experiments. A) An image of the rotating screen used for Experiment 1, 2, 4, and 5 with labels to show components of the apparatus and examples of stimuli for one of the test conditions. B) A diagram of the Y-maze used in Experiment 3 with labels showing the components of the apparatus, and examples of stimuli for one experimental condition. A bee enters through the small hole into the decision chamber where it is presented with two stimulus options and must make a decision on which pole to land to potentially collect a reward for a correct choice.

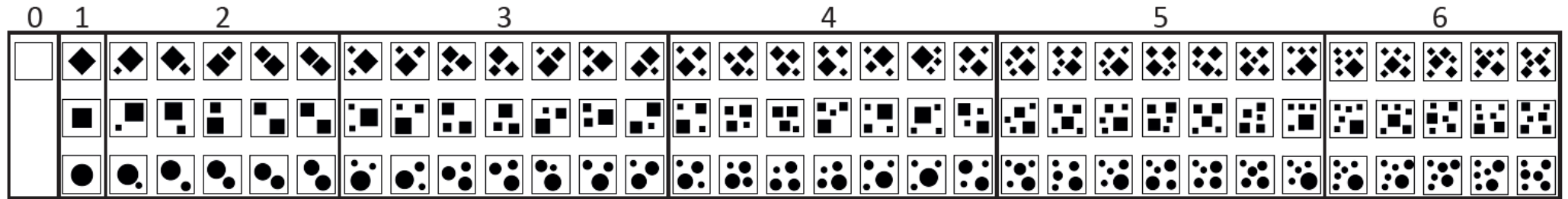
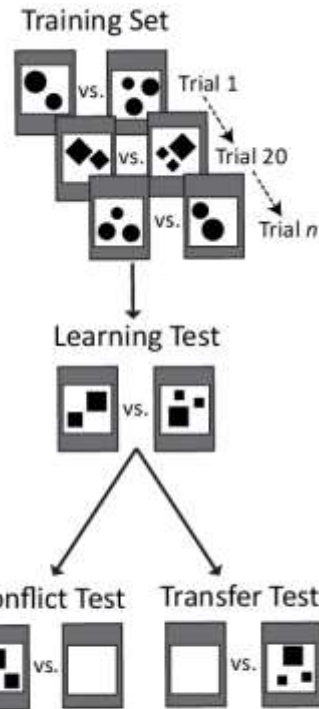


Fig. S2.

The 97 different stimuli which could be presented to bees during learning and testing phases in all experiments. These stimuli are separated by number of elements and shape categories. Stimuli were controlled for colour balance, spatial frequency, surface area, pattern, shape, and element sizes. In addition, stimuli were randomly rotated to one of four possible positions (numbers 2 – 6) to further exclude low-level cues resulting in 376 possibilities.

Experiment 4

A)



B)

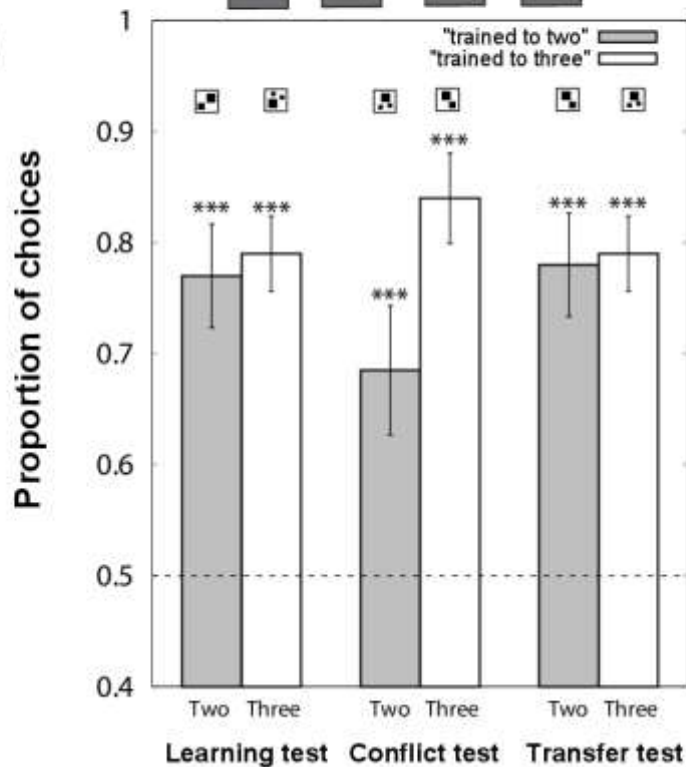


Fig. S3

A graphic representation of the method and results in Experiment 4. A) Example of possible combinations during trials and tests. B) Performance during the three non-reinforced testing phases during Experiment 4: learning test, conflict test with a rewarding stimulus vs. 0, and transfer test with a punishing stimulus vs. 0. In the conflict and transfer tests, data shown are the bee's choices for stimuli containing elements. Broken black line at 50 %

indicates chance level performance. Significant from chance level performance is indicated by * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.0001 .

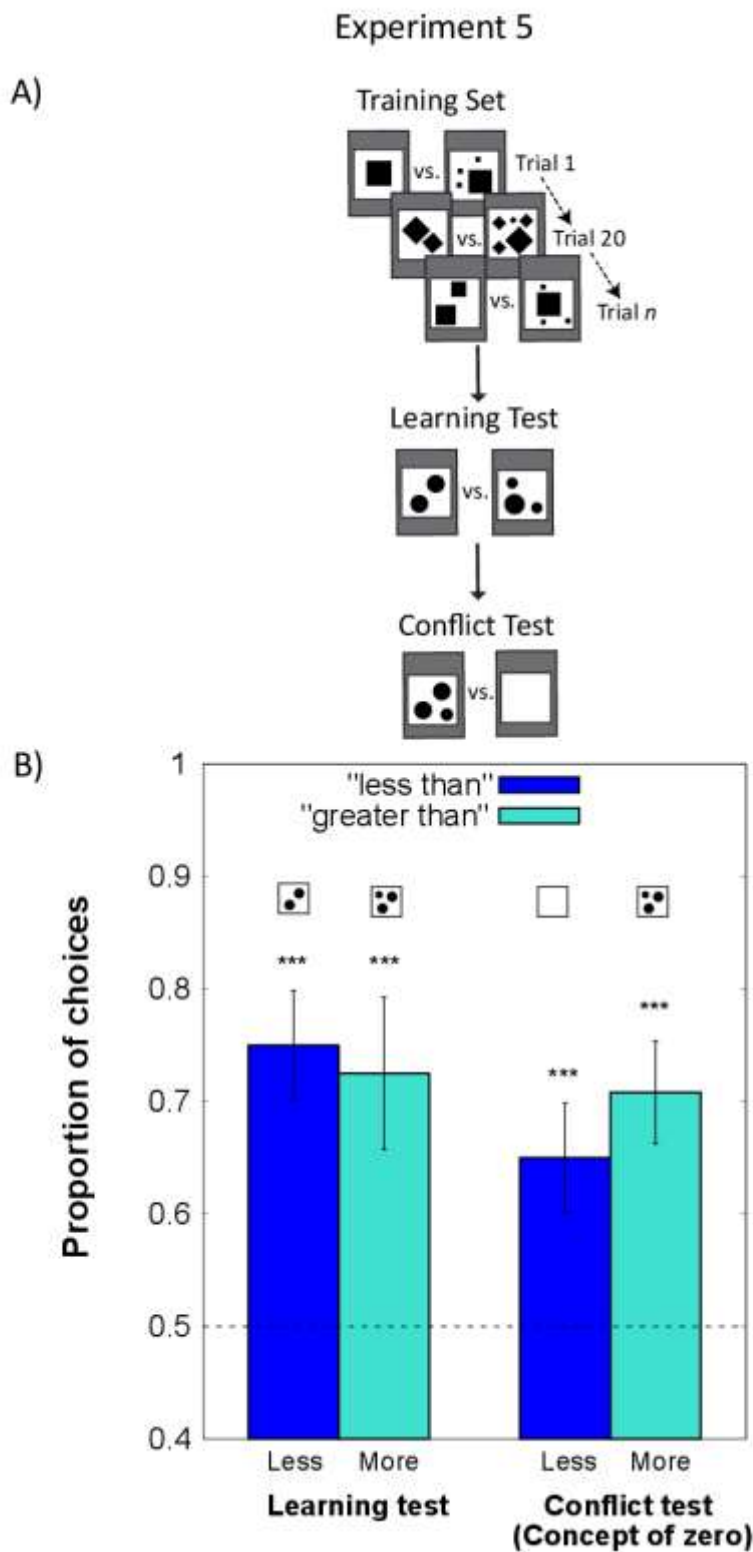


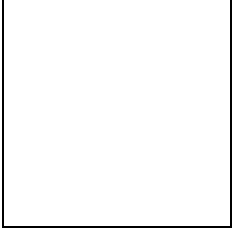
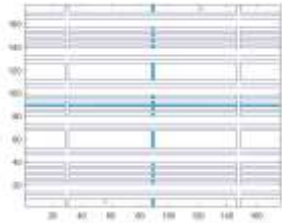
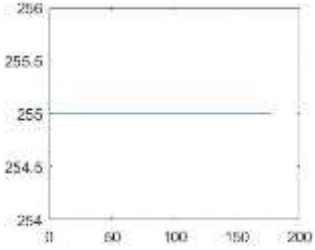
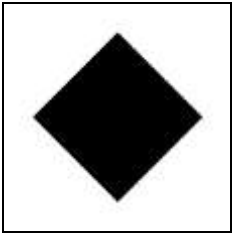

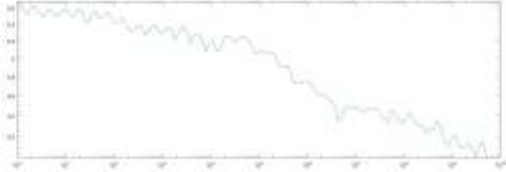
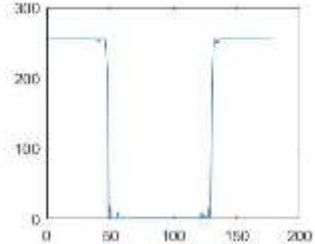
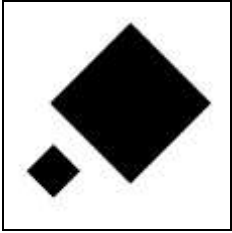
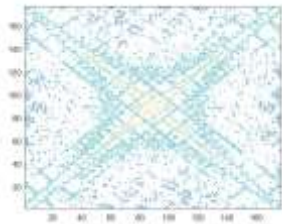
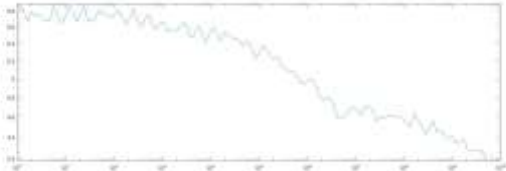
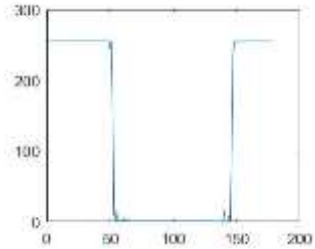
Fig. S4

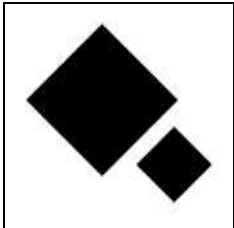
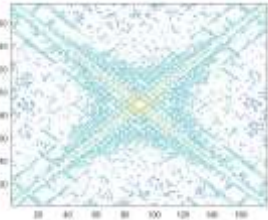
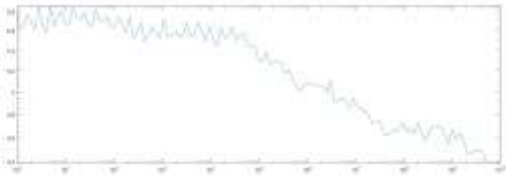
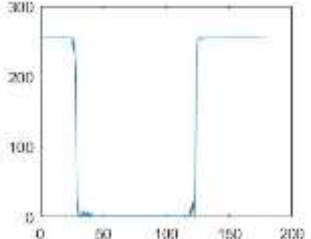
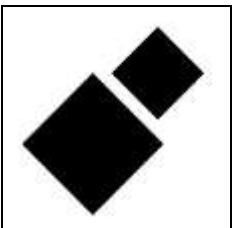
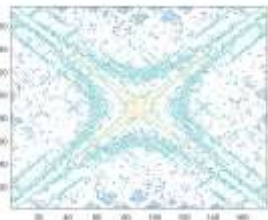
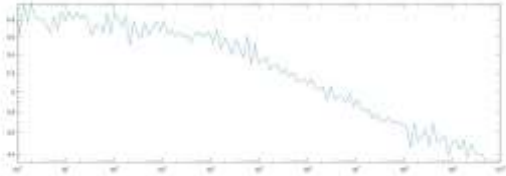
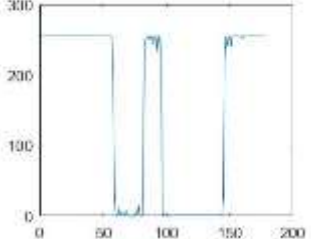
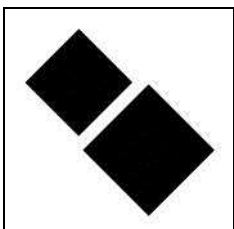
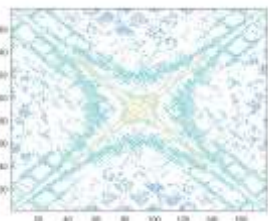
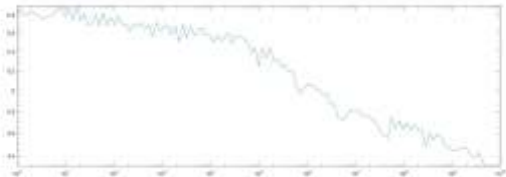
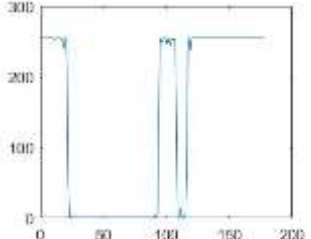
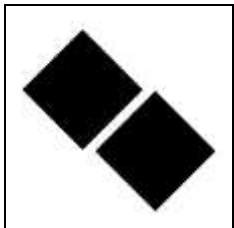
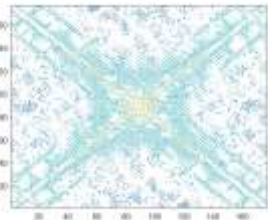

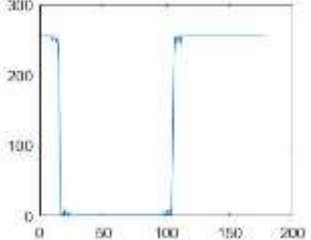
A graphic representation of the method and results in Experiment 5. A) Example of possible combinations during trials and tests. B) Performance during the two non-reinforced testing phases during Experiment 5: learning test and conflict test. In the conflict test, data shown for the 'less than' group are bee's choices for an empty set while data shown for the 'greater than' group are the bee's choices for stimuli containing elements. Results for bees trained to 'less than' are shown in dark blue, while results for bees trained to 'greater than'

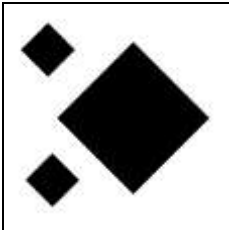
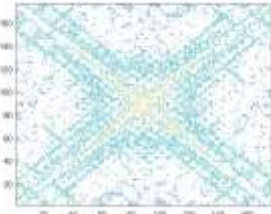
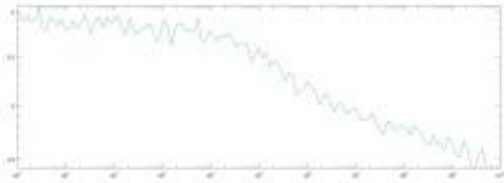
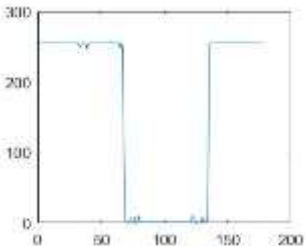
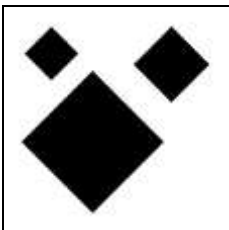

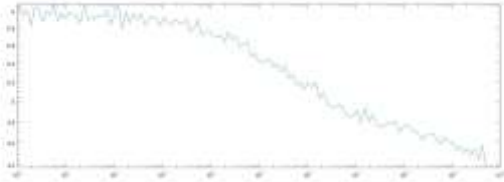
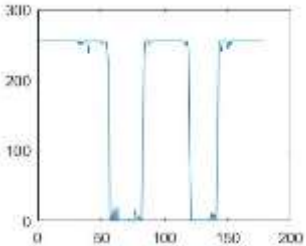
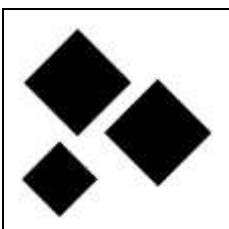

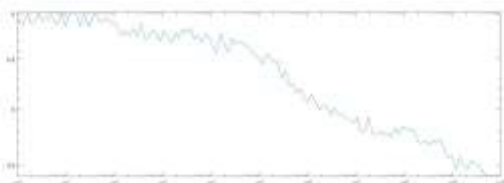
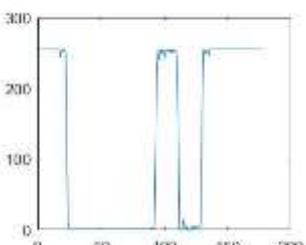
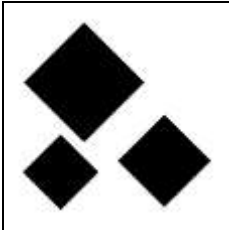

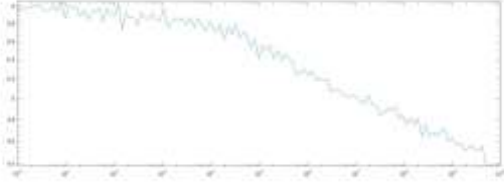
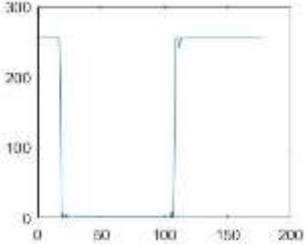
are shown in turquoise. Broken black line at 50 % indicates chance level performance. Significant from chance level performance is indicated by * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.0001 .

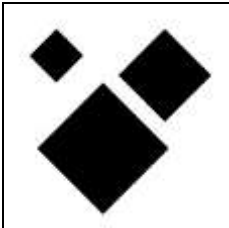

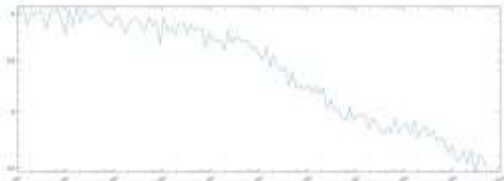
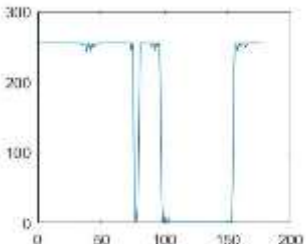
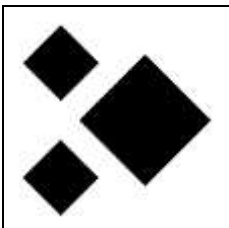

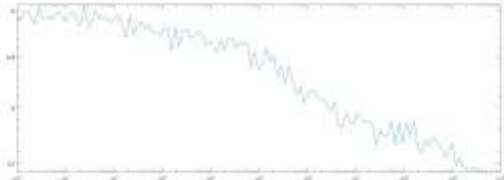
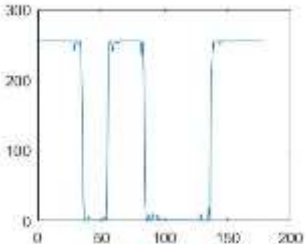
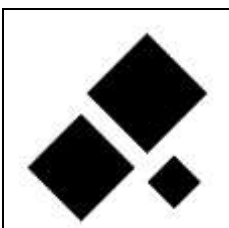

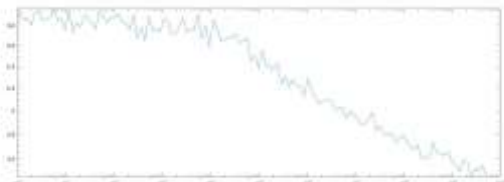
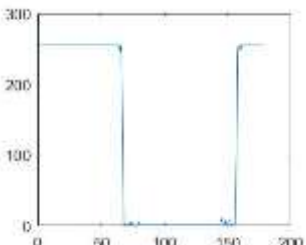
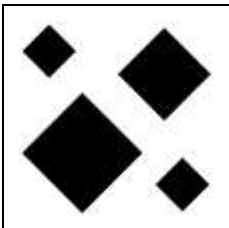

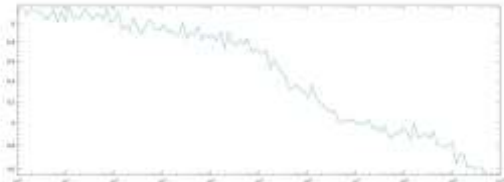
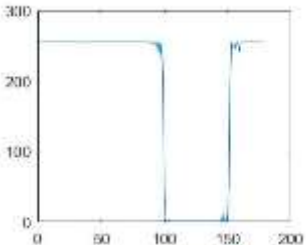
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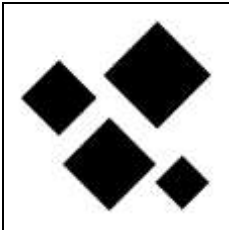
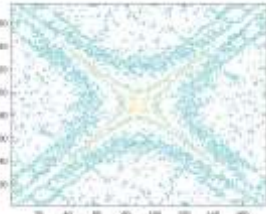
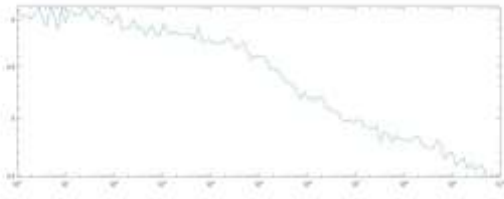
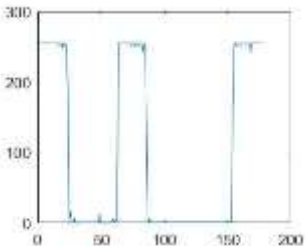
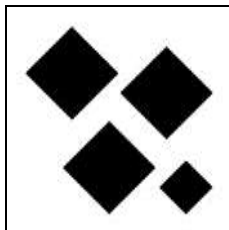

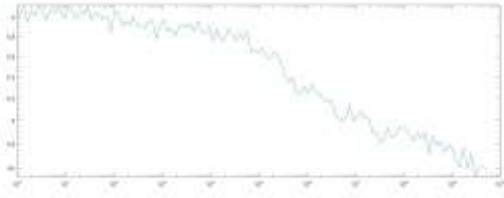
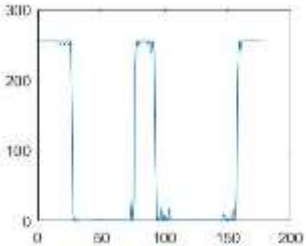
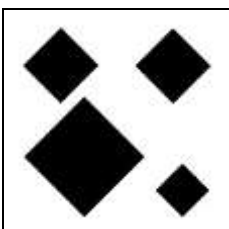

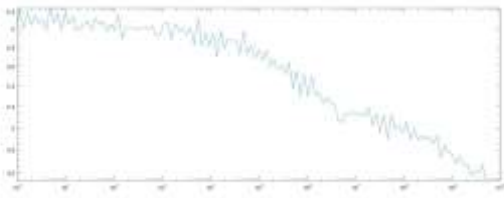
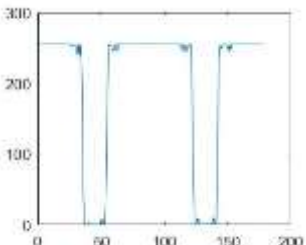
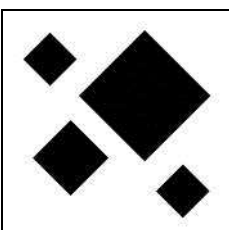
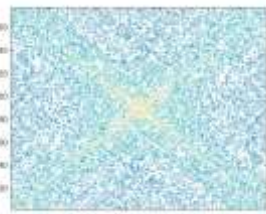
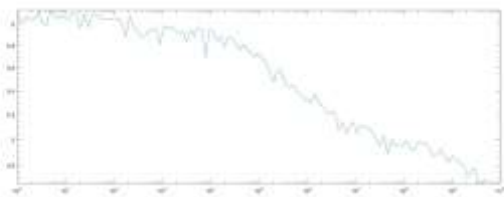
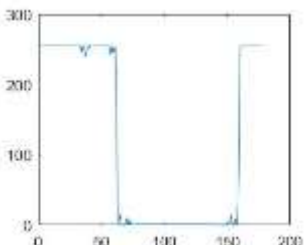
For all 97 stimuli there is a spatial frequency image, a power spatial graph, and a transect graph produced. Column 1: element shape; column 2: stimulus ID; column 3: stimulus image; column 4: spatial frequency image; column 5: power spatial graph with frequency (Hz) on the x-axis and power on the y-axis; column 6: transect graph with position on the x-axis and spectrum on the y-axis (*see below*).

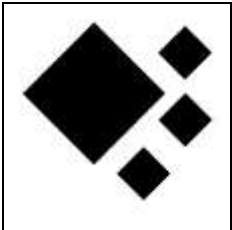
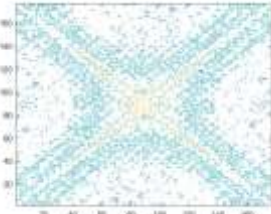
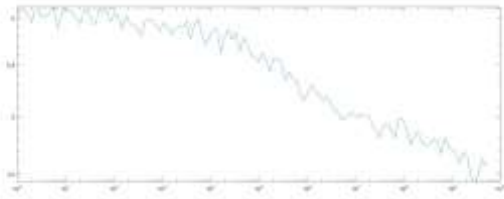
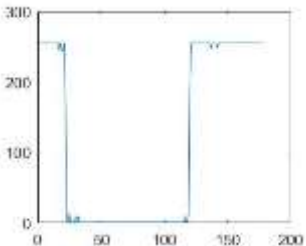
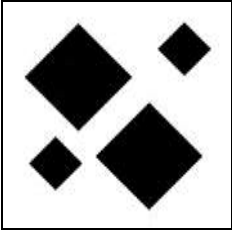
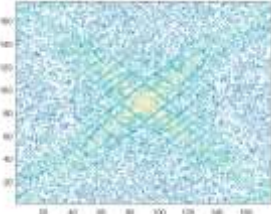
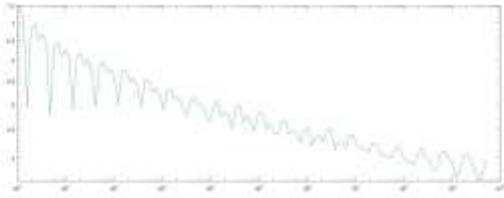
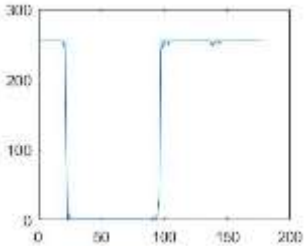
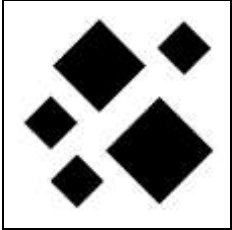
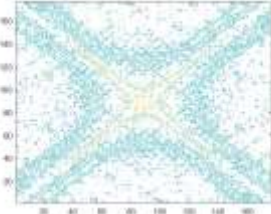
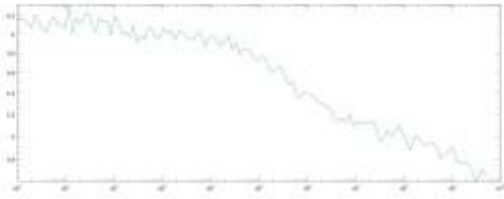
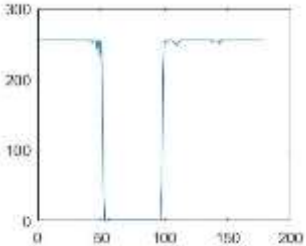
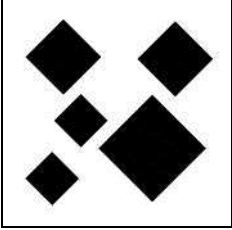
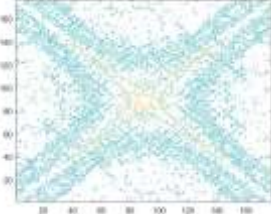
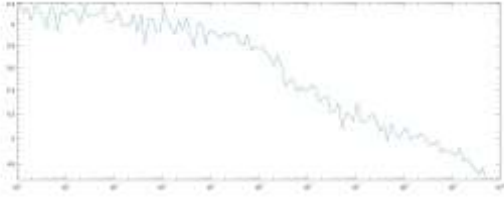
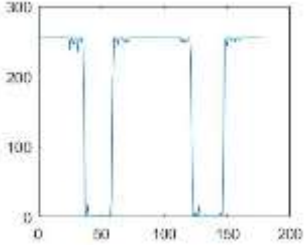
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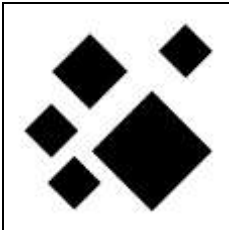
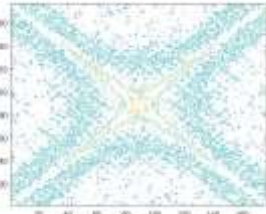
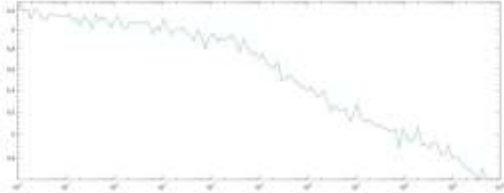
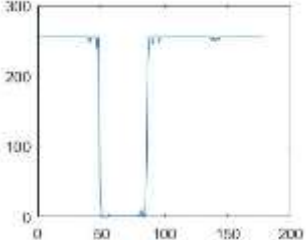
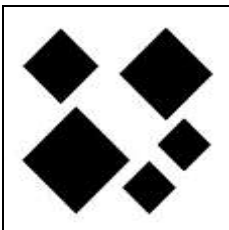

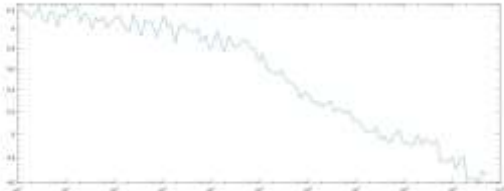
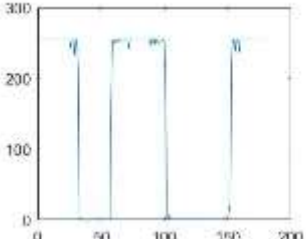
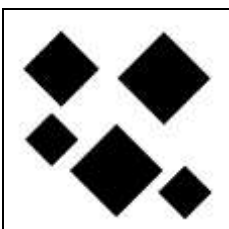
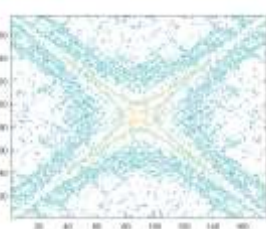
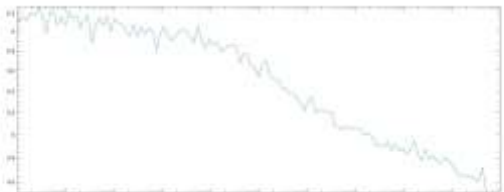
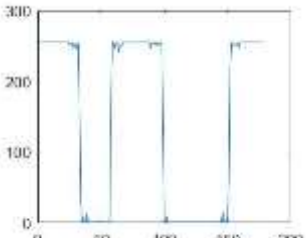
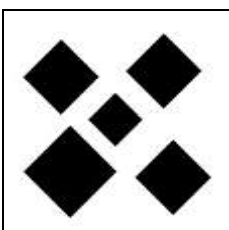

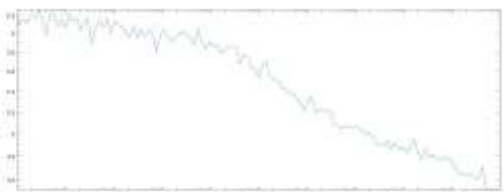
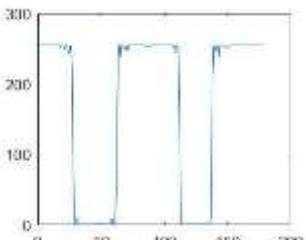
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Diamond	2.3				
Diamond	2.4				
Diamond	2.5				

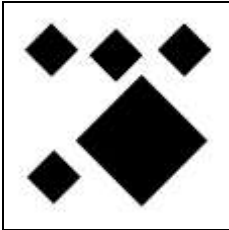
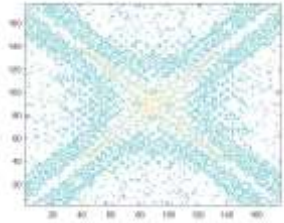
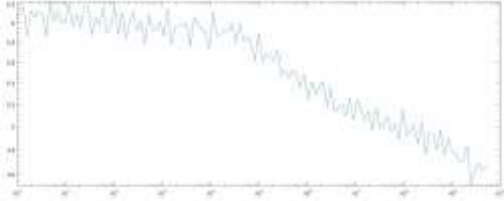
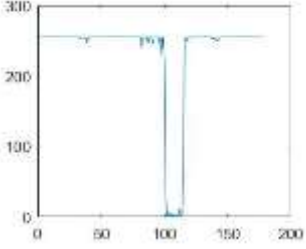
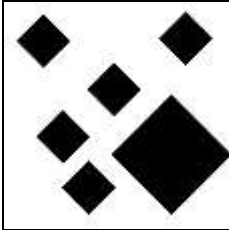
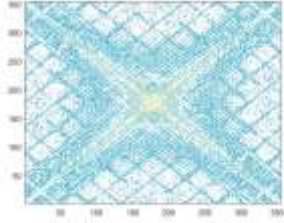
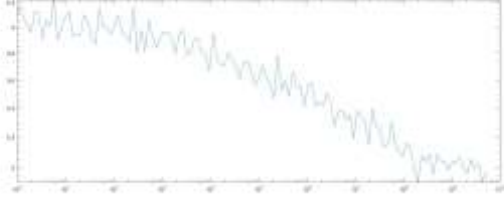
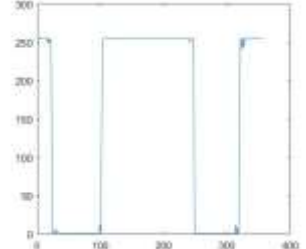
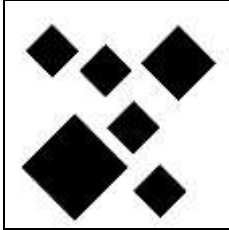
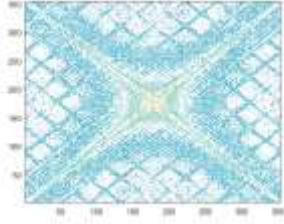
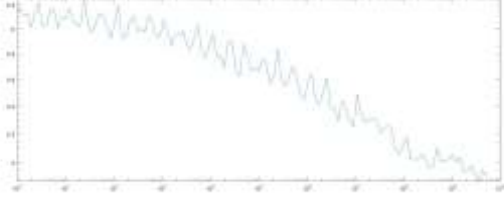
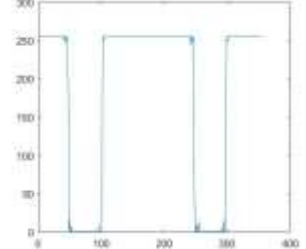
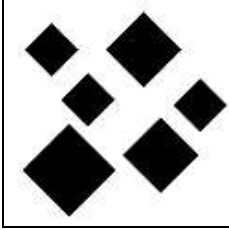
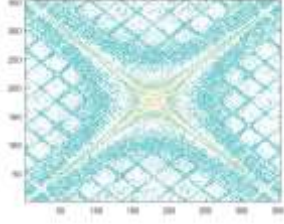
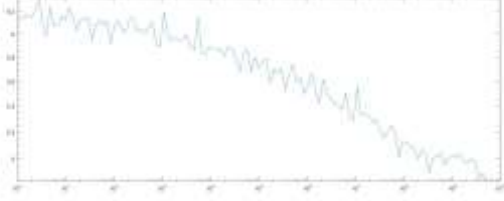
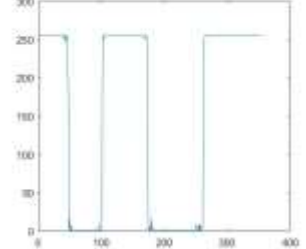
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Diamond	3.2				
Diamond	3.3				
Diamond	3.4				

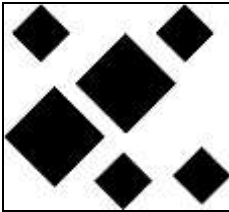
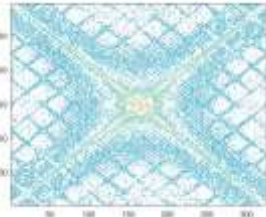
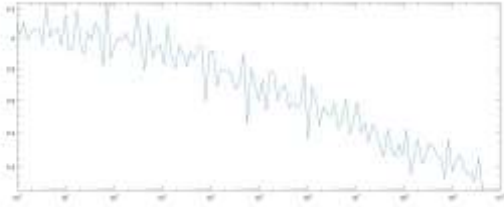
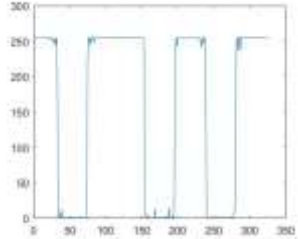
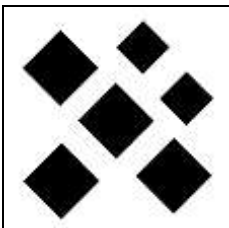

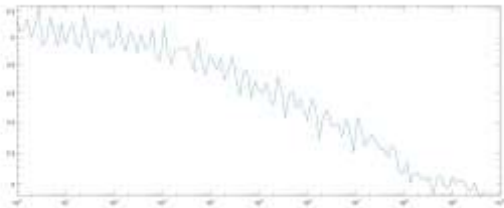
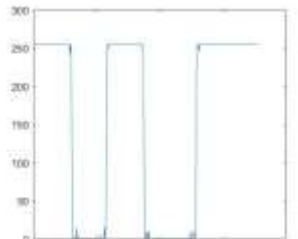
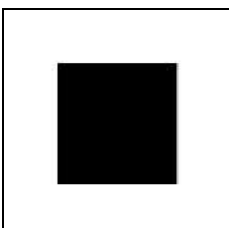
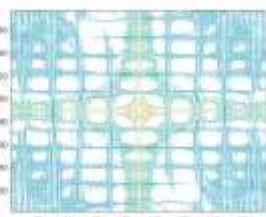
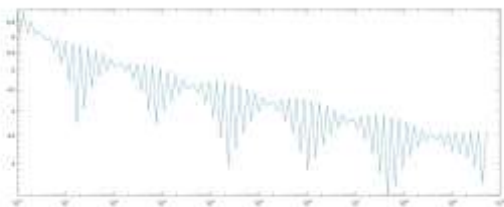
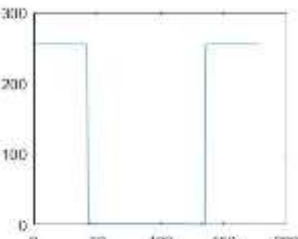
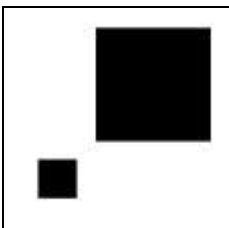
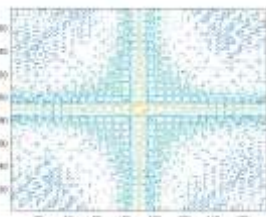
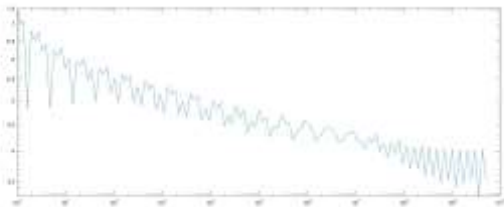
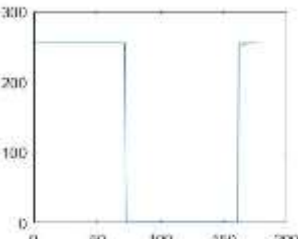
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Diamond	3.6				
Diamond	3.7				
Diamond	4.1				

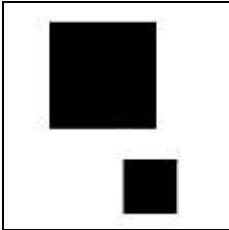
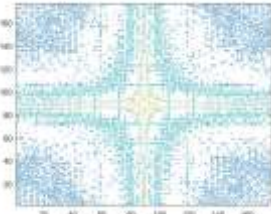
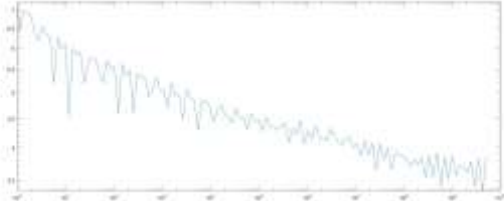
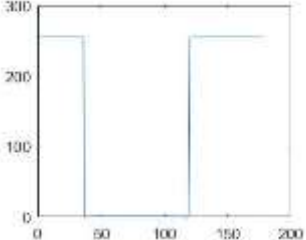
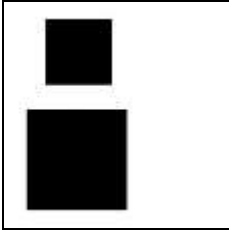

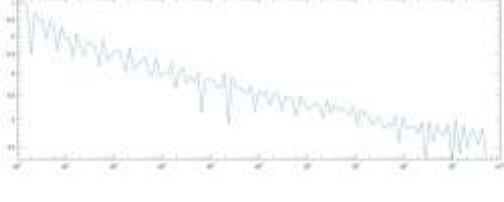
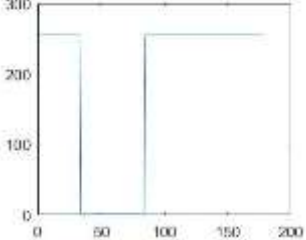
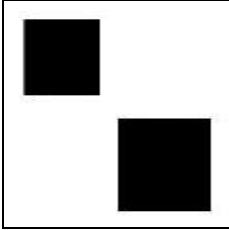

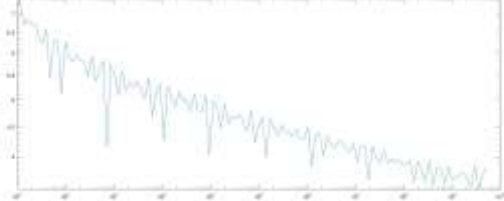
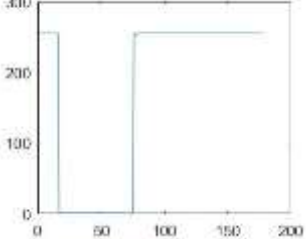
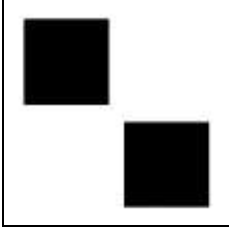
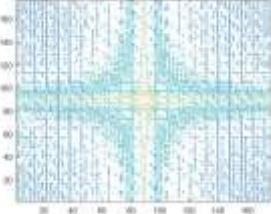
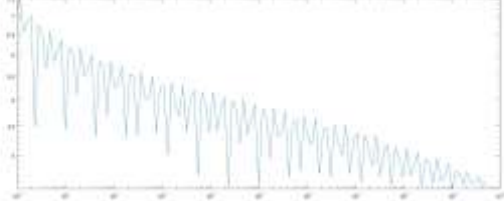
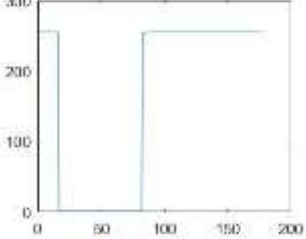
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Diamond	4.3				
Diamond	4.4				
Diamond	4.5				

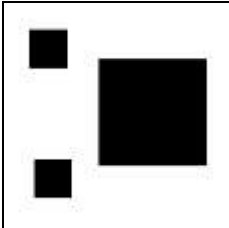
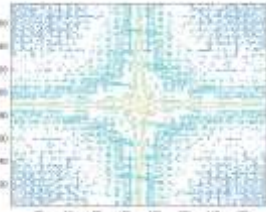
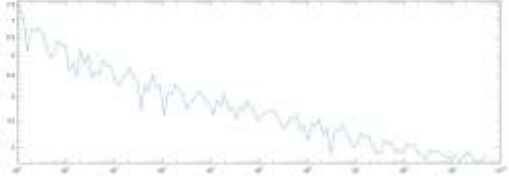
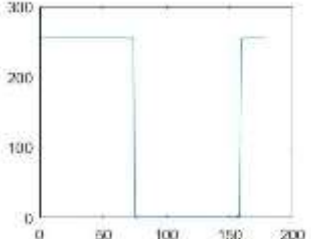
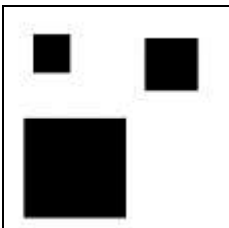
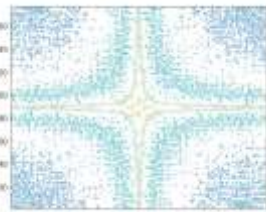
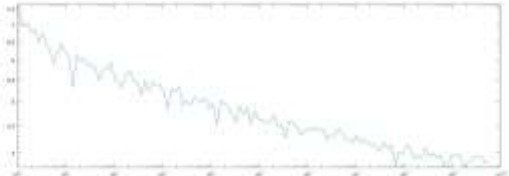
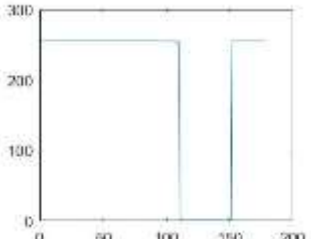
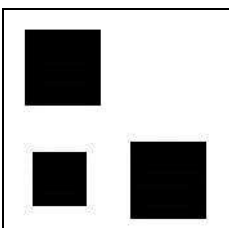

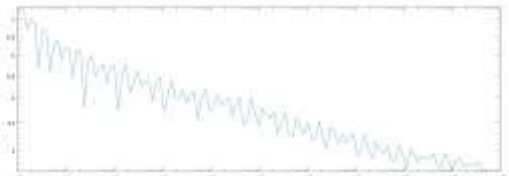
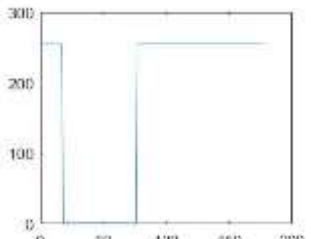
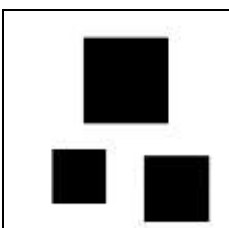

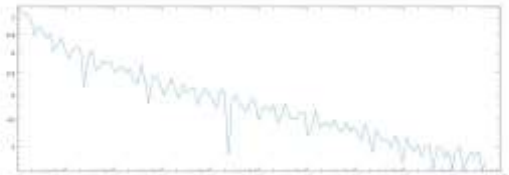
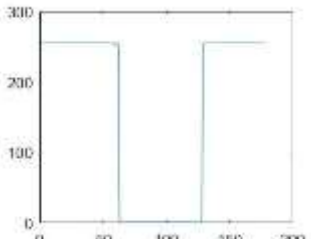
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Diamond	5.1				
Diamond	5.2				

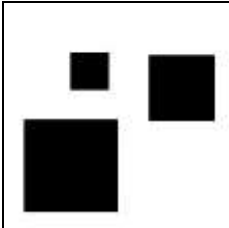

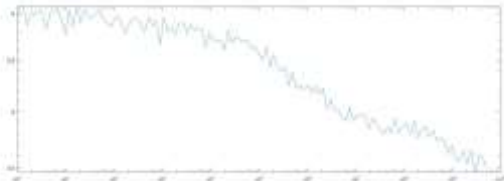
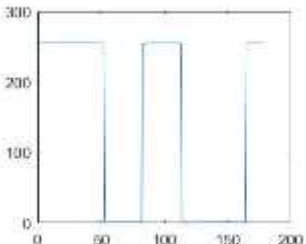
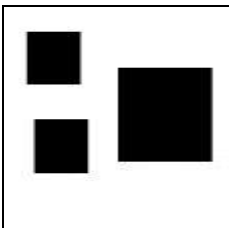
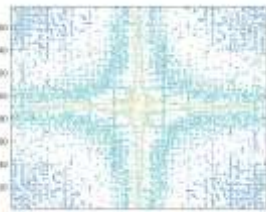
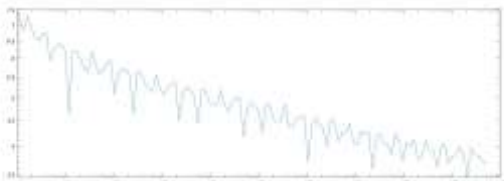
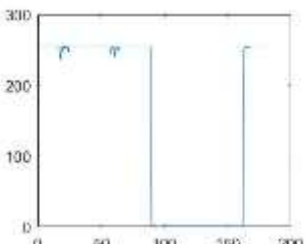
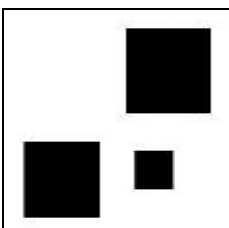

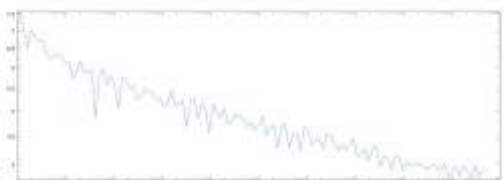
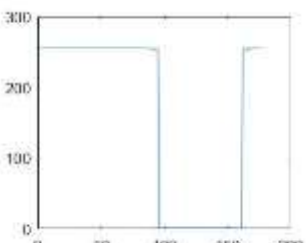
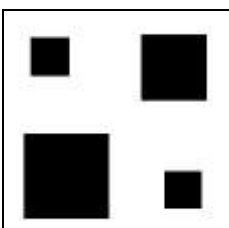

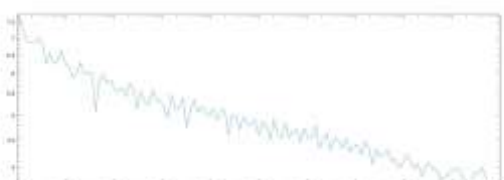
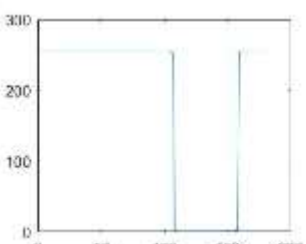
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Diamond	5.5				
Diamond	5.6				

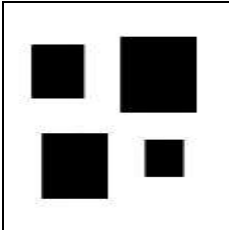
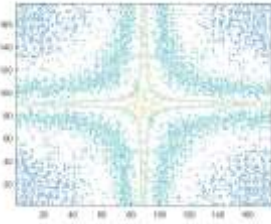
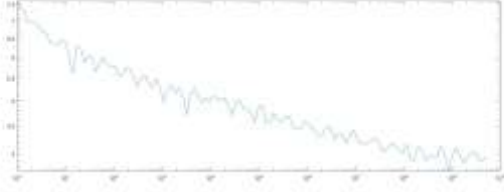
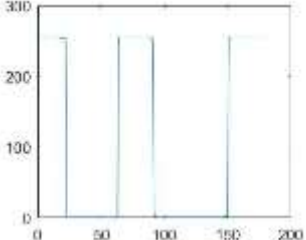
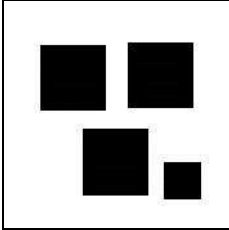
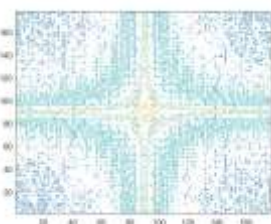
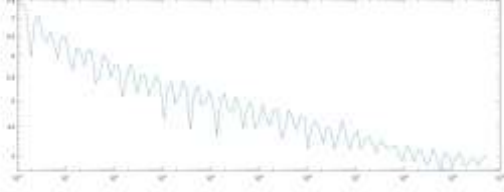
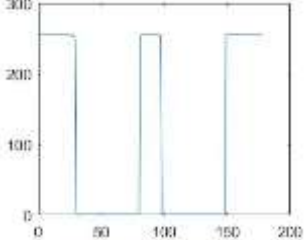
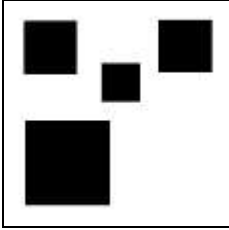
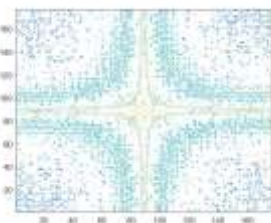
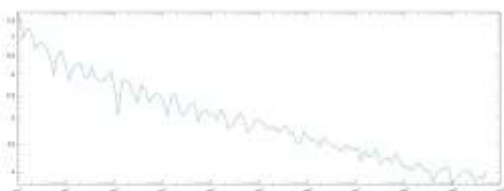
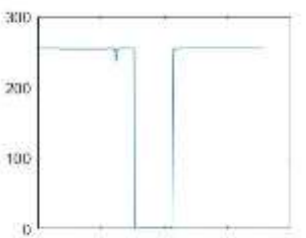
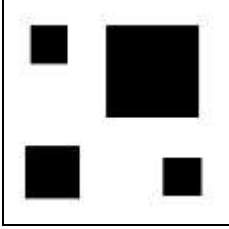
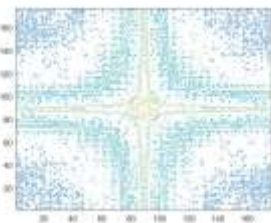
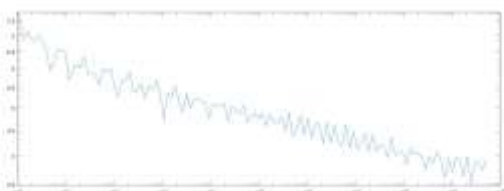
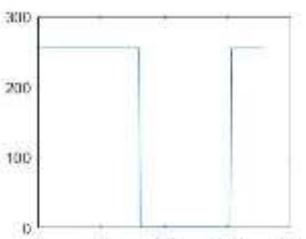
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Diamond	6.2				
Diamond	6.3				

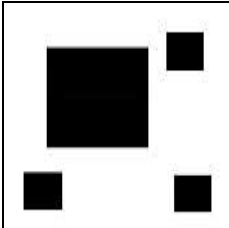
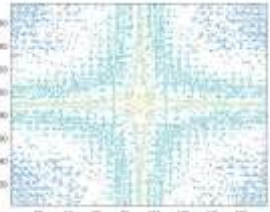
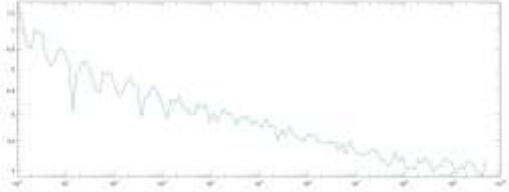
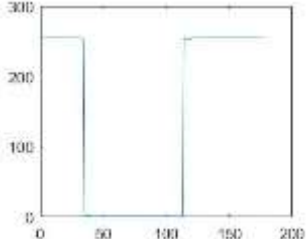
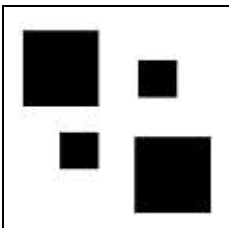
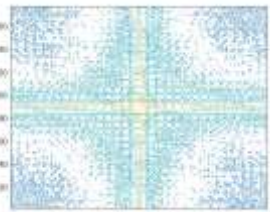
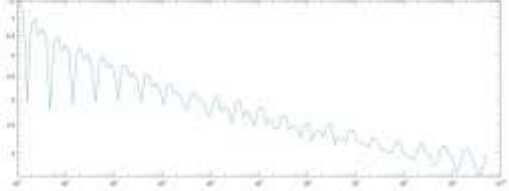
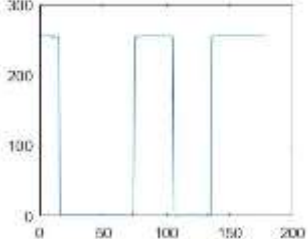
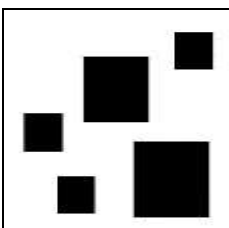

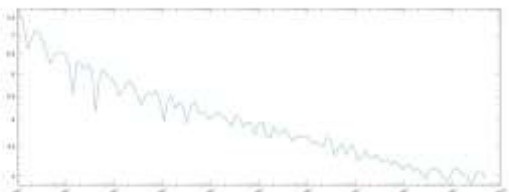
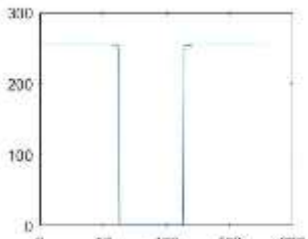
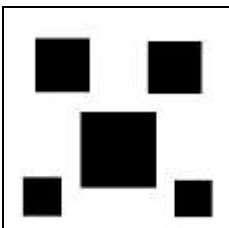

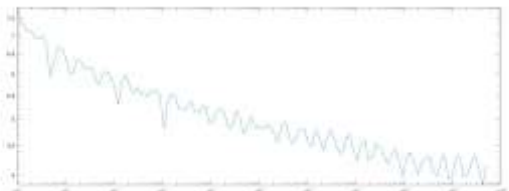
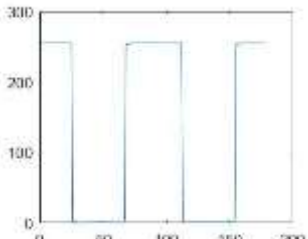
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Square	2.1				

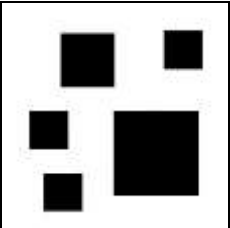
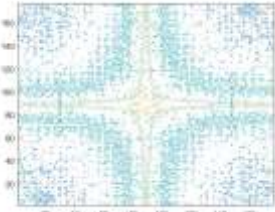
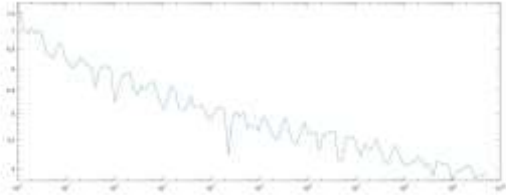
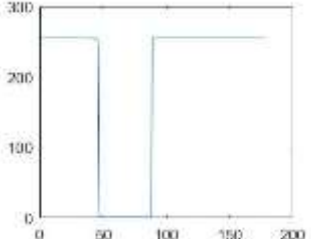
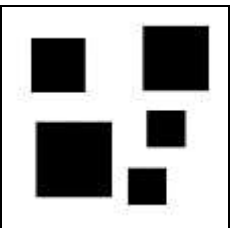
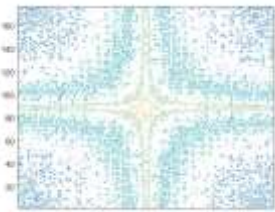
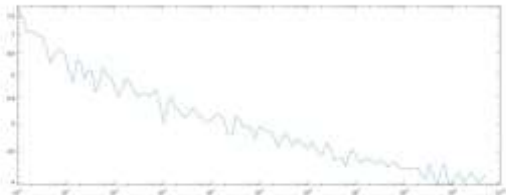
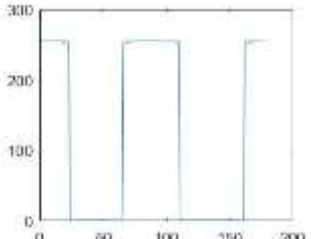
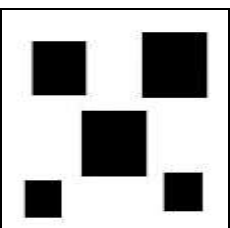
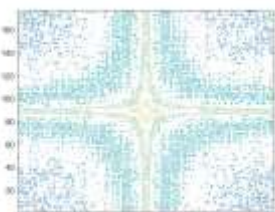
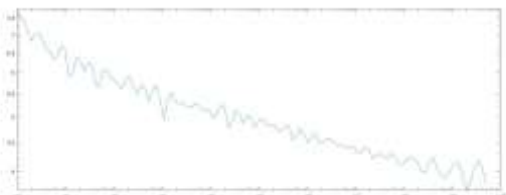
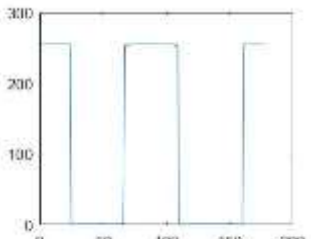
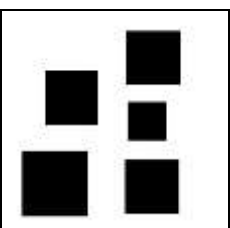

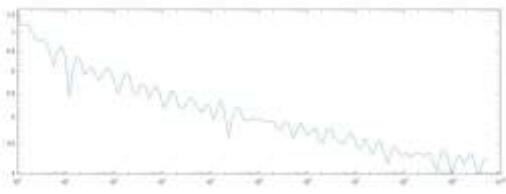
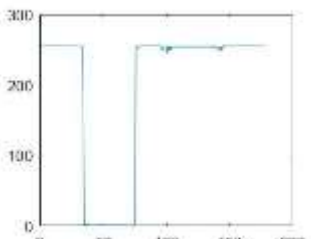
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Square	2.4				
Square	2.5				

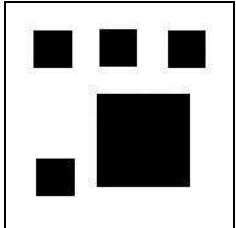
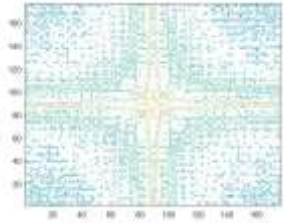
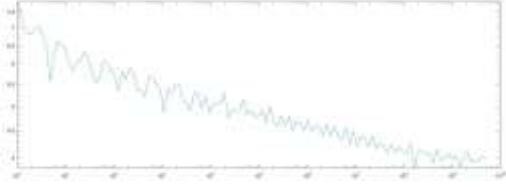
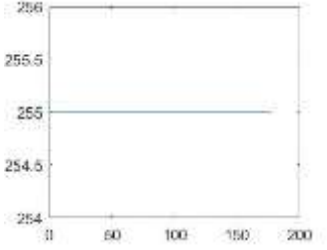
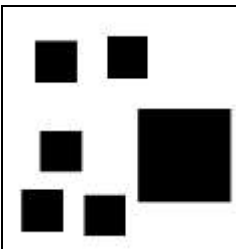
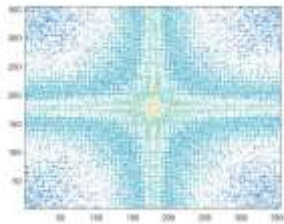
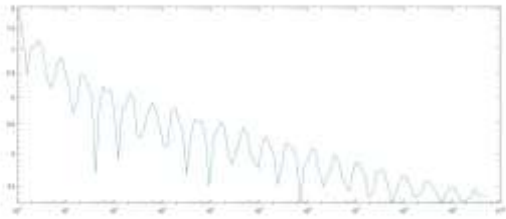
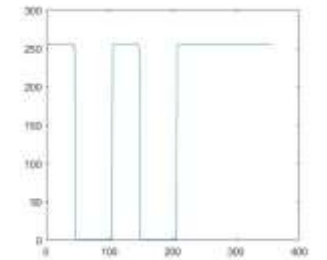
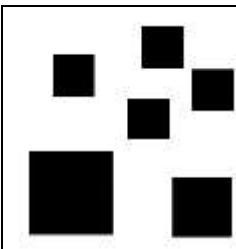
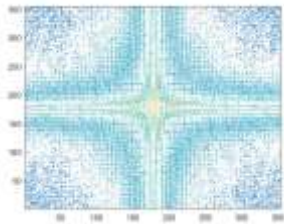
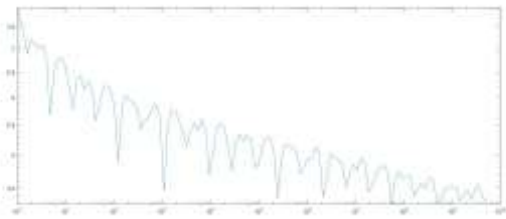
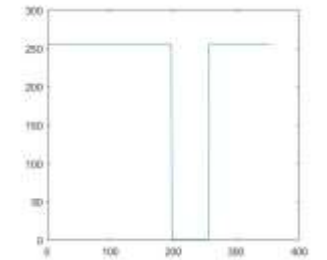
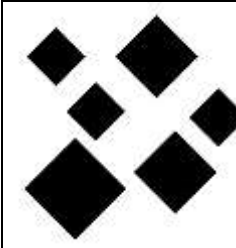
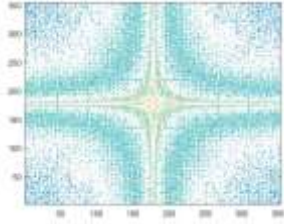
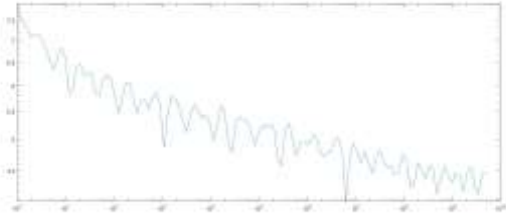
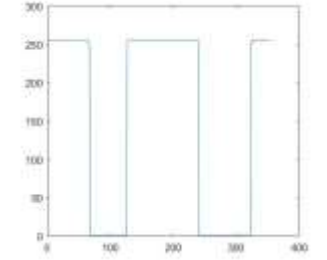
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Square	3.2				
Square	3.3				
Square	3.4				

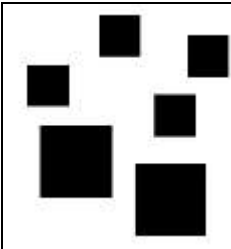
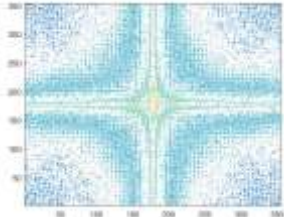
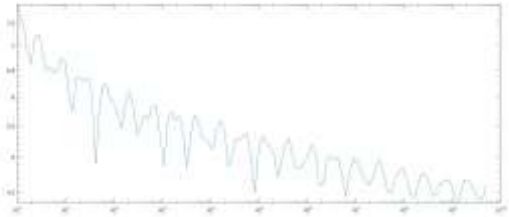
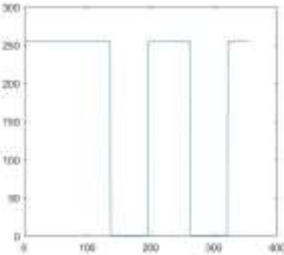
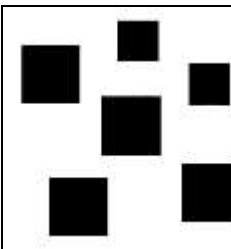
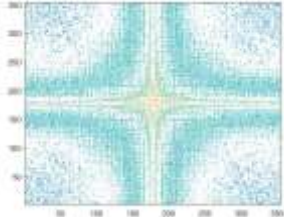
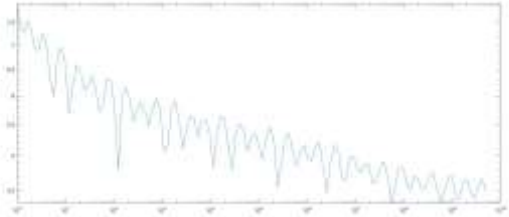
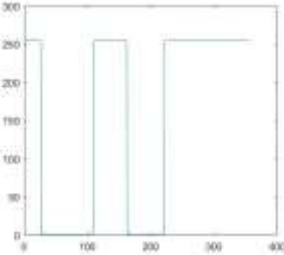
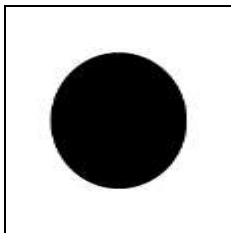
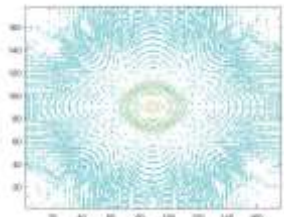
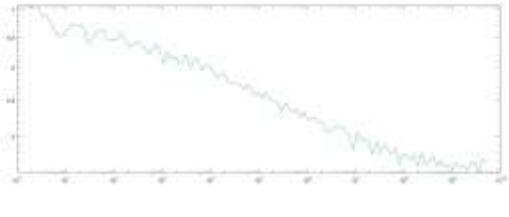
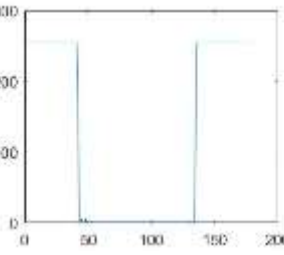
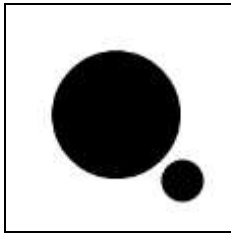
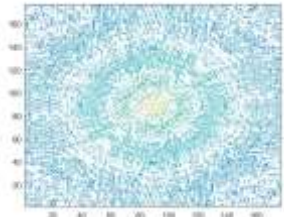
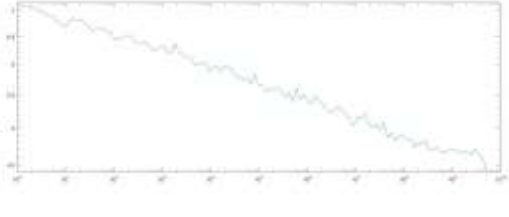
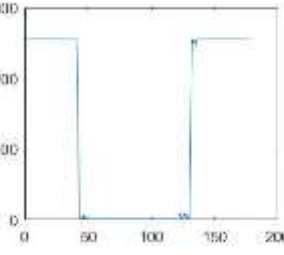
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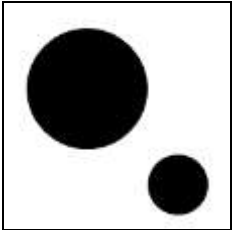
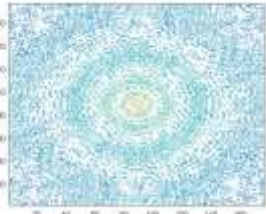
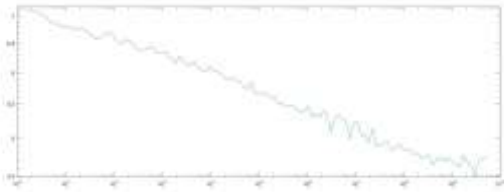
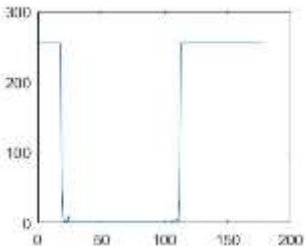
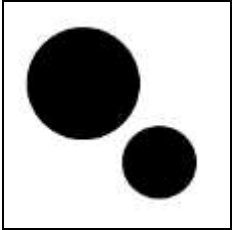
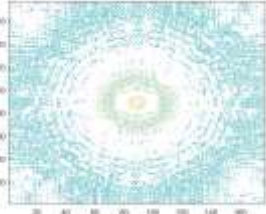
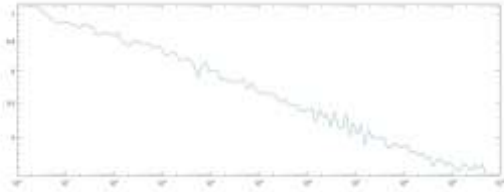
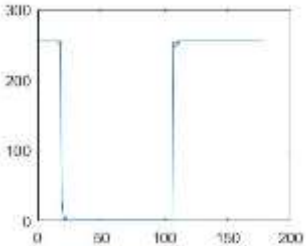
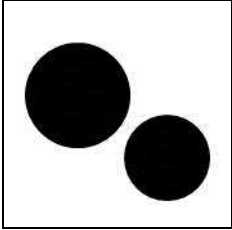
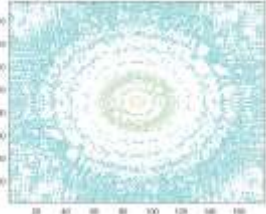
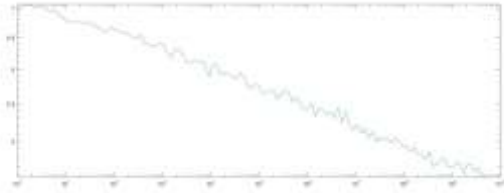
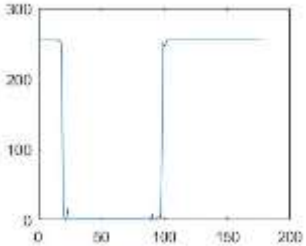
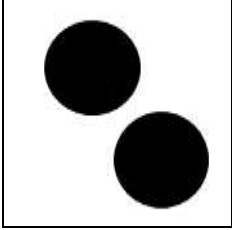
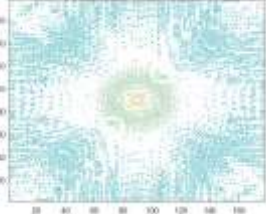
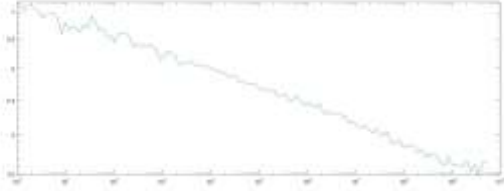
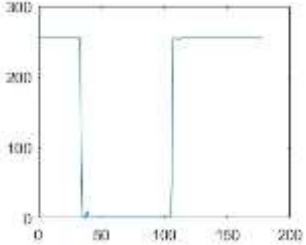
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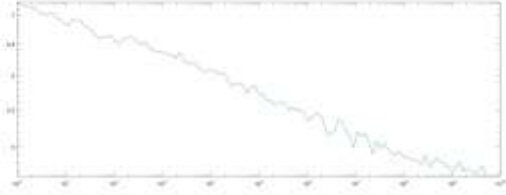
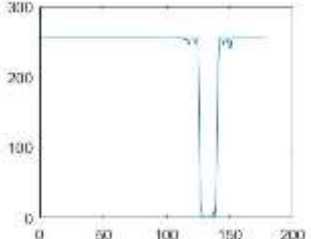
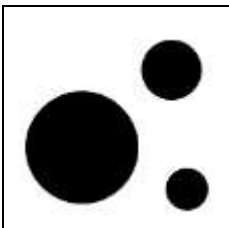

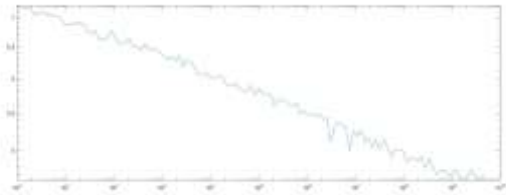
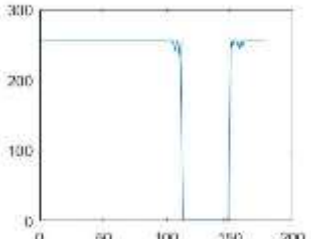
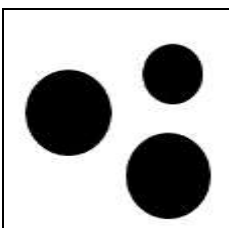

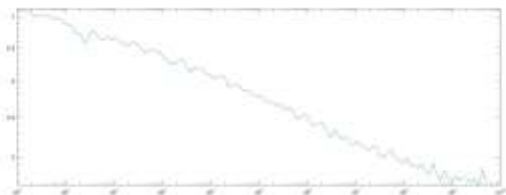
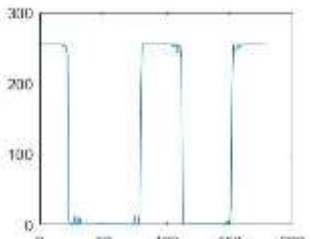
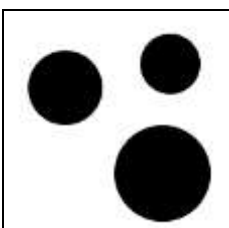

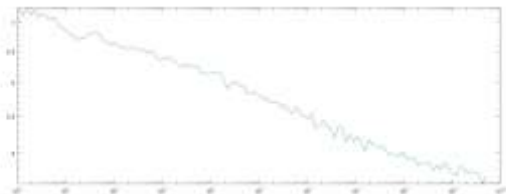
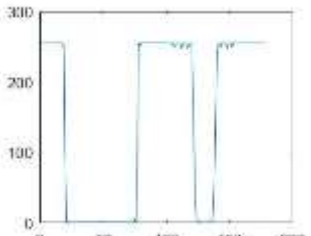
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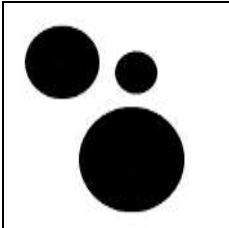

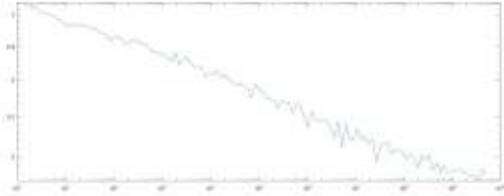
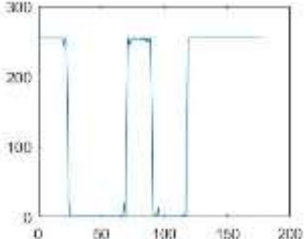

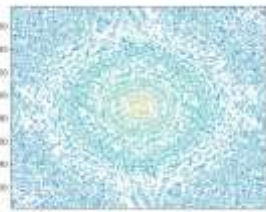
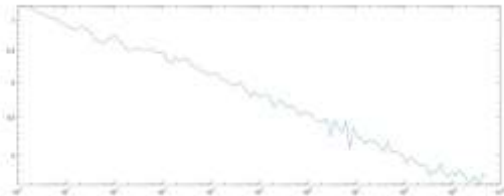
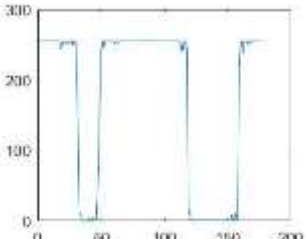
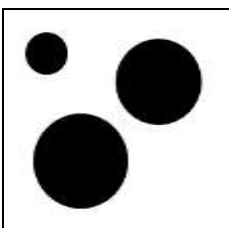

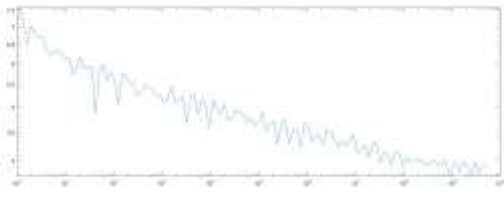
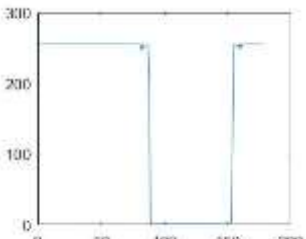
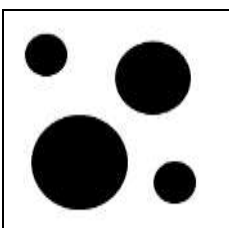

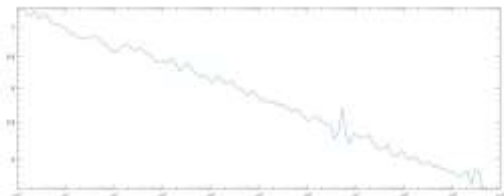
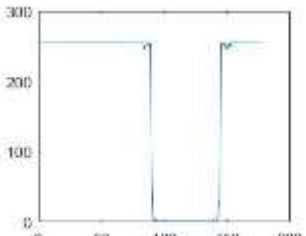
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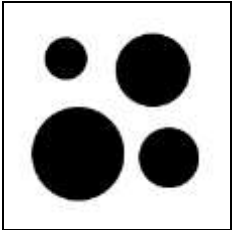
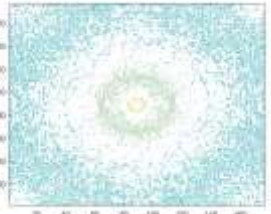
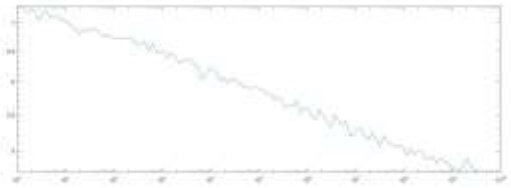
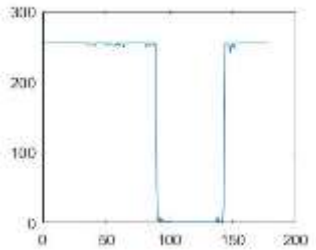
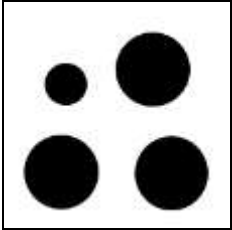
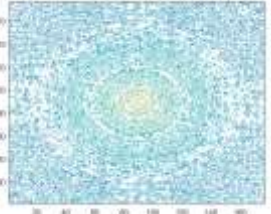
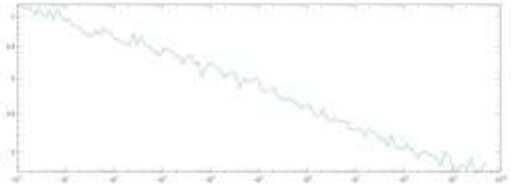
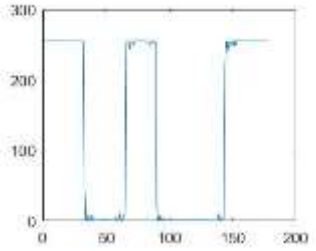
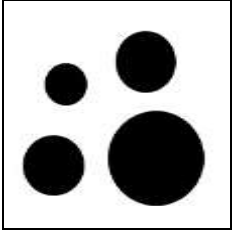
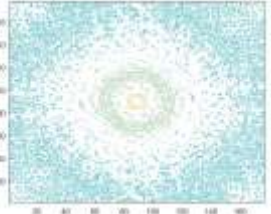
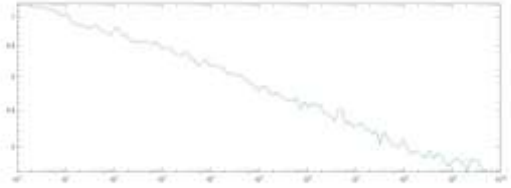
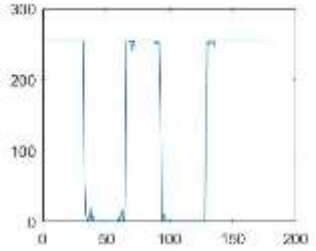
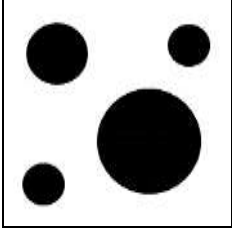
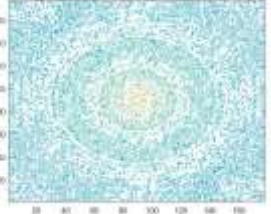
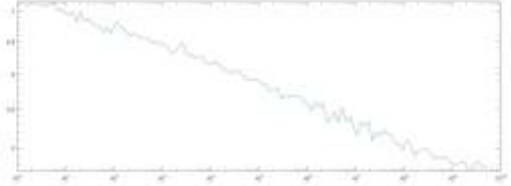
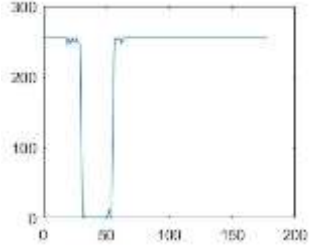
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Square	6.3				

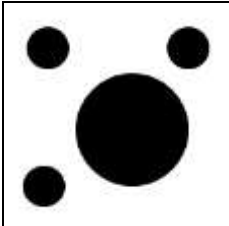
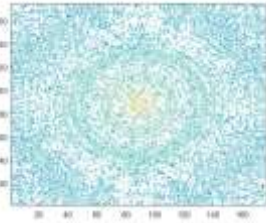
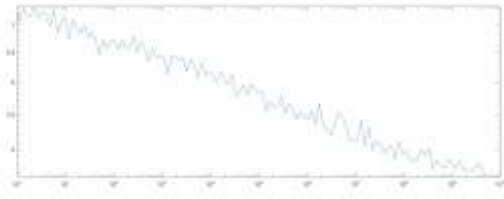
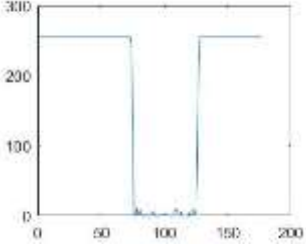
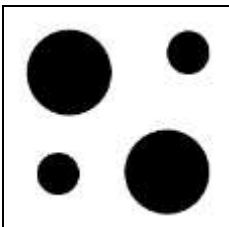
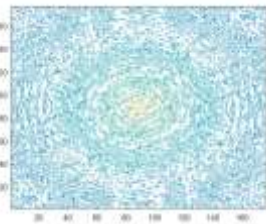
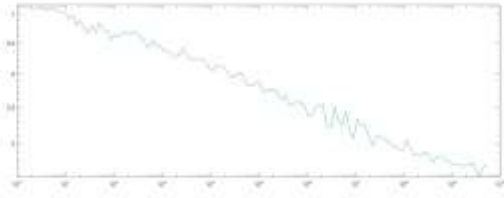
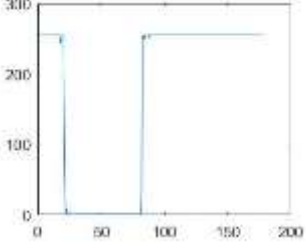
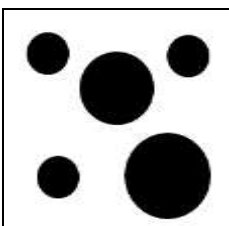
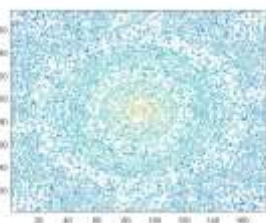
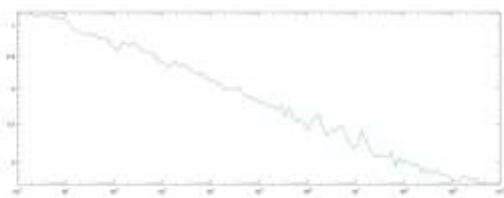
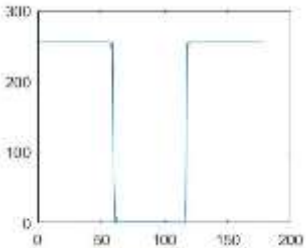
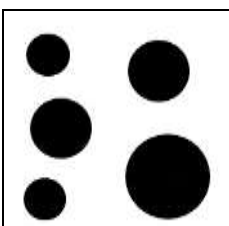
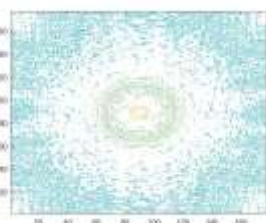
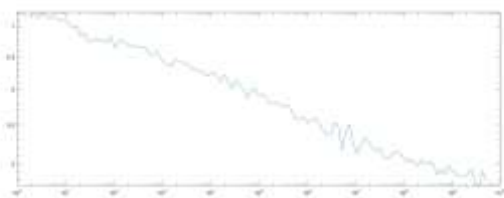
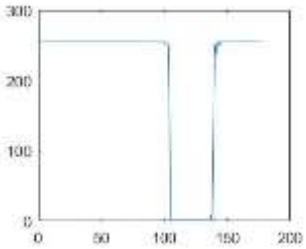
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Circle	2.1				

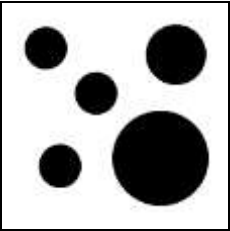
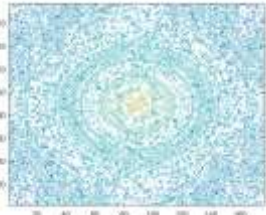
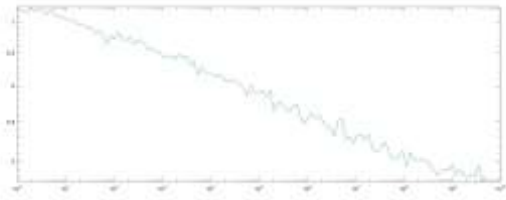
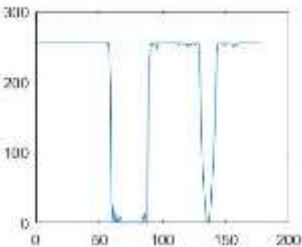
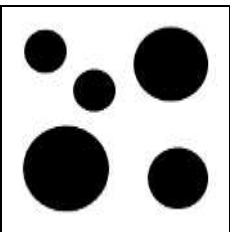

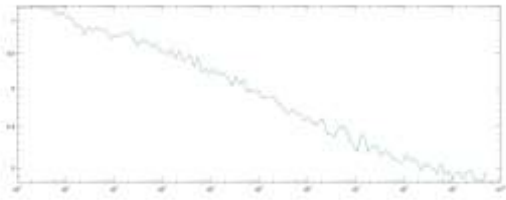
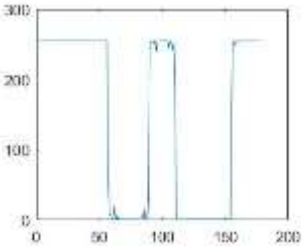
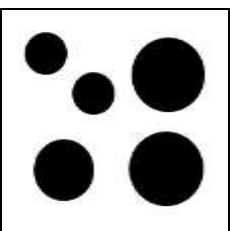

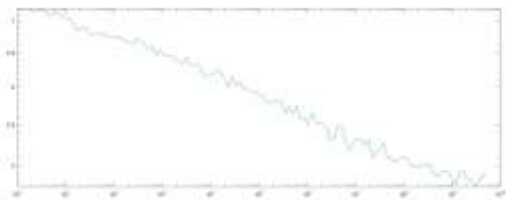
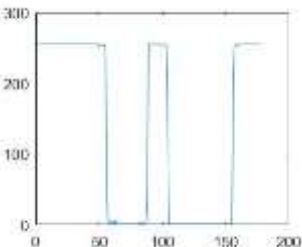
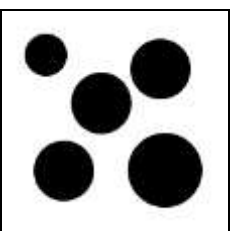

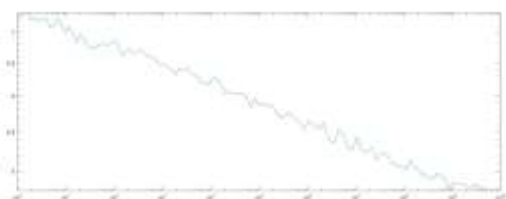
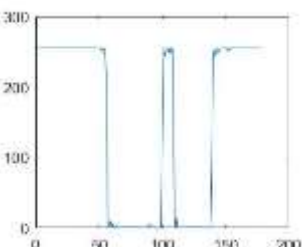
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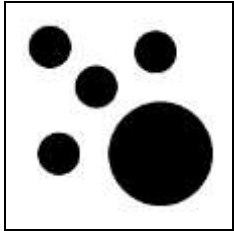
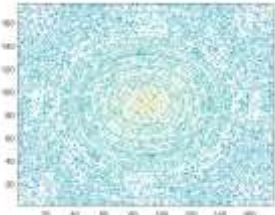
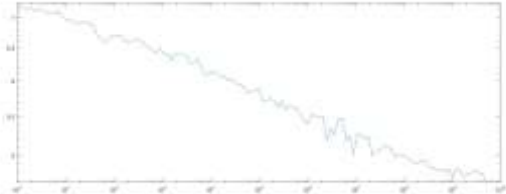
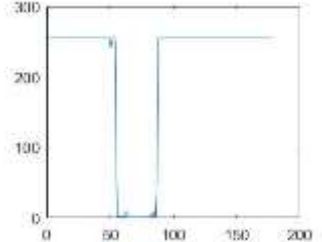
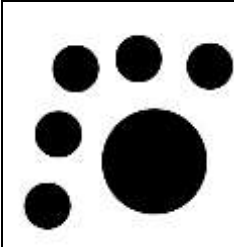
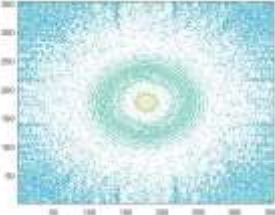
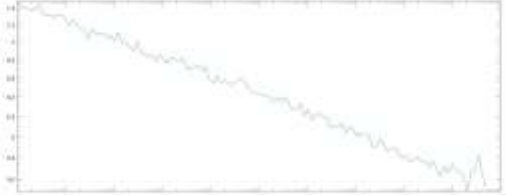
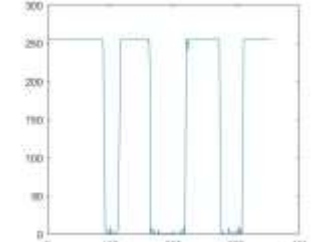
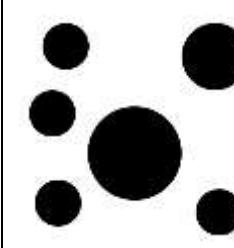
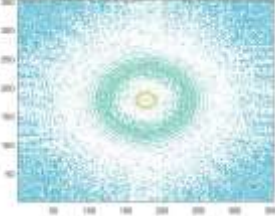
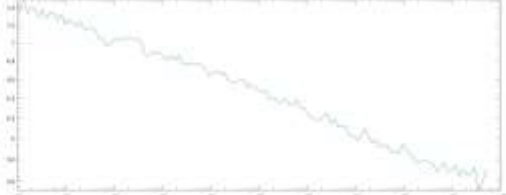
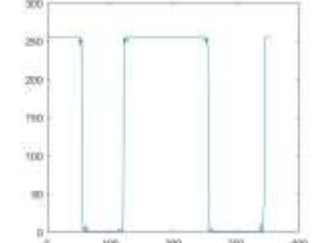
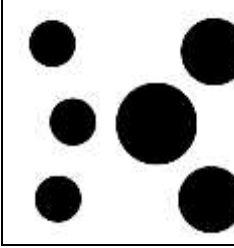
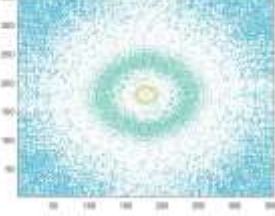

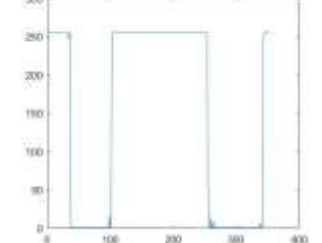
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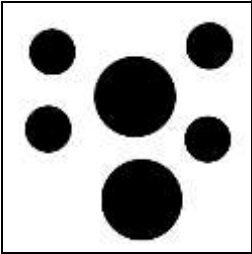
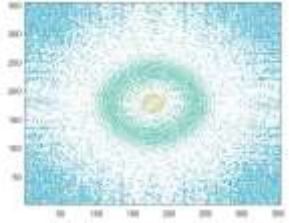
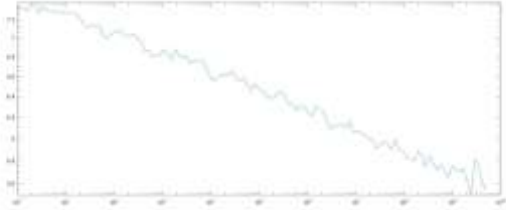
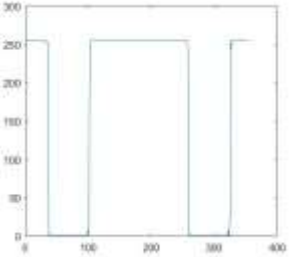
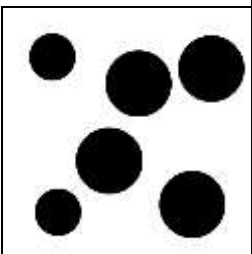
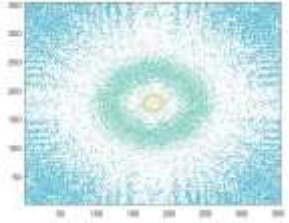
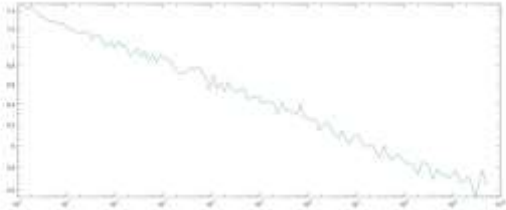
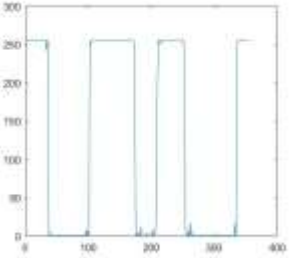
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Chapter 4

Numerical cognition in honeybees enables addition and subtraction

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Title

Numerical cognition in honeybees enables addition and subtraction

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Abstract

Many animals understand numbers at a basic level for use in essential tasks such as foraging, shoaling, and resource management. However, complex arithmetic operations, such as addition and subtraction, using symbols and/or labelling have only been demonstrated in a limited number of non-human vertebrates. We show that honeybees, with a miniature brain, can learn to use blue and yellow as symbolic representations for addition or subtraction. In a free-flying environment, individual bees used this information to solve unfamiliar problems involving adding or subtracting one element from a group of elements. This display of numerosity requires bees to acquire long term rules and use short term working memory.

Given that honeybees and humans are separated by over 400 million years of evolution, our findings suggest that advanced numerical cognition may be more accessible to non-human animals than previously suspected.

Keywords: addition; *Apis mellifera*; arithmetic; numerical cognition; quantal cognition; subtraction

One-sentence summary: Honeybees learn to add or subtract one item from a set using colour cues and can interpolate operations to a novel number.

MAIN TEXT

Introduction

Currently, there is considerable debate surrounding the ability of animals to possess or learn complex number skills (1-5). A distinction is made between species which are able to use quantal (e.g. quantity discrimination) and numerical (exact, symbolic) cognition (2). While many species are able to use quantal cognition to forage, make decisions, and solve problems, it is debated whether any non-human or non-primate animals could reach the level of *numerical cognition*, such as exact number and arithmetic operations, for example solving addition and subtraction problems (1, 2). Such a capacity would require complex management of quantities in both working memory and longer term rule based memory (6). There are studies which demonstrate that vervet monkeys (6), chimpanzees (7-9), orang-utans (10), rhesus monkeys (11), one African grey parrot (12, 13), pigeons (14), spiders (15, 16), and human infants (17, 18) have the ability to add and/or subtract. Some studies show very sophisticated addition and subtraction abilities such as in the case of a chimpanzee and an African grey parrot that could both label the result of an addition sum using Arabic symbols or an English label respectively which would constitute exact numerical cognition (7, 12, 13).

Other studies show that some species are able to perform addition and subtraction problems spontaneously, without training, in a more naturalised task, such as spiders which can count prey items and notice when prey is added or subtracted (15), and rhesus monkeys which choose to approach obscured food in the wild when a subtraction sum should result in food being present (11).

Honeybees are a model for insect cognition and vision (19, 20). Bees have demonstrated the ability to learn a number of rules and concepts to solve problems such as ‘left/right’ (21), ‘above/below’ (22), ‘same/different’ (23), and ‘larger/smaller’ (24-26). Honeybees have also shown some capacity for counting and number discrimination when trained using an appetitive (reward-only) differential conditioning framework (27-30). Recent advancements in training protocols reveal that bees perform significantly better on perceptually difficult tasks when trained with an appetitive-aversive (reward-punishment) differential conditioning framework (31). This improved learning capacity is linked to attention in bees (31), and attention is a key aspect of advanced numerosity and spatial processing abilities in the human brain (32, 33). Using this conditioning protocol, honeybees were recently shown to acquire the numerical rules of ‘greater than’ and ‘less than’ and subsequently apply these rule to demonstrate an understanding that an empty set, zero, lies at the lower end of the numerical continuum (34). Thus to inform the current debate on number skills in animals, research on insects with miniature brains enables valuable comparisons of what brains of different sizes and architectures can achieve .

The capacity of honeybees to learn complex rules and concepts (20) alongside evidence of their number-sense (29, 34) suggests they are a good model for testing numerical cognition. We trained bees to identify a salient colour (blue or yellow) as a symbolic representation of whether to follow a rule based on addition (blue) or subtraction (yellow), and thus choose the correct result of an arithmetic operation.

In this study, honeybees were trained to enter a Y-maze and view a visual sample stimulus presented vertically containing a set of elements in isolation (Fig. 1). Bees would then fly through an opening into a decision chamber and choose between two possible options (Fig. 1). The sample stimulus could contain 1, 2, 4, or 5 elements (1, 2, or 4 elements if blue/addition; 2, 4, or 5 elements if yellow/subtraction). If the elements were blue, bees would need to choose the stimulus option in the decision chamber which was one element greater than the sample, however, if the elements were yellow, bees would need to choose the stimulus which contained one less element than the sample number (Fig. 1). The colour of the elements, and thus the arithmetic problem to be solved, was randomly assigned per bee for each trial. Correct and incorrect options during experiments ranged from 1 to 5 elements and the incorrect option could be higher or lower than the correct option (which also included the sample number as a possible incorrect option). The sample number of three elements was never shown during training and was only used as a novel sample number during testing. See Materials and method section below for more information.

Results

Training phase

Over the course of 100 appetitive-aversive (reward-punishment) reinforced choices (31), honeybees were trained to add or subtract one element based on the colour of a sample stimulus (Fig. S1). Bees were provided with a 10 μ L drop of either a 50 % sucrose solution (CS+) or a 60 mM quinine solution (CS-) as rewarding or punishing outcomes for a correct or incorrect choice, respectively (See Materials and Method). In this learning phase, there was a significant increase in the number of correct choices made over the 100 conditioned choices ($z = 8.14$; $P < 0.001$) demonstrating that bees learnt to simultaneously add or subtract by one based on the colour of the sample stimulus (Fig. 2A). Each individual bee appears to

learn differently, possibly due to the random presentation of stimuli and/or individual differences in cognitive abilities (SI Material, Methods and Results; Fig. S2).

Testing phase

We subsequently tested bees during non-reinforced tests (no reward or punishment) on their ability to interpolate the learnt concepts of addition and subtraction to the novel sample stimulus of three elements (See Materials and method). We conducted four tests: two addition operations and two subtraction operations. Two of these four tests presented an incorrect option in the same numerical direction as the correct option, and the other two presented an incorrect option in the opposite numerical direction of the correct option:

1. Addition: incorrect option in same numerical direction as correct option

Sample = 3; Correct = 4; Incorrect = 5

2. Addition: incorrect option in opposite numerical direction as correct option

Sample = 3; Correct = 4; Incorrect = 2

3. Subtraction: incorrect option in same numerical direction as correct option

Sample = 3; Correct = 2; Incorrect = 1

4. Subtraction: incorrect option in opposite numerical direction as correct option

Sample = 3; Correct = 2; Incorrect = 4

In each of the four tests, bees performed at a level that was significant from chance. In the addition (same direction) test, bees chose the correct option of 4 in 72.1 ± 3.20 (mean \pm standard error of the mean (S.E.M.)) % of choices ($z = 5.05$, $P < 0.001$; Fig. 2B). In the other addition (opposite direction) test, bees chose the correct option of 4 in 66.4 ± 2.69 % of choices ($z = 3.81$, $P < 0.001$; Fig. 2B). In the subtraction (same direction) test, bees chose the correct option of 2 in 63.6 ± 2.89 % of choices ($z = 3.17$, $P = 0.002$; Fig. 2B). In the other

subtraction (opposite direction) test, bees chose the correct option of 2 in 67.9 ± 3.66 % of choices ($z = 4.13$, $P < 0.001$; Fig. 2B). There was no significant difference between the performance of bees in any of the four tests ($z = -0.887$, $P = 0.375$), demonstrating that bees performed equally well on all tests.

Discussion

Honeybees were able to use colour as a symbolic representation of the addition and subtraction signs and learnt, during 100 appetitive-aversive trials, to thus add or subtract one element from different samples. Furthermore, bees could successfully interpolate the learnt operations of addition and subtraction to an unfamiliar sample number and shape during tests.

Arithmetic operations such as addition and subtraction problems are known to involve complex cognitive processes as they require two levels of information processing. The first is the representation of numerical attributes, and the second is the mental manipulation of those representations in working memory (6). In the current study, bees not only succeeded in performing these processing tasks, but also had to perform the arithmetic operations in working memory as the number to be added or subtracted (one element) was not visually present, but rather an abstract concept which bees had to resolve over the course of training. This important step into combining the arithmetic and symbolic learning abilities of an insect has identified numerous areas of research to expand into and also poses the question of whether such complex numeric understandings may be accessible to other species without large brains, such as the honeybee (35). While the posterior parietal cortex and the prefrontal cortex are key areas for numerical processing in primates (32), we are yet to determine where number representation and processing may occur in honeybee brains, however we do show that the comparatively large and complex brain areas required in primates are not necessary for an insect to process number problems.

While the specific task of addition/subtraction may not directly be apparent in the honeybee's natural environment, the skills and cognitive plasticity required for performing the arithmetic task are likely to be ecologically advantageous. For example, the ability of bees to acquire and manipulate learnt information to make decisions using multiple memory phases (23) is useful in foraging to remember which flower traits (e.g. colour, shape, size) may provide essential resources, and which flower traits may not (35). Thus rule learning involving linking visual traits to reward quantification, such as in the arithmetic task, is likely to be beneficial to a honeybee's foraging lifestyle.

The debate regarding a non-human animal's capacity to demonstrate numerical cognition suggests that either numerical skills are biologically evolved traits (1), or that animals only possess limited quantical abilities and human culture is necessary for more complex numerical abilities (2, 4). However, this debate also inspired a third important argument; Verguts and Chen (2017) suggest that at the very least we must consider the rapid evolution of individual learning of numerical cognition which occurs during an animal's lifetime. In this regard the honeybee is a proficient learner of many tasks including sameness and differences judgements (23), mazes (21, 36), face stimuli (37), and spatial relationships (38), and the results of the current study demonstrate that honeybees are capable of learning and applying numerical cognition as individuals. Our results suggest the possibility that honeybees and other non-human animals may be biologically tuned for complex numerical tasks, but such possibilities are of high value to be further explored, particularly in insects.

Interestingly, human infants with no language for number have demonstrated large number addition and subtraction (18), and native speakers of Mundurukú from Brazil, a language that has no words for large numbers, can add large approximate numbers far beyond their naming range (39). While speakers of the Mundurukú language demonstrated exact arithmetic with small numbers (< 4 and 5), they failed at exact arithmetic for large numbers

(> 4 or 5), but were able to use approximation to calculate solutions. These studies demonstrate that human language is not necessary for arithmetic operations such as addition and subtraction. Combined with the results from our current study, we propose that language and prior advanced numerical understandings are not a prerequisite necessary to calculate addition and subtraction solutions. In the current study, bees were tested on the number range of 1 – 5 for their ability to add and subtract, thus it would be valuable to examine bee performance on large number quantities to determine if they could use approximation or exact arithmetic to solve similar large number arithmetic problems.

Materials and Methods

Study design:

We aimed to determine if free-flying honeybees could learn to add or subtract one element from an array of elements in a delayed-matching-to-sample task. To do this, we trained bees to use different colours (blue or yellow) as a prompt to perform either addition or subtraction. Bees were trained to use a Y-maze (described below; Fig. 1) to view a sample stimulus containing a certain number of coloured elements on a grey background. Once they had viewed this stimulus they could fly into a decision chamber to choose the correct option resulting from the arithmetic problem encountered (Fig. 1).

Study species:

We used 14 free-flying honeybees (*Apis mellifera*) foragers for this experiment. All bees were marked with a coloured dot on the thorax to identify individuals. An *ad-lib* von Frisch type gravity feeder providing ca. 10 – 30 % sucrose was set-up to maintain a regular number of bees.

Apparatus:

Individual honeybees were trained to enter a Y-maze (as described in (22); Fig. 1). The bees had to fly through an initial entrance hole to enter a chamber where they would view the sample stimulus. This stimulus would contain either blue or yellow elements on a grey background. Each bee could then fly through another hole into the decision chamber where it would be presented with two different options in each arm of the chamber. If the sample stimulus had been blue, the bee would need to choose the stimulus with a number of elements which was one more than the sample number, however, if the sample stimulus had been yellow, the bee would need to choose the stimulus with a number of elements which was one less than the sample number (Fig. 1). This delayed matching-to-sample method using a Y-maze apparatus is the standard methodology for testing honeybee learning, and specifically quantity matching in honeybees (30), and has been validated through producing consistent learning outcomes to alternative apparatus (20).

Stimuli were presented on grey backgrounds located 15 cm away from the decision lines. Two stimuli, one correct and one incorrect, were presented simultaneously in each arm of the Y-maze on the grey plastic background (Fig. 1). A 10 μ L drop of either a 50 % sucrose solution (correct choice) or a 60 mM quinine solution (incorrect choice) were used as rewarding and punishing outcomes respectively during the training phase as this promotes enhanced visual discrimination performances in free-flying honeybees. Each stimulus had a grey landing pole located below it which held either the drop of sucrose under the correct option or quinine under the incorrect option so that bees would learn to associate stimuli with either a reward or punishment. Poles were replaced when touched by a bee and cleaned with 20 % ethanol to exclude olfactory cues. The sides of the correct and incorrect stimuli were randomly changed between choices (38). If a bee made an incorrect choice and started to imbibe the quinine; it was allowed to fly to the pole in front of the correct stimulus to collect

sucrose to maintain motivation; but only the first choice was recorded for statistical analysis (38). Once the bee was finished imbibing the sucrose, it was allowed to fly back to the hive if satiated or make another decision by re-entering the maze. During the non-reinforced tests, a drop of water was placed on each of the poles placed in front of the stimuli. Ten choices (touches of the poles) were recorded for each of the four tests to enable statistical comparisons.

Stimuli:

Each stimulus was a 6 x 6 cm grey square with either blue (addition) or yellow (subtraction) elements presented on it (Fig. S1), and was covered with 80 μm Lowell laminate. The chosen colours were spectrally different and salient considering honeybee vision. Elements could be one of four shapes: square, diamond, circle, or triangle. Three of these shapes were used in training, and the other novel shape was used for testing to ensure patterns and shapes were unfamiliar to bees during tests. To control for surface area, each pattern (cumulated surface area of black elements) was $10 \pm 0.3 \text{ cm}^2$ regardless of shape, pattern, or number of elements; and each element was above the minimum resolution threshold for honeybee vision as based on previous psychophysics findings (SA range: Circle: 1 cm^2 to 9.95 cm^2 ; Square/Diamond: 1 cm^2 to 6.32 cm^2 ; Triangle: 1 cm^2 to 10 cm^2). There were 216 stimuli in total, 108 for addition and 108 for subtraction (Fig. S1). Element size, line length, and convex hull for all stimuli were not consistently correlated with increasing or decreasing numbers of elements.

There were a total of 108 different patterns, comprising of 1 - 5 elements of the four different shapes (square; diamond; circle; triangle) which could be presented throughout the experiment, this was done to control for the potential use of an associative mechanism by bees to learn the outcomes of each stimulus. There were no low-level cues which could be used to solve the problem as the correct answer could be lower or higher than the original

number depending on sample element colour and the incorrect answer could be the same number as the sample or any (non-correct) number above or below the sample. Thus the correct answer was not predicted by visual similarity to the original sample number or numerical closeness to the sample number.

Training procedure:

Bees were incrementally trained to enter the Y-maze and both arms of the apparatus over 30 – 60 minute periods. Once each bee was able to fly into the entrance hole and the hole that led to the decision chamber and could find the poles in both Y-maze arms, the experiment began.

After entering the Y-maze, bees would be in the initial chamber where they could view the sample number. To solve the task the bees were required to either add or subtract the value of one to this sample number depending on the colour of the elements (Fig. 1). Bees would then fly through the next hole in the Y-maze and into the decision chamber where they could simultaneously view two stimuli in a dual choice test. If the sample number was blue, the bee would need to choose the option which was one element greater than the sample stimulus to receive a reward, whilst if the sample number was yellow, the bee would need to choose the option which was one element less than the sample number to receive a reward. The incorrect option was randomly selected and could be any number from 1 – 5; including the sample number itself which controlled for bees choosing the correct option based on visual similarity, and incorrect choices were associated with a bitter tasting quinine solution.

Each bee thus completed 100 appetitive-aversive (31) reinforced trials presenting either addition or subtraction arithmetic problems. Whether a trial would involve adding or subtracting one element from the sample number was randomised.

Throughout the training, the numbers which could be used for the sample in the addition trials were 1, 2, 4. Thus correct answers could be 2, 3, 5 and the incorrect answers could be 1, 2, 3, 4, 5. During the subtraction trials, the numbers which could be used for the sample number were 2, 4, 5. Thus correct answers could be 1, 3, 4 and the incorrect answers could be 1, 2, 3, 4, 5. The number 3 was never shown as a sample number during training for any bee and was thus used as the sample number for all unreinforced tests to ensure the sample number was novel during tests.

Testing procedure:

Once bees had completed the training, there were four tests of 10 unreinforced choices. Between each of the four tests there were 10 refresher reinforced choices to maintain bee motivation. The sequence of these tests was randomised. These tests were non-reinforced (no reward or punishment) and used a 10 μ L drop of water instead of quinine or sucrose to motivate bees to land. We conducted four tests where two arithmetic operations were addition and two were subtraction. As the sample stimulus of three elements had never been presented during training, bees had not previously received reinforcement on the number four for addition, or two for subtraction trials. Two of these four tests presented an incorrect option in the same direction as the correct option, and the other two presented an incorrect option in the opposite direction of the correct option. Two of the tests required addition and two required subtraction using the novel sample number of three.

Two of the tests involved the incorrect answer being the same direction as the correct answer (addition: sample = 3, correct = 4, incorrect = 5; subtraction: sample = 3, correct = 2, incorrect = 1).

Two of the tests involved the incorrect answer being in the opposite direction of the correct answer and thus also one element different from the sample (addition: sample = 3, correct = 4, incorrect = 2; subtraction: sample = 3, correct = 2, incorrect = 4).

Statistical analysis:

To test for the effect of training on bee performance (number of correct choices), data from the learning phase of 100 choices were analysed with a generalized linear mixed-effect model (GLMM) with a binomial distribution using the ‘glmer’ package within the R environment for statistical analysis. We fitted a full model with trial number as a continuous predictor, and subject as a random factor to account for repeated choices of individual bees.

To determine whether bees were able to learn to follow addition and subtraction rules, we analysed the test data by employing a GLMM including only the intercept term as fixed factor and subject as a random term. The proportion of ‘correct’ choices (MPCC) recorded from the tests were used as response variable in the model. The Wald statistic (z) tested if the mean proportion of correct choices recorded from the learning test, represented by the coefficient of the intercept term, was significantly different from chance expectation, *i.e.* $H_0: \text{MPCC} = 0.5$.

A separate analysis was performed to determine if there were any differences between the four tests regarding the performance of bees. We analysed the test data using a GLMM including only the intercept term as fixed factor and subject as a random term. The proportion of ‘correct’ choices during the tests and the test type (addition test 1; addition test 2; subtraction test 1; subtraction test 2) were used as response variable in the model. The z statistic tested if the mean proportion of correct choices recorded from the tests differed based on test type.

All analyses were performed within the R environment for statistical analysis.

H2: Supplementary Materials

Supplementary Materials, Methods and Results

Fig. S1. The full set of stimuli used ($n = 216$) for the addition (blue; $n = 108$) and subtraction (yellow; $n = 108$) training and test phases.

Fig. S2. The Bayesian determined bias for each of the bees, averaged over $n_t = 10$ trials (except for the first 10 experiments, which were evaluated with respect to all previous experiments).

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Author contributions: SRH, AAW, JEG, and AGD designed the experiment. SRH performed data collection. SRH, JEG, and ADG analysed data. All authors were involved in the interpretation of results and drafting of the manuscript.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The raw choice data of individual bees that support the findings of this study are available in Dryad Data Repository with the identifier doi: doi:10.5061/dryad.56r4rv4. Additional data related to this paper may be requested from the authors.

Ethics statement

All animal care was in accordance with institutional guidelines.

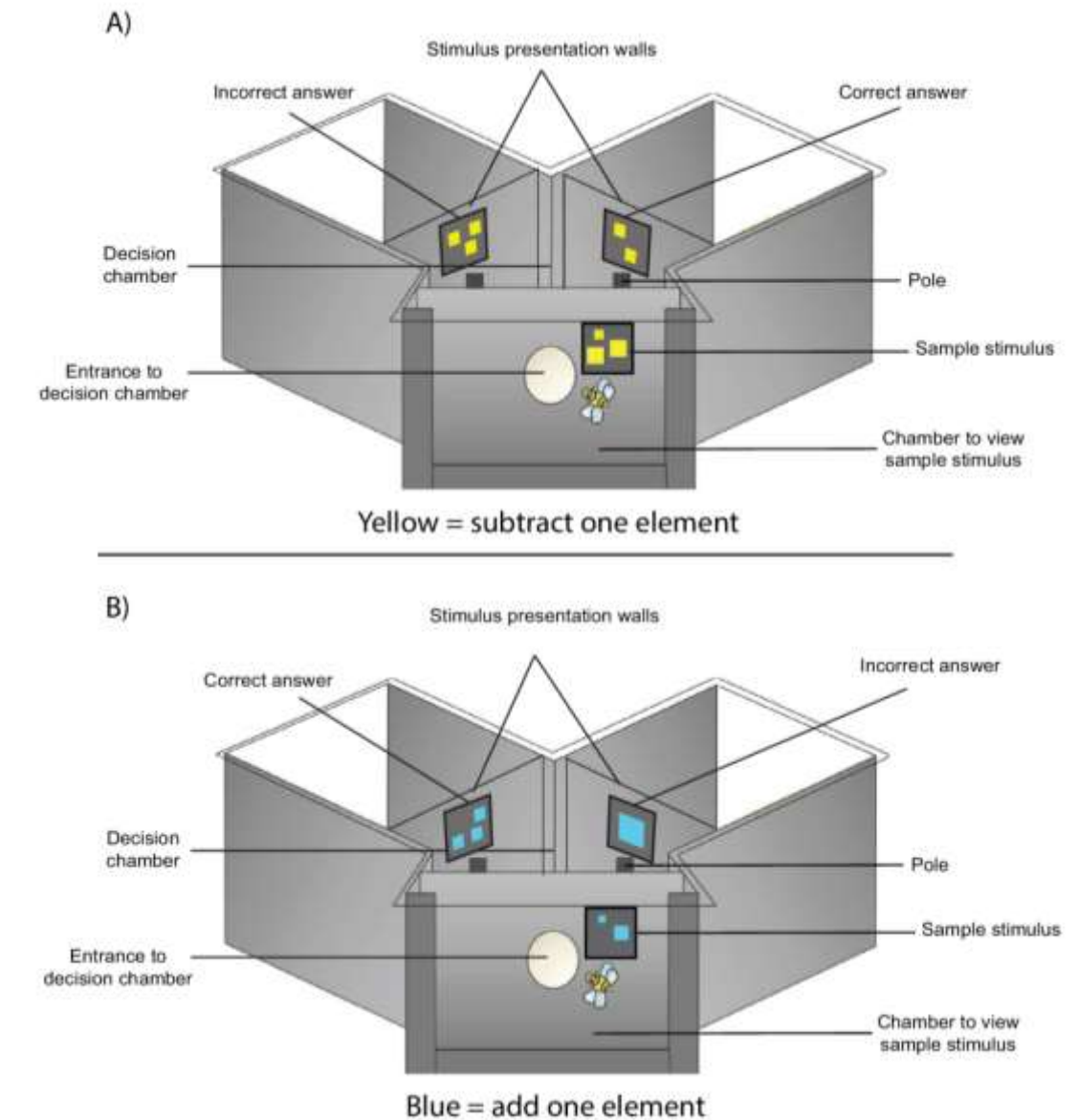


Fig. 1. Apparatus set-up for A) subtraction and B) addition trials. Diagram shows parts of the y-maze and the stimuli positions. When bees view a yellow sample stimulus (A) they must subtract one element from it and when bees view a blue sample stimulus (B) they must add one element to it. (Not visible in this diagram is the entrance wall into the first chamber).

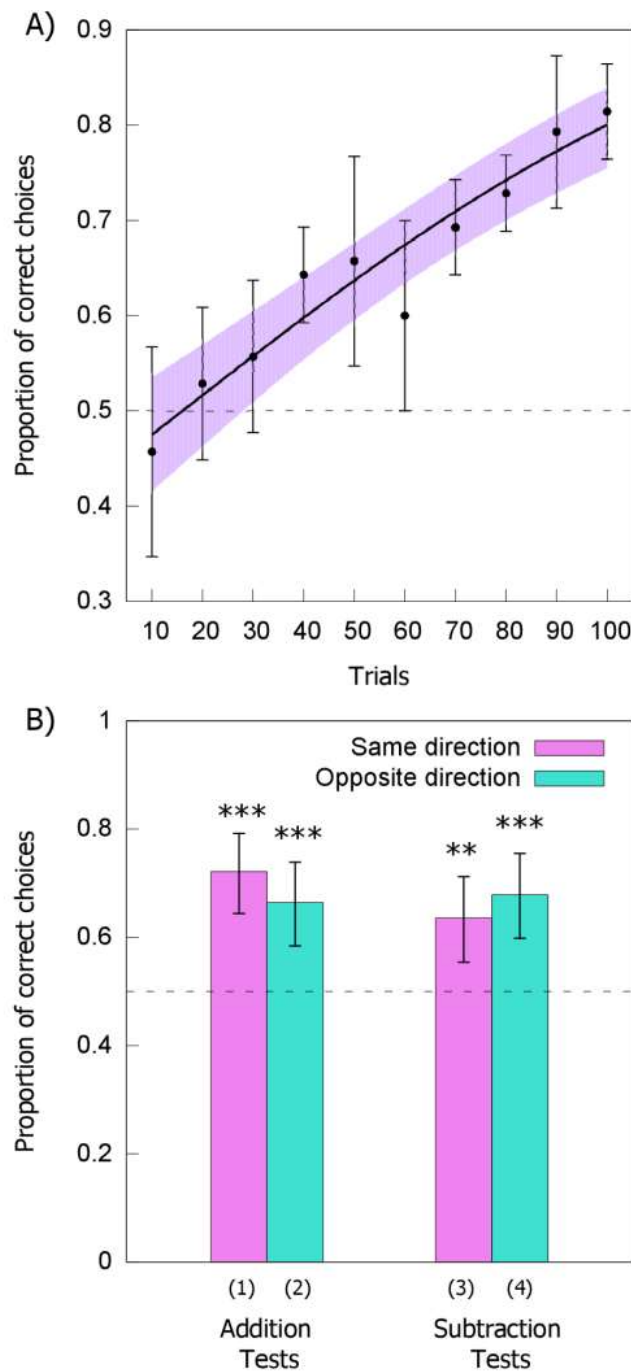


Fig. 2. Results of the learning and testing phases. A) Performance during the learning phase. Dashed line at 0.5 indicates chance level performance. Solid black line represents a function describing the learning phase of $n = 14$ bees as modelled by a generalised linear mixed model (glmm). Points (closed circles) along the curve indicate the observed mean \pm 95 % CIs (purple) of correct choices for the bees. Increase in performance during the learning phase was significant. B) Performance

during the testing phases for addition and subtraction. Pink columns (left) show results when the incorrect answer was in the same direction as the correct answer and blue column (right) show results when the incorrect answer was in the opposite direction as the correct answer. Numbers under columns (1, 2, 3, and 4) correspond to the operations in main text. Dashed line at 0.5 indicates chance level performance. Significance from chance level performance is indicated by * ≥ 0.05 , ** ≥ 0.01 , *** ≥ 0.001 . Data shown are means \pm 95 % CI boundaries for all tests.

Supplementary Materials

Supplementary Materials, Methods and Results

Although the results obtained in the main text show a clear learnt ability for the bees to correctly identify the operations “add one” and “subtract one”, over 100 appetitive-aversive conditioning choices, the mechanism by which these rules are learnt is much harder but important (28) to ascertain. In particular, the averaged population results presented earlier mask the fact that each individual bee must learn at a different rate conditioned either by the random presentation of stimuli and/or by individual differences in cognitive abilities. This effect is accounted for in the main manuscript by the use of random effect of individual in our statistical modelling for both the training phase and the testing phase, but the understanding of decision making at an individual level is also important for understanding processes which we additionally consider below.

The complexity of the addition/subtraction task is difficult, and requires the bee to identify several steps before a correct determination of the result is obtained. These steps include at least the following:

- 1) The output ports of the Y-maze correspond to a ‘solution’
- 2) One of the ‘solutions’ is correct, the other incorrect
- 3) The input port is connected to the solution identified above
- 4) The number of symbols at the input relates to the number in the solution
- 5) Colour denotes the operation plus one or minus one

Operations 4) and 5) can likely not be solved independently of each other.

As the bee attempts to negotiate this highly complex space, it is likely that she applies certain investigative behaviours in attempting to achieve a solution.

Accordingly it is not surprising that we sometimes find bees achieving results worse than chance during the acquisition stage (see individual results below in Fig. S2). For example, a bee may first try to always choose one direction in the Y-maze, or try to choose the higher number presented before determining the correct strategy required for solving the addition and subtraction tasks.

It is also likely that the bee's learning is strongly influenced by the (presumably random) choices that the bee initially makes.

Another important factor is that the appetitive-aversive conditioning applied in the current experiment likely provides more feedback than appetitive-only conditioning. Appetitive-only conditioning provides a positive outcome for the correct result, but no outcome for an incorrect result. Conversely, appetitive-aversive conditioning provides an outcome for both correct and incorrect results, thereby providing more feedback to the bee, which may be significant in the ability of the bee to learn the task (31).

In the present experiment, if the bee makes an incorrect choice, then it is allowed to subsequently visit the solution and thus experience the correct option associated with sucrose. This is important to retain motivation with appetitive-aversive conditioning (31), but also permits more information to enable exploratory learning over multiple trials. In such a case, the bee learns that the incorrect choice is aversive and costly while also learning the correct choice is rewarding. This provides the bee with more information to assist in their development of the addition/subtraction rules above what is expected for a correct choice.

To highlight the individuality of the learning curves of the bees, here we show the full results, bee by bee, of the addition/subtraction experiments. We assume that an unconditioned bee in the Y-maze is likely to make random choices. Hence we perform a Bayesian analysis of the experimental data, treating the bee choices as a biased coin, where the bias indicates the degree to which the task has been learnt. A random outcome is identified by a bias of 0.5, whereas a bee solving the task perfectly results in a bias of 1.0. A consistently anti-correlated result would result in a bias less than 0.5.

To determine each individual bee's ability, we seek the effective bias B_{eff} of the bee on the basis of a certain number of experiments, n_t , which is less than the total number of experiments to take into account the change in the bee's understanding of the arithmetic task through the experiment. There are two results, "Success" and "Failure", to which we assign the numerical values 1 and 0 respectively. The Bayesian approach allows the determination of the probability of the bee succeeding in the arithmetic task, which is the posterior probability.

Fig. S2A – n shows the Bayesian determined bias for each of the bees, averaged over $n_t = 10$ trials (except for the first 10 experiments, which were evaluated with respect to all previous experiments). The Bayesian analysis allows for bias probability from 0 to 1 with 101 levels (i.e. $B_{\text{eff}} = 0.00, 0.01, 0.02, \dots, 1.00$) and the colour bar shows the probability of that bias determined by the Bayesian update. The red line superimposed on the colour plot shows the conventional running average of the last 10 trials, which accords with the maximum bias probability as expected. The lower plot in each of the figures shows the bees actual results for each trial.

The individual traces show a considerable amount of noise, which highlights that although the addition/subtraction task is certainly learnt, it is not learnt to perfect

accuracy, nor is it immediately obvious when the bee has learnt the task (Figs. S2A – N). We had initially expected that the individual bee performance would indicate an ‘aha!’ moment (40), which would manifest in a significant and sustained increase in performance at a given experiment. Again, although the population averaged result clearly shows progressive learning (Fig. 2), individual performance shows bees with initially successful results that can only be due to chance (see for example Fig. S2I), or drops in performance (see for example Fig. S2C). Longer term statistics are necessary to determine the extent to which the bees are able to retain, although given the complexity of the learning task, it is difficult to conduct such an experiment without the confounding effects of studies on the same individual for more than one day.

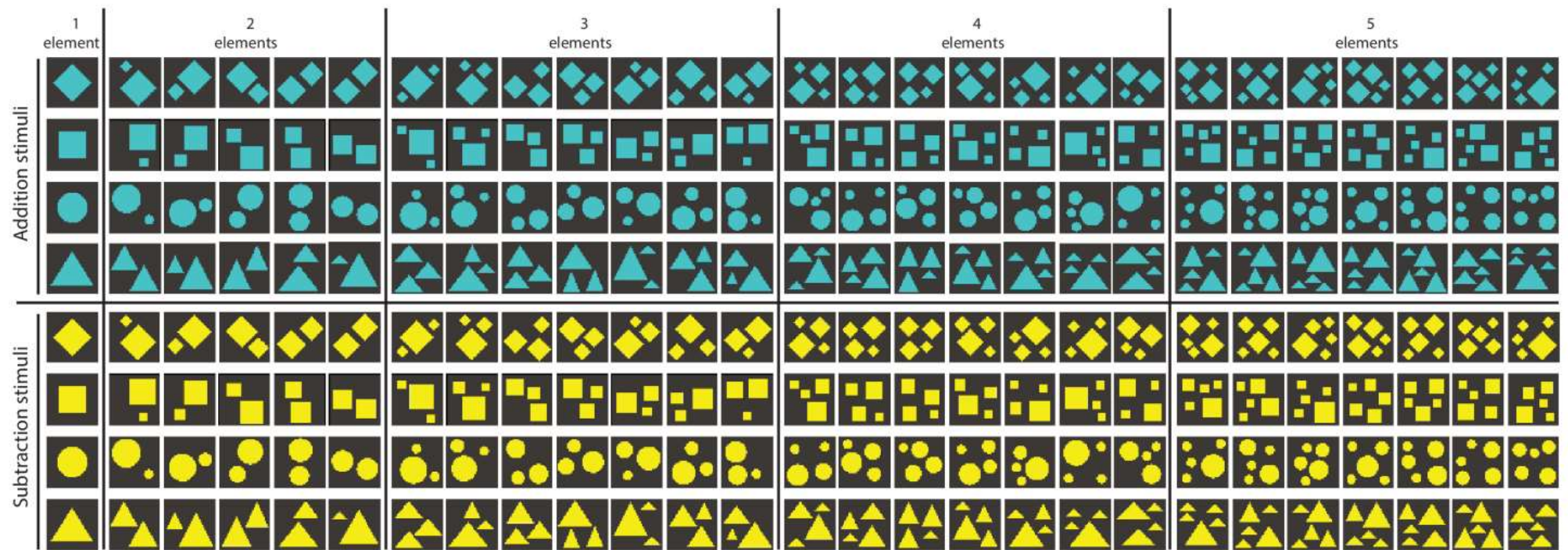


Fig. S1. The full set of stimuli used ($n = 216$) for the addition (blue; $n = 108$) and subtraction (yellow; $n = 108$) training and test phases. The stimuli contained between one and five elements with four possible shapes and there were 108 patterns for each of the addition and subtraction groups of stimuli.

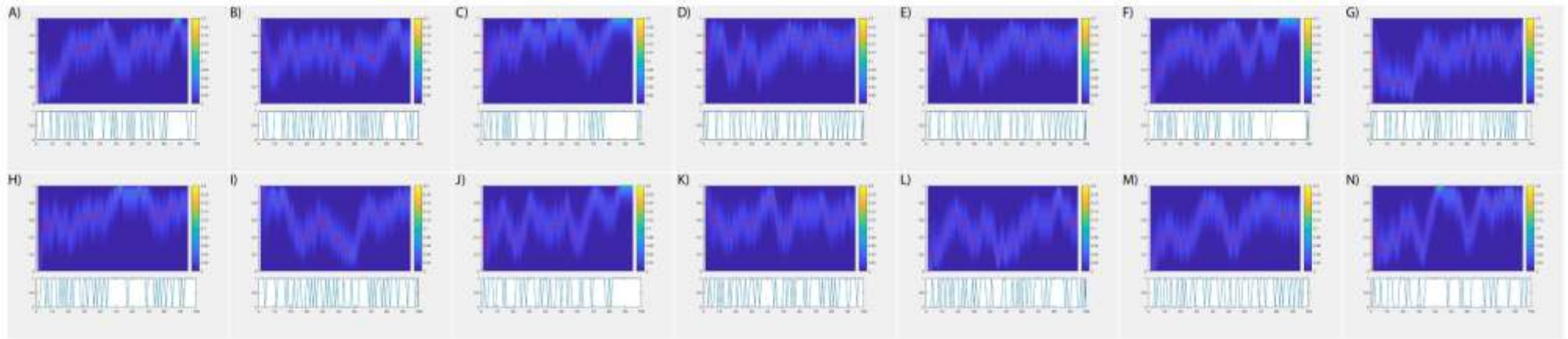


Fig. S2. The Bayesian determined bias for each of the bees, averaged over $n_t = 10$ trials (except for the first 10 experiments, which were evaluated with respect to all previous experiments). The Bayesian analysis allows for bias probability from 0 to 1 with 101 levels (i.e. $B_{\text{eff}} = 0.00, 0.01, 0.02, \dots, 1.00$) and the colour bar shows the probability of that bias determined by the Bayesian update. The red line superimposed on the colour plot shows the conventional running average of the last 10 trials, which accords with the maximum bias probability as expected. The lower plot in each of the figures shows the bees actual results for each trial.

Chapter 5

Symbolic representation of number in the honeybee (*Apis Mellifera*)

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Abstract

The assignment of a symbolic representation to a specific numerosity is a fundamental requirement for humans solving complex mathematical calculations used in diverse applications such as algebra, accounting, finance, physics, and everyday commerce. However, there is no evidence for such symbolic representations of quantity arising spontaneously in non-human animals. Here we show that honeybees are able to learn to match a symbol to a number or a number to symbol and subsequently transfer this knowledge to novel numerosity stimuli changed in colour properties, shape, and configuration. While honeybees learnt and applied the associations between the quantities two and three and symbols (an 'N' and an inverted 'T' respectively), they failed at reversing their specific task of symbol-to-number-matching to number-to-symbol-matching and vice-versa (i.e. a honeybee that learnt to match a symbol to a number of elements was not able to invert this learning to match the number of elements to a symbol). This demonstrated that while bees could learn the association between a symbol and number, it was linked to the specific task and they could not spontaneously extrapolate the association to a novel, reversed task. Our study therefore reveals that the basic requirement for numerical symbolic representation can be fulfilled by an insect brain suggesting that the absence of its spontaneous emergence in animals is not due to cognitive limitation.

Introduction

Matching specific numbers with symbolic representations of that number, such as Roman or Arabic numerals, is valuable for representing and manipulating numbers¹. True counting requires the presence of symbolic representations of number and ordinality^{2,3}, where the former is the assignment of a symbol to a specific quantity and the latter is the ability to order numeric symbols².

Some non-human animals have demonstrated symbolic number representation, ordinality, or even both. Pigeons have been able to demonstrate learned symbolic number representation through peck number on a symbol representing that number^{4,5}. The combination of ordinality and symbolic number representation, thereby demonstrating true counting, has been demonstrated in an African grey parrot⁶⁻⁸, chimpanzees⁹⁻¹⁷ and rhesus monkeys¹⁸. Alex, a single African grey parrot, was able to learn the names of numerals and subsequently sum the quantities, thereby demonstrating both numerical skills required for true counting^{7,19,20}. In addition, chimpanzees have been able to learn Arabic numerals and order them quantitatively⁹⁻¹⁷, and Rhesus monkeys have learned to choose the larger of two Arabic numerals to receive a corresponding number of food pellets¹⁸ as well as match numbers of items to the correct Arabic numeral²¹. Nevertheless, there is no evidence that any species apart from *Homo sapiens* have ever *spontaneously* developed symbolic representations of numbers, which opens the question of which animals are capable of *learning* symbolic number representations, which are capable of *generating* such representations, and whether this implies a fundamental difference in the mental processing of *Homo sapiens* compared with other animals²²⁻²⁵.

Honeybees are considered a model species for studying numerical cognition in insects²⁶⁻³¹. Previously, honeybees have demonstrated the capacity to evaluate quantities by counting the number of landmarks to reach a feeder^{28,29}, or matching the number of objects in pictures³¹. We recently demonstrated that bees can correctly value quantities up to six by correctly choosing a stimulus with fewer elements between two alternative quantities³². Interestingly bees spontaneously placed an empty set, zero numerosity, at the lower end of the numerical scale without specific training on the task. This discovery challenges the postulate of zero being an unnatural abstract concept only reachable by large brained animals^{32,33}. Honeybees have also demonstrated an ability to learn and perform simple addition and subtraction

operations of ‘plus one’ or ‘minus one’ using symbolic prompting and arrays of one to five objects³⁰. This recent study was the first step in determining if insects could use symbolic representation in numerical problem solving. The current study aimed to extend on this area of research by training honeybees to learn to associate numerosities with symbols.

In the current study, honeybees were examined on their ability to match two numerical tags/icons (symbols) to arrays of two or three elements specifically. Bees were trained to perform either a symbol-to-number-matching task (Group 1) or a number-to-symbol-matching task (Group 2), and were subsequently tested on their ability to reverse their learnt association.

Results

Using a delayed-matching-to-sample procedure³¹ in a Y-maze apparatus (Fig. 1ab), honeybees were trained during 50 appetitive-aversive trials to match a N-shape symbol to pictures containing two elements while an inverted T-shape symbol was matched to pictures presenting three elements. Bees were either trained with the symbols as samples and quantities as choices (Group 1, Fig. 1c) or quantities as samples and symbols as choices (Group 2, Fig. 1c). The stimuli containing two or three elements were made up of three sets: elements of equal surface area (set 1), elements of the same surface area (set 2), and randomised shapes (set 3; for use in the transfer test only; see ³⁴; Fig. 2; see ‘Stimuli’ subsection in Methods section for more information; Extended Data Fig. 1). In each trial, a correct match was reinforced by a sucrose appetitive reward and incorrect choices were associated with an aversive quinine solution. Bees in both groups demonstrated significant learning over the course of the appetitive-aversive trials to match either symbols to numbers (Group 1: $z = 4.460$; $P < 0.001$; Fig. 2c) or numbers to symbols (Group 2: $z = 4.387$; $P <$

0.001; Fig. 2d), demonstrating that bees learnt the two symbols had corresponding matching quantities associated with them.

Following the acquisition of the symbol and number matching task, we conducted four unreinforced tests (no reward or punishment for choices) to determine the level of performance bees had achieved following training and whether they could transfer that knowledge to a novel inversed matching situation. When bees were asked to transfer the matching task to completely novel stimuli, with chromatically coloured backgrounds and elements which were of novel shape and arrangement (set 3), displaying quantities of two or three (transfer test), bees in both groups performed this task at a level higher than expected by chance. Bees in Group 1 choose the correct number at a proportion of 0.650 (95 % confidence intervals (CIs) = 0.554, 0.739; $z = 2.95$, $P = 0.003$; Fig. 2e). Bees in Group 2 choose the correct symbol at a proportion of 0.620 (CIs = 0.506, 0.741; $z = 2.19$, $P = 0.029$; Fig. 2f).

Bees were also tested on transferring their learnt matching tasks to stimuli which were similar to the training set but of novel configuration. Bees in both groups were reliably able to choose the correct number or symbol in these tests. In the learning test, using novel stimuli from the set which had an equal surface area for quantities of both two and three, bees in Group 1 chose the correct option at a proportion of 0.620 (CIs = 0.523, 0.711; $z = 2.38$, $P = 0.018$; Fig. 2e) and bees in Group 2 chose the correct option at a proportion of 0.660 (CIs = 0.564, 0.748; $z = 3.14$, $P = 0.002$; Fig. 2e). In the learning test, using novel stimuli from the set in which all elements were of equal size for quantities of both two and three, bees in Group 1 chose the correct option at a proportion of 0.630 (CIs = 0.533, 0.722; $z = 2.57$, $P = 0.010$; Fig. 2f), and bees in Group 2 chose the correct option at a proportion of 0.610 (CIs = 0.513, 0.702; $z = 2.18$, $P = 0.029$; Fig. 2f).

Finally, bees were tested on their ability to transfer either the learnt symbol-to-number-matching task to a number-to-symbol-matching task (Group 1), or their number-to-symbol-matching task to a symbol-to-number-matching task (Group 2). Stimuli used in this test were randomly selected from Set 1 or Set 2 using the stimuli which were not presented to bees during the training. Bees in both groups were unable to perform this reversal test at a level significant from chance expectation with bees in Group 1 choosing the correct symbol at a proportion of 0.550 (CIs = 0.452, 0.645; $z = 1.00$, $P = 0.318$; Fig. 2e) and bees in Group 2 choosing the correct number at a proportion of 0.520 (CIs = 0.390, 0.649; $z = 0.24$, $P = 0.346$; Fig. 2f).

Discussion

Our findings show that independent groups of honeybees can learn and apply either a symbol-to-number-matching task or a number-to-symbol-matching task and subsequently apply acquired skills to novel stimuli. Interestingly, despite bees demonstrating a direct number and symbol association, they were unable to transfer the acquired skill to solve a reverse matching task. While independent groups of bees are able to learn the association in either direction, it seems the association itself is not reversible.

Piaget's Theory on children's actions providing the basis for their own cognitive development gives some insight into our results³⁵. Piaget considered 'operations' or 'reversible actions' as most conducive to a child's mental development. Operations are where an action can be undone by other actions, such as being able to turn a light on using a switch, and reverse this action by flicking the switch the other way³⁵. Children will develop 'operational schemes' throughout their learning which allows them to think about the ability to reverse their actions³⁵. If we consider a child knocking over a glass of milk, they will learn that this is an irreversible action and the milk cannot easily be put back into the glass.

However, as in the previous example, a child is able to acquire the ability to understand reversing the state of a light as on or off via experimentation with these relative states, and individual actions.

If we consider Piaget's theories in terms of the results of the current study, it seems that bees have not learnt 'operations' or 'operational schemes' in this instance. Our experiments show that bees can learn to perform the association of symbol and number in either direction but neither group can reverse the association. Thus, bees are not easily able to reverse a learnt task unless they have had experience with the association in a specific direction. Thus, for cognitively demanding tasks like symbol and number matching, spontaneous reversion of the association appears to be beyond the capacity of an insect brain.

In a previous study by Gross *et al.* (2009), honeybees were able to match two identical quantities differing by shape, colour, and configuration in a delayed-matching-to-sample framework³¹, such as the method used in the current study. A study by Zhang *et al.* (1999) showed that bees were able to navigate by visual stimuli and memory recall of different groups of stimuli. Thus, bees were able to use symbol-like stimuli to inform decision on which direction to choose in a maze³⁶. Our current study takes the task of numerical matching and problem solving to a higher cognitive problem by training bees to match symbols and quantities within a delayed-matching-to-sample framework. In addition, honeybees were recently shown to learn simple addition and subtraction operations using this same DMTS framework³⁰. When bees saw a blue array of elements, they learnt to add one element to the quantity, however when the array of elements was yellow, the bees learnt to subtract one element from the quantity. The study demonstrated the use of colour as a symbolic cue as well as showing that honeybees had the capacity to perform simple arithmetic by using the symbolic colour cues.

While it is currently unknown where and how numerical information may be processed in insect brains³³, evidence from vertebrates can inform us of how different numerical formats and abstract concepts may be formed, processed, and stored. Monkeys performing spatial or temporal enumeration tasks activated different populations of neurons in the intraparietal sulcus, however, after the task was completed, a different neural population stored the cardinality information, regardless of whether the original enumeration task was temporal or spatial³⁷. As the processing and final storage of temporal and spatial numerical formats are different in monkeys, the authors conclude on distinct processing stages for different numerical formats³⁷. The lack of reversibility in the bees' understanding of the symbol-number matching tasks suggests that the symbolic and numerical information is processed and/or stored by different populations of neurons. Thus symbol and number matching tasks are not able to be reversed without training in both the forward and backward directions, i.e. with two different neuronal populations for the processing of the two directions of associations. Recent work on symbol and number associations in Rhesus monkeys shows that with training over several months, monkeys learn the association between Arabic numerals and their corresponding quantities. Eventually this training resulted in the symbols being responded to in a similar way to the quantities in the prefrontal cortex, and to a lesser extent in the parietal cortex²¹. Thus, perhaps with similar training on the quantitative value of multiple symbols, honeybees would demonstrate a similar association and neuron populations could respond to the symbolic representation and quantities in the same way, which may facilitate the reversal task. However, currently such a complex conditioning paradigm has not been developed for training and testing bees as it would require a very complex experimental design within subject, using an animal that has a short lifespan. Bees would need to be trained on the valuation of different quantities (e.g. ³²), then to match those quantities with symbolic representations (as in the current study). The next phase of the experiment would then be to

design a way to train bees to associate the quantitative value of the numerosity with the symbol. Finally, bees would need to be tested on their ability to value the symbols numerically. Considering the lifespan of the bee and the complexity of such a design, it is currently not possible to test this hypothesis.

The results of the current study, combined with previous studies on honeybee numerical ability²⁸⁻³², suggest that bees may be capable of true counting^{2,3}. Our above results have shown that bees are able to learn symbolic representations of number and extrapolate this knowledge to novel stimuli, which constitutes two aspects of true counting. It remains to be explored as to whether bees can learn specific number and symbol associations for more than two relations and use symbolic representations to order numbers by value, which would demonstrate both symbolic number representation and ordinality, evidence of true counting^{2,3}. Thus, this task does not put honeybees on par with the use of symbolic representations of number in pigeons, chimpanzees, Rhesus monkeys, and Alex the parrot, as these other species were able to learn more than two symbol-number associations and even give the symbols (or labels) quantitative value. The current study is valuable for refining our understanding of numerical abilities in insects and shows that at least the learning of symbolic numerical representations is not restricted to vertebrates. Furthermore in previous studies, bees were able to quantitatively value numbers as ‘greater’ or ‘lesser’ than other numbers correctly; demonstrating ordinality of sets³². A final demonstration of the ability to order the symbolic tags associated with specific numbers would constitute true counting^{2,3} in bees. Understanding how such apparently complex numerical skills are acquired by miniature brains will help enable our understanding of how mathematical and cultural thinking evolved in humans, and possibly, other animals^{1,24,25,38,39}.

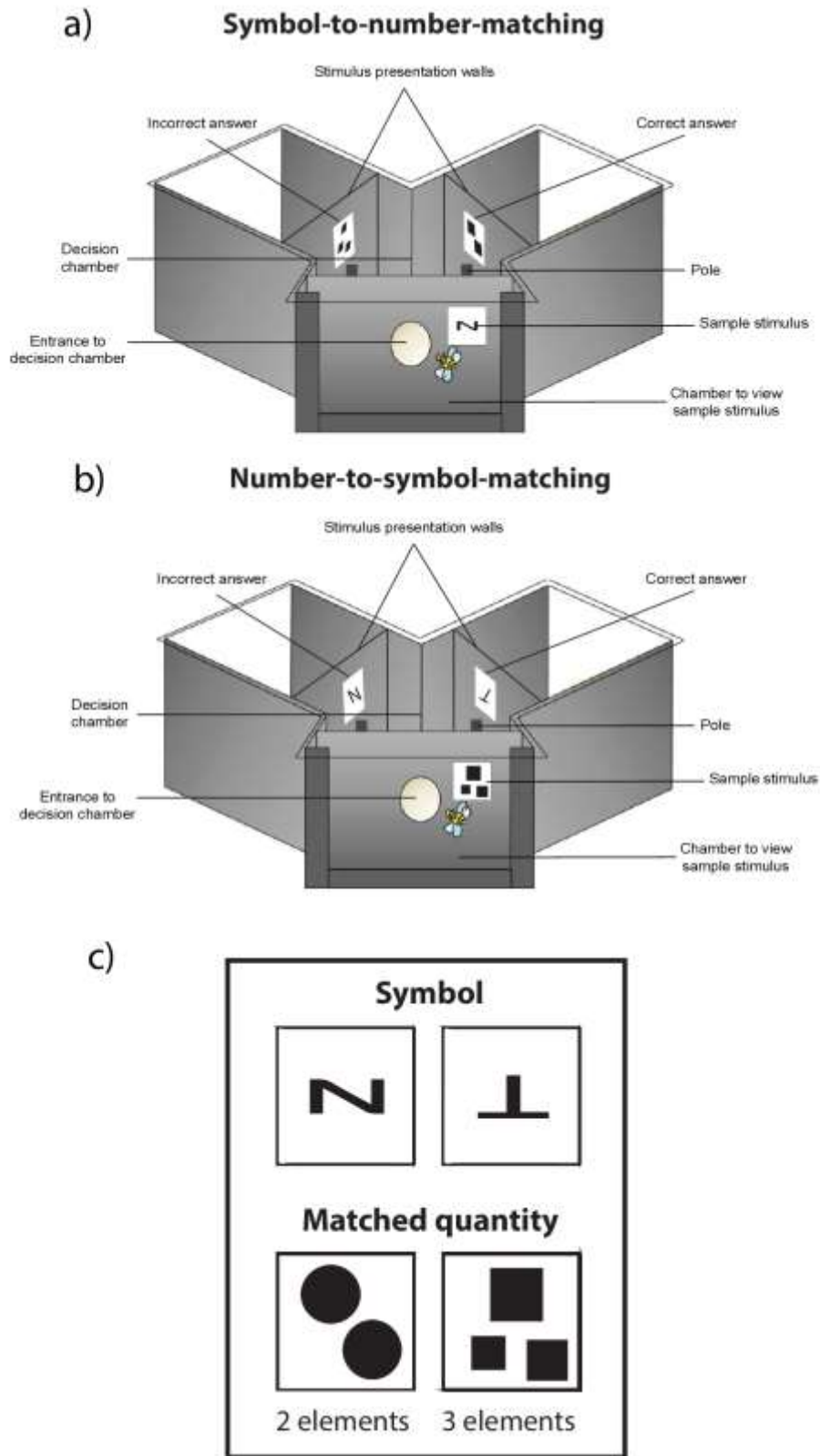


Figure 1: Apparatus set-up and stimuli examples for the a) symbol-to-number-matching task and b) number-to-symbol-matching task. Diagram shows parts of the y-maze and the stimulus positions. a) In the symbol-to-number-matching task, when bees view a sample

symbol (N-shape or inverted T-shape), they must match it to the correct quantity of two or three elements. b) In the number-to-symbol-matching task, when bees view a sample quantity (two or three elements), they must match it to the correct symbol (N-shape or inverted T-shape). (Not visible in this diagram is the entrance hole and wall into the first chamber and the plexiglass cover for the entire apparatus). c) An example of the symbols being matched to their corresponding correct quantity (N-shape to two elements; inverted T-shape to three elements).

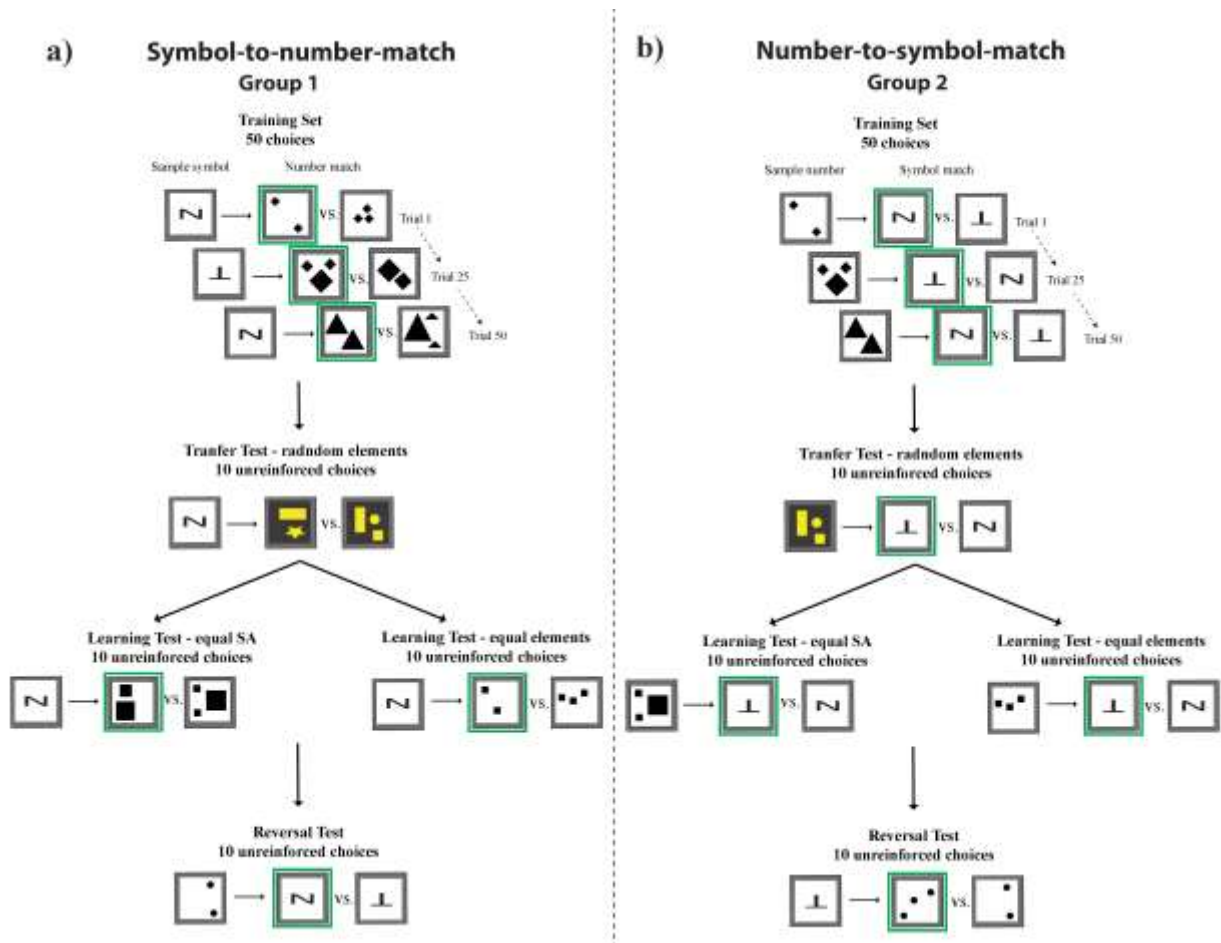


Figure 2: Examples of possible stimulus combinations during trials and tests in experiments on a) symbol-to-number-matching tasks and b) number-to-symbol-matching tasks. The diagrams show the sample stimulus (Group 1: symbol; Group 2: number) and the matched correct or incorrect options which would be presented in the two arms of the Y-maze (Fig. 1ab). The four tests were conducted after the 50 appetitive-aversive trials had been completed and were conducted in the order of: transfer test, learning tests (pseudo-randomised order), and then the reversal test. The green square on the right hand side of the arrow indicates which option is the correct answer for each of the example combinations.

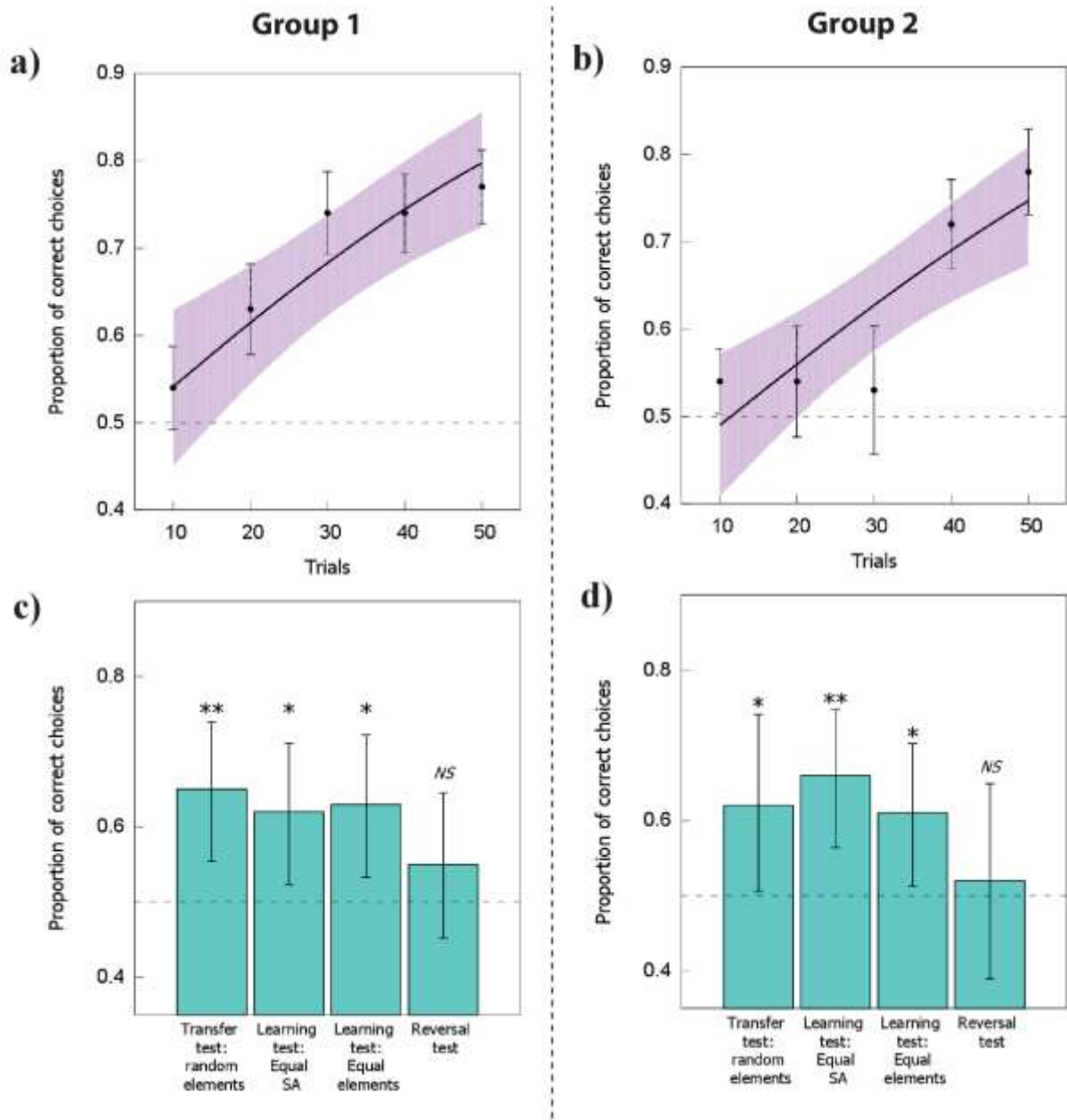


Figure 3: Results of the training and testing phases for the (a) group of bees trained to match a symbol with a quantity (Group 1) and for the (b) group of bees trained to match a quantity with a symbol (Group 2). a-b) Performance during the training phase for Group 1 (a) and Group 2 (b). Dashed line at 0.5 indicates chance level performance. Solid black line represents a function describing the learning phase of $n = 10$ bees as modelled by a generalised linear mixed model (GLMM). Points (closed circles) along the curve indicate the

observed mean \pm 95 % CIs (purple) of correct choices for the bees. Increase in performance during the learning phase was significant. c-d) Performance during the testing phases for both Group 1 (c) and Group 2 (d). Dashed line at 0.5 indicates chance level performance. Significance from chance level performance is indicated by * ≥ 0.05 , ** ≥ 0.01 , *** ≥ 0.001 , *NS* > 0.05 . Data shown are means \pm 95 % CI boundaries for all tests.

Method

Study species:

We used 20 free-flying honeybees (*Apis mellifera*) foragers for this experiment. All bees were marked with a coloured dot on the thorax to identify individuals. A gravity feeder providing ca. 10 – 30 % sucrose near the experimental set-up (distance: 20 m) maintained a regular number of bees for testing.

Apparatus:

Individual honeybees were trained to enter a Y-maze (as described in ^{40,41}; Fig. 1ab) through a small entrance hole (approximately 5 cm diameter). The Y-maze arms were 40 x 20 x 20 cm (L x W x H). The bees had to fly through an initial entrance hole to enter a chamber where they would view the sample stimulus. This sample stimulus would be either a symbol or quantity depending on the group being trained. Each bee could then fly through another hole into the decision chamber where it would be presented with two different options in each arm of the chamber. If the sample stimulus was a symbol then the two options would be quantities of two vs. three elements, if the sample stimulus was a quantity of two or three elements, then the two options would be the two symbol options (Fig. 1).

Stimuli were presented on grey backgrounds located 5 cm away from the decision lines. Two stimuli, one correct and one incorrect, were presented simultaneously in each arm of the Y-maze on the grey plastic background (Fig. 1ab). A 10 μ L drop of either a 50 % sucrose solution (correct choice) or a 60 mM quinine solution (incorrect choice) were used as rewarding and punishing outcomes respectively during the training phase as this promotes enhanced visual discrimination performances in free-flying honeybees. Each stimulus had a grey landing pole located below it which held either the drop of sucrose under the correct

option or quinine under the incorrect option so that bees would learn to associate stimuli with either a reward or punishment. Poles were replaced when touched by a bee and cleaned with 20 % ethanol then dried to exclude olfactory cues. The precise order by which stimuli were arranged in the Y-maze was randomly allocated between choices. If a bee made an incorrect choice and started to imbibe the quinine; it was allowed to fly to the pole in front of the correct stimulus to collect sucrose to maintain motivation; but only the first choice was used for statistical analysis. When the bee landed on the pole with sucrose, both the pole and bee were removed from the Y-maze; then the pole was replaced with a clean pole, and stimuli, positions of target and distractor were randomised. Once the bee was finished imbibing the sucrose, it was allowed to fly back to the hive if satiated, or make another decision by re-entering the maze. During the non-reinforced tests, a drop of water was placed on each of the poles placed in front of the stimuli. Ten choices (touches of the poles) were recorded for each of the four tests to enable statistical comparisons.

Stimuli:

Each symbol stimulus consisted of a 6 x 6 cm white square with a black symbol (either an N-shape or an inverted T-shape), and stimuli containing quantities were 9 x 9 cm white square cards with elements presented on them, all covered with 80 μm Lowell lamina (Fig. 1). Symbol stimuli had a surface area of 36 cm^2 (visual angle of 149° from the decision line) while quantity stimuli had a surface area of 81 cm^2 (visual angle of 165° from the decision line). Symbols were based on the Xia et al. (2000) paper on pigeons matching numbers to symbols⁴. The stimuli containing two or three numbers of elements were split into three different sets: 1) a set where surface area of all elements was equal ($n = 32$); 2) a set where each element was the same size ($n = 32$); 3) a set where random chromatic objects/shapes were used (for transfer test only; $n = 8$). Sets 1 and 2 consisted of squares, diamonds, circles, or triangles which was pseudo-randomised between trials (Extended Data Fig. 1). Training

sets used in each trial were randomised in terms of continuous predictors of numerosity as much as possible, however set 3 was used in testing as a control for variables correlating with numerosity, such as density, size of elements, convex hull, area of elements, line length). Stimulus sets 1 and 2 were achromatic (white background with black elements) and set 3 was chromatic (grey background with salient yellow elements⁴¹). Stimulus sets were based on sets from³⁴.

Training:

We trained two groups ($n = 10$ per group) of bees to fly into a Y-maze apparatus and used a delayed-matching-to-sample (DMTS) framework for conditioning³¹. Upon flying into the Y-maze, one group of bees (Group 1) were presented with one of two possible symbols (Fig. 1). After the bees flew into the next chamber, the decision chamber, they were presented with two options, one correct (two or three elements) and the other incorrect (two or three elements). The second group of bees (Group 2) were trained using the opposite DMTS framework, where they were first showed a quantity of elements in the first chamber and then presented with two symbols in the arms of the Y-maze in the decision chamber.

We used sets of stimuli with controlled surface area (set 1) and equal element size area (set 2) with achromatic properties (black elements on white background). The choice of stimuli for each trial was pseudo-randomised with regards to set (1 or 2), shape, and arrangement of elements. Bees were trained for 50 choices using appetitive-aversive differential conditioning.

Testing:

After the 50 training choices were completed, bees underwent four tests in the following order:

A transfer test was conducted for 10 unconditioned choices. In Group 1 this test consisted of showing a symbol as the sample and then a randomised set of abstract objects not previously seen by bees (Set 3: random set with chromatic properties). In Group 2 this test consisted of showing the random set of two or three elements and then both symbols as the options.

Two learning tests were conducted afterwards for 10 unconditioned choices in a pseudo-randomised order [two controlled sets: set 1) equal surface area of elements; set 2) equal element size; both with achromatic properties].

A reversal test was finally conducted for 10 unconditioned choices. The Group 1 test consisted of showing a quantity as the sample and having the bee choose a corresponding symbol. The Group 2 test consisted of showing a symbol as the sample and having the bee choose a corresponding quantity. This test aimed to determine whether bees could extrapolate the task to the reverse matching situation. Stimuli in this test were also achromatic and pseudo-randomly chosen from stimuli within set 1 or set 2 which had not previously been presented to bees during training.

Statistical analysis:

To test for the effect of training on bee performance (number of correct choices), data from the learning phase of 50 choices were analysed with a generalized linear mixed-effect model (GLMM) with a binomial distribution using the ‘glmer’ package within the R environment for statistical analysis. We fitted a full model with trial number as a continuous predictor, and subject as a random factor to account for repeated choices of individual bees.

To determine whether bees were able to learn to match a symbol with the correct corresponding quantity, we analysed the test data by employing a GLMM including only the intercept term as fixed factor and subject as a random term to account for the repeated

measures. The proportion of ‘correct’ choices (MPCC) recorded from the tests were used as response variable in the model. The Wald statistic (z) tested if the mean proportion of correct choices recorded from the tests, represented by the coefficient of the intercept term, was significantly different from chance expectation, *i.e.* $H_0: \text{MPCC} = 0.5$.

All analyses were performed within the R environment for statistical analysis⁴³.

Data availability:

The raw choice data from training and tests of individual bees that support the findings of this study will be made available on Dryad Data Repository.

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Author contributions:

SRH, AAW and AGD designed the experiment. SRH collected and analysed the data, and wrote the manuscript. All authors were involved in data interpretation and gave final approval for submission.

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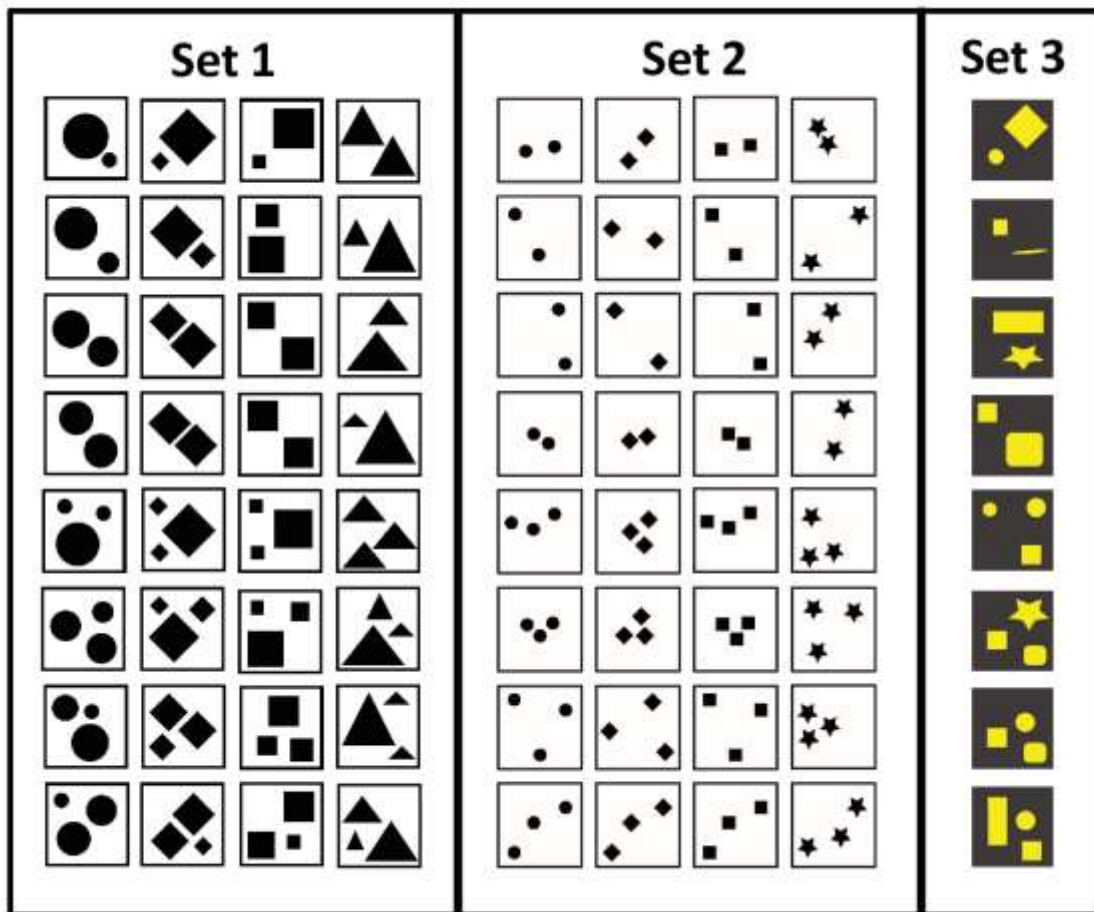
Competing interests:

We declare no competing interests.

Materials & Correspondence:

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Extended Data



Extended Data Figure 1: The three sets of numerosity stimuli. Set 1 consisted of 6 x 6 cm white cards containing either two or three elements. Shapes consisted of circles, diamonds, squares, or triangles. All elements contained in each stimulus in Set 1 had a combined black surface area of $10.0 \pm 0.3 \text{ cm}^2$ regardless of how many elements they contained. Set 2 consisted of 6 x 6 cm white cards containing either two or three elements. Shapes consisted of circles, diamonds, squares, or stars. Each element in Set 2 had a surface area of 1 cm^2 , resulting in stimuli containing two elements having an overall black surface area of 2 cm^2 and stimuli containing three elements having an overall black surface area of 3 cm^2 . Set 3 consisted of grey cards containing either two or three salient yellow elements. Shapes, configurations, and element sizes were pseudo-randomised in Set 3. Sets 1 and 2 were using

in training, the learning tests and the reverse test, while Set 3 was only shown during the transfer test and thus was completely novel to bees.

Chapter 6

6. Discussion

Honeybees have previously demonstrated the ability to count (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008) and discriminate between (Gross et al., 2009) up to four objects. My research (Chapters 2 – 5) has shown an extended numerical capacity in honeybees including learning, application, and extrapolation of different numerical concepts and rules with the use of appetitive-aversive differential conditioning (Chapter 2; Avarguès-Weber et al., 2010; Chittka et al., 2003).

Over the course of my research, honeybees have demonstrated improved quantity discrimination (Chapter 2), the ability to apply ‘less than’ and ‘greater than’ numerical rules to quantitatively value zero numerosity (Chapter 3; Howard et al., 2018), simple addition and subtraction (Chapter 4), and symbol and number associations (Chapter 5). My research has thus enabled a further understanding of what the honeybee brain can do when motivated, suggested a new training procedure for understanding numerical skills and cognition in other animals, and opened up the question of how numbers might be processed by an insect brain (Nieder, 2018). Furthermore, the demonstration of complex numerical capacities in an invertebrate, which is separated from humans by 600 million years, suggests that numerical capacity may be a result of convergent evolution (Nieder, 2016a) and has an important adaptive function for animals.

The results reported above, in connection with other studies, give us an insight into questions such as: whether language is required for numerical skills, whether non-human animals can demonstrate quantal and numerical cognition, how an insect is able to learn, remember and

apply numerical concepts and skills, how conditioning can modulate numerical ability, and what sort of real-world applications, ecological and technological, this research may provide.

There is a debate surrounding the potential capacity of non-human animals to possess or even learn complex number skills (Nieder, 2005; Nieder, 2016a; Nieder, 2017; Núñez, 2017a; Núñez, 2017b; Verguts and Chen, 2017). While Núñez (2017a; 2017b) argues that number skills are an artefact of human evolution and the development of culture and language, Nieder (2017) contends that studies which demonstrate complex number skills in non-human animals suggest numerical ability has also evolved in non-human animals. The arguments supporting the evolution of numerical capacity in non-human animals are further strengthened by the discovery of neurons selective to number detection in the brains of different species of animals (discussed below; Bongard and Nieder, 2010; Dehaene, 2003; Diester and Nieder, 2007; Ditz and Nieder, 2015; Ditz and Nieder, 2016b; Nieder, 2012; Nieder and Dehaene, 2009; Nieder et al., 2006; Nieder et al., 2002; Nieder and Merten, 2007; Nieder and Miller, 2003; Nieder and Miller, 2004; Ramirez-Cardenas et al., 2016; Sawamura et al., 2002; Thompson et al., 1970; Tudusciuc and Nieder, 2007; Viswanathan and Nieder, 2013). My research informs the current debate by showing that advanced numerical concepts, such as the understanding and valuation of zero numerosity, performing simple arithmetic, and symbol-number matching, are accessible to honeybees, provided there are adequate conditioning procedures and experimental design. The research into numerical ability in honeybees provides evidence that number sense has evolved in non-human animal species as it can be observed across different taxa from primates to insects. My research also supports the position of Verguts and Chen (2017) that ‘evolution’ also occurs at an individual level throughout an animal’s lifetime. ‘Evolution’ at an individual level is classified as a “much faster evolutionary time scale, operating within an individual’s lifetime” in which “individuals learn to accommodate cognition (via instruction and trial-and-error learning) to

their culture, constrained by their biology” (Verguts and Chen, 2017). The capacity to cognitively learn by trial and error on an ‘individual evolutionary timescale’ is observed in honeybees, as bees rapidly learn to use numbers, symbols, and symbolic representations of numbers to solve complex numerical problems (Chapter 2 – 5).

6.1 Honeybee numerical capacity

In Chapter 1, I reviewed the literature on the numerical capacities of different species to summarise what is currently known about the level of complexity of numerical ability animals are able to display (Figure 1). In this section I will discuss the ability of honeybees to reach these levels using the same categories and tasks presented in the introduction.

6.1.1. Subitizing/ Object File System (OFS)

Honeybees have previously demonstrated the ability to subitize (Figure 1), the quick and accurate enumeration of four and fewer items ‘at a glance’ (Agrillo et al., 2008; Kaufman et al., 1949; Piazza et al., 2002; Tomonaga and Matsuzawa, 2002; Trick and Pylyshyn, 1994). This quantification mechanism is also known as using the object file system or parallel individuation system (Hyde, 2011). Gross et al. (2009) demonstrates that bees can match quantities of abstract elements of two and three, and discriminate between quantities up to three vs. four. In my research, I did not seek to re-assess subitizing ability in honeybees, however in Chapter 2 and Chapter 3 bees used in my experiments demonstrated the ability to discriminate between numerosities above and below the subitizing range. In Chapter 2, bees demonstrated an ability to learn to discriminate numerosities one to ten from four elements during training, and were successfully able to discriminate between 4 vs. 5, 4 vs. 6, 4 vs. 7, and 4 vs. 8 in tests when trained with appetitive-aversive differential conditioning. In Chapter 3, bees were trained and tested on their ability to discriminate between quantities of zero to

six and were successfully able to learn these discriminations and apply the learning to novel numerosities.

6.1.1. True counting

While true counting is not considered accessible to non-human animals (Núñez 2017b), honeybees have demonstrated some aspects of the criteria necessary for true counting.

6.1.1.1. Extrapolation to novel representations of numerosity and novel numerosities:

Previous research demonstrated that bees could perform a few aspects required for true counting. As shown in Figure 1 and Figure 2, true counting requires the ability to extrapolate numerical tasks to novel representations of numerosity (Dacke and Srinivasan, 2008; Davis and Pérusse, 1988). In Gross et al. (2009), bees demonstrated two attributes of true counting which are considered of mid-range complexity (Figure 1). Honeybees in this study were able to extrapolate quantity discrimination to novel abstract elements, known as the ‘abstraction principle’ (Dacke and Srinivasan, 2008; Davis and Pérusse, 1988).

Gross et al. (2009) also demonstrated the extrapolation of quantity discrimination to novel numerosities, which is an ability required for the demonstration of true counting (Davis and Pérusse, 1988; Lazareva and Wasserman, 2017). Honeybees could learn to match the sample stimulus of two elements to the correct option in the comparison of two vs. three elements, bees could then perform the matching of three to the correct option of three vs. four elements without further training (Gross et al., 2009), showing extrapolation of the task using a novel numerosity.

Figure 1: A schematic describing the relative complexities of different **numerical concepts** (overall titles) and **numerical tasks** (smaller titles), how the numerical concepts relate, and which species have demonstrated each task and concept. The diagram shows which tasks belong at varied levels of complexity ranging from non-numerical through to basic, mid-range, and high complexity tasks. The tasks are grouped into overall concepts which are defined across the literature. In some cases all tasks need to be demonstrated for a concept to be shown (e.g. true counting requires all criteria to be met), in other cases a single task needs to be shown to demonstrate the concept (e.g. any task in quantical cognition is evidence of using quantical cognition). The interaction between concepts is also shown by the dark areas of the diagram. Some concepts have overlapping tasks needed to demonstrate more than one concept. Beside or below each task I have shown the animal species which have successfully performed the task. The star in the key classifies the task as having been demonstrated by all species shown on the schematic. *I have included this figure from Chapter 1 for easy reference in the current section.*

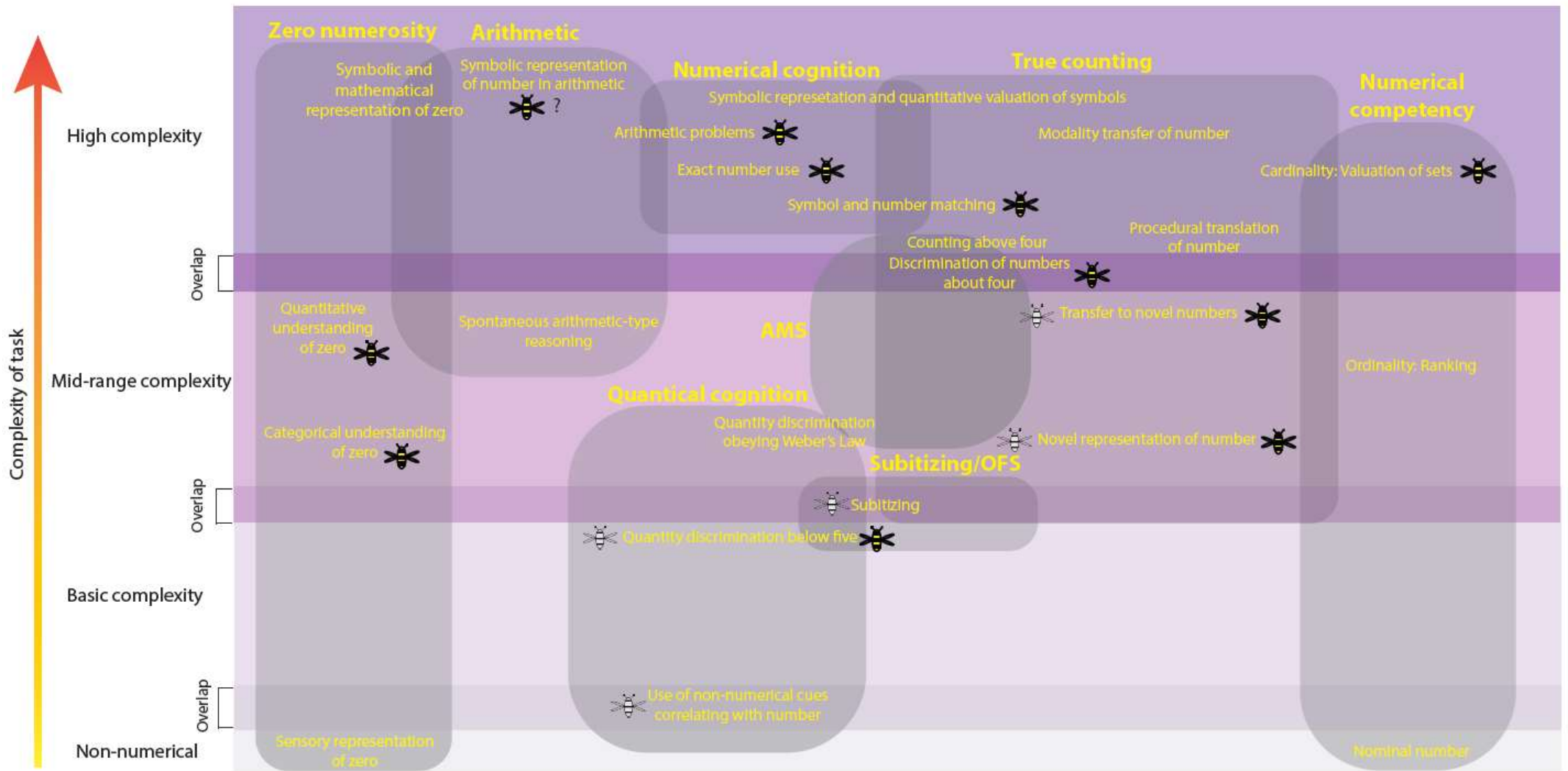


Figure 2: A schematic describing the relative complexities of different **numerical concepts** (overall titles) and **numerical tasks** (smaller titles), how the numerical concepts relate, and which species have demonstrated each task and concept, as above in Figure 1. In this diagram the icons (white/black bee and yellow/black bee) indicate which tasks have been performed by honeybees. The white/black bee icon represents what previous studies have shown in terms of numerical abilities of bees, while the yellow/black bee icon indicates the numerical tasks honeybees have performed as described in my experimental chapters (Chapters 2 – 5). The question mark bee icon denotes where bees have met some criteria possibly constituting the demonstration of a task but may not have fully reached that level (discussed in section 6.1.5.2).

I have demonstrated that honeybees are able to extrapolate number tasks to novel representations of numerosity and novel numerosities (Chapters 2 – 5). In each experimental chapter, the testing phases required bees to translate learnt number tasks to novel elements differing in colour, shape, pattern, and/or element size as well as transfer learning to novel numerosities. In Chapter 2, bees were able to learn to discriminate between numerosities of 4 : 5 to 4 : 8 (0.80 to 0.50) using appetitive-aversive conditioning and extrapolate this discrimination ability to novel shapes and patterns. In Chapter 3, bees were able to learn the numerical rules of ‘less than’ and ‘greater than’ and subsequently apply these rules to the novel numerosities of five elements and an empty set, as well as extrapolating the rules to novel shapes and patterns in the testing phases. In Chapter 4, bees were first able to learn to either add or subtract one element based on the colour of the sample stimulus; subsequently bees were able to extrapolate both rules to a novel numerosity, novel patterns, and novel shapes. In Chapter 5, bees were able to learn to match two quantities with two symbols and were subsequently able to extrapolate these associations to stimuli of novel pattern and colour. Thus my research has shown the ability of bees to apply multiple learnt numerical tasks to both novel representations of stimuli and novel numerosities, thereby demonstrating two aspects of true counting.

6.1.1.2. Discrimination of numerosities above four:

Another aspect of true counting requires the serial counting of five and more elements, identified as a complex level of numerical capacity (Figure 1; Figure 2; Agrillo et al., 2008; Davis and Pérusse, 1988; Kaufman et al., 1949; Lazareva and Wasserman, 2017; Piazza et al., 2002; Tomonaga and Matsuzawa, 2002; Trick and Pylyshyn, 1994). A building block for performing the serial counting task is the ability to discriminate between numerosities at and above the subitizing range (Figure 1; Figure 2). In Chapter 2, bees demonstrated the ability to

successfully discriminate between numerosities of 4 vs. 5, 4 vs. 6, 4 vs. 7, and 4 vs. 8, thus surpassing the previous threshold of numerosity discrimination reported in Gross et al. (2009). Additionally, Chapter 3 results demonstrate that bees were able to learn ‘less than’ and ‘greater than’ numeric rules when trained on the numerosities one to six. The results reported in Chapter 2 and Chapter 3 were achieved with appetitive-aversive differential conditioning, while the previous discrimination limit of 3 vs. 4, shown in Gross et al. (2009), was determined using appetitive-differential conditioning. While my experiments do not demonstrate the ability of bees to count serially, they show that bees are able to process and discriminate between quantities above four.

6.1.1.3. Cardinality:

Cardinality is the quantitative assignment of value to differing amounts of items, such as the number of elements in a set (Nieder, 2005). Honeybees have demonstrated this capacity through their ability to correctly value novel numerosities with the learnt rules of ‘less than’ and ‘greater than’, which involved the relative valuation of two numerosities and the ordering an empty set by value along the number line (Chapter 3; Howard et al., 2018). To a lesser extent, cardinality is demonstrated in Chapter 2 as honeybees were able to discriminate between sets of numbers from one – ten.

6.1.1.4. Symbol and number matching:

The ability to assign a numerical tag to a specific numerosity and subsequently value that symbol is another aspect of true counting (Lazareva and Wasserman, 2017). While my research has not demonstrated that honeybees can reach this level of symbolic representation, bees in Chapter 5 did demonstrate the precursor for this capacity, namely the ability to match numerosities with symbols (Figure 1; Figure 2). The results from Chapter 5 have provided a mechanism for bees to learn symbol and numerosity associations thus enabling further

research into their ability to give symbols quantitative values. The possibility of training bees to give quantitative value to symbols would be a very complex experiment. The first stage of such an experiment would require bees to learn the numeric valuation of different numerosities (e.g. as demonstrated in Chapter 2 and Chapter 3). Bees would then need to learn the associations between numerosity and symbol, as done in Chapter 5. The next phase of the experiment would then be to design a way to train bees to associate the quantitative value of the numerosity with the symbol. Finally, bees would need to be tested on their ability to value the symbols numerically. This is a very complex experiment to design and conduct within subject, using an animal that has a short lifespan. Despite the challenges involved in this experimental design, the learning and combination of these different stages of the experiments may be possible as honeybees have demonstrated an ability to learn dual concepts (Avarguès-Weber et al., 2012). Honeybees were able to simultaneously learn two rules (e.g. left/right and same/different) and then combine those rules to form a dual concept which could be applied to novel problems and stimuli. Thus, the study by Avarguès-Weber et al. (2012) provides an insight into how bees could learn symbolic valuation in stages. However, linking all of these stages, within subject, in an experimental study would currently be challenging to achieve.

6.1.1.5. True counting criteria:

While true counting is not considered accessible to non-human animals, due to its' quantification of numerosity using symbolic reference, the studies reported above demonstrate that bees can learn and perform some aspects of true counting, such as extrapolation to novel representations of number and novel numerosity, discrimination of numerosities above the subitizing range, cardinality, and symbol-number associations, but have not demonstrated all criteria (Figure 2). However, all criteria required for the demonstration of true counting has not been shown in a single non-human animal species

(Figure 1). It appears experimental access to this question is difficult within subject due to the substantial list of criteria needed to show true counting.

6.1.2. Quantical cognition

6.1.2.1. Non-numerical cues:

Some species are able to use non-numerical cues which correlate with numerical cues in order to judge quantity. In previous studies of honeybees, bees demonstrated that when trained to forage on a feeder at a certain number of landmarks, some individuals preferred to use sequential of counting landmarks to find the feeder while others preferred to use distance cues, or possibly other cues correlated with distance such as energy use or other landmarks (Chittka and Geiger, 1995). Another study also found a preference of bees to use information other than landmark counting to forage efficiently (Menzel et al., 2010).

In my research I was careful to control for different elements of the stimuli to ensure bees could not use non-numerical cues in order to solve the numeric tasks presented (Chapter 2 – 5). The supplementary material in Chapter 3 presents an extensive methodology of how this was achieved. Stimuli used in Chapter 3 were controlled for spatial frequency, surface area of colour, patterns, shapes, and element sizes. To ensure spatial frequency was not a predictor of the results in Chapter 3, we compared the spatial frequency of the images, power spectra, and the transect graphs produced for each of the 97 stimuli used and found that spatial frequency could not allow bees to transfer a learnt ‘spatial frequency rule’, rather than a numerical rule, to correctly select the empty set stimulus in the experiments, thus bees must have been using the learnt numerical rules of ‘greater than’ and ‘less than’ to solve the tasks. In addition to this, all experiments in my research (Chapters 2 – 5) involved learning and transfer tasks which tested the ability of bees to extrapolate the numerical tasks to novel stimuli of different shapes, patterns, element sizes, numerosities, and/or colours without reinforcement for

choices. The controls employed for the stimuli properties in all experiments demonstrate that bees were not able to utilize non-numerical cues in order to solve numeric tasks and thus must have been solving the tasks based on numeric information alone.

6.1.2.2. *Quantity discrimination:*

Quantity discrimination of four or less elements has been demonstrated in a delayed-matching-to-sample task by Gross et al. (2009). In Gross et al. (2009), honeybees demonstrated the ability to differentiate between quantities of 2 vs. 3 and 3 vs. 4 but not 4 vs. 5 or 5 vs. 6.

In Chapters 2 – 5, honeybees have demonstrated some level of quantity discrimination. In Chapter 2, honeybees were able to learn to successfully discriminate between numbers below and above the subitizing range. Honeybees also learnt to discriminate between the relative quantities of zero to six, demonstrating quantity discrimination below and beyond the subitizing range in Chapter 3. In Chapter 4, honeybees were able to discriminate between the correct and incorrect options of an arithmetic problem which ranged from one to five. Additionally, bees in Chapter 5 demonstrated the ability to differentiate between the quantities of two and three when represented as different abstract shapes, sizes, patterns, and colours.

Therefore, honeybees have demonstrated both basic and mid-range learning of numerical tasks, which show they are able to perform quantical cognition.

6.1.3. Numerical cognition

6.1.3.1. Symbol and number matching

As discussed in section 6.1.2.4, honeybees in Chapter 5 displayed the ability to match symbols and numbers. Thus, bees are displaying an aspect of exact number use and an aspect of numerical cognition (Figure 1; Figure 2; Núñez, 2017a).

6.1.3.2. Arithmetic

Arithmetic, including abilities such as addition, subtraction, multiplication, and division, is a numerical task which demonstrates exact number use and numerical cognition (Núñez, 2017a). In Chapter 4, honeybees demonstrate the ability to perform the addition and subtraction tasks of $+1$ and -1 , thus demonstrating arithmetic, exact number use, and numerical cognition (Figure 2). Whilst Chapter 4 shows some capacity of bees to add and subtract by one element using symbolic representation of operators, future work could explore if arithmetic in bees extends to $+2/-2$, multiplication, and/or division. The framework created in Chapter 4 now allows for these experimental possibilities.

6.1.4. Numerical competency

6.1.4.1. Cardinality

Cardinality, the ability to order sets of different numerosities (Nieder, 2005), was demonstrated by honeybees in Chapter 3, and to a lesser extent Chapter 2, as discussed in section 6.1.2.3. In Chapter 3, bees were able to give relative value to two different sets of numerosities using ‘greater than’ and ‘less than’ rules. The bees could subsequently apply these numeric rules to novel numerosities, demonstrating cardinality. Similarly, in Chapter 2 bees were able to discriminate four elements from stimuli containing one to ten elements, which demonstrates cardinality to an extent.

6.1.5. Arithmetic

6.1.5.1. Arithmetic problems using abstract objects

As described in section 6.1.4.2 honeybees displayed some level of arithmetic in Chapter 4. Honeybees were able to add or subtract one element from the numerosities one to five. The ability of bees to perform this task was able to be transferred to novel numerosities and novel representations of the stimuli, thus demonstrating this level of arithmetic.

6.1.5.2. Symbolic representation of numerosity for use in arithmetic

Bees are potentially able to demonstrate symbolic representation in arithmetic (Figure 1). Honeybees were able to learn that a colour represented whether the bee had to add or subtract an element from the sample stimulus in each trial. Bees used this symbolic representation of an operation to determine which numerical task to perform (Chapter 4). While bees were not using symbolic representation of numerosity in arithmetic, they were able to use some level of symbolic representation in a numeric task (Figure 2). Further research into the use of symbols in arithmetic by bees should address this outstanding question.

6.1.6. Zero numerosity

Honeybees in Chapter 3 demonstrated the third level of understanding zero numerosity as defined by Nieder (2016b). Honeybees learnt the relational numerical rules of ‘less than’ and ‘greater than’ and were subsequently able to apply these rules to value zero at the low end of the numerical continuum. Furthermore, bees displayed numerical distance effects with zero numerosity (Nieder, 2016b), thereby demonstrating an understanding of zero as a quantitative numerosity.

6.1.7. Numerical concepts and tasks in honeybees

Honeybees have displayed numerical ability ranging from the basic use of numerical information correlated with non-numerical cues (Chittka and Geiger, 1995; Menzel et al., 2010) through to the complex task of arithmetic using symbolic representations of addition and subtraction rules (Chapter 4). Honeybees have also been able to demonstrate a variety of numerical categories (defined in Figure 1; Figure 2) including quantal cognition, subitizing aspects of true counting, use of the AMS/ANS, numerical competency, numerical cognition, zero numerosity tasks, and arithmetic. Quantification and discrimination of numerosities above four elements demonstrates that honeybees are able to use the approximate magnitude system (ANS), also known as the analog number system (AMS), which is the mechanism human and non-human animals use to process numbers past the subitizing/OFS range (Brannon and Merritt, 2011; DeWind et al., 2015; Feigenson et al., 2004; Núñez 2017b). The ANS is a non-symbolic quantification system used by species, as opposed to true counting which is symbolic and considered only accessible to humans.

6.2. Explanation for the honeybee's capacity to learn and perform numerical tasks

There are various reasons why a honeybee may be able to learn and apply complex numerical concepts. We observe a spontaneous ecological need for quantal and numerical abilities in other animals. These tasks include addition and subtraction of food items in rhesus monkeys (Sulkowski and Hauser, 2001; Tsutsumi et al., 2011) and spiders (Nelson and Jackson, 2012; Rodríguez et al., 2015), allowing them to keep track of food resources. Another example is with quantity discrimination between shoals in angelfish (Gómez-Laplaza and Gerlai, 2011a; Gómez-Laplaza and Gerlai, 2011b) and mosquitofish (Agrillo et al., 2007; Agrillo et al., 2008; Dadda et al., 2009) in order for them to choose to shoal with a higher number of fish for predation avoidance (Hamilton, 1971). Landmark counting in honeybees is used for

navigation to locations of food sources (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008). In my research bees have demonstrated an advanced capacity for an insect to learn and apply complex numerical concepts (Figure 2), and so we must ask, how and why are they able to perform these tasks? Has numerical capacity evolved in bees?

Bees are exceptional learners when it comes to flower traits. They learn flower characteristics such as colour (de Ibarra and Giurfa, 2003; Dyer and Arikawa, 2014; Dyer and Neumeyer, 2005; Dyer et al., 2008; Rusch et al., 2017), scent (Reinhard et al., 2004a; Reinhard et al., 2004b), symmetry (Giurfa et al., 1996), and size (Avarguès-Weber et al., 2014; Howard et al., 2017a; Howard et al., 2017b; Martin, 2004). Due to a lifestyle of needing to quickly learn which flowers provide rewards of nutrition like pollen and nectar, as bees have a relatively short lifespan, they may have evolved a capacity to learn tasks quickly and efficiently. When their ability to rapidly learn tasks is combined with appetitive-aversive conditioning, which is known to modulate accuracy (Chapter 2; Avarguès-Weber et al., 2010; Chittka et al., 2003), bees appear to learn both quickly and accurately. Thus, one explanation for the honeybee's ability to learn and apply numerical rules to solve problems may be that they are evolved to learn and apply information in foraging. Perhaps their ability to learn many different tasks, information, and rules is a direct result of an evolved ability to learn flower traits (Dyer, 2012).

It is possible that bees may need certain numerical skills to survive and forage in the complex environments in which they live. For example, Chittka and Geiger (1995), and Dacke and Srinivasan (2008) have shown that bees can learn to count landmarks in order to find the correct position of a food source. With the amount of foraging and navigating that honeybees perform, counting and other numerical skills would be useful in different environments to remember and navigate to the locations of highly rewarding flower patches (Chittka and

Niven, 2009). Potentially numerical skills could also help bees to recognise flowers by the number of petals they contain, which is not a farfetched notion considering we know bees use colour, scent, symmetry and size to identify flowers, thus perhaps petal number differences between species of flowers could also be an additional cue (Gross et al., 2009; Leppik, 1953).

It is important for any animal to understand the concept of zero at the basic levels (i.e. no water, no food, no nectar, no predators; Nieder, 2016b). Thus, perhaps another possibility for their ability to understand zero at some level is ecologically relevant to their need to keep track of honey storage for winter months, flower absences in different seasons, or even which flowers provide no nectar compared to which flowers do provide nectar. Additionally, completing tasks when foraging or maintaining the hive may need an element of arithmetic-type reasoning as we observe in food tracking in vertebrates (Sulkowski and Hauser, 2001; Tsutsumi et al., 2011) and invertebrates (Nelson and Jackson, 2012; Rodríguez et al., 2015), such as with the collection and use of pollen and nectar in the hive.

6.3. Evolutionary implications

In Chapter 1, I reviewed the literature of numerical ability in humans and non-human animals (Figure 1), which suggested a paucity of experimental studies testing the numerical ability of invertebrates compared to vertebrates (Carazo et al., 2009; Dacke and Srinivasan, 2008). I noted the importance of further studies testing the ability of invertebrates to display complex numerical ability and reviewed the honeybee as a model species for this purpose. The importance of determining the numerical capacity of an invertebrate pertains to informing the evolutionary pathways by which numerical ability may have evolved. The honeybee is a model species for the study of cognition and rule-learning as demonstrated by previous studies (for reviews see: Avarguès-Weber and Giurfa, 2013; Dyer, 2012; Srinivasan, 2010; Zhang, 2006). In addition, the honeybee is a good comparative model as its full genome is

mapped (Consortium, 2006), and it is a species separated from humans for 600 million years (Consortium, 2006; Grimaldi et al., 2005). Thus, the ability of honeybees to quickly learn (within a few hours) and perform numerical tasks of varied complexity (Figure 2; Chapters 2 – 5) suggests a convergent evolutionary pathway for numerical abilities. As humans and honeybees are very evolutionarily separated, it is most-likely that convergent evolution has occurred to provide different species (Figure 1) with numeric capacity relevant to their particular environments. My conclusions on a convergent evolutionary pathway for numerical abilities are also supported by studies demonstrating that number neurons appear to have evolved independently in vertebrates, despite the very different neuroanatomy of their brains (Nieder, 2016a). Non-human primates and birds are known for their advanced cognitive ability, including their numerical capacity (Scarf et al., 2011), however they have evolved very distinct brain structures through the parallel evolution of endbrains (Dugas-Ford and Ragsdale, 2015; Dugas-Ford et al., 2012). The last common ancestor for mammals and primates, a reptilian-like species without a neocortex, existed over 300 million years ago (Evans, 2000), thus the evidence that numerical tasks activate similar neural populations in such different brain structures (Ditz and Nieder, 2015) supports the theory of convergent evolution of numerical capacity.

The next step in collecting evidence for the evolutionary pathway of numerical capacity is to determine the neuron populations and structures involved in numerical tasks performed by invertebrates. My research has demonstrated that the honeybee is capable of learning and performing complex numerical tasks, thus brain recordings of which areas of the bee brain are involved will provide important evolutionary and neurobiological data to answer further questions.

6.4. Neurobiological inferences

Nieder (2018) identified an important topic of consideration arising from my work (Chapter 3; Howard et al., 2018; Nieder, 2018). “Number neurons” (Dehaene, 2002; Nieder, 2016a) activate in response to a specific number of elements. There is some recent work in monkeys showing that understanding “nothing”, an empty set, as having a quantitative null value is actively represented by cortical neurons (Ramirez-Cardenas et al., 2016). Ramirez-Cardenas et al. (2016) showed that neurons are activated in a similar way to ‘nothing’ as they are to numbers, for example in non-human primates (Nieder et al., 2002; Nieder and Miller, 2003; Nieder and Miller, 2004; Sawamura et al., 2002), corvids (Ditz and Nieder, 2015; Ditz and Nieder, 2016a; Ditz and Nieder, 2016b), and cats (Thompson et al., 1970). Thus, when trained monkeys demonstrate a similar response in the brain for empty sets, we can determine that non-human primate brains are capable of understanding and representing numerosity zero as “something”. Nieder (2018) notes that no neurophysiological understanding of numerical competence in insects is known, thus not only does this raise the questions of how and where honeybees may process zero and which neurons may activate, but also the question of how a bee (or other insect) represents number in the brain currently remains unknown (Nieder, 2018). The above studies demonstrate that honeybees are able to perform a number of basic and complex numerical skills such as understanding the quantitative value of an empty set (Chapter 3; Howard et al., 2018), demonstrating learning of quantitative values of cardinal sets, symbol and number matching, and simple arithmetic (Figure 2). Where this may occur in the insect brain is still unknown but poses an interesting question for future work now that I have established numerical competency in insects along with an appropriate training paradigm. Insights into possible mechanisms of colour learning are starting to emerge using mapping of how bee brains process visual colour stimuli depending upon

conditioning (Sommerlandt et al., 2016), but methods have not yet been developed for examining complex cognitive-like tasks in bee brains.

One of the outstanding questions identified in a recent review of the concept of zero in human adults, children, and non-human animals was:

“How are zero-like concepts neurophysiologically encoded in animals that did not evolve a layered neocortex (cerebral cortex), such as birds?” (Nieder, 2016b).

This question is still outstanding considering animals such as birds and insects. Indeed, I have answered the question:

“Are animals in taxa only remotely related to humans, such as fish or even insects, able to represent empty sets as a quantitative category?” (Nieder, 2016b).

6.5. Bees as a model for bio-inspired technology

In addition to being a model species for the study of vision and cognition (Avarguès-Weber et al., 2011; Avarguès-Weber and Giurfa, 2013; Dyer, 2012; Srinivasan, 2010), honeybees are also a model species for the development of bio-inspired technologies. For example, honeybees have been useful for the development of artificial intelligence, aerial machine navigation, flight control, successful landing procedures, accurate distance estimation, and the regulation of flying height (Bukovac et al., 2013; Srinivasan, 2006; Srinivasan, 2011; Srinivasan et al., 1999). Through combining the numerical capacity of a miniature and seemingly simplistic honeybee brain with computational technology, research may be able to determine a new bio-inspired mechanism for processing numbers using a simulated honeybee brain.

Honeybee processing has also been applied to neuromorphic systems which are computational models of biological systems. They are of use for computing, sensory

processing in robots, and concept learning in machines (Helgadóttir et al., 2013; Kleyko et al., 2015; Sandin et al., 2014). Neuromorphic systems are designed to cope with uncertainty and use brain-like computations for processing real-world problems. As honeybees live in complex environments and can learn concepts to solve problems, they are considered an ideal model for neuromorphic systems machines (Helgadóttir et al., 2013; Kleyko et al., 2015; Sandin et al., 2014). Overall, insects demonstrate many goal-directed and plastic behaviours that are currently beyond the capacity of today's artificial systems (Helgadóttir et al., 2013).

Schmicker and Schmicker (2018) recently built a simple neural network which could replicate the result of honeybees understanding the quantitative value of an empty set (Chapter 3; Howard et al., 2018). The simple neural network, consisting of three convolutional layers, could learn and apply 'greater than' and 'less than' rules when trained using the same stimuli and protocol, as in Chapter 3, and apply it to understand the value of zero items (Schmicker and Schmicker, 2018). However, this study also demonstrates that we still have a lot to learn from biologically evolved processing systems, such as the honeybee brain, as while bees in Chapter 3 took less than 100 trials to learn the task, the simple neural network took about 4 million trials to learn the same task (Chapter 3; Howard et al., 2018; Schmicker and Schmicker, 2018). The study by Schmicker and Schmicker (2018) illustrates how the findings of my thesis are already having an impact on computer design principles.

6.6. Conclusions

The honeybee has proven an excellent model for testing whether conditioning procedure can allow the extension of previous limits of numerical competency and to determine what level of numerical ability can be reached by an insect model. While the honeybee was not able to pass all tests presented, for example the reverse task test (Chapter 5) or differentiating a correct choice consistent with rule learning compared to an incorrect choice consistent with

associative mechanisms (Experiment 2 in Chapter 3), the success of bees to discriminate challenging ratios of number (Chapter 2), learn numerical rules and apply them to value zero numerosity (Chapter 3), perform simple arithmetic (Chapter 4), and learn to associate a symbol and a specific quantity (Chapter 5) has surpassed their previous known numerical competency threshold (Figure 2). However, there are still many questions remaining.

Outstanding questions include: Whether bees can demonstrate all aspects of true counting; whether bees are able to extrapolate number ability cross-modally or cross-procedurally; and whether bees are able to learn to understand the quantitative value of different symbols matched with specific quantities and order those symbols correctly. Future research also needs to explore what part of the insect brain, specifically honeybees, numerical tasks activate, and whether bees, like primates, corvids, and cats, have number neurons. My research suggests that numerical competency is not a result of the evolution of human culture and language and may be due to convergent evolution of numerical capacity. Further work into this topic, enabled by the new findings within my thesis, will aid in understanding how and when different levels of numerical ability may have evolved.

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Appendices

Appendix 1:

Howard SR, Avarguès-Weber A, Garcia JE, Greentree AD, Dyer AG (2018) Numerical ordering of zero in honey bees. *Science*. **360**: 1124-1126.

Appendix 2:

Dyer AG, **Howard SR**, Garcia JE (2016) Through the Eyes of a Bee: Seeing the World as a Whole. *Animal Studies Journal* **5**: 97-109.

Appendix 3:

Howard SR, Avarguès-Weber A, Garcia JE, Dyer AG. (2017). Free-flying honeybees extrapolate relational size rules to sort successively visited artificial flowers in a realistic foraging situation, *Animal Cognition* **20**: 627-638.

Appendix 4:

Howard SR, Avarguès-Weber A, Garcia JE, Stuart-Fox D, Dyer AG. (2017). Perception of contextual size illusions by honeybees in restricted and unrestricted viewing conditions, *Proceedings of Royal Society B* **284**: 20172278.

Appendix 5:

Guntarik O, Garcia JE, **Howard SR**, Dyer AG. (2018). TRACES: Mobile Eye Tracking Captures User Sensory Experience in an Outdoor Walking Tour Environment, *Leonardo* **51**: 163-164.

Appendix 6:

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Appendix 7:

Garcia JE, Shrestha M, **Howard SR**, Petersen P, Dyer AG. Signal or cue: the role of structural colours in flower pollination. *Current Zoology*.

Appendix 8:

Shrestha M, Garcia JE, Chua JHJ, **Howard SR**, Tscheulin T, Dorin A, Nielsen A, Dyer AG. (2019). Fluorescent pan traps affect the capture rate of insect orders in different ways, *Insects*.

EVOLUTIONARY COGNITION

Numerical ordering of zero in honey bees

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Some vertebrates demonstrate complex numerosity concepts—including addition, sequential ordering of numbers, or even the concept of zero—but whether an insect can develop an understanding for such concepts remains unknown. We trained individual honey bees to the numerical concepts of “greater than” or “less than” using stimuli containing one to six elemental features. Bees could subsequently extrapolate the concept of less than to order zero numerosity at the lower end of the numerical continuum. Bees demonstrated an understanding that parallels animals such as the African grey parrot, nonhuman primates, and even preschool children.

Four stages are used to describe the acquisition of understanding zero in human history, psychology, animal cognition, and neurophysiology (1). The first is the ability to define zero as nothing—the absence of a stimulus. The second is the categorical classification of zero as “nothing” versus “something.” The third stage is understanding zero as a quantity at the low end of the positive integer numerical continuum. The fourth, and currently designated as the most advanced stage of understanding zero, is the symbolic representation of zero, as with an Arabic number and as used in modern mathematics and calculations (2).

Several ancient human civilizations lacked the full understanding and importance of zero, leading to constraints in their numeric systems (3). Interestingly, some vertebrate animals have recently demonstrated a capacity to acquire and understand this numerical concept. Rhesus monkeys learned that empty sets of objects occupy a position on a numerical continuum (2, 3), vervet monkeys used subtraction-like reasoning to determine if food was present or absent (4), a chimpanzee reached near-perfect performance on zero-concept tasks with training (5), and an African grey parrot spontaneously labeled absent objects as “none” (6).

Honey bees have previously demonstrated the capacity to count and discriminate up to four objects (7–10) in experiments that use classic conditioning techniques. Recent advancements in conditioning protocols (11) reveal that bees can acquire rule-based relational concepts (12, 13), thus enabling remarkable plasticity to acquire

and apply seemingly advanced concepts such as size ordering (14). In this study, we tested the capacity of honey bees to extrapolate the acquired concepts of “greater than” and “less than,” as shown in primates (15, 16), and thus formally demonstrate that an invertebrate can understand the concept of zero numerosity.

We designed a set of experiments to test the extent to which honey bees may understand the concept of zero numerosity (17). In the first experiment, we trained bees to understand the concepts of less than and greater than using appetitive-aversive differential conditioning (11). Bees were trained to the respective concepts using white square stimuli containing one to four black elements (Fig. 1A, fig. S1, and table S1). After reaching a criterion of $\geq 80\%$ accuracy, bees demonstrated in nonreinforced tests that they had learned the concept of “numerically less” [$75.0 \pm 4.1\%$ (mean \pm SEM); logistic regression with individual as random term tested differences between observed proportion of bee choices and chance level, $y = 0.5$, z score = 5.08, $P < 0.001$, $n = 10$] and “numerically greater” ($75.5 \pm 3.3\%$; z score = 6.556, $P < 0.001$, $n = 10$) when presented with novel stimuli of one to four elements. Furthermore, bees were able to apply these concepts to determine that five elements were greater than two or three elements (less-than group: $68.0 \pm 5.0\%$, z score = 3.411, $P < 0.001$, $n = 10$; greater-than group: $75.0 \pm 3.9\%$, z score = 5.333, $P < 0.001$, $n = 10$). Interestingly, bees demonstrated an understanding that zero numerosity lies at the lower end of the numerical continuum by choosing an “empty set” stimulus containing no elements if trained to less than ($64.0 \pm 5.4\%$; z score = 2.795, $P = 0.005$, $n = 10$; Fig. 1C) or by choosing unfamiliar stimuli containing elements if trained to greater than ($74.5 \pm 2.6\%$; z score = 6.609, $P < 0.001$, $n = 10$; Fig. 1C).

In the second experiment, we tested the extent to which bees may understand the quantitative concept of zero in comparison with other animals. As some animals find it challenging to differentiate between the numbers zero and one

(5, 6, 18), we trained bees to less than using stimuli containing two to five elements and then tested their ability to differentiate between the unfamiliar numerosities of one and zero (Fig. 1B). After reaching a criterion of $\geq 80\%$ accuracy, bees demonstrated the learned concept of numerically less when presented with the numbers two to five ($73.8 \pm 1.9\%$; z score = 10.18, $P < 0.001$). When presented with the unfamiliar numbers of one versus zero, bees chose the lower number of zero ($63.0 \pm 2.9\%$; z score = 4.23, $P < 0.001$; Fig. 1D), showing an understanding that an empty set is lower than one, which is challenging for some other animals (5, 6, 18).

When bees were presented with two conflicting pieces of information, two versus zero, where the two-element stimuli had always been rewarded in training and zero was the correct lower number, bees chose the empty set at a frequency level that was not significant from chance ($56.2 \pm 3.4\%$; z score = 1.64, $P = 0.101$; Fig. 1D); thus, bees perceived both plausible alternatives as consistent with their conditioning experience. These results demonstrate that bees were using both an associative mechanism for choosing two elements and a concept-based mechanism for choosing zero numerosity. This phenomenon was also observed in a dolphin trained to choose the numerically less option by using white dots on a black background. This result is explained in terms of an artifact of training-set conditioning causing a bias toward consistently rewarding stimuli (19).

To test if bees understood an empty set quantitatively along the numerical continuum, we evaluated numerical-distance effects, where accuracy of performance potentially improves as the difference in magnitude between two respective numbers increases (1). In the third experiment, we trained and tested bees on the less-than concept using the numbers zero to six. If bees considered zero numerosity as a number along the numerical continuum, we would expect accuracy of decisions to be the greatest with zero versus six and poorer for lower numbers versus zero numerosity (Fig. 2). After reaching a criterion of $\geq 80\%$ accuracy during training, bees demonstrated in tests that they could discriminate an empty set from numbers one to six accurately (supplementary text S1 and Fig. 2B). Although bees could accurately discriminate all numbers from zero numerosity, there was a significant effect of numerical distance on accuracy (Fig. 2B). Bees were more accurate when numbers were numerically more distant (zero versus five and zero versus six) than when numerically closer (zero versus one), showing that bees are affected by number magnitude and thus exhibit numerical-distance effects.

An alternative explanation for our results could be that bees have a preference for the unfamiliar presentation of an empty-set stimulus. However, control experiments showed that the bees’ understanding that zero belongs at the lower end of the numerical continuum was rule based and not driven by an unfamiliar preference (supplementary text S2 and fig. S2). The spatial

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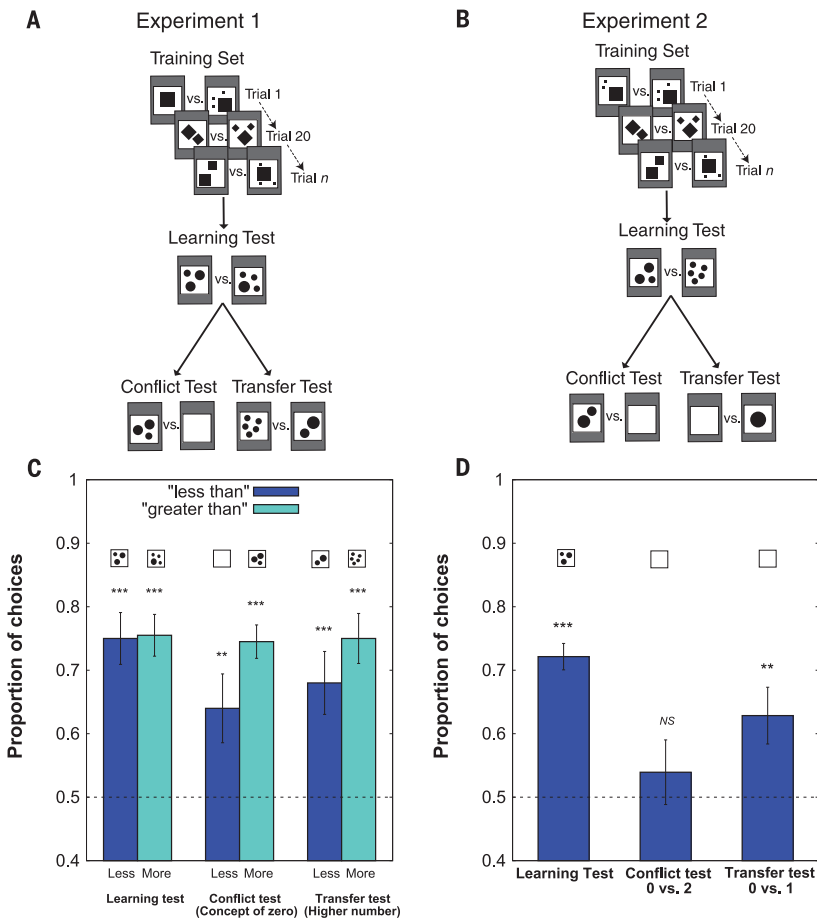


Fig. 1. Graphic representation of the method and results of experiments 1 and 2. (A and B) Examples of possible stimuli combinations during trials and tests in experiments 1 and 2. (C and D) Performance during the unreinforced testing phases during experiments 1 and 2. Data shown are means \pm SEM for both treatment groups. Bees trained to less than are shown in dark blue; bees trained to greater than are shown in turquoise. Stimuli above the columns represent the choices for those stimuli in the data. In experiment 1, in the conflict test evaluating the bees' concept of zero, data shown for the less-than group ($n = 10$) are choices for zero, and data shown for the greater-than group ($n = 10$) are choices for stimuli containing elements. In the transfer test to a higher number, data shown for bees trained to less than are choices for a lower number, and data shown for bees trained to greater than are choices for the higher number of five. In experiment 2 ($n = 25$), the conflict and transfer tests show the bees' choices for zero. Dashed black line at 0.5 indicates chance-level performance. Significance from chance-level performance is indicated by ** $P \geq 0.01$ and *** $P \geq 0.001$. NS, not significant.

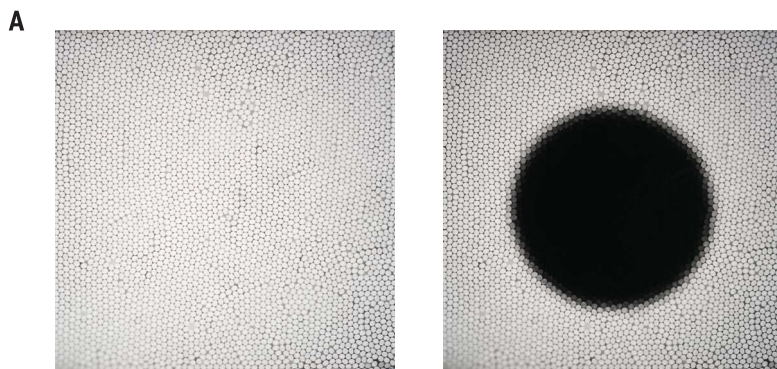
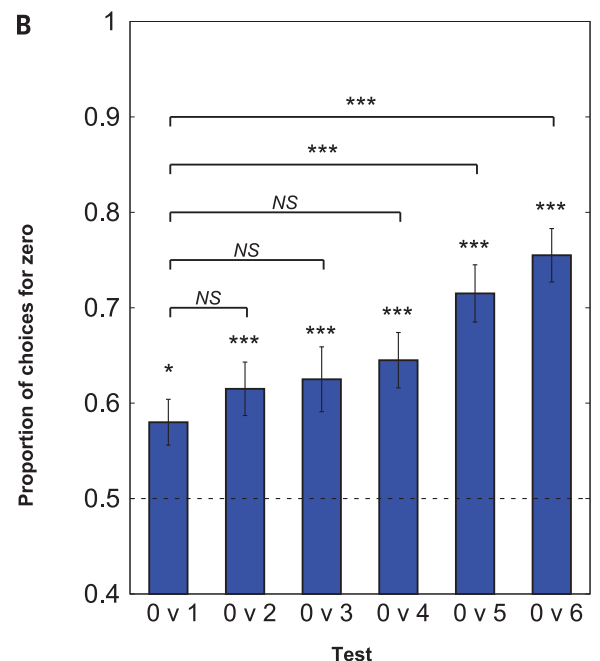


Fig. 2. Photographic representation of stimuli and results from experiment 3. (A) Representation of honey bee spatial vision when viewing stimuli of either zero or one (22). (B) Honey bee performance during experiment 3, testing the behavioral effects of numerical distance of numerosity zero. Data shown are means \pm SEM for the choice of the zero stimuli. Dashed black line at 0.5 indicates chance-level performance. Significance from chance-level performance and from other tests is indicated by * $P \geq 0.05$ and *** $P \geq 0.001$. NS, not significant.



frequencies of stimuli are also ruled out as a potential explanation for results (supplementary text S1 and table S1). We additionally conducted further control experiments to exclude the possibility that bees learn to match pairs of num-

bers during training (supplementary text S3 and fig. S2).

Our findings show that honey bees can learn and apply the concepts of greater than and less than to interpret a blank stimulus as represent-

ing the conceptual number of zero and place zero in relation to other numerical values. Bees thus perform at a level consistent with that of non-human primates by understanding that zero is lower than one (5).

An open question remains as to whether such advanced numerical understandings may be widespread across many animals that deal with complexity in their environments or if our findings are the result of independent evolution in honey bees. Recent comparative studies of primate and crow brains found that similar levels of numeric processing are facilitated by very different brain structures, suggesting independent evolution of numeric processing (20, 21). Because it can be demonstrated that an insect, with a different brain structure from primates and birds, can understand the concept of zero, it would be of high value to consider such capacities in other animals.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/360/6393/1124/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S4
Table S1
References (23–26)
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Through the Eyes of a Bee: Seeing the World as a Whole

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***Abstract:** Honeybees are an important model species for understanding animal vision as free-flying individuals can be easily trained by researchers to collect nutrition from novel visual stimuli and thus learn visual tasks. A leading question in animal vision is whether it is possible to perceive all information within a scene, or if only elemental cues are perceived driven by the visual system and supporting neural mechanisms. In human vision we often process the global content of a scene, and prefer such information to local elemental features. Here we discuss recent evidence from studies on honeybees which demonstrate a preference for global information. We explore insights from imaging studies suggesting why a global preference may be important for foraging in natural environments where a holistic representation of elemental factors is advantageous. Thus we aim to provide a brief new insight into how animal vision may perceive the complex world in which we must all operate and suggest further ways to test this.*

***Keywords:** Vision; local-global; Gestalt; Holistic; Flower; Wurmbea; Photography*

We often view and understand the environment around us in context (Torralba et al.), and humans are adept at visually perceiving a global construct or Gestalt of a complex scene (Sayim, Westheimer and Herzog). Although a review of these two concepts is beyond the scope of this work, here we employ the two terms interchangeably adhering to the terminology used by the authors of the cited papers. The Gestalt phenomenon is nicely illustrated in the famous finding of Navon (1977), aptly named ‘Forest before trees: the precedence of global features in visual perception’, which showed humans prefer the global construct compared to local elemental information within a scene. This work has been well replicated in several studies on human subjects, but surprisingly, most animals tested to date show a preference for local information (Navon ‘The Forest Revisited: More on Global Precedence’; Deruelle and Fagot; Fagot and Tomonaga; Spinozzi, De Lillo and Truppa; Kelly and Cook).

Insect visual processing is somewhat complicated by the design of their eyes which consist of an array of many, thousands in some species, individual light sensing units (*ommatidia*) packed in a small, compound eye (Land and Chittka). As a consequence of this design, insect vision has relatively poor resolution, ‘image sharpness’ compared to the larger human eye consisting on a single lens (Land and Nilsson).

In a well-studied insect species like the honeybee, it has typically been assumed that visual processing was relatively simple and mediated by elemental evaluation of cues (Horridge). However, for an animal with limited optical resolution, this may not be a very efficient solution as local elemental information available in a complex environment could easily be confounded with many other cues of similar appearance. This makes orientation and discrimination decisions difficult in complex environments (Adrian G. Dyer). For example, Figure 1 shows a photograph of a bunch of flowers imaged in the human visible spectrum and imaged through a mechano-optical device made of thousands of thin, black tube ‘drinking straws’ simulating the ommatidia present in a honeybee’s compound eye. This device allows us to obtain a visual representation closely simulating the resolution attainable by a honeybee’s eye (Dyer and Williams) as measured through behavioural experiments (Srinivasan and Lehrer).

The upper panel in Figure 1 shows a magnified section of the image representing what an elemental processing type system might perceive in isolation, and how localised elemental information could be easily confused with different components within the entire image

(Figure 1, lower panel). This observation thus raises the question of whether honeybees indeed perceive a visually complex world by local elemental processing or if their visual system is capable of representing information more globally.

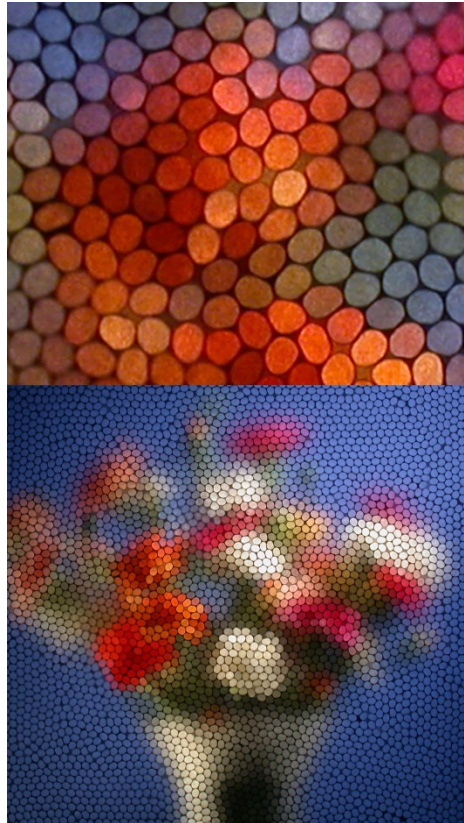


Figure 1. A human visible spectrum image of a bunch of flowers that have been captured through a mechano-optical device that closely matches the resolution of honeybee spatial vision (Dyer and Williams). The upper panel shows a segment of the image that would be easily confused with several parts of a scene, whilst the lower panel shows how a holistic view reveals the true shape and structure of the flowers. Whilst humans easily process the Gestalt of a scene, it was assumed insects like bees might only use elemental information. New work now shows that bees actually prefer global type processing, but can also pay attention to local elements within a complex scene (Aurore Avarguès-Weber, Adrian G Dyer, et al.).

The question of whether bees can perceive holistic information started to emerge as a strong possibility following a seminal publication by Stach et al. which showed that free-flying honeybees could indeed assemble local information to solve novel problems about the overall pattern of a more complex scene. Around this time, several studies also started testing the capacity of free-flying honeybees to learn very complex visual problems like different flower patterns (Stejskal et al.; Zhang et al.), landscapes (Dyer, Rosa and Reser; Zhang et al.), human artwork (Wu et al.) and even human faces (Dyer, Neumeyer and Chittka; Dyer and Vuong); whilst studies on other insect species like wasps also reported a strong capacity to recognise complex patterns like conspecific faces (Sheehan and Tibbetts; Tibbetts).

These studies on invertebrate vision were largely possible because of improved training and/or conditioning techniques employed by the researchers to train free-flying wild bees to visual tasks. Stimuli were presented vertically to control for orientation angle, and were learnt in relation to perceptually similar stimuli; termed differential conditioning (Giurfa et al.). Somewhat surprisingly, using these training techniques revealed a capacity in free-flying honeybees to process complex patterns including face-like stimuli, where bees showed evidence of configural type processing (A. Avarguès-Weber et al.). Indeed, prior to these studies configural processing was thought to be a mechanism requiring a large mammalian brain (Parr et al.; Tanaka and Sengco), and so it became high value to understand what else bees could learn to see if appropriate training regimes were employed. Despite having a miniature brain with less than one million neurons (compared to 100 billion in the human brain) (Srinivasan; Aurore Avarguès-Weber, Adrian G. Dyer, et al.), it has recently been shown that bees can learn rules such as the relative position of an object; above or below a given reference (Avarguès-Weber, Dyer and Giurfa), how relative size can allow for accurate recognition (Aurore Avarguès-Weber, Daniele d'Amaro, et al.), or even how multiple rules like above-below/left-right and same/different can be combined to solve novel visual problems (Aurore Avarguès-Weber, Adrian G. Dyer, et al.). The capacity to process information by applying rules such as above/below would enable faster and more reliable visual processing by bees when operating in complex environments (Chittka and Jensen), and strongly suggests that bees could simultaneously process more complex images than would be allowed by elemental processing (Adrian G. Dyer).

To test if bees could process complex scene information, it was possible to use hierarchical visual stimuli (Figure 2) as proposed by Navon (1977), which were presented to honeybees in a Y-maze to enable training and then testing with novel stimuli in which local and/or global information content was manipulated (Aurore Avarguès-Weber, Adrian G Dyer, et al.). In their experiment, free-flying honeybees were individually trained (using a sugar reward) to enter a Y-maze, which consists of an entrance hole, a long tunnel, and then a decision-making chamber which forks into two tunnels presenting two different options. The choice of a bee is counted once they choose which fork to fly down in order to hopefully receive a reward of sucrose (sugar water).

Somewhat surprisingly in the context of what is currently known about animal vision, bees learnt both local, the upright and inverted triangles in Figure 2, and global cues, the overall square or diamond shapes in Figure 2, from complex patterns during the training phase. Subsequently in the transfer tests where bees were presented with novel stimuli, bees preferred to rely on the global information to make decisions; but could also use local information if pre-trained to use the local elements to solve visual tasks. (Aurore Avarguès-Weber, Adrian G. Dyer, et al.).

This shows that a miniature brain can holistically process complex information and the reason why different animals may or may not share this capacity may be to do with environmental factors rather than brain size and complexity (Aurore Avarguès-Weber, Adrian G. Dyer et al.; Truppa et al.).

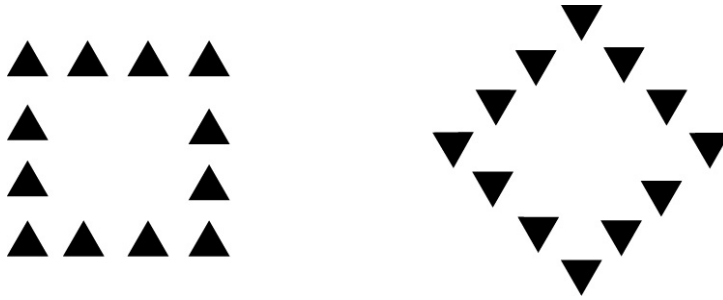


Figure 2. Visual stimuli used to test whether an animal will prefer to use global elements, the overall shape of each stimulus, or local information, the individual elements making up the overall shape, by (Aurore Avarguès-Weber, Adrian G. Dyer et al.) in their 2015 study.

This new evidence that bees can holistically process visual information leads to fascinating new questions about whether insect-pollinated flowers evolved certain shapes or morphologies to attract bee pollinators in a similar process to flower pigment colour, which has evolved to suit specific pollinator vision (Chittka and Menzel; Dyer et al.).

Honeybees demonstrate a preference for specific geometric traits such as radiating elements and symmetrical patterns (Lehrer et al.). In flowers, honeybees also prefer symmetry to asymmetry and radial symmetry over bilateral symmetry (Wignall et al.). To date, it has been questioned as to whether insects have the required optical resolution to perceive fine-scale differences across various levels of symmetry (Wignall et al.). With the aid of the mechano-optical device, we can now obtain insight into the level of symmetry fluctuations that a honeybee may be able to detect. The degree to which honeybees may perceive other morphological differences in flowers could also be examined using a mechano-optical device.

Interestingly, many insect-pollinated flowers have nectar guides or other striking patterns that would probably require the processing of multiple elements to perceive the overall pattern (Adrian G. Dyer; Guldberg and Atsatt). For example, Figure 3 shows a mechano-optical image of an Australian native flower, *Wurmbea dioica*, which has complex patterns that likely evolved for promoting visits by important pollinators such as native bees. *W. dioica* is a dioecious

species, meaning plants are either male or female (Vaughton and Ramsey), presenting flower size dimorphism whereby male plants have larger and ‘more showy’ flowers than females (Barrett ‘The Evolution of Mating Strategies in Flowering Plants’; Barrett ‘Understanding Plant Reproductive Diversity’). Another sexual dimorphic trait of *W. dioica* is that male plants will have more flowers than females; both of these sexually dimorphic male characteristics result in attracting a higher number of bees (Vaughton and Ramsey). Using the mechano-optical device, we are now able to gain insight into how well a pollinator can differentiate between plants with more flowers and flowers of a larger size. We can examine this more closely by simulating different distances bees are at when making foraging choices, such as between sexual dimorphic individuals of *W. dioica*.

By developing new image processing techniques based on recent advances in digital imaging, it is now possible to dissect real floral patterns into their different spectral components, *i.e.* the colour channels in a digital image, in relation to their particular spatial configurations (Garcia, Girard, et al.; Garcia, Greentree, et al.). Indeed, the relationship between the different elements defining the spatial configuration of an object such as a flower, its variability within a species, and the visual background against which it is observed are the ultimate causes of visual perception by an animal (Troscianko et al.). However, data allowing for the understanding of these relationships from the point of view of an animal such as an insect pollinator are scarce. For example, a recent survey of Australian flowers using digital cameras calibrated for quantitatively assessing colour (Garcia, Greentree et al.), revealed a significant amount of within subject colour variability, which is very likely to be perceived by a pollinator. How then can an insect with relatively poor resolution cope with this variability and still recognise their target species in spite of slight changes in chromatic appearance? Or, can it be that the low resolution of the compound eye helps the insect by filtering out small variations thus facilitating object detection? The use of devices such as the mechano-optical device gives an insight into what a bee may see from a flower and helps us to answer these and other questions regarding the evolution of flower patterns in flowers.

By understanding how insect pollinators such as the honeybee see the world, it is also possible to improve current farming practices. For example, in agroecosystems using traditional farming techniques in developing countries, different plant species are planted along with economically important crops to reduce risk, manage pests and improve production (Altieri). In these

contexts, knowledge of the visual appearance of insect pollinated plants from the point of view of a bee may assist on species selection in order to increase the saliency of target plant species, thus potentially improving pollination rate and therefore increasing harvest yield.

The design of optical devices simulating invertebrate vision such as the mechano-optical device described here creates new and exciting possibilities to better understand how animals see and interact with the world. In the case of bees, this information is of high value due to the contribution of these important pollinators to agriculture and food production.

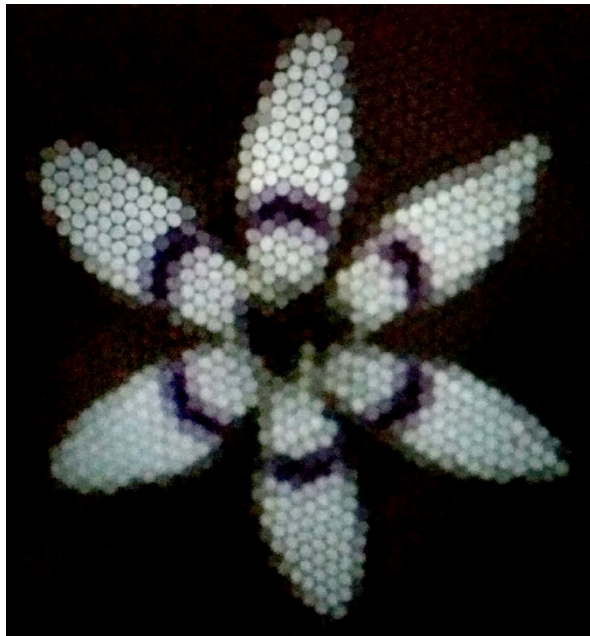


Figure 3. A bee's eye view of an Australian native *Wurmbea dioica* flower photographed through a mechano-optical device (Dyer and Williams 2005), showing the optics of an insect's compound eye can easily resolve details within a flower that may serve to improve recognition or orientation.

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Free-flying honeybees extrapolate relational size rules to sort successively visited artificial flowers in a realistic foraging situation

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Abstract Learning and applying relational concepts to solve novel tasks is considered an indicator of cognitive-like ability. It requires the abstraction of relational concepts to different objects independent to the physical nature of the individual objects. Recent research has revealed the honeybee's ability to rapidly learn and manipulate relations between visual stimuli such as 'same/different', 'above/below', or 'larger/smaller' despite having a miniature-sized brain. While honeybees can solve problems using rule-based relative size comparison, it remains unresolved as to whether bees can apply size rules when stimuli are encountered successively, which requires reliance on working memory for stimuli comparison. Additionally, the potential ability of bees to extrapolate acquired information to novel sizes beyond training sets remains to be investigated. We tested whether individual free-flying honeybees could learn 'larger/smaller' size rules when visual stimuli were presented successively, and whether

such rules could then be extrapolated to novel stimulus sizes. Honeybees were individually trained to a set of four sizes such that individual elements might be correct, or incorrect, depending upon the alternative stimulus. In a learning test, bees preferred the correct size relation for their respective learning group. Bees were also able to successfully extrapolate the learnt relation during transfer tests by maintaining the correct size relationships when considering either two smaller, or two larger, novel stimulus sizes. This performance demonstrates that an insect operating in a complex environment has sufficient cognitive capacity to learn rules that can be abstracted to novel problems. We discuss the possible learning mechanisms which allow their success.

Keywords Extrapolation · Concept learning · Working memory · Foraging · Cognition · *Apis mellifera*

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Introduction

Relational knowledge and conceptualization ability play an important role in human cognition (Domas et al. 2008; Halford et al. 2010). The application of learnt relational rules allows for 'flexibility and adaptability that are central to intelligent behaviour' (Wallis et al. 2001, p. 953) and is controlled by the prefrontal cortex in the primate brain among other executive functions (Miller et al. 2003; Wallis et al. 2001). Evidence of relational rule learning in non-human primates (Wallis et al. 2001), cetaceans (Kilian et al. 2003; Mercado et al. 2000), and birds (Blaisdell and Cook 2005; Pepperberg 1987) has revealed the capacity of vertebrates to learn concepts such as 'same' and 'different', and apply the respective rule to similar tasks within the boundary limits of a familiar learning set, or to interpolate.

Moreover, some animals [e.g. lemurs (Merritt et al. 2011), dolphins (Mercado et al. 2000), pigeons (Edwards et al. 1983; Spetch and Friedman 2003), and corvids (Wilson et al. 1985)] demonstrate a capacity to also apply rules to previously unseen stimuli beyond the limits of the original task, a process known as extrapolation. The capacity to extrapolate information is typically considered a harder task than interpolation as in both humans and monkeys, and interpolation tasks yield a higher frequency of correct choices than extrapolation tasks (Bülthoff and Edelman 1992; Logothetis et al. 1994; Srinivas and Schwoebel 1998).

Honeybees can learn a number of cognitive tasks with extended visual experience that were previously assumed to require a large mammalian brain (Chittka and Niven 2009; Dyer 2012). Bees are able to apply a number of relational rules and abstract concepts to forage efficiently (Avarguès-Weber and Giurfa 2013; Zhang 2006). For example, they can solve problems using rules such as ‘left/right’ (Zhang et al. 2000), ‘above/below’ (Avarguès-Weber et al. 2011, 2012), ‘same/different’ (Giurfa et al. 2001; Zhang et al. 2005), and numerical processing (Chittka and Geiger 1995; Gross et al. 2009) and can also choose to opt-out of difficult decisions (Perry and Barron 2013). The capacity of bees to learn and apply relational rules creates many new questions, not only in the field of cognitive neuroscience but also in a more ecological framework. Indeed, how such abilities might apply and be adaptive in natural contexts for an insect pollinator remains unknown (Avarguès-Weber and Giurfa 2013).

In some plant–pollinator systems, flower size plays a role in communicating the value of rewards to potential pollinators. There is evidence that some plant species are under selection pressure to use flower size as an indication of reward quality (Benitez-Vieyra et al. 2010, 2014), which may have resulted in larger flowers of some species rewarding pollinators with a higher quantity of food than smaller flowers (Ashman and Stanton 1991; Campbell et al. 1991; Cohen and Shmida 1993). For example, honeybees have previously been observed to have a preference for larger flowers of *Mimulus guttatus* (Martin 2004). While this phenomenon may be due to the higher visibility of larger target colours (Spaethe et al. 2001), it is clear that for bees, flower size has a significant role to play in plant–pollinator interactions. Other studies have demonstrated that bumblebees are able to learn to associate one large artificial flower with a reward and one smaller flower with an aversive substance (Blarer et al. 2002; Essenberg et al. 2015). These studies suggest that bees can discriminate between two sizes and learn to use specific size as an informative cue.

Avarguès-Weber et al. (2014) previously established that honeybees could learn the size rules of ‘larger than’ and ‘smaller than’. Through a series of conditioned choices to variable-sized stimuli presented on a vertically

orientated rotating screen, either a ‘larger than’ or ‘smaller than’ relationship could be learnt as the correct rule in independent groups of test bees. Honeybees could apply an acquired size rule to novel stimuli of different shape and colour, providing that such stimuli modulated contrast to the bee long wavelength ‘green’ receptor, thus driving an achromatic channel implicated in spatial learning tasks (Avarguès-Weber et al. 2014). However, experiments such as this, testing honeybee cognition and rule learning, often use rotating screens and Y-maze apparatuses, which potentially allow stimuli to be compared simultaneously (Srinivasan et al. 1993; Avarguès-Weber et al. 2011, 2014), and thus do not stress working memory.

Successive viewing is an important ecological factor to consider when designing experiments as honeybees forage in environments where flowers are often spatially separated. Due to the poor visual acuity of insect compound eyes (Land 1997; Srinivasan and Lehrer 1988; Srinivasan 2010; Williams et al. 2008), flowers often cannot be viewed and compared at exactly the same point in time. For this reason, it is likely that bees most often view flowers successively before making a decision about where to land (Dyer and Chittka 2004; Dyer and Neumeyer 2005). Successive comparison is considered more difficult than simultaneous comparison as it requires the loading of visual information about a given stimulus into working memory, then the subsequent recall of stimulus properties and comparison of this information to an alternative stimulus when encountered. In addition, within a complex environment it is potentially challenging for a bee to know the exact distance at which it is viewing a particular stimulus (Dyer and Griffiths 2012). Thus, visually choosing targets in complex environments using rule-based criteria may require the interaction between longer-term rule-based memory and shorter-term working memory (Brown and Demas 1994; Brown et al. 1997; Greggers and Menzel 1993; Giurfa et al. 2001; Zhang et al. 2005).

Considering the potential importance of size information for foraging bees in natural environments, we investigated whether free-flying honeybees could use a size rule in a foraging context by testing the potential capacity of bees to (i) extrapolate a size rule to stimuli outside of an initial training set and (ii) learn to use a relational size rule to solve a problem when stimulus options must be viewed successively. This will reveal how bees might use rule-based information for solving novel visual problems in natural contexts.

Materials and method

Study species and recruitment

Experiments were conducted with free-flying honeybee (*Apis mellifera*) foragers ($n = 20$). An *ad-lib* von Frisch-

type gravity feeder provided 5–30% sucrose to maintain a regular number of bees for use in the experiments (Whitney et al. 2008).

Apparatus and experimental method

An individual bee was recruited from the gravity feeder and trained to visit the experimental apparatus. Each bee was marked on the thorax for identification and could freely return to the hive when satiated (Dyer 2012). The apparatus consisted of a cube-shaped, multifaceted surface (35 cm width \times 25 cm height; Fig. 1). It was constructed such that four stimuli (two identically sized correct targets and two identically sized distractor stimuli) could be presented on pseudo-random sides of the four vertical faces during each bout (one return visit from apparatus to hive by a bee), but could not be viewed simultaneously by free-flying bees. For example, a bout could consist of 3 versus 4 and then 5 versus 6 in the next bout. Two sets of baffles on each face of the apparatus obscured the view of stimuli except when viewed front on; thus, bees were unable to view more than one stimulus at a time (Fig. 1). The cube was covered with laminated grey card that acted as a neutral adaptation background for colour modelling (Online resource 1; Fig S1; Table S1). Individual stimuli were placed on each vertical side of the cube, 3 cm above a landing platform which was used to place either a 10 μ l drop of sucrose or quinine during training (Fig. 1). This kept the distance between the platform and stimulus edge constant independent of stimulus size. The apparatus could be freely rotated on a ball-bearing pivot base to exclude potential position orientation cues.

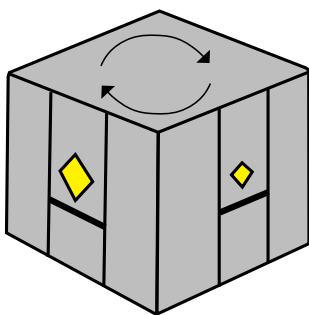


Fig. 1 The rotating successive stimulus presentation apparatus used during the experiments. The cube had four vertical faces, which each displayed one stimulus and had a landing platform. Each vertical face also had two sets of baffles (position of *vertical black lines* on cube) such that a bee could only view one stimulus at a time. The schematic diagram shows two of the vertical faces, while the other two faces are on the far side of the cube. The *arrows on top* of the diagram illustrate that the cube could be freely rotated and that bees could also fly around the cube to successively view each of the four faces, each presenting a visual stimulus

We used 50% sucrose solution to reward a bee for a correct choice, while aqueous 60 mM quinine HCl was used as an aversive substance for an incorrect choice to promote attentional learning in bees (Avarguès-Weber et al. 2010). Each bee was initially pre-trained to land on platforms without visual stimuli present on the apparatus, so as to associate each of the four sides of the apparatus with a potential sucrose reward. This process took bees between 30 and 60 min to learn. Once each bee was able to land on all sides of the apparatus, the visual training stimuli were introduced.

Stimuli

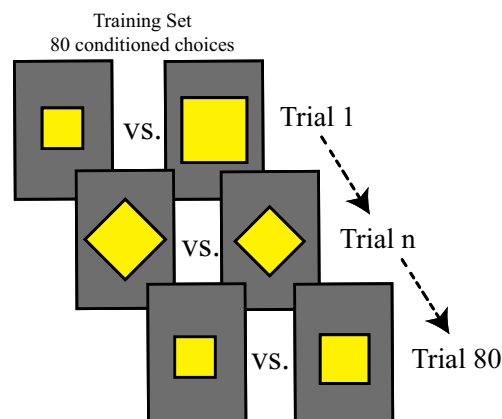
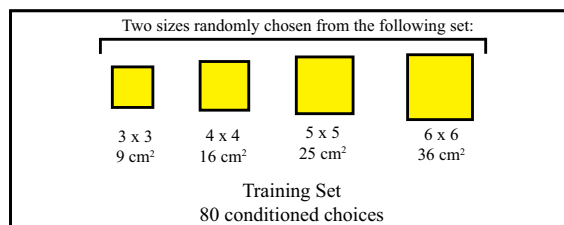
Training and test stimuli (artificial flowers) consisted of square card, covered with 80- μ m Lowell laminate, appearing ‘yellow’ to a human observer (Online resource 1; Fig S1; Table S1). The stimuli were of a biologically relevant size range considering the flower sizes a foraging bee may typically encounter (Guldberg and Atsatt 1975; Dyer 1996; Avarguès-Weber et al. 2014). In the learning phase and learning test, side edges ranged from 3 to 6 cm, yielding four different possible sizes (Fig. 2). Side edges 1 and 2 cm, or 7 and 8 cm were only used for the respective transfer tests (Fig. 2). To ensure the task was potentially consistent with rule-based learning, stimuli were pseudo-randomly changed by both size and orientation (orientation was kept constant for all stimuli in each bout). Stimuli were thus presented as squares or diamonds by orientating them by 45° between bouts in a pseudo-random fashion to further promote potential rule learning (Avarguès-Weber et al. 2014). Bees were exposed to squares or diamonds approximately 50% of the time each, and there was no difference in the correct choices made when stimuli were either diamond or square shaped.

Training and testing phases

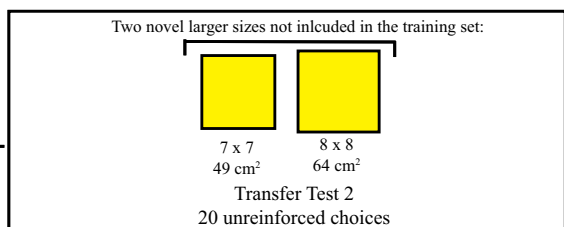
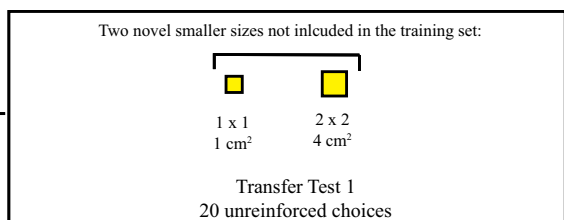
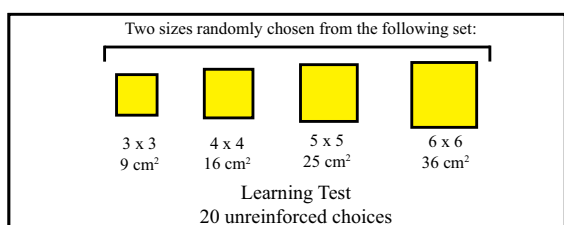
A counterbalanced design was used for this experiment, where one group of bees was trained to associate relatively larger-sized stimuli with a reward ($n = 10$), while a second group of bees was trained to associate relatively smaller-sized stimuli with a reward ($n = 10$). Testing order was pseudo-randomized between groups. To exclude olfactory scent-marking cues, stimuli and apparatus were cleaned with 30% ethanol solution and then water between each bout and test (Avarguès-Weber et al. 2010). In addition, stimuli were replaced if touched by a bee.



The experiment consisted of four phases (Fig. 2): (i) during the initial learning phase, bees were presented with the training set of stimuli (side edges: 3, 4, 5, 6 cm; Fig. 2) and each correct or incorrect response was recorded for 80 choices (tasting substance on platforms), which is a

Learning Phase



Testing Phases



 OR  pseudo-randomly between bouts and tests

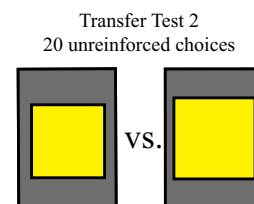
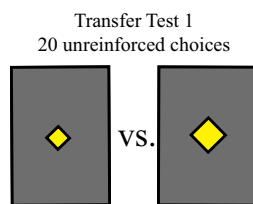
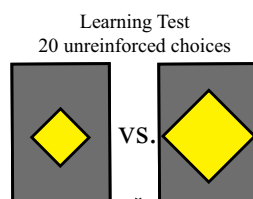


Fig. 2 The learning phase conducted over 80 conditioned choices with stimuli presented from the training set of four sizes (size edges: 3, 4, 5, 6 cm). The testing phase consisted of three tests: the learning test conducted over 20 unreinforced choices (side edges: 3, 4, 5, 6 cm), transfer test 1 conducted over 20 unreinforced choices with stimuli smaller than the training set (side edges: 1, 2 cm), and transfer

test 2 conducted over 20 unreinforced choices with stimuli larger than the training set (side edges: 7, 8 cm). Dimensions and surface area of all stimuli are shown. On the right size of the figure, a representation of how the learning and testing phases would appear in sequence with rotating stimuli is provided

training length previously established for size learning in honeybees (Avarguès-Weber et al. 2014). In the learning phase, the nature of a given stimulus was ambiguous as correct or incorrect since it depended on the size of the other stimulus presented within a given trial (Fig. 2). Each

time a bee made a correct choice and landed to drink the sucrose it was collected from the platform with a Plexiglas spoon and held above the apparatus (completely obscuring any view of the stimuli), while the area was cleaned and the apparatus was rotated. Bees generally made between 3 and

6 correct choices during each bout before they became satiated and returned to the hive. When a bee made an incorrect choice, it was allowed to continue flying around the apparatus without rotation of the sides until a correct choice was made. Bees returned from the hive within 5–10 min during which time the apparatus was cleaned, new stimulus sizes were placed on the sides, and drops of sucrose and quinine were replenished. After bees had made 80 choices, they were collected onto a Plexiglas spoon with sucrose and drank until satiated and returned to the hive so the testing stimuli could be introduced. Bees could then return the experiment. Two bees which took more than one day to complete the training and testing were given 20 additional rewarded refresher choices during the learning phase on the second day.

(ii) Following the learning phase, we conducted a learning test to determine whether honeybees had learnt the size rule. The learning test was an unreinforced phase used to evaluate bee performance without further reinforcement. Water was used in place of sucrose or quinine in all unreinforced tests. We recorded 20 unreinforced choices (any touch of the platform) for each bee which was scored as the dependant variable for analysis. In this test, bees were presented with two identical small and two identical large stimuli randomly chosen from the initial training set (side edges: 3, 4, 5, 6 cm; Fig. 2). (iii, iv) Finally, two unreinforced transfer tests were conducted to determine whether knowledge acquired during the learning phase could be transferred to novel stimulus sizes. The order of these transfer tests was pseudo-randomized.

Transfer tests included stimuli that were smaller (side edges: 1 and 2 cm) or larger (side edges: 7 and 8 cm; Fig. 2) than the stimuli presented during training. The training and test stimuli were chosen in the context of the physiological limits of bee visual resolution (Srinivasan and Lehrer 1988), what sizes could feasibly be presented in the experiment, and what was a good representation of an ecologically relevant size range of flowers in a natural environment (Guldberg and Atsatt 1975; Dyer 1996). Non-rewarded transfer tests were conducted without rewarding or punishing outcomes for correct or incorrect choices. Each test lasted for 20 choices during a single bout (less than 5 min), and refresher training was also presented for one bout between all tests to maintain bee motivation as is typical for honeybee experiments (Avarguès-Weber et al. 2012, 2014). The orientation of the stimuli as square or diamond was randomly determined for all unreinforced tests.

Viewing distance of bees

Since the main experiment required a bee to fly freely around a cubed apparatus, it was only possible to make

observational estimates of distance of bees when decisions were made without potentially interfering with bee behaviour. Our observations suggested bees made their decisions at the same point, independent of stimulus size. To confirm this, we tested an additional bee where a camera was used to record the bee's distance relative to a known scale. We used the methods described above, while an Olympus EP3 digital camera with an Olympus 18 mm f2.8 lens recorded bee decisions. The camera was mounted on a tripod and recorded one face of the apparatus, where a calibrated background grid provided a size scale. It has previously been shown with this method that honeybees fly towards a distractor stimulus and make aborts at a set angle (Dyer et al. 2008). We used segmented stills from the video to quantify when the bee was stationary in flight while inspecting a stimulus, before aborting (Dyer et al. 2008) using the program ImageJ. For the training phase sizes, the bee inspected and turned away from stimuli at 4.9 ± 0.6 cm. During the transfer test to smaller stimulus sizes, the distance aborts occurred was 4.6 ± 0.4 cm, and during the transfer test to larger sizes the inspection distance was 4.8 ± 0.6 cm. Figure 3 and the supplementary video show the position of a bee just before aborting the choice of stimuli in the transfer test to smaller sizes (Fig. 3a), the training set (Fig. 3b), and the transfer test to larger sizes (Fig. 3c; Video S1). The use of aborts in analysing a bee's distance from the stimulus was only implemented in video analysis and not used for counting choices in the learning phase or tests.

Statistics

Learning phase

To test for the effect of training on bee performance (number of correct choices), data from the learning phase of 80 choices were grouped into eight blocks of ten choices and analysed with a generalized linear model (GLM) with a logit link function. To evaluate bee performance during the learning phase, we initially fitted a full model with trial block (ten choices) as a continuous predictor, and group as categorical predictor with two levels: larger and smaller, plus an interaction term between predictors with subject as a random factor to account for repeated measures. We then applied standard model selection by simplification, i.e. backward selection, (Zuur et al. 2009). The process began with the most complex model which included trial, group, and an interaction term. Model simplification was then performed by formulating simpler models after excluding the non-significant terms. The process continued until all remaining factors were significant. Furthermore, we validated the adequacy of the model using the Akaike information criteria (AIC) value.

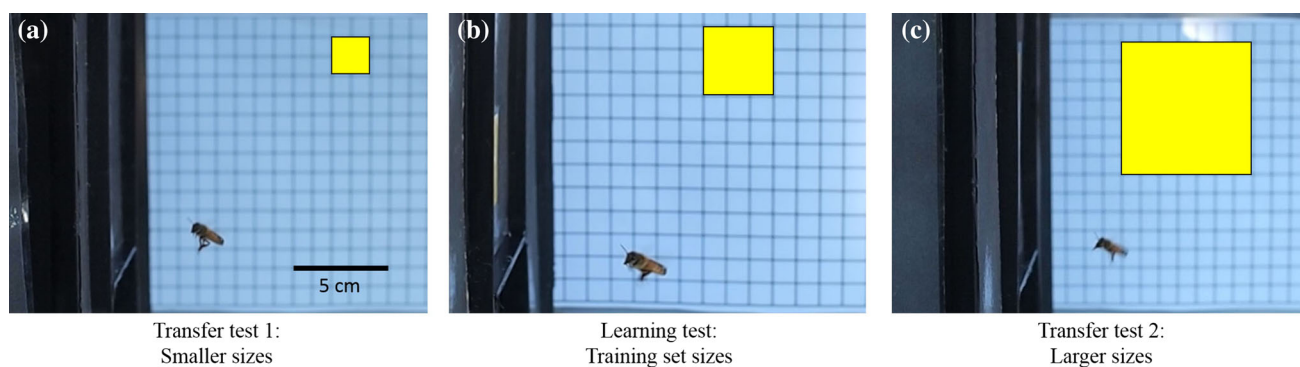


Fig. 3 Panels **a–c** show a honeybee just before aborting a stimulus choice. **a** The position of a bee viewing a 1×1 cm stimulus in the transfer test to smaller stimuli at 4.6 ± 0.4 cm [mean \pm standard error of the mean (s.e.m)]. **b** The position of a bee viewing a 3×3 cm stimulus in the training set at 4.9 ± 0.6 cm. **c** The position

of a bee viewing a 7×7 cm stimulus in the transfer test to larger sizes at 4.8 ± 0.6 cm. These stills are taken from the provided video footage (Supplementary video). The yellow squares overlaid on the still images show the size of the stimulus the bee is viewing relative to the image (colour figure online)

Testing phases

To determine whether bees were able to learn the size rules of ‘smaller than’ and ‘larger than’ for the learning test data, we estimated the mean of ‘correct’ choices, represented by the intercept of a generalized linear mixed model using bee response (correct or incorrect) as a (binary) predictor. To account for the repeated measurements, we included subjects as a random factor in the model.

To evaluate whether bees could extrapolate the rules to novel sizes which were smaller or larger than the training set, the two unreinforced transfer tests [transfer test 1 (smaller sizes); transfer test 2 (larger sizes)] were analysed with the same statistic. These statistical tests and models were performed on the R environment for statistical analysis (R Core Team 2016).

Consistency of tests

Separate analyses were performed to determine whether there were any asymmetries in the behavioural data that might be expected if an associative mechanism were used by bees to solve the different visual problems presented in respective tests. For this analysis, a mixed between–within-subjects ANOVA (split-plot) design was conducted with target size as a fixed factor with two levels (smaller; larger), and the tests as a within-subjects factor with three levels (learning; transfer 1; transfer 2). For this analysis, proportion data were converted into angles by means of an arcsine angular transformation. This test was conducted to determine whether the three tests (learning; transfer 1; transfer 2) differed in the number of correct choices across both groups (trained to larger/smaller). Tests were carried out in SPSS Statistics (SPSS 2011).

Results

Learning phase

We initially tested for the effect of trial block, group, and an interaction between trial and group using a generalized linear model (GLM). There was no effect of the interaction predictor between trial and group (deviance (G) = 0.163, df = 1, P = 0.687); therefore, this term was excluded from the subsequent model. We then tested for the main effects of trial and group. Both variables significantly predicted the number of correct choices (trial: G = 31.493, df = 1, P < 0.001; group: G = 6.025, df = 1, P = 0.014) so no further model simplification was possible.

Since groups trained to larger and smaller stimuli learnt at significantly different rates, we analysed these data separately. Subsequent analyses suggested a significant increase in the proportion of correct choices over the eight blocks of ten choices during the learning phase in bees trained to both smaller and larger sizes (smaller than: (G = 17.558, df = 1, P < 0.001); larger than: (G = 14.100, df = 1, P < 0.001); Fig. 4a). However, bees trained to smaller sizes learnt at a quicker rate as shown by the significance of the group factor. The apparent difference in learning rate appears to be in the earlier stages of acquisition, and after 80 learning events the respective groups appeared to reach a similar level of performance as evidenced by the asymptotic behaviour of the acquisition curves in Fig. 4a.

Testing phases

Figure 4b shows the results of the respective bee groups in the three tests conducted. In the learning test, bees trained

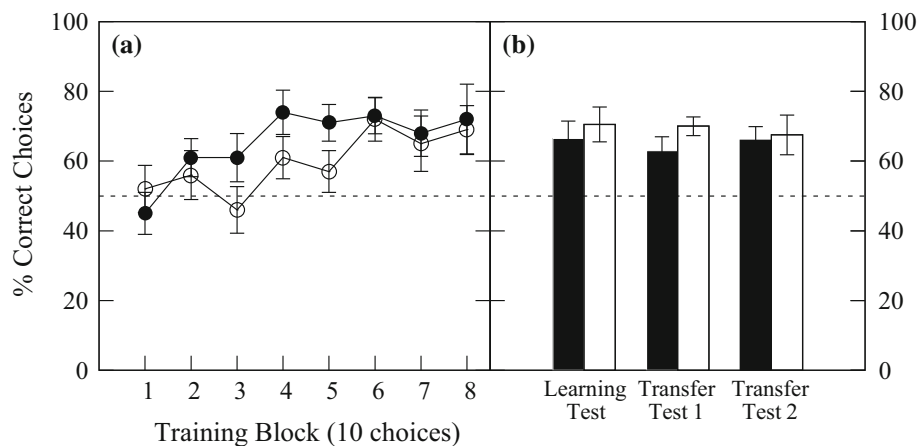


Fig. 4 Performance of bees during the learning (a) and test phases (b). Black dashed line at 50% indicates the chance level performance. **a** Performance during learning phase. Data points shown are mean \pm s.e.m. for each group (smaller trained is closed circle and larger trained is open circle) throughout appetitive and aversive learning. Ten bees were in each group. **b** Performance during the

to smaller sizes chose the correct stimulus in $66.5 \pm 5.1\%$ [mean \pm standard error of the mean (s.e.m)] of choices which was significantly different from chance level ($H_0 = 50\%$, $Z = 3.095$, $P < 0.010$; mean proportion of correct choices = 0.677, confidence intervals (CIs) 0.560, 0.787), while bees trained to larger sizes chose the correct stimulus in $70.0 \pm 5.1\%$ of choices ($Z = 3.766$, $P < 0.001$; mean proportion of correct choices = 0.712, CIs 0.601, 0.812). In the transfer test where bees were presented with stimuli smaller than the training set, bees trained to smaller sizes chose the correct stimulus in $63.0 \pm 4.0\%$ of choices ($Z = 3.254$, $P < 0.01$; mean proportion of correct choices = 0.632, CIs 0.548, 0.714), while bees trained to larger sizes chose the correct stimulus in $70.0 \pm 2.8\%$ of choices ($Z = 5.491$, $P < 0.001$; mean proportion of correct choices = 0.700, CIs 0.634, 0.762). In the transfer test where bees were presented with stimuli larger than the training set, bees trained to smaller sizes chose the correct stimulus in $66.0 \pm 3.9\%$ of choices ($Z = 3.962$, $P < 0.001$; mean proportion of correct choices = 0.662, CIs 0.579, 0.745), while bees trained to larger sizes chose the correct stimulus in $67.5 \pm 5.7\%$ of choices ($Z = 2.916$, $P < 0.010$; mean proportion of correct choices = 0.693, CIs 0.557, 0.816).

Consistency of tests

There was no significant main effect between the proportion of correct choices between bees trained to large and small sizes in the split-plot ANOVA analysis ($F_{18,1} = 0.727$, $P = 0.425$). Considering the within-subjects factor, we found no significant difference between the

three testing phases: learning test, transfer test 1, and transfer test 2. Bees trained to ‘smaller than’ are shown in *black* and bees trained to ‘larger than’ shown in *white*. Data shown are mean \pm s.e.m. for both treatment groups. Ten bees were used in each group for each test; thus, 20 bees were used in each test. All tests were significantly different from chance level ($P < 0.010$)

different tests ($F_{18,2} = 0.200$, $P = 0.820$), nor a significant interaction between tests and subjects ($F_{18,2} = 0.119$, $P = 0.888$). This shows bees reliably transferred the acquired rule from the learning set to both smaller and larger novel stimuli. This performance is consistent with the application of rule-based problem solving, but would not be expected if bees were using an associative mechanism to solve the different visual problems.

Discussion

In our experiment, honeybees demonstrated that they could learn the size rules of ‘larger/smaller’ when encountering stimuli in successive viewing conditions, as might be typical in natural settings where bees forage. Bees trained to smaller stimuli initially learnt at a quicker rate to those trained to larger stimuli, but following 80 trials of appetitive–aversive conditioning (Avarguès-Weber et al. 2010; Stach and Giurfa 2005), the final test outcomes were independent of training size (Fig. 4). Bees were able to extrapolate a learnt rule to previously unseen sizes from outside of the training set, and this capability was observed in both directions when including stimuli larger than and stimuli smaller than the training set sizes. Such a capacity was independent of whether bees were trained to small or large stimuli in respective learning groups. Additionally, there was no difference in performance between interpolation of size rules during the learning test and extrapolation of the rules during the transfer tests. This result, with our current method, is consistent with studies of pigeon interpolation and extrapolation, but is in contrast to what is

observed in humans (Spetch and Friedman 2003). Pigeons perform the tasks of interpolation and extrapolation at a similar level of success (accuracy and speed), while humans find extrapolation a more difficult task than interpolation but seem to generally perform better than pigeons (Spetch and Friedman 2003). The difference in success for humans performing interpolation and extrapolation of rotated views of objects could be due to the potential benefit of view-combination mechanisms. For example, when training subjects on two rotated images of an object, a novel interpolated image of a rotated object is more similar to the trained images than a novel extrapolated rotation (Friedman et al. 2005). In the current experiment, bees successfully extrapolated and interpolated the size tasks at the same level demonstrating that similar to pigeons, honeybees find the tasks of interpolation and extrapolation equally challenging.

While our study suggests that the learning and application of a size-based rule in bees is achieved by conceptual learning, there were other possible mechanisms that needed to be considered. These include the possible effects of an associative mechanism, peak shift, or retinotopic matching. The experimental design of our study rules out the effect of associative learning as a possible mechanism for bees to learn and extrapolate a size rule. Indeed, with an associative mechanism, bees could potentially learn to associate probabilities of reward with the frequencies of encountering stimuli of certain sizes. In this study, if the correct choice is ‘smaller than’, then stimuli from the smaller-sized half of the training set present rewards more often; even if occasionally such stimuli might be incorrect when presented against the very smallest size (Avarguès-Weber et al. 2014). Our study has been able to account for this potential confound through the use of the dual transfer tests. An associative mechanism predicts that when presented with stimuli from outside of the training set, bees should, if anything, choose the stimulus that is closest to the learning parameters encountered during the learning phase, while a conceptual rule-based solution to size perception predicts the opposite for one of the tests in order to maintain and apply the relative size rule encountered during training (Avarguès-Weber et al. 2014). Bees showed a significant preference for maintaining the correct size relationship acquired during the learning phase in both transfer tests, thus ruling out the possibility of an associative mechanism explaining results.

Another potential perceptual phenomenon that might explain how honeybees extrapolated learnt size relationships to stimuli outside of the size parameters encountered in the learning phase could be peak shift, an observed behavioural response bias arising from discrimination learning in which animals display a directional, but limited, preference for or avoidance of novel stimuli after

differential conditioning (Andrew et al. 2014; Chittka and Raine 2006; Hanson 1959; Leonard et al. 2011; Lynn et al. 2005; Martínez-Harms et al. 2014). The peak of the response gradient is sometimes moved slightly in a direction away from the less desired stimulus, such that a stimulus in a perceptual dimension further away from the desired stimulus is actually preferred by an animal (Leonard et al. 2011; Lynn et al. 2005; Martínez-Harms et al. 2014). We regard it as unlikely that peak shift could be a major explanation mediating bee decisions in our two respective transfer tests since peak shift is typically evidenced by a limited shift away from an actual target stimulus, so peak shift would probably not explain a capacity to extrapolate acquired learning to both ends of a learning spectrum. However, the testing of animals with such complex stimuli sets as used in the current study and the observed capacity to extrapolate information would benefit from further investigations to establish whether peak shift could operate at both ends of a continuum.

It was also important to consider whether bees may change their individual viewing angle to avoid the potentially challenging task of extrapolation and/or reduce the difficulty of the initial learning tasks, as may be predicted by retinotopic matching. Also known as the ‘template hypothesis’, retinotopic matching predicts that the visual template of a stimulus is stored in visual memory, preserving the retinotopic coordinates. Subsequently, when tested on novel stimuli bees should prefer options which overlap the greatest with the stimuli previously learnt in training (Dittmar et al. 2010; Efler and Ronacher 2000). The results of this study are not consistent with retinotopic matching as bees trained to ‘smaller than’, with 3×3 cm stimuli always being rewarding, chose 1×1 cm stimuli at a level significant from chance when shown against 2×2 cm stimuli in the extrapolation task. Retinotopic matching predicts that bees should choose the 2×2 cm stimuli during extrapolation as this overlaps more greatly with the rewarding size of 3×3 cm. Similarly, for bees trained to larger stimuli, with size 6×6 cm always rewarding, in an extrapolation test to larger stimulus sizes of 7×7 and 8×8 cm, bees should prefer 7×7 cm stimuli as they visually overlap more greatly with the rewarding size of 6×6 cm. However, this is not the case and bees demonstrated an understanding of the ‘larger than’ rule by choosing 8×8 cm stimuli at a level significantly higher than chance. Additionally, we can also rule out a retinotopic matching mechanism explaining results as bees were observed to make decisions at the same distance regardless of the size of stimuli, while a retinotopic type mechanism would predict that bees should change their viewing distance for different sizes in order to maintain a constant image size on the retina (Efler and Ronacher 2000). For example, quantification of the viewing distance

for a bee when viewing stimuli shows that individuals did not change their position when making a decision, despite even large changes in stimulus size (Fig. 3; Video S1).

While we can quantitatively and qualitatively demonstrate that bees were not using a simple associative mechanism or retinotopic matching to successfully learn and generalize a size rule, we cannot pinpoint the exact mechanism bees use to solve the problem. For example, bees may be using the colour ratio of each side (grey ratio coverage vs. yellow ratio coverage), or even the distance from the edge of the side to the stimulus; however, these would both still be an example of size rules, as comparing small yellow coverage to large yellow coverage, and large distance to the edge compared to a small distance to the edge are both still relational rules which requiring judging size of a colour or a distance. We think these two scenarios are unlikely compared to the varied sizes of the stimuli we used which were of the necessary green contrast essential for size rule learning (Avarguès-Weber et al. 2014). We suggest concept learning, defined as the learning of relations between objects rather than the absolute physical features (Perry et al. 2013), as a likely explanation for our results. The ability of bees to learn and generalize a rule to novel stimulus sizes may be due to an integration of multiple learning mechanisms too complex to pull apart into a single explanation to describe their success in this experiment.

During the learning phase, bees trained to the ‘smaller than’ rule learnt at a significantly quicker rate than those trained to the ‘larger than’ rule. After 80 conditioned trials during the learning phase, a split-plot ANOVA revealed that there was no significant effect of group (trained to smaller/larger) on test results. However, the asymmetry observed in the learning phase data is unexpected considering previous studies show that honeybees have a preference for larger flower sizes (Martin 2004). One explanation for this asymmetry could be due to individual differences among bees (Chittka et al. 2003). However, we minimized the impact of individual differences by using a long training regime to promote the bee’s ability to learn the task before testing. In addition, we had a consistent number of choices during the learning phase to make analysis robust. Therefore, by the first test there was no significant difference in performance between bees trained to larger or smaller sizes demonstrating that the asymmetry in the learning phase data did not impact the test results.

Our results parallel rule learning and extrapolative abilities in vertebrate species such as primates (Merritt et al. 2011; Spetch and Friedman 2003; Wallis et al. 2001), cetaceans (Kilian et al. 2003; Mercado et al. 2000), and birds (Blaisdell and Cook 2005; Edwards et al. 1983; Spetch and Friedman 2003). Classically, it has been thought that rule learning and extrapolative ability required

a prefrontal cortex, or an analogous structure in birds (Miller et al. 2003; Wallis et al. 2001). While the importance of the prefrontal cortex for rule learning and extrapolation has been demonstrated in other studies (Miller et al. 2003), our current work shows that the capacity to abstract can also occur in alternative brain structures for species lacking a prefrontal cortex, such as the honeybee. Learning and memory are believed to be linked to the mushroom bodies in arthropod brains (Capaldi et al. 1999; Devaud et al. 2015; Hammer and Menzel 1995; Hourcade et al. 2010; Strausfeld et al. 1995, 1998), a structure sharing analogies with the higher cortical centres of vertebrate brains (Farris 2008). There is also some evidence that ‘cortical-like’ processing needed for extrapolation may occur in the mushroom body of the arthropod brain (Chittka and Niven 2009; Srinivasan et al. 1993). Thus, as bees can perform a cognitive task like extrapolation, this would suggest that a task’s ecological relevance to an animal may be more important (Chittka and Niven 2009; Dyer 2012) than absolute brain size for mediating what functionality a brain can achieve.

Nevertheless, it has to be noted that, by comparison with other insects such as *Drosophila*, a classical insect model, honeybees (among other Hymenoptera), do possess particularly large and elaborate mushroom bodies. In honeybees, the calyces are doubled and expanded in relation to the lobes while receiving novel afferences from the visual part of the brain (Avarguès-Weber and Giurfa 2013). Such relative development started back with ancestral parasitoid wasps that shared spatial, visual, or olfactory learning facilities with honeybees (Farris and Schulmeister 2011). The social brain hypothesis (Dunbar 1998) is therefore questioned by insect studies and replaced by an adaptive role of mushroom bodies to allow for an increase in learning flexibility. However, elaborate mushroom bodies might not be sufficient to trigger conceptual learning abilities as to date there is no evidence of such capacity in close species (Brown and Sayde 2013; Moreno et al. 2012; Thompson and Plowright 2014), although more experimental effort is still required to understand the potential specificity of honeybees for conceptual learning among hymenoptera. For example, some evidence suggests that the capacity of the wasp, *Polistes fuscatus*, to recognize other wasp faces may rely on the processing of spatial relations between facial features (spatial configuration; Chittka and Dyer 2012; Sheehan and Tibbetts 2011) which would be a first step towards relational concept use (Avarguès-Weber and Giurfa 2013). Other animals like the archerfish, *Toxotes chatareus*, have been shown to recognize large numbers of human faces, despite lacking cortical processing (Newport et al. 2016), and recent work in the Neotropical ant, *Gigantiops destructor*, shows that acquired size relationships can be interpolated to solve a

novel visual task, even when stimuli are encountered sequentially (Beugnon and Macquart 2016). Further work on concept-type processing in insects of different brain sizes will add a lot to our understanding of what brain size and structures may be required for solving problems in complex environments.

It is important to note that our training and testing methodology allowed honeybees to move freely in their environment before making a landing decision. By using free-flying honeybees and not restricting viewing distance, we were able to make inferences about the use of relational rules in a natural foraging setting. Often problems occur in experiments when viewing distance is controlled and animals are unable to move freely, or choose the most appropriate distance to make decisions from (Salva et al. 2014). For example, in humans, limiting the field of vision can cause a significant reduction in perception and performance (Alfano and Michel 1990; Biersdorf et al. 1963). In our experiment with free-flying honeybees, viewing angles and distances were not mediated by our apparatus; thus, bees were able to choose the best distance for decision-making. Our approach further pushed the limits of what level of cognitive performance is accessible to a miniature insect brain.

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Authors Contributions Scarlett Howard was involved in the design of the experiment, data collection, data analysis and interpretation, and drafted the manuscript. Aurore Avarguès-Weber was involved in the experimental design, interpretation of data, and drafting of the manuscript. Jair Garcia was involved in the data analysis and drafting of the manuscript. Adrian Dyer was involved in the design of the experiment, data collection, data analysis, and drafting the manuscript. All authors gave final approval for submission.

Compliance with ethical standards

Conflict of interest There were no conflicts of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Research



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Perception of contextual size illusions by honeybees in restricted and unrestricted viewing conditions

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How different visual systems process images and make perceptual errors can inform us about cognitive and visual processes. One of the strongest geometric errors in perception is a misperception of size depending on the size of surrounding objects, known as the Ebbinghaus or Titchener illusion. The ability to perceive the Ebbinghaus illusion appears to vary dramatically among vertebrate species, and even populations, but this may depend on whether the viewing distance is restricted. We tested whether honeybees perceive contextual size illusions, and whether errors in perception of size differed under restricted and unrestricted viewing conditions. When the viewing distance was unrestricted, there was an effect of context on size perception and thus, similar to humans, honeybees perceived contrast size illusions. However, when the viewing distance was restricted, bees were able to judge absolute size accurately and did not succumb to visual illusions, despite differing contextual information. Our results show that accurate size perception depends on viewing conditions, and thus may explain the wide variation in previously reported findings across species. These results provide insight into the evolution of visual mechanisms across vertebrate and invertebrate taxa, and suggest convergent evolution of a visual processing solution.

1. Introduction

Our visual system allows us to process and assess our environment by providing information such as object size, shape, texture, colour and movement [1]. Visual illusions, classified as errors of perception, are informative for understanding variation in visual processing in both human and non-human animals [1]. One illusion which has been studied extensively in humans is the Ebbinghaus illusion (e.g. [2,3]). It is considered one of the strongest geometric illusions that humans perceive [1,4–6] and occurs where environmental context causes an object to appear relatively larger when surrounded by smaller objects, or relatively smaller when surrounded by larger objects [1,4,7] (figure 1). Humans generally perceive the world using global processing, which is the tendency to process the overall image of a scene rather than separately processing the individual elements which form it, which is known as local processing [8]. Global processing has been proposed to promote the perception of illusions, while local processing does not [1].

The ability to perceive contextual size illusions varies across vertebrates. Among those species currently known to be able to perceive the Ebbinghaus illusion are bottlenose dolphins [9], retdail splitfins [10], bower birds [11,12] and domestic chicks [13]. Baboons, however, do not perceive this illusion,

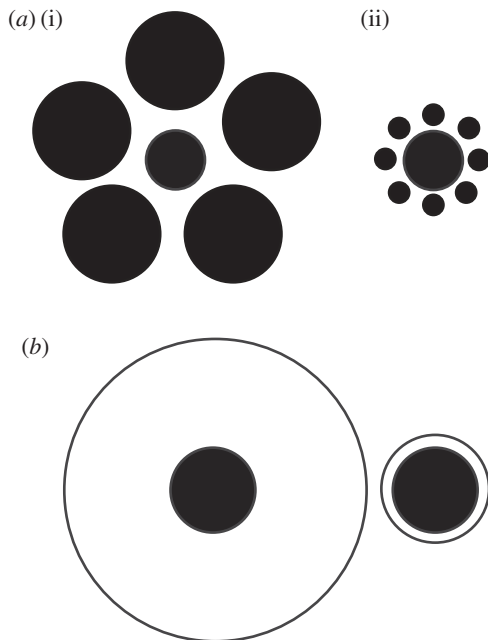


Figure 1. (a) A well-known example of the Ebbinghaus or Titchener circle illusion: two identical central targets are made to look smaller (i) when surrounded by large, distant circles (inducers) than when surrounded by small and close inducers (ii). (b) A representation of the Delboeuf Illusion: a larger circle (annulus) surrounds a central target, resulting in it appearing smaller than when surrounded by a smaller annulus [7].

thereby allowing them to accurately judge size regardless of context [6]. Interestingly, some species such as pigeons, bantams and domestic dogs perceive the opposite illusion, an assimilation illusion, where the central target size is perceived as being closer in size to the inducers which surround it [14–16]. Remarkably, not only does the Ebbinghaus illusion vary between species, but also within a single species. The Himba, an isolated remote human culture from northern Namibia, experience a strongly reduced effect of the size illusion compared with Western and urbanized populations [17–19].

Similar in nature to the Ebbinghaus illusion is the Delboeuf illusion [7] (figure 1*b*). The Delboeuf illusion relies on the misperception of size due to context [7]. A well-known example of this illusion is the tendency for identical meal portions to look smaller on a large plate and larger on a small plate [20,21]. Humans, chimpanzees [20,22], capuchin monkeys and rhesus monkeys [23] are vulnerable to this illusion, while domestic dogs are not [16,24]. In humans, this size illusion is thought to involve region V1 cortical representations of target size and context [25]. The differences regarding susceptibility to size illusions observed between species is potentially due to the ability of species to process visual images locally or globally [8], as baboons and Himba people do not perceive the illusion [6,17] and demonstrate a local precedence [17,18,26]. Interestingly, pigeons can flexibly shift between local and global processing [27], and, as mentioned, perceive the illusion as an assimilation illusion [15].

Another potential explanation for the differences in perceiving these size illusions is the variance in testing methods for respective studies, specifically the restriction of the viewing distance (as discussed in [10]). For example, studies on pigeons, bantams and domestic dogs required the participants to touch the correct stimulus with their nose or beak (dogs [16,24]; birds [14,15]), forcing subjects to view illusions at a close range [10]; and for baboons, the

viewing distance was restricted to 49 cm away from the screen displaying illusions [6]. Indeed, in humans, the Ebbinghaus illusion is reduced or reversed to an assimilation illusion when participants are forced to view the illusions at close range [28,29]. Thus, it appears that promoting a restricted distance on animals and humans may have a significant impact on whether size illusions are able to be perceived, although this has not been formally evaluated using a within-species study.

The honeybee is an important model species for testing visual and cognitive tasks due to the readily accessible way in which individuals can be trained [30–34], which permits high-value comparative analyses to vertebrate systems [35]. Honeybees can accurately discriminate stimulus sizes when presented on homogeneous backgrounds, and have the capacity to learn and apply ‘larger/smaller’ size rules [36,37]. While this demonstrates sophisticated visual cognition in a miniature brain with regard to size perception, stimuli in both previous size discrimination experiments were presented on a background of consistent colour, shape and size [36,37]. Thus, the bee’s ability to judge size in variable contexts remains unresolved; yet bees forage in complex, dynamic environments where the context in which flowers are encountered often changes. Honeybees express a global preference [38] when processing complex stimuli made up of multiple elements, and may therefore be sensitive to size illusions based on variation of the contextual surrounding cues.

In this study, we use contextual size illusions based on the Delboeuf illusion to determine how context and self-regulation of the viewing distance may impact a bee’s ability to accurately judge size. We consequently trained bees to choose larger- or smaller-sized stimuli, and tested contextual size judgement considering either restricted or unrestricted viewing conditions by employing stimuli potentially promoting size illusions.

2. Material and method

(a) Study site and species

Experiments were conducted at the University of Melbourne between April 2015 and May 2017. Free-flying *Apis mellifera* foragers (experiment 1: $n = 10$; experiment 2: $n = 10$) were marked with a coloured mark on the thorax to identify individuals used in respective experiments [38].

(b) Experimental procedure

Training and test stimuli were composed of a central black square target presented on a white square acting as the surrounding annulus/inducer (figure 2). All stimuli were covered with 80 μm Lowell laminate. One bee was tested at a time during the training and testing phases. A counterbalanced design was used for both the unrestricted and restricted viewing distance experiments (see below), where in each experiment one group of bees was trained to associate larger stimulus sizes with a reward ($n = 5$), while a second group was trained to associate smaller stimulus sizes with a reward ($n = 5$), on a background of constant size. Previous work established that bees learn either size relation to a similar performance level [36,37]. Thus, the pseudorandomized counterbalance was done to exclude any potential preference effects on the test results. We used a rotating screen apparatus to promote an unrestricted viewing condition and a Y-maze apparatus to create the restricted viewing condition. Previous work has demonstrated

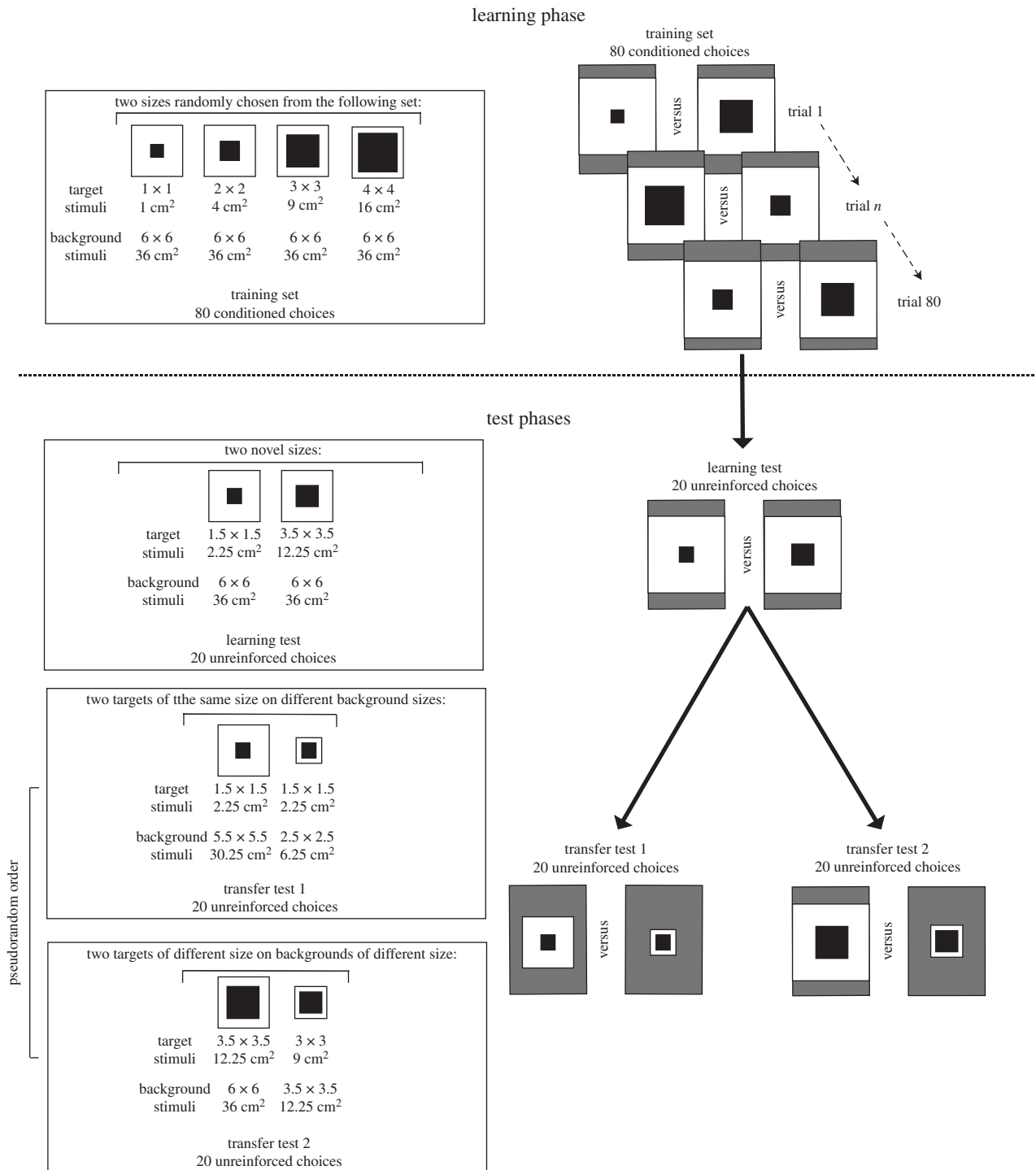


Figure 2. The stimuli used for the training and testing phases of both experiments showing the dimensions and surface area of target and background stimuli. In the learning phase, 80 appetitive and aversive choices were conducted with four stimulus sizes (side edges: 1, 2, 3, 4 cm) presented on a consistent background (side edge: 6 cm). Two different sizes of target stimuli were simultaneously presented to bees during the learning and test phases. The unreinforced learning test presented bees with novel sizes (side edges: 1.5, 3.5 cm). In transfer test 1, two central targets of the same size (side edges: 1.5 cm) were presented to bees on a background of different sizes (side edges: 2.5, 5.5 cm) to create the effect of a visual illusion where the central target on the larger background appeared smaller in context, and the central target on the smaller background appeared larger in context. In transfer test 2, a larger central target (side edge: 3.5 cm) was displayed on a larger background (side edge: 6 cm), while a smaller central target (side edge: 3 cm) was displayed on a background which was small (side edge: 3.5 cm) in order to create the effect of the smaller central target appearing larger and the larger central target appearing smaller due to the surrounding context.

that there is no significant difference in results for processing of complex visual patterns (non-illusionary stimuli) between a rotating screen and Y-maze [39].

(i) Experiment 1: unrestricted viewing distance

Apparatus

Honeybees were trained to visit a vertical rotating screen made of grey Plexiglas, 50 cm in diameter (figure 3*a*). By using this

screen, the spatial arrangement of stimulus choices could be randomly changed, thus excluding positional cues. Stimuli were presented vertically on 6 × 8 cm grey Plexiglas hangers with a landing platform attached below the presentation area. Hangers and surrounding screen areas were washed with 30% ethanol between foraging bouts and before each test to prevent the use of olfactory cues. Consistent with protocol for the rotating screen [39], four stimuli (two identical correct stimuli; two identical incorrect stimuli) were presented simultaneously above

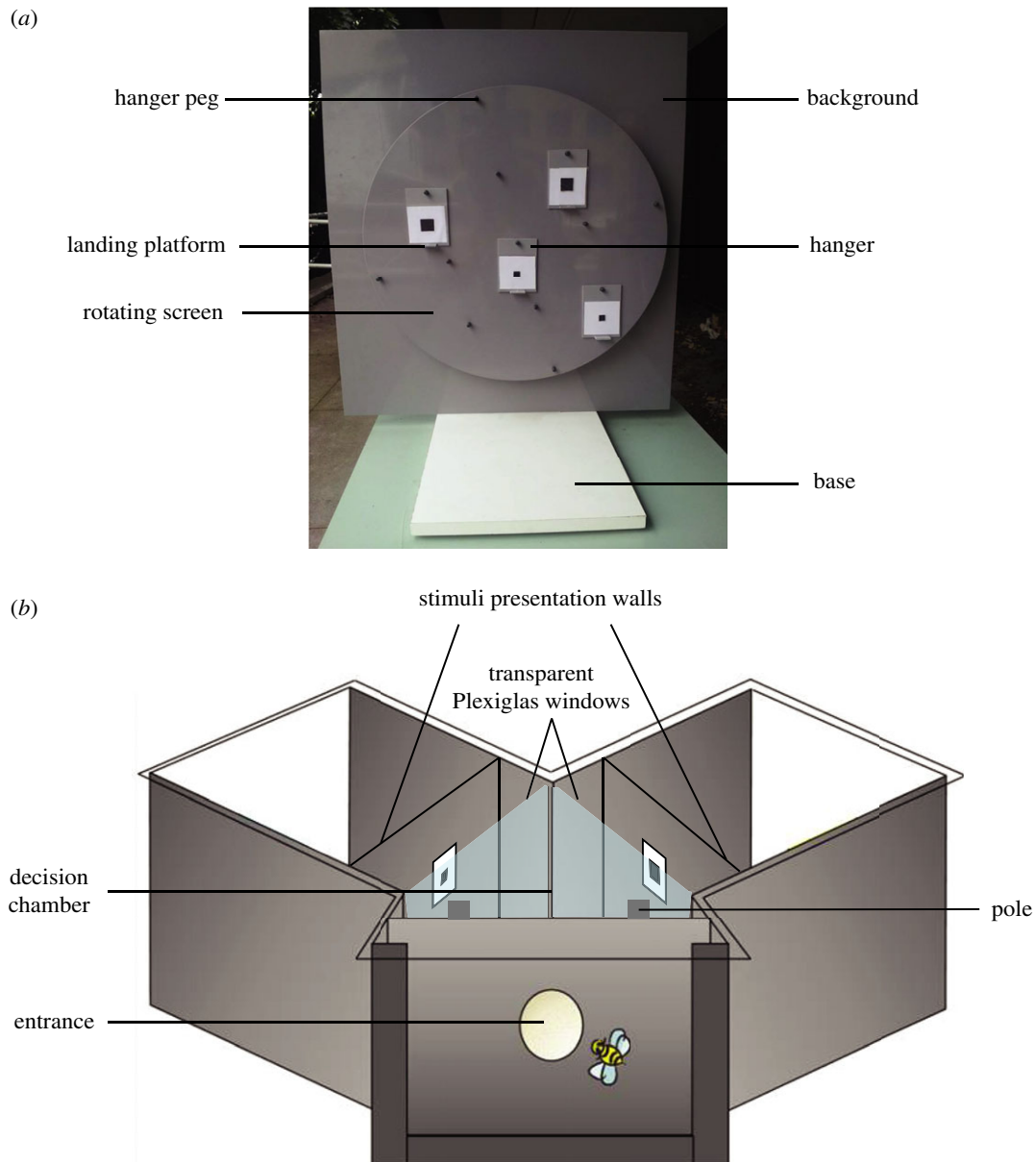


Figure 3. (a) An image of the rotating screen used for Experiment 1 with labels to show basic parts of the apparatus. (b) A diagram of the Y-maze used in Experiment 2 with labels showing the basic parts of the apparatus. A bee enters through the small hole into the decision chamber where it is presented with two stimuli behind Plexiglas windows and must make a decision on which pole to land on for a reward. (Online version in colour.)

landing platforms on the hangers, which could be positioned in different random spatial positions and were randomly changed between choices [36].

Procedure

Bees were first trained to land on platforms without stimuli present. Once individual bees were able to land on the platforms, the training stimuli were introduced. By using the rotating screen, bees were able to make choices at any distance from the stimuli, flying as far away or as close as they elected prior to making a decision on where to land. By using this design, we intentionally did not control the viewing distance for bees, but allowed individuals to self-regulate their distance prior to making a decision (figure 3*a*).

The experiment consisted of four parts (figure 2). During the learning phase, the target stimuli varied in size (side edges: 1, 2, 3, 4 cm; figure 2) but were displayed on a background of consistent size (side edge: 6 cm). Bees were presented with two different target sizes during each bout (return from hive to apparatus) and we recorded each correct or incorrect response for a total of 80 appetitive and aversive choices. Stimulus sizes and positions were pseudorandomized and changed between bouts. The

sizes of the target stimuli were determined by rolling a die and stimulus positions on the rotating screen were determined by rotating the screen. Stimulus sizes always maintained the size rule for respective groups. A 10 μ l drop of either a 50% sucrose solution (correct choice) or a 60 mM quinine solution (incorrect choice) were used as rewarding and punishing outcomes, respectively, during the training phase (figure 2), as this promotes enhanced visual discrimination performances in free-flying honeybees [40]. The procedure followed the logic of size-rule learning [36] where target stimulus sizes were pseudorandomly allocated such that the exact nature of a given stimulus (e.g. side edges: 2 or 3 cm) was ambiguous as correct or incorrect depending on whether it was larger or smaller in size compared with the alternative stimulus presented in a given phase of the conditioning. This training protocol is a form of differential conditioning which promotes processing of the entire image [38,41,42]. Once a bee made a correct choice, it was collected onto a Plexiglas spoon providing 50% sucrose solution and placed behind an opaque barrier 1 m away from the screen while stimulus sizes and positions were pseudorandomly changed, and platforms and surrounding areas were cleaned. If a bee made an incorrect choice, it would taste the bitter quinine

solution and then was allowed to continue making choices until a correct choice was made, at which point the same procedure for a correct choice would be followed.

Following the learning phase, we conducted an unreinforced learning test for 20 choices to determine if honeybees had learnt the relational size rule (figure 2). Stimulus target sizes were chosen to be novel and interpolated from the training set (side edges: target, 1.5, 3.5 cm; background, 6 cm). Novel sizes were used to determine if rule learning, rather than a simple associative mechanism [36], was responsible for observed performance.

Following the learning test, two transfer tests were conducted in pseudorandom order. The role of the two sets of stimuli in each of the transfer tests was to induce the potential perception of an illusionary image as demonstrated in other animal models described above (figures 1 and 2). In transfer test 1, bees were presented with central targets of the same size (side edges: 1.5 cm), displayed on backgrounds of different sizes (side edges: 2.5, 5.5 cm). We hypothesized that if bees could accurately judge size regardless of context, there would be no significant difference in the number of choices between the two stimuli. However, if bees perceived an illusion and were trained to associate smaller-sized stimuli with a reward, they should choose the central target on the larger background, as it looks smaller in context. Similarly, if the bees trained to associate larger stimuli with a reward perceived an illusion, they should choose the central target on the smaller background as it looks larger in context. In transfer test 2, bees were presented with a small central target on a small background (side edges: target: 3 cm; background 3.5 cm) against a larger central target on a larger background (side edges: target: 3.5 cm; background 6 cm). This test was designed to determine the potential strength of the illusion in bees. Indeed, in the first transfer test, the target size was identical and therefore ambiguous for the bees, which could potentially facilitate the perception of the illusion. In the second transfer test, a difference in sizes between targets is maintained but might be compensated by the illusion triggered by the difference in the background sizes. In this test, if bees trained to both smaller and larger rules did not perceive an illusion, they should choose the respective stimulus in which the central target maintains the correct size relationship, regardless of the size of the background. However, if bees perceived a size illusion, those trained to a 'smaller than' rule should choose the stimulus on the larger background, as it looks smaller in context, and bees trained to a 'larger than' rule should choose the stimulus on the smaller background as it appears larger in context [43–45]. If bees perceived an assimilation illusion, central targets would appear more similar in size to the surroundings (inducer). Thus bees in the respective transfer tests trained to larger sizes would choose the stimulus with a larger surrounding as the target size also appeared larger, and *vice versa* for bees trained to smaller sizes.

During all three tests (learning test, transfer test 1 and transfer test 2) stimuli were presented without rewarding or punishing outcomes for respective choices, as we used water drops as a neutral substance for tests. We recorded 20 choices (touches of a platform) for each of the three tests. The sequence of the transfer tests was randomized and refresher choices were given between tests for the duration of one bout to maintain bee motivation [36,46].

(ii) Experiment 2: restricted viewing distance

The procedure for experiment 2, testing potential illusionary perception with restricted viewing distance, was largely the same as experiment 1 except for the apparatus mediating viewing conditions. Honeybees were trained to enter a Y-maze (figure 3*b*; as described in [47,48]). Stimuli were presented on grey backgrounds located 6 cm away from the decision lines. At the position of the decision lines, a transparent Plexiglas barrier was placed such that individual bees could view stimuli at the

set distance of 6 cm but were unable to fly any closer, thus restricting their viewing distance to 6 cm (potential maximum distance from the entrance hole is 12 cm). Sucrose or quinine was placed on respective poles directly in front of the Plexiglas barrier so that bees would learn to associate stimuli with either a reward or punishment. Poles were replaced when touched by a bee and cleaned with ethanol to exclude olfactory cues. Two stimuli, one correct and one incorrect, were presented simultaneously in each arm of the Y-maze on the grey plastic background. The size and side of correct and incorrect stimuli were randomly changed between choices. If a bee made an incorrect choice and started to imbibe the quinine, it was allowed to fly to the pole in front of the correct stimulus to collect sucrose to maintain motivation; but only the first choice was recorded. Once the bee had finished drinking the sucrose, it was free to fly back to the hive or make another decision by re-entering the maze. During the unreinforced tests, a drop of water was placed on each of the poles placed in front of the stimuli. Twenty choices (touches of the poles) were recorded.

(c) Statistical analysis

(i) Learning phase:

To test for the effect of training on bee performance (number of correct choices), data from the learning phase of 80 choices were analysed with a generalized linear mixed-effects model (GLMM) with a binomial distribution using the 'glmer' package within the R environment for statistical analysis [49]. We initially fitted a full model with trial number as a continuous predictor, and group as a categorical predictor with two levels (trained to larger or smaller), plus an interaction term between predictors with subject as a random factor to account for repeated choices of individual bees. As the interaction term was not statistically significant in experiment 2, it was excluded from the final model.

(ii) Testing phase:

To determine whether bees were able to learn the size rules of 'smaller than' and 'larger than' from the learning test data, we employed a GLMM including the intercept term as a fixed factor and subject as a random term. The proportion of 'correct' choices (MPCC) recorded from the learning tests were used as a response variable in the model. The Wald statistic (z) tested if the mean proportion of correct choices recorded from the learning test, represented by the coefficient of the intercept term, was significantly different from chance expectation (i.e. 50% of correct choices).

The two unreinforced transfer tests (transfer test 1: targets of the same size on different background sizes; transfer test 2: large target on large background and small target on small background) were analysed using the same analyses employed for the learning test. For this analysis a 'correct choice' was defined as the choice for a stimulus suggesting the perception of a contrast illusion.

(iii) Comparison between experiments:

To determine if there was a significant difference between the learning curve functions of the learning phases in experiments 1 and 2, we used a GLMM with bee response (correct or incorrect) as a binary predictor, and trial number, viewing condition and the interaction term as fixed factors. Subject (bee) was included as a random factor. We also tested for differences between the pairs of unreinforced tests (learning test, transfer test 1 and transfer test 2) using the same model structure with bee response as a predictor and experiment (unrestricted or restricted viewing distance) as a factor. All statistical analyses were performed in the R environment using the 'nlme' and 'mass' packages [49].

3. Results

(a) Experiment 1: unrestricted viewing distance

There was a significant increase in the number of correct choices made over the 80 conditioned choices during the learning phase (trial number: $z = 3.823$, $p < 0.001$; figure 4a), with a significant interaction between group and trial ($z = -2.087$, $p = 0.037$) and no significant effect of group ($z = 1.184$, $p = 0.236$). For individual bee performance see electronic supplementary material, figure S1.

In the learning test, bees consistently chose the correct stimulus in $66.5 \pm 3.0\%$ (mean \pm s.e. of the mean) of choices, significantly higher than chance expectation ($z = 4.577$, $p < 0.001$; mean proportion of correct choices (MPCC) = 0.665, 95% confidence intervals (CIs): 0.595, 0.731; figure 4b).

During transfer test 1 presenting target stimuli of identical sizes, bees chose the stimulus suggesting contrast illusionary perception in $63.0 \pm 3.8\%$ of choices, significantly higher than chance ($z = 2.592$, $p < 0.010$; MPCC = 0.630, CIs: 0.524, 0.725; figure 4b).

Likewise, in transfer test 2, bees presented with two central targets of different sizes on backgrounds of different sizes chose the contrast illusion stimulus in $64.7 \pm 4.1\%$ of choices ($z = 3.506$, $p < 0.001$; MPCC = 0.647, CIs: 0.565, 0.735; figure 4b). For individual bee performance see electronic supplementary material, figure S2.

Group was not a significant factor for any of the tests ($p > 0.05$ in all cases).

(b) Experiment 2: restricted viewing distance

As in experiment 1, there was a significant increase in the number of correct choices made over the 80 conditioned choices during the learning phase (trial number: $z = 5.411$, $p < 0.001$; figure 4a) and no difference between groups ($z = 0.321$, $p = 0.748$). For individual bee performance see electronic supplementary material, figure S3.

In the learning test, bees selected the correct stimulus in $66.5 \pm 2.0\%$ of trials. The mean number of correct choices was significantly different from chance ($z = 4.310$, $p < 0.001$; MPCC = 0.655, CIs: 0.587, 0.719; figure 4b).

During transfer test 1 presenting target stimuli of identical size on backgrounds of different sizes, bees chose the contrast illusion stimulus in $50.0 \pm 1.1\%$ of choices, which did not differ significantly from chance expectation ($z = 0.000$, $p = 0.944$; MPCC = 0.500, CIs: 0.431, 0.569; figure 4b).

During transfer test 2 presenting two central targets of different size on backgrounds of different size, bees chose the contrast illusion option based on their training group in $35.0 \pm 2.2\%$ of choices ($z = -4.176$, $p < 0.001$; MPCC = 0.350, CIs: 0.286, 0.418; figure 4b), thus choosing the correct relative target size in $65.0 \pm 2.2\%$ of choices regardless of the annulus size. For individual bee performance see electronic supplementary material, figure S4.

Group was not a significant factor for any of the tests ($p > 0.05$).

(c) Comparison of experiments

There was no significant difference between the slopes of the learning phase in experiments 1 and 2 (viewing condition \times trial number: $z = -1.749$, $p = 0.080$; figure 4a), and in the performance of bees ($z = -1.023$, $p = 0.306$; figure 4b) during the training phase, but there was a significant effect of trial on

bee performance ($z = 3.520$, $p < 0.001$). There was a significant difference between experiments in the results of transfer tests (transfer test 1: $Z = -2.406$, $p < 0.020$; transfer test 2: $Z = -5.824$, $p < 0.001$; figure 4b) as bees trained using the rotating screen perceived illusions in both tests, whereas bees trained using the Y-maze did not.

4. Discussion

While the ability to perceive size illusions varies across vertebrate species, it appears that the experimental method, specifically the restriction of the viewing distance, may influence results in some experiments [6,14–16,24]. We formally tested and compared the potential ability of honeybees to perceive size illusions under restricted or unrestricted viewing conditions. Bees in the unrestricted viewing conditions perceived contrast illusions, while independent bees under restricted viewing distance conditions did not exhibit choices consistent with the perception of an illusion. These results demonstrate that visual perception is influenced by the ability of bees to choose their own viewing distance and show that context is a relevant factor in accurate size discrimination.

Differences in perception can potentially be explained by the capacity of a species or individual to process visual cues locally or globally [6,8,26]. Local (or featural) processing seems to allow species to accurately judge size by ignoring surrounding information (inducers), while global processing allows the perception of illusions whereby the surrounding information is incorporated into the overall image [1,6]. Honeybees have demonstrated the ability to process both locally and globally, but do show a preference for global processing [38,50]. The honeybee's preference to process globally could explain why bees were able to perceive illusions in an unrestricted viewing context. Indeed, the current study shows that illusion effects are influenced by viewing conditions, and thus suggests that local–global processing effects observed in different animal species may be strongly influenced by viewing context.

Illusionary size perception in the unrestricted viewing condition may also be influenced by visual angle. Bees could have been mediating their distance during the transfer tests in the unrestricted viewing condition in order to place the white square surroundings at an equivalent visual angle, and thus choose the target with the larger or smaller visual angle [51] (see electronic supplementary material, S1). However, this is very unlikely due to the very large or very small distances and visual angles (below the minimum threshold for detection [52]) bees would need to view stimuli from to match the visual angles of the white surroundings. In addition, if bees were mediating visual angle to match background sizes, this would mean bees were 'fooled' into trying to match visual angles to make decisions on relative size. We could thus still conclude that context is a relevant factor for free-flying bees to judge size. Additionally, the ratio of white to black area could potentially have been a cue for bees; however, we consider this unlikely for three reasons. First, bees were trained to the difference in the local cues (targets) with a white background of consistent size, which promoted size-rule learning of the target [36,37]. Second, during the learning test bees would have needed to be able to discriminate a very small difference of 5.7% between black–white ratios for success in this experiment, which is unlikely in a rule-learning context. Finally, bees in both viewing conditions were trained using the same stimuli and

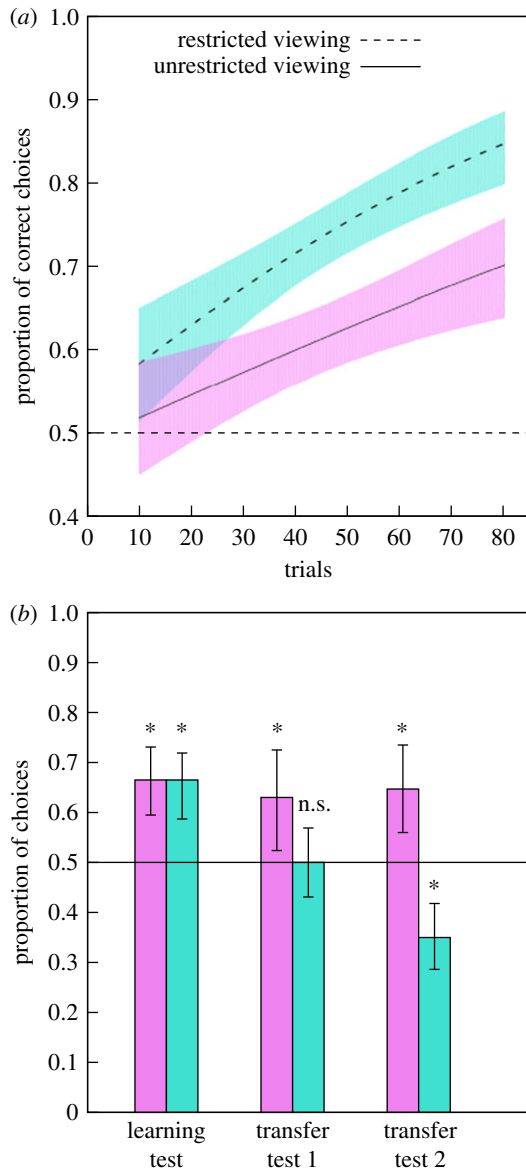


Figure 4. (a) Performance during the learning phase in experiments 1 and 2. Dashed line at 0.5 indicates the chance level performance. Solid black line indicates the line of best fit for data points in the unrestricted viewing condition and the dashed black line indicates the line of best fit for the restricted viewing condition. The surrounding violet (unrestricted condition; solid line) and blue (restricted condition; dashed line) areas indicate 95% CI boundaries. Increase in performance during the learning phase was significant for both experiments but learning regression lines were not significantly different from each other. (b) Performance during the three testing phases: learning test, transfer test 1, and transfer test 2 for bees in experiments 1 (violet, left bars) and 2 (blue, right bars). For the learning test, performance is measured by proportion of choices for the correct size option; for the transfer tests, performance is measured by the proportion of choices for the illusory option. Dashed line at 0.5 indicates chance level performance and * indicates performance significantly different from chance. Data shown are means \pm 95% CI boundaries for all three unreinforced tests. Ten bees were used for each test in each experiment. (Online version in colour.)

conditioning framework; however, the test results from the two viewing conditions differed significantly. The bees in the restricted viewing condition could not be using the white–black ratio, and so it seems likely that the bees in the unrestricted viewing condition were learning the same cues as those in the restricted condition.

Studying comparative perception of visual illusions allows us to make inferences about the evolution of the

visual sensory system. Parallels found across species for the ability to perceive similar illusions suggests a conserved or convergent visual processing solution [5,10]. In mammals and primates, current research suggests that the ability to perceive the Ebbinghaus illusion through specific perceptual mechanisms is due to the recent evolution of this trait [6,10,15]. Illusory perception in primates is potentially due to the neural substrate located in the neocortex, where the dorsal and ventral streams, two independent neural pathways, are responsible for visual awareness and action control [53]. In non-mammalian species, such as birds, these neural circuits are organized differently, perhaps due to evolutionary differences [54]. This may also have resulted in differences among species regarding the ability to perceive a size illusion and, additionally, the type of illusion which is perceived (contrast or assimilation) in the Ebbinghaus illusion [10]. However, as discussed, this may simply be due to differences in testing procedure [5,10]. Some research suggests that the perception of visual illusions is indeed a conserved ability in both ‘lower’ and ‘higher’ vertebrates. [5,10]. With the addition of honeybees to species with known perception of size illusions, we suggest that convergent evolution of a visual processing solution is more likely to be the explanation as to why we see this error of perception in both vertebrates and now an invertebrate. However, this hypothesis requires testing, particularly the ability of other invertebrate species to perceive the Ebbinghaus illusion.

Coupled with studies of other illusions perceived by bees, our research provides additional insight into the honeybee’s visual system and cognitive processing. Honeybees perceive illusory contours [55–57], the Benham illusion [58] and the Craik–O’Brien–Cornsweet illusion [59], which are spatial, movement and colour illusions, respectively. Illusory perception is potentially important for honeybee perception and assessment of the complex, dynamic environments in which they live. For example, perceiving the relative size of an object is important for assessing distance, thus manipulation of object size can impact distance estimation [1]. However, illusions may also be perceived because it is difficult to process all of the sensory information available in a complex environment. Focusing on a small number of reliable cues can be used to inform behaviour; thus, the information immediately surrounding an object of interest can result in the distortion of sensory cues, such as size [1]. Size perception is a classic problem in animal perception [6,36,37,60], and our new finding that viewing context promotes very different outcomes within species provides for new avenues for exploration in future studies.

Compliance with ethical standards. All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Data accessibility. Behavioural data: Dryad doi: <https://doi.org/10.5061/dryad.mc02p> [61].

Authors’ contributions. All authors contributed to experimental design. S.R.H. and A.G.D. collected the data. J.E.G. and S.R.H. analysed the data. S.R.H. wrote the first draft of the manuscript. All the authors contributed to interpretation of the data and editing the manuscript. All the authors gave their final approval for submission.

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TRACES: MOBILE EYE TRACKING CAPTURES USER SENSORY EXPERIENCE IN AN OUTDOOR WALKING TOUR ENVIRONMENT

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Abstract

This article explores the use of mobile eye tracking to provide insights on the dynamics of haptic (touch/sense) and visual experience. We created a digital cultural walking trail (TRACES), designing an app to explore user experiences of their environment, and as a way to reveal the multilayered interactions between places and technology. Using mobile eye tracker technology to trace a person's place-based engagements, we show how the design of tablet-friendly apps can enrich experience by guiding viewers through an immersive and interactive environment with valuable information. We also highlight how this activity may negatively impact on experience by demanding attention away from real world engagement.

Seeing the Context

We begin this article by framing our discussion within broader concerns about how knowledge is documented and circulates across complex digital environments, including developing new engagements with people and places at greater risk of marginalization and exclusion [1]. By drawing attention to interactions with the environment, we explore the sensory relationship between haptic and visual dimensions for improving understandings of historical context. We show how eye tracking recorded different ways in which a user viewed their environment through the haptic and visual features of our app.

Walking as a Form of Relational Thinking

We created a cultural walking trail (TRACES) using recent innovations in locative mobile media and mapping systems as part of a 2017 RMIT University-hosted symposium, *Connect/Disconnect: Experiences of Body and Place in the Networked City*. Performance artist Stelarc, in his keynote address noted how the body is “accelerated and augmented to perform beyond the boundaries of its skin and beyond the local space it inhabits” [2]. This suggests we are simultaneously elsewhere and here, our bodies already marked by excess through our everyday digital encounters.

We examined how digital mechanisms might enrich our experience with cultural and heritage material by identifying four key locations for interactive exploration: William Barak Building, Old Melbourne Gaol, Ngarara Place Indigenous Garden and the Fig Tree with Visible Roots. Users of the app were able to sequentially explore each site that was chosen to engage the user in detailed and historically changing narratives about the custodians of the land on which the site was situated, and the cultural and historical significance of that place from the perspectives of traditional owners. As a person approached each location, the media on their mobile device would be “triggered” through identified GPS-

coordinates by their geographic location. Layers of information would appear as augmented features on their device, highlighting a mix of factual and political content.

The app incorporated digital videos, Indigenous oral histories, images of material culture and user-generated content. The app also allowed us to capture a user's movements and location-based experiences along the trail, which users could share in visual and/or textual form through their social media networks (Instagram, Facebook, Twitter). We engaged technologies including the mobile applications Aurasma and Mobile Learning Academy and a Tobii2 eye tracker and documented the actions of one of our team members (SRH). We measured where her attention was focused, in what sequence, and for what period of time whilst using the app. We did not initially disclose the full purpose of our study to the participant, so as not to influence how she would use the app. She read the instructions provided on the app, responded to questions posed on the screen and followed the walking trail presented on the tablet's map, while the eye tracker captured her eye movements as she was engaged with the contextual environment. Through follow-up interviews and collating feedback recorded by the app, we were able to collect sensory data on how important sound, image, touch and movement were to her visual and interactive experience. Following Tim Ingold, we define the “sensory” as information received by an individual's senses through movement, hearing, sight, smell, touch, taste, intuition and equilibrium [3].

We found that touch, sound, seeing and reading text could be dynamically incorporated through the technology to increase concentration and immersion and to bring focus to an object/environment. The analyses provided insights on the connections between the haptic and visual experience of the user, suggesting pathways for future studies on such diverse factors as how to present content meaningfully, how to collect participant data to improve the design of educational software, what kind of data can be generated through an app to support environmental and historical literacy, how to understand the extent to which people retain, memorize and are affected by the information they are presented with in visual, textual, aural, oral and haptic forms.

Eye trackers have been used to understand the sensory impact of music [4] and virtual reality environments [5] and have elaborated on the kinds of data that can be extracted through the exhibition interactions of museum visitors, including what people stop to look at (fixations) and what can spontaneously be overlooked (saccades or blind spots), helping to form a connection (scan pattern) to identify viewer intentions [6]. Other research highlights ways in which environment design for tracking studies can be established to generate knowledge on participant engagement with artwork [7].

In our research, an eye tracker enabled us to interpret both qualitative and quantitative data. We were able to document a gaze path, which indicated the configuration of movement followed by the subject's eyes while experiencing signed and contextual information in one of TRACES's environments. Numeric representation of this experience revealed more than 450 information pathways over a 146 second period; we could capture how the subject was absorbing environmental information once the instructions to search for specific details on the app were read. Hence, the seeing pathways could be understood as the user's interpretation of contextual content or her response to questions presented on the app. Once we were able to match this quantitative data with the qualitative findings, we could determine that when the participant was asked to answer a question posed on the app, eye actions around the environment (which she was asked to search for the answer) were more frequent, faster and erratic. There were also moments when the eyes would scan over different features of the environment with greater and lesser intensity (Fig. 1). Another outcome was that the user spent a substantial percentage of her time

looking at the tablet (43%) versus the surrounding environment (57%). This result was unexpected given that the aim of the app was to encourage participants to concentrate on exploring and absorbing the physical site in preference to scrutinizing the app screen.

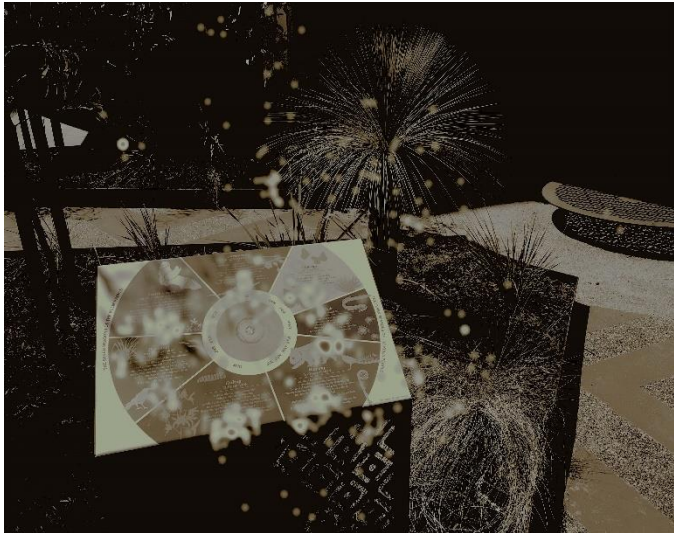


Fig. 1. Summary of eye tracking analysis over a period of 146 seconds. Dark heat spots indicate where the user looked the longest; lighter shapes represent shorter bursts of looking. (© Photo: Bio Inspired Digital Sensing (BIDS)-Lab).

The findings allowed us to revise the information on the app based on user feedback and through software testing. This allowed us to create a politics of representation that sought to generate new agencies for places that could easily be erased, forgotten or overlooked. In this sense the haptic interactive and sensorial immersive dimensions of the walking trail were unashamedly political. The work aimed to intimate knowledge of what was once there and what is no longer, of things now present built over to erase a silenced history through the passage of time and made palpable through the omnipresence of virtual worlds. TRACES strived to evoke a sense of disquiet and displacement, while aspiring to stir moments of discovery and curiosity that would resonate long after leaving a location.

This understanding provided new momentum to our research encompassing digital humanities, creative practice, visual ecology and ethnography. The study carries implications for future research by revealing the ways in which we can understand relationships between multimodal visual, auditory and haptic senses whilst engaged in important cultural experiences. The crossover of this interdisciplinary exposure allowed us to develop collaborative techniques applied to ethical dilemmas in the process of digital design, sensory data analytics and representations of people and place. Our intent was to invite thoughtful reflection and purposive considerations about each site and to provide insights on the potential of the eye tracker to chart new directions on how the presentation of digital material can be creatively re-thought by tracing the movements between the eye of an observer and observed. Understanding eye movements in relation to other movements of the hand (haptic touch) and legs (walking through place) was crucial to effective app design.

Ways of Seeing/Knowing

Archival evidence proves that the eye tracker resonated in artistic circles as early as the 1990s. Jochem Hendricks's longtime

fascination with using infrared sensors to draw with his eyes led to *Newspaper*, a series of artworks based on the path his eyes traveled while reading a newspaper. The normally invisible act of reading is reproduced as navigation lines on a printed newspaper to convey the absorption of information; here, complex patterns become a leitmotif of the mind. Convoluted traces where lines appear as messier, darker shapes express the returning to, re-reading and re-seeing of content, while empty spaces are veiled markers for what was overlooked, omitted or perceived as unimportant.

For Hendricks, the eye tracker was the “interface between myself and the world”, articulating the liminal space where the inner and outer experience are always in negotiation, where information is remediated, redefined, reabsorbed or left aside [8]. This response parallels John Berger's theory that ways of seeing are socially conditioned, leading him to conclude that the “relation between what we see and what we know is never settled” [9], and as Alfred Yarbus shows, often context dependent [10]. When using TRACES, the eye tracker was an interface, allowing two systems to encounter one another and interact, changing the *gestalt* of receptivity and opposition (eye/environment, mind/body, self/other, present/past, digital/physical, seeing/believing); opening a gateway into the other, allowing a person viewing the tracking to embody the person wearing the tracker.

Despite the intentions for playful exploration, what surprised us most was the user's attachment to the screen over the surroundings. This tells us that mechanisms to engage people with real world events can sometimes be perversely counterintuitive. We learnt that seeing with the human eye is a complex interaction of collected light, modulated color and intensity within a changing landscape, and a window on what the mind is prepared to comprehend. We were also reminded of how difficult it can be to disconnect and shift away from our own perspective and to move toward another person's point of view.

Acknowledgments.

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Special Column

Honeybees prefer novel insect-pollinated flower shapes over bird-pollinated flower shapes

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Abstract

Plant–pollinator interactions have a fundamental influence on flower evolution. Flower color signals are frequently tuned to the visual capabilities of important pollinators such as either bees or birds, but far less is known about whether flower shape influences the choices of pollinators. We tested European honeybee (*Apis mellifera*) preferences using novel achromatic (gray-scale) images of 12 insect-pollinated and 12 bird-pollinated native Australian flowers in Germany; thus, avoiding influences of color, odor, or prior experience. Independent bees were tested with a number of parameterized images specifically designed to assess preferences for size, shape, brightness, or the number of flower-like shapes present in an image. We show that honeybees have a preference for visiting images of insect-pollinated flowers and such a preference is most-likely mediated by holistic information rather than by individual image parameters. Our results indicate angiosperms have evolved flower shapes which influence the choice behavior of important pollinators, and thus suggest spatial achromatic flower properties are an important part of visual signaling for plant–pollinator interactions.

Key words: angiosperm, *Apis mellifera* (European honeybee), bird-pollinated, flower, insect-pollinated, pollinator

Studies on the co-evolution of pollinators and angiosperms have found that floral phenotypes may have evolved due to their selection by different functional groups of pollinators (Fenster et al. 2004, 2006). Flowers utilize a variety of signals, cues, and traits in order to attract or deter specific pollinators (Lunau et al. 2011; van der Kooi et al. 2018) as animals exhibit different sensory capabilities. Plant communication has developed specific plant–pollinator relationships which maximize signal quality and reception (Chittka and Menzel 1992).

The difference in evolutionary pathways of flower color for plants that have evolved for insect or for bird pollination has been observed in different sites around the world (Chittka and Menzel 1992; Rausher 2008; Des Marais and Rausher 2010; Dyer et al. 2012; Shrestha et al. 2013). Bird-pollinated flowers generally reflect long wavelength radiation (Raven 1972), which has been shown as evidence of spectral signals tuning to important pollinators, independent of phylogenetic constraints (Shrestha et al. 2013). Analogous type changes also occur

at the short wavelength (UV) region of the spectrum for insect pollinators (Lunau et al. 2011).

Pollinators have preferences for shapes, sizes, and patterns of real and artificial flowers (Lehrer et al. 1995; Johnson and Dafni 1998; Dafni and Kevan 1997). For example, beetles prefer “bowl-shaped” flowers, while small bees prefer flowers which consist of broken outlines (Dafni and Kevan 1997). Bee-flies prefer larger dissected flower models (Johnson and Dafni 1998), and honeybees prefer larger flowers to smaller ones (Martin, 2004). Studies demonstrate that the preference of pollinators for spatial characteristics of flowers may be a driver of flower evolution (Giurfa et al. 1999; Lázaro and Totland 2014; Gómez et al. 2016). Furthermore, the morphology of flowers constrains access to morphologically complex flower species (Krishna and Keasar 2018). Bees recognize a number of different flower characteristics which they use to make decisions on which flowers to forage from. These signals, cues or traits include scent (Raguso 2008), color (Giurfa et al. 1995), shape (Lehrer et al. 1995), size (Martin 2004), or symmetry (Giurfa et al. 1996). Given that honeybee foragers have shown preferences for flower-like shapes (Lehrer et al. 1995), symmetry (Lehrer et al. 1995; Giurfa et al. 1996), larger sizes (Martin 2004), and/or different spatial frequencies (lower spatial frequencies when viewing images from a distance and higher spatial frequencies when viewing images at close range; Lehrer et al. 1995), which represent the resolution of bee vision, we tested whether such preferences may indeed exist for real-flowers.

As a number of floral spectral signals have evolved to attract birds or bees for pollination, we hypothesize that differences in flower morphology between insect- and bird-pollinated flowers could be an additional signal which may be used to attract pollinators. While some insect- and bird-pollinated flowers may share similar morphologies, there are some flowers for respective pollinator groups that appear different in morphologies (Cronk and Ojeda 2008) and thus in the current study we randomly selected flowers from our Australian flower data base to test the potential preference question. By using achromatic images of Australian native flowers (Shrestha et al. 2013), which exclude confounding factors of flower color and scent, it is possible to get insights into whether honeybees have a preference for certain natural flower shapes. Research has demonstrated that bumblebees view flowers and images of flowers as similar (Thompson and Plowright 2014), which validate the use of 2D-printed pictures in our study. European honeybees (*Apis mellifera*) were tested in Germany as within this region there are no bird-pollinated flowers and no occurrence of the Australian native flowers used in this study, thus enabling insights into how innate preferences may influence the pollinator decisions for choosing flowers.

Materials and Methods

Study site and species

Experiments were conducted in the bee training facilities at the Johannes Gutenberg University in Germany with free-flying honeybee foragers (*A. mellifera*). Individual bees were marked on the abdomen or thorax with a colored mark for identification. One bee was tested at a time and overall a total of 422 individual honeybees were tested. A gravity feeder which provided 5–10% sucrose solution was used to maintain a regular number of bees available for testing. Foragers from different hives were recruited to the feeder to use as a food source and individuals in our experiments were collected from this feeder. We collected 1 individual at a time for participation in the experiments. To collect a honeybee from the feeder, the bee was picked-up using a plexi glass spoon containing a higher concentration of sucrose than

the feeder (50% sucrose solution). The bee was taken to the rotating screen apparatus and placed on one of the platforms which contained 50% sucrose solution (Figure 1). Once bees were consistently coming back to the apparatus instead of the feeder for a higher reward, the experiments began.

Apparatus

Honeybees were trained to visit a vertical rotating screen, 50 cm in diameter (Dyer et al. 2008; see Figure 1). By using this screen, the spatial arrangement of stimulus choices could be randomly arranged, thus excluding position cues. The apparatus was able to be rotated between choices and bouts to randomize the position of the stimuli, but was not constantly rotating. Stimuli were presented vertically on 6 × 8 cm hangers with a landing platform attached below the presentation area (Figure 1). A standard gray plastic was used for the screen, hangers, and landing platforms (Dyer et al. 2008). Hangers and surrounding screen areas were cleaned with 20% ethanol solution and then dried between landings and before each test were conducted to exclude the use of olfactory cues.

Experiment 1: Preference for bird- versus Insect-pollinated flowers

Stimuli

Stimuli used for the study consisted of 24 achromatic photographs of Australian native flowers with known pollinators chosen from our databases (Shrestha et al. 2013; Burd et al. 2014). Flowers were chosen for the experiment based on the quality of the collected images from previous field work to exclude photographer bias for the current study (Shrestha et al. 2013; Burd et al. 2014). Twelve of the flowers were identified as exclusively insect-pollinated (Figure 2A) and 12 were exclusively bird-pollinated (Figure 3A). As these flowers were novel to European honeybee pollinators in Germany, we could determine that results were not caused by familiarity with flowers from previous foraging experience. Images of flowers were cropped to 6 × 6 cm squares. The color images were transformed into achromatic grayscale images using the program ImageJ (version 1.50) by discarding the red and blue layers of the original RGB images and keeping only the layer produced by the green channel (Figures 2A and 3A and Supplementary Figure S1). We selected the green channel as the wavelengths sensed by this channel map closely between camera and bee green photoreceptor sensitivities (Garcia et al. 2014) which are known to be important for how free-flying bees perform spatial tasks (Giger and Srinivasan 1996; Hempel de Ibarra and Giurfa 2003; Stach et al. 2004; Morawetz et al. 2013; Avarguès-Weber et al. 2014). The images were printed on EPSON Archival Matte Paper, Super A3, 192 g/m² and laminated with Avery Dennison® DOL 1480 3D Matte (Matte Clear Super Conformable Cast Overlamine). A radiometer (Instrument Systems SPECTRO 320 Optical Scanning Spectrometer) was used to ensure stimuli were monochromatic images in the green-receptor channel. Chromatic contrast (0.05 units for the white paper) was also calculated in a Hexagon color space (Chittka 1992) and was well below the threshold of 0.11 Hexagon units that bees perceive as different from an achromatic background (Dyer et al. 2012). For information on flower size, see Supplementary Table S1.

Priming phase

We primed 138 individual honeybees over 24 rewarded choices to land on platforms and become familiar with the apparatus using a 10 µL drop of 50% sucrose solution placed on each of the 8 hanger

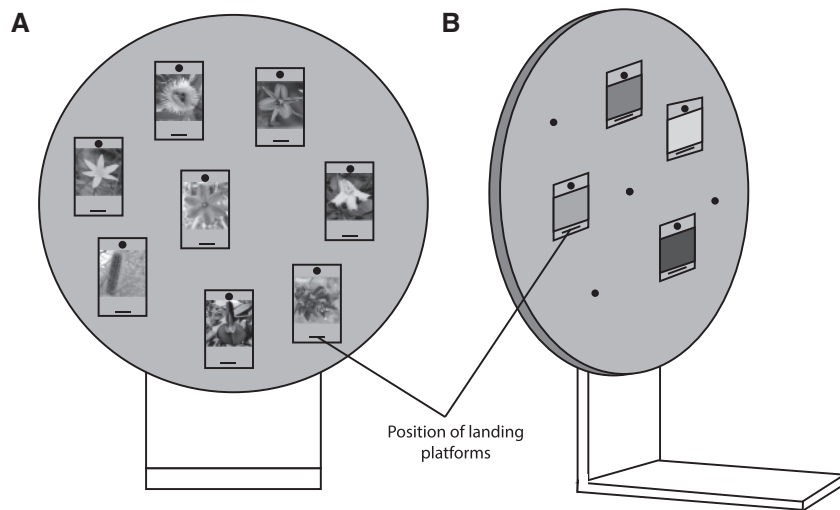


Figure 1. Schematic of the rotating screen apparatus where the (A) achromatic flower images were presented to bees (front view). Shown are examples of insect- and bird-pollinated flower images presented on hangers with landing platforms located below images on the hangers. (B) The rotating screen with control stimuli presented to bees (side view). Shown is the test for brightness preference.

platforms. This type of priming was found necessary during pilot studies to enable a very high level of motivation from the bees for the subsequent non-rewarded testing. We began counting these priming choices when bees could land on the hanger platforms without assistance. During the priming phase, 6×6 cm squares of sand-blasted aluminium were presented on hangers as a spectrally neutral stimulus. The sand blasted aluminium reflects radiation equally from 300 to 650 nm (Dyer et al. 2016) and are thus achromatic for bee perception. After individual bees landed and imbibed the sucrose, they were gently removed from the apparatus using a transparent spoon with sucrose on it and placed behind an opaque screen about 1 m from the rotating screen while the apparatus and hangers were cleaned (Dyer et al. 2008). After this procedure, bees could either choose to land on the apparatus hangers for a reward again or return to the hive to deposit the sucrose.

Testing phase

After the priming phase, we conducted 1 test with 8 pseudo-randomly chosen flower image stimuli from our image database of 24 flowers by using dice rolls (Figure 2A: 4 different insect-pollinated flowers; Figure 3A: 4 different bird-pollinated flowers, Figure 1A). The flower stimuli were placed on the hangers and $10 \mu\text{L}$ drop of water was used instead of sucrose in the associated platforms as the test was unrewarded. We recorded the number of choices (touches of platforms or images) for a total of 24 choices in this test thus each image had an equal chance of being chosen. A touch was defined as any contact to the platform or flower image during the test.

Statistical analysis

Bee preference analysis

To determine whether bees had any preference to insect- or bird-pollinated flower images, we estimated the mean of the insect-pollinated choices from the intercept of a generalized linear mixed model only including the intercept as predictor. Choices were recorded as binary responses giving a value of 1 for choices made to insect-pollinated flowers and zero otherwise. Subject (individual bees) was included as a random variable to account for the repeated measurements. The model was estimated using the routine “glmer”

available as part of the “lme4” package written for the R statistical language (R Core Development Team 2016).

Image analysis

We also analyzed the flower images to determine if contrast or line length of the flower images used were significantly different in terms of insect-pollinated (Figure 2B) or bird-pollinated images (Figure 3B). For all images, the brightness profiles were constructed from pixel values of a linear transect sampling going from the leftmost pixel location to the rightmost location along the central axis of the image. Contrast for each image was calculated as the root mean square of the pixel intensity values (Bex and Makous 2002) for the entire image. Contrast values for the 2 image groups (bird-pollinated or insect-pollinated) were compared by means of an independent *t*-test. Contrast analyses were performed in MATLAB release 2016b. The flowers line length was analyzed using ImageJ by tracing the perimeter of the flowers and measuring the line length. The line lengths of the bird-pollinated and insect-pollinated flowers were then compared by means of a 2-tailed *t*-test. The *t*-tests were carried out in SPSS version 24.

Experiment 2: Honeybee preferences to different aspects of the flower images

Stimuli

There were 5 control tests that were conducted to check preferences for (i) 4 different levels of brightness, (ii) 3 different elongations of a typical flower-shape, (iii) 3 different flower sizes, (iv) a preference for 1 versus 3 flower shapes, and (v) a preference for 1 versus 11 flower shapes in an image (Figures 1 and 4). The stimuli for this control experiment were developed using the previous tested images of flowers in Experiment 1. We tested for a preference to brightness using 4 stimuli of different levels of brightness 10%, 20%, 35%, and 50% (Figure 4A) which mirror the biologically relevant range of reflectance values for the most common flowers (Chittka et al. 1994; van der Kooij et al. 2016). We tested for a preference to shape using a familiar flower-like star shape (Lehrer et al. 1995), which was elongated, using 3 different stimuli: $1 \times$ elongation (none), $2 \times$ elongation, and $3 \times$ elongation (Figure 4B). We tested for a flower size preference in the image using 3 differently sized flower-like

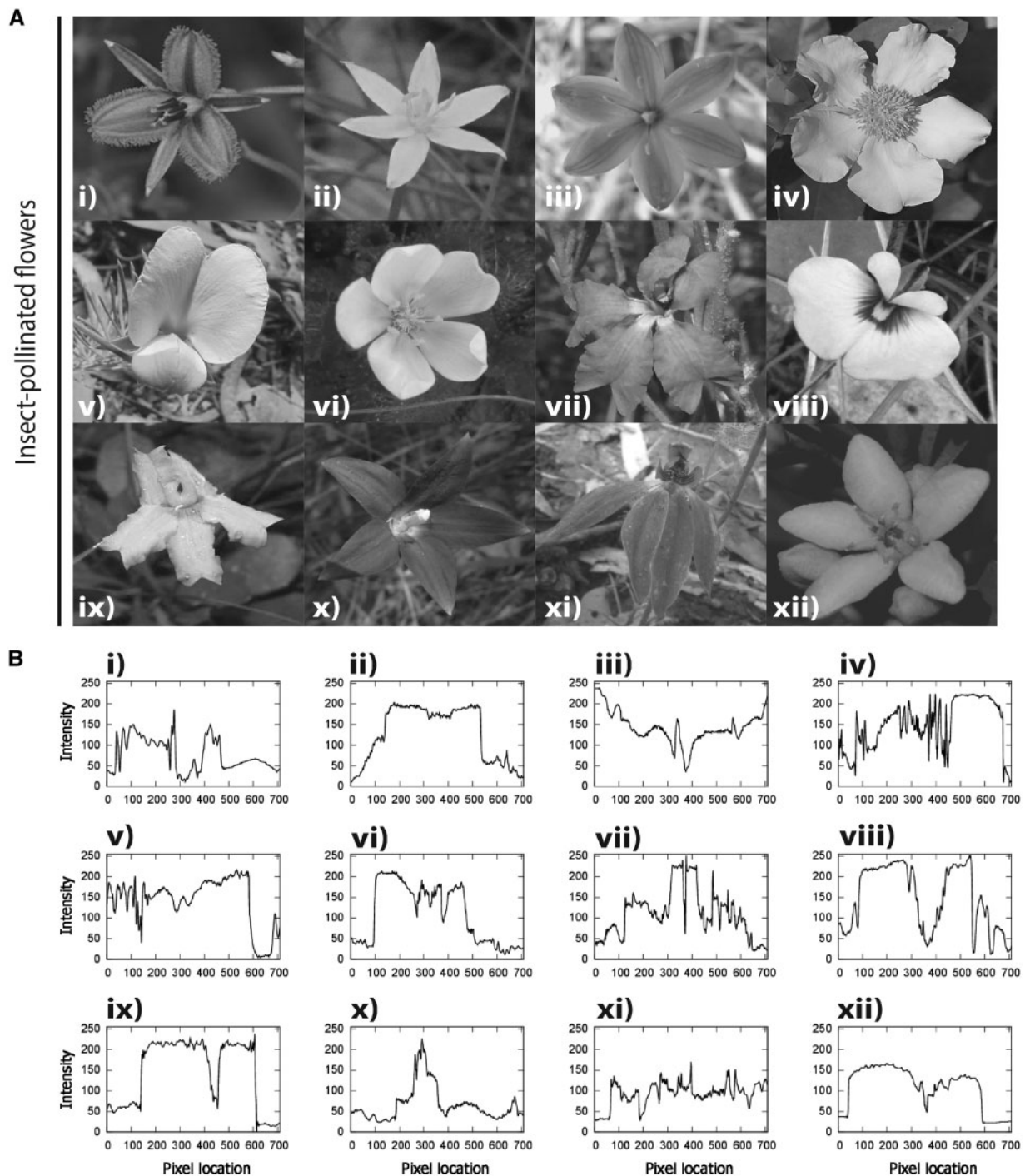


Figure 2. (A) The 12 insect-pollinated flowers used in the experiments which are native to Australia. The color images of the flowers (i–xii) were converted into achromatic grayscale images by selecting the layer corresponding to the green channel of the original RGB images. (B) The corresponding brightness profiles for the insect-pollinated flower images taken along a linear transect sampled across the middle of the image on the horizontal axis in (A). Species names: (i) *Thysanotus junceifolius*, (ii) *Tricoryne elatior*, (iii) *Chamaescilla corymbosa*, (iv) *Hibbertia scandens*, (v) *Gompholobium huegellii*, (vi) *Drosera whittakeri*, (vii) *Dampiera stricta*, (viii) *Eutaxia microphylla*, (ix) *Goodenia lanata*, (x) *Wahlenbergia gloriosa*, (xi) *Caladenia carnea*, and (xii) *Philotheca myoporoides*. See Supplementary Figure S1A for full color images.

stimuli: small, medium, and large (Figure 4C). We also assessed in 2 tests the preference for images containing 1 flower-like stimulus versus 3 (few; Figure 4D) or 1 versus 11 (many; Figures 1 and 4E) flower-like stimuli. We tested the bees' preferences for number of flower-like elements in an image as insect-pollinated flowers in our stimuli set (Figure 2) typically consist of 1 large flower-shaped

element in an area of the plant, while bird-pollinated flowers in our stimuli set (Figure 3) often have inflorescence (multiple flowers in a single area).

Priming phase

The priming phase was identical to Experiment 1.

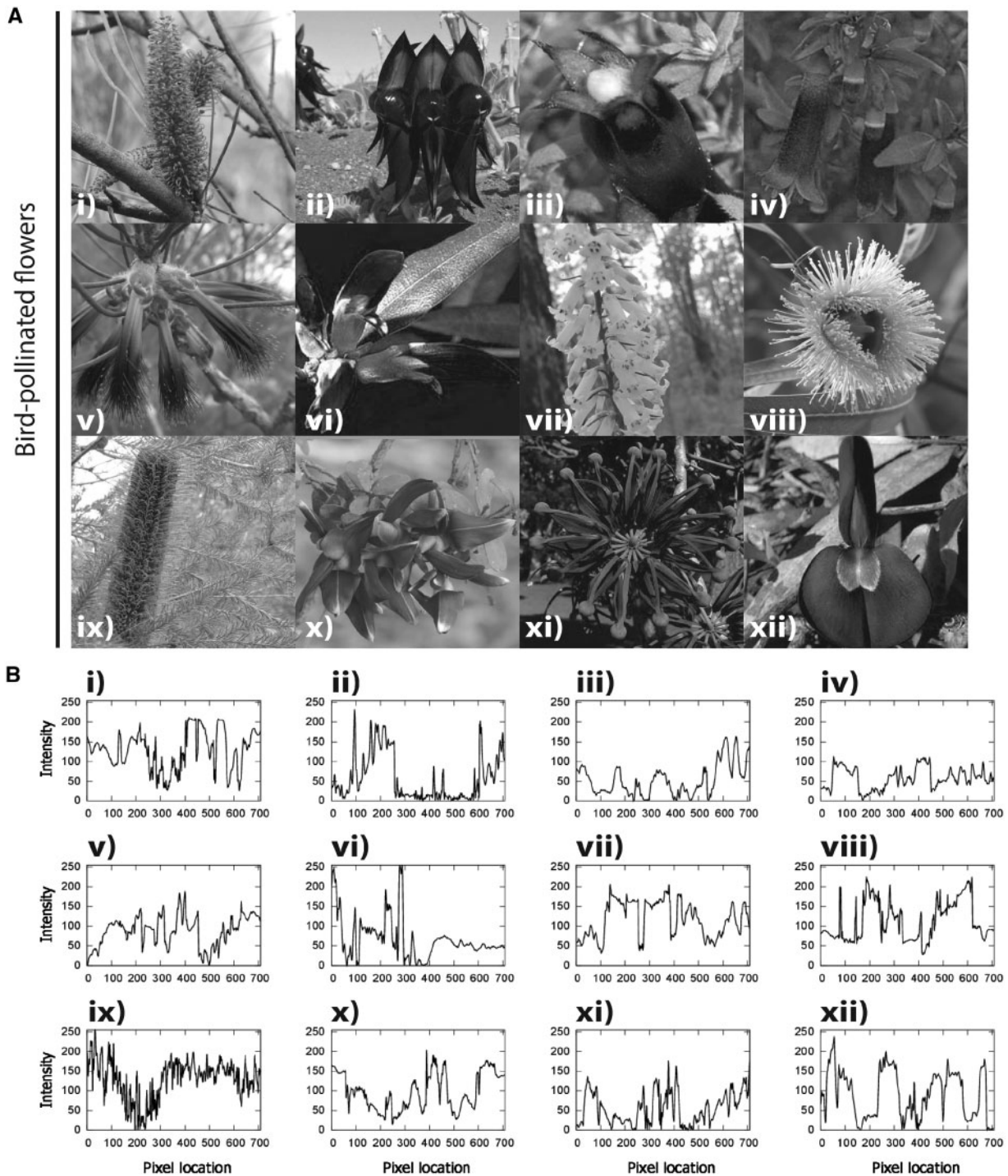


Figure 3. (A) The 12 bird-pollinated flowers used in the experiments which are native to Australia. The color images of the flowers (i–xii) were converted into achromatic grayscale images by selecting the layer corresponding to the green channel of the original RGB images. (B) The corresponding brightness profiles for the bird-pollinated flower images taken along a linear transect sampled across the middle of the image on the horizontal axis in (A). Species names: (i) *Hakea francissiana*, (ii) *Swainsona formosa*, (iii) *Astroloma ciliatum*, (iv) *Corea pulchella*, (v) *Calothamnus rupestris*, (vi) *Gastrolobium celsianum*, (vii) *Epacris impressa*, (viii) *Eucalyptus* sp., (ix) *Banksia ericifolia*, (x) *Templetonia retusa*, (xi) *Stenocarpus sinuatus*, and (xii) *Kennedia prostrata*. See [Supplementary Figure S1B](#) for full color images.

Testing phase

After the priming phase, a total of 280 bees participated in one of the control tests in which stimuli were either manipulated for (i) brightness ($n = 78$), (ii) shape elongation ($n = 61$), (iii) size ($n = 65$),

or (iv–v) number of elements (1 versus 3: $n = 34$; 1 versus 11: $n = 42$) and were placed on the hangers. Testing order was random. Ten choices were recorded per bee. A choice was defined as any contact to the platform or stimulus during the test.

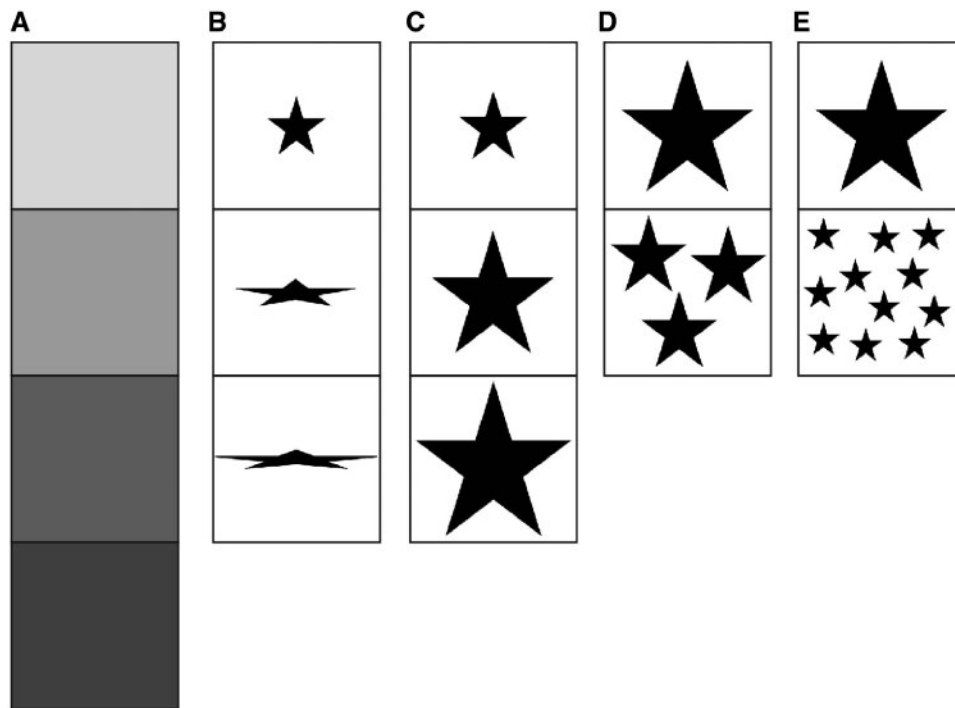


Figure 4. Samples of the control stimuli used in experiments. (A) Representation of brightness stimuli (10%, 20%, 35%, and 50%). (B) Shape stimuli with elongation of a star-shaped flower-like image at 1× elongation, 2× elongation, and 3× elongation. (C) Size stimuli showing small, medium, and large surface areas of flower-like images with the areas derived from the flower sizes used in part 1. (D) Stimuli used for the flower number experiment of 1 versus 3. (E) Stimuli used for the flower number experiment of 1 versus 11.

Statistical analysis

In Experiment 2, we used a set of generalized linear mixed models (glmm) initially including choice number (sequence) and stimuli parameter as fixed terms to test for potential bee preferences for different visual aspects of the flower images and a potential effect of choice number (sequence of choices). We followed a classical model reduction analytical framework to test for significant effects of the 2 fixed factors. Bees participating on tests for brightness, amount of elongation, and size could select from more than 2 options; therefore, we assumed that the response variable, that is, the stimulus chosen on each trial, followed a multinomial distribution (Faraway 2005). Models for the flower number experiments assumed a binomial distribution for the response variable. Subject (individual bees) was included as a random effect on all models to account for the repeated measurements (Zuur et al. 2009).

The stimulus options with (i) a brightness level of 20%, (ii) 1× elongation, and (iii) medium size were selected as baseline for the multinomial models. The baselines were chosen as (i) 20% as this was similar to the priming brightness level, (ii) 1× elongation as this means there was no elongation in this stimulus, and (iii) medium size as this was the average size of flowers in the images in Experiment 1. Images depicting 1 flower were designed as the “correct” answer for the (iv–v) binomial models. All choice comparisons were done relative to the baseline following standard protocols (Faraway 2005).

Multinomial models were fitted using Bayesian interference with Monte Carlo Markov Chain methods with the routine MCMCglmm (Hadfield 2010), available for the R statistical language. Multivariate normal distributions with mean vector zero and large variance were used as diffuse priors for the fixed and random terms (Hadfield 2010). Models were run with 210,000 iterations, a

thinning interval of 1,000 and discarding the first 10,000 iterations as burnin phase. By the end of the simulation phase, chains in all models had an autocorrelation value <0.1.

Binomial models were also fitted using Bayesian techniques. Diffuse normal priors were assumed for the fixed terms while half-Cauchy priors were assumed for the random terms (Zuur et al. 2015). Fitting of the binomial models was done in JAGS (Hornik et al. 2003) for R using the same number of iterations, thinning, and burnin parameters used for the multinomial model.

Posterior distributions of the regression model coefficients were subsequently used to evaluate if the magnitudes of the model’s coefficients were different from zero. For the multinomial models, coefficient values including zero demonstrate that there is no difference between the number of choices observed for the respective trait and the chosen baseline (Supplementary Table S2).

Results

Experiment 1

Bee preference analysis

Honeybees ($n = 138$) significantly preferred insect-pollinated flower images compared with bird-pollinated flower images at a level of $53.8 \pm 1.1\%$ (mean \pm standard error of the mean) which was significantly different from chance level ($H_0 = 50\%$, $z = 3.556$, $P < 0.0001$). Thus, honeybees had a significant preference for novel insect-pollinated flower images (Figure 5A).

Image analysis

The contrast values of the images ($n = 12$) were normally distributed for both insect-pollinated images ($W = 0.960$, $df = 12$, $P = 0.780$; Figure 2B and Supplementary Figure S2) and also for the 12 images

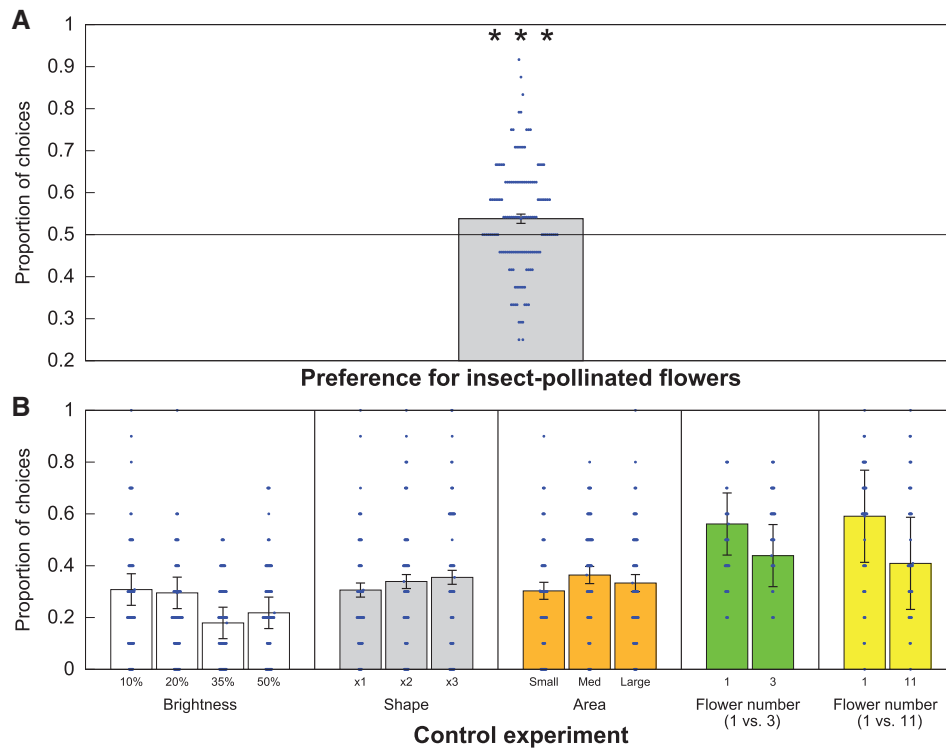


Figure 5. The results of the preferences tests for Experiments 1 and 2. **(A)** The mean proportion of choices made for insect-pollinated flower images (gray) during the preference test. This column shows the mean \pm 95% confidence intervals (CIs). The solid black line shows chance expectation at 50%. Significance from chance level performance is indicated by $***\geq 0.001$. Blue dots indicate the raw data, depicted as a bee-swarm plot, of each individual bee's preference for insect-pollinated flowers ($n = 138$). **(B)** The mean proportion of choices made for each of the 5 preference control experiments: brightness (white; $n = 78$); shape (gray; $n = 61$), area (orange; $n = 65$), flower number test 1 versus 3 (green; $n = 34$), and 1 versus 11 (yellow; $n = 42$). The columns show the mean \pm 95% CIs. Blue dots indicate the raw data, depicted as a bee-swarm plot, of each individual bee's preference for each option in the tests.

of bird-pollinated flowers ($W = 0.958$, $df = 12$, $P = 0.753$; Figure 3B and Supplementary Figure S3). We conducted an independent-sample t -test between the contrast values for the 2 groups and found no significant differences ($t = 1.692$, $df = 17.255$, $P = 0.109$).

The line length of the bird-pollinated and insect-pollinated flowers was not significantly different (independent samples t -test: $t = -0.728$, $df = 22$, $P = 0.475$). The area of flowers was also not significantly different (independent samples t -test: $t = 0.928$, $df = 22$, $P = 0.364$); thus, the contrast nor the line length nor the area could be considered a driver of bee preference.

Experiment 2

Zero was included in all the 95% credible intervals for the trial coefficient in all models. This suggests that bees were generally showing similar choices at the beginning and end of the 10 choices in the tests. Therefore, reduced models only including the intercept were subsequently fitted to the data to test for differences in the total number of choices for each trait modification relative to the baseline chosen for each trait. Analyses revealed that bees did not choose any of the modified traits for shape ($n = 61$), brightness ($n = 78$), or number of petals (1 versus 3: $n = 34$; 1 versus 11: $n = 42$) (Figure 5B). However, bees chose the small flowers less frequently relative to the normal sized images ($n = 65$; Figure 5B and Table 1).

Discussion

Considering flowers presented to honeybees were novel (flowers were native to Australia whereas our honeybee population was

Table 1. Percentage of bee choices for each option in each of the 5 tests

Brightness			
10%	20%	35%	50%
30.77%	29.49%	17.95%	21.79%
Shape			
x1	x2	x3	
30.65%	33.87%	35.48%	
Size			
Small	Medium	Large	
30.30%	36.36%	33.33%	
1 versus 3 shapes			
1 shape	3 shapes		
56.10%	43.90%		
1 versus 11 shapes			
1 shape	11 shapes		
59.09%	40.91%		

located and tested in Germany), we propose that the preference for insect-pollinated flowers was not a direct result of familiarity with flowers through foraging. Based on our results, we thus suggest that the choice for insect-pollinated flowers based on shape is an effect due to an evolved preference rather than through familiarity with specific flowers. This position would be consistent with theories of innate shape preference present in bees proposed by Lehrer et al. (1995). In addition, our control tests suggest that honeybees prefer to choose flowers based on an overall, global view of the flower images rather than on a single parameter. This interpretation fits

with how honeybees are known to prefer to process visual input using global holistic information rather than local elemental features (Zhang et al. 1992; Avarguès-Weber et al. 2015, 2018; Howard et al. 2017b). However, we acknowledge that it is also possible that the observed preference for insect-pollinated flowers could alternatively be a result of familiarity of foraging on “similar” insect-pollinated flowers throughout an individual bee’s lifetime. For example, Verguts and Chen (2017) suggested that an individual animal undergoes “evolution” at an individual level throughout its lifetime as it learns and experiences its own environment, thus bees in our experiment may demonstrate a preference for insect-pollinated flowers due to their previous individual experience. Future work with fully naïve bees could help inform the mechanisms underpinning the observed effect of a preference for certain flower morphologies.

Consistent with the current study, honeybees have previously demonstrated a preference for larger flowers of the species, *Mimulus guttatus* (Martin 2004). In both studies, the selection by honeybees against smaller sizes is possibly due to the lower visibility of the smaller flower-like shape. Other previous works have demonstrated that flower size plays a significant role in plant–pollinator interactions. For example, larger flower sizes may be caused by selection pressures to advertise a higher reward quality or quantity (Ashman and Stanton 1991; Campbell et al. 1991; Cohen and Shmida 1993; Benitez-Vieyra et al. 2010, 2014), thus resulting in a preference against smaller flowers. This is evident in flowers of *Turnera ulmifolia* L., where nectar production and petal length (an indication of flower size) were positively correlated in an environment where signal accuracy was selected for by pollinators (Benitez-Vieyra et al. 2010). Bees can reliably learn and process size (Howard et al. 2017a) but the size factor alone could not explain the observed preference for insect-pollinated flowers as there were no significant size differences between the images of the flower types. Our investigation of potential elemental factors that might influence bee preferences did not find any significant effect of flower elongation, nor brightness on bee choices. This result is consistent with recent findings that image brightness is not processed by honeybees when using color vision to detect flowers, and indeed brightness appears an unreliable visual cue in complex environments (Ng et al. 2018; van der Kooi et al. 2018).

The results in our current study suggest 2 potential evolutionary mechanisms. The first involves the evolution of flowers to suit pollination by insects such as honeybees due to the bees preference for certain morphologies. This possibility is supported by previous research demonstrating that evolution of flower color occurred through flowers tuning to the relative sensitivity of the plant’s most important pollinators (Chittka and Menzel 1992; Rausher 2008; Des Marais and Rausher 2010; Dyer et al. 2012; Shrestha et al. 2013). The second possible mechanism would be the evolution of bees to prefer morphologies of insect-pollinated flowers as those are the flowers from which it would be easiest to receive nutrition compared with bird-pollinated flowers. As a result, over time bees may have developed evolutionary relevant recognition of insect-pollinated flowers and be able to generalize that familiarity to novel flower comparisons, as discussed above. The preference for insect-pollinated flower shapes could also be a result of a combination of these 2 mechanisms, where insect-pollinated plants and insects, specifically bees, co-evolved.

Our results suggest that the recognition and preference for insect-pollinated flowers by honeybees is innate as bees in Germany had not previously encountered the species of flowers which we presented. In addition, if flowering plants have evolved to suit

morphological preferences of bees, Europe and Australia have been separated for many millions of years (with honeybees arriving in Australia within the last 200 years; Paton 1993, 1996), meaning the coevolution of this plant–pollinator system is a deep rooted evolutionary occurrence. Such a phylogenetically conserved effect of the visual system of bee pollinators is plausible as flower colors in Australia have evolved to suit color discrimination of native bee pollinators; and the distribution of colors is the same as regions of the world where honeybees were the dominant influence on flower coloration evolution (Chittka and Menzel 1992; Dyer et al. 2012). Thus, our new evidence suggests that native Australian pollinators may also have a similar preference for flower-shape.

Author Contributions

S.R.H., M.S., J.S., and A.G.D. were involved in experimental design and creation of the stimuli. S.R.H., J.S., J.E.G., and A.G.D. were involved in data collection. S.R.H. and J.E.G. were involved in data analysis. S.R.H. wrote the manuscript. All authors were involved in the interpretation of data and editing of the manuscript.

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Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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Article

Signal or cue: the role of structural colors in flower pollination

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Abstract

Angle dependent colors, such as iridescence, are produced by structures present on flower petals changing their visual appearance. These colors have been proposed to act as signals for plant–insect communication. However, there is a paucity of behavioral data to allow for interpretations of how to classify these colors either as a signal or a cue when considering the natural conditions under which pollination occurs. We sampled flowers from 6 plant species across various viewpoints looking for changes in the visual appearance of the petals. Spectral characteristics were measured with different instruments to simulate both the spectral and spatial characteristics of honeybee’s vision. We show the presence of color patches produced by angle dependent effects on the petals and the calyx of various species; however, the appearance of the angle dependent color patches significantly varies with viewpoint and would only be resolved by the insect eye at close distances. Behavior experiments with honeybees revealed that pollinators did not use angle dependent colors to drive behavior when presented with novel flower presentations. Results show that angle dependent colors do not comply with the requirements of a signal for plant–pollinator communication since the information transmitted by these colors would be unreliable for potential, free-flying pollination vectors. We thus classify angle dependent colors produced by micro- and ultra-structures as being a cue (a feature which has not evolved for communication), and observe no evidence supporting claims of these angle dependent colors having evolved as visual signal.

Key words: approach angle, color, flower, iridescence, photography, pollination, vision

Flowering plants around the world have evolved a wide range of flower types displaying a striking gamut of colors using a variety of different pigments (Faegri and Pijil 1966; Scogin 1983; Rausher 2008; Tanaka et al. 2008; Dyer et al. 2012; Ng et al. 2018). Petal colors attract flower visitors, like bees or birds (Varassin et al. 2001; Shrestha et al. 2013), which facilitate the efficient transfer of pollen between conspecific plants (Chittka and Menzel 1992; Chittka et al. 1999). In recent times, there has been an increasing number of reports of different optical phenomena producing angle dependent coloration through the interaction of optical radiation with microstructures on flowers belonging to distantly related clades to produce visual effects

including iridescence (Whitney et al. 2009b; Vignolini et al. 2015), mirror-like reflectance (gloss) (Vignolini et al. 2012; van der Kooi et al. 2017), and “halos” (Moyroud et al. 2017). The optical principles leading to the production of angle dependent colorations such as iridescence and mirror-like reflection (gloss) are produced by interference of incident light caused by the presence of nano- and ultra-structures of different refractive order regularly or quasi-regularly ordered on the petal surface (van der Kooi et al. 2018, 2017); for this reason, such colors are commonly referred to as structural colors to differentiate them from colors produced by the selective absorption of light as those produced by pigments (Srinivasarao 1999; Nassau

2001). In the present manuscript, we will thus refer interchangeably to both angle dependent and structural colors, as our primary question is how such colors may be used by bee pollinators in a way that would fit the formal definition for signal.

Accordingly to various authors, angle dependent colors have evolved to produce visual signals to potential pollinators (Whitney et al. 2009b; Moyroud et al. 2017). However, it currently remains unresolved as to whether such optical effects are indeed biologically significant when considering the sensory capabilities of important pollinators like bees (Morehouse and Rutowski 2009; van der Kooi et al. 2015). To understand if angle dependent color in flower can be classified as a signal when considering plant–pollinator interactions, it is essential to recognize in what circumstances visual information can be effectively transferred to a potential pollinator. Thus, to understand flower evolution, it is necessary to understand bee–pollinator perception.

The use of the term *signal* when referring to angle dependent colors in plants implies that these colors allow for an effective visual communication between plant (sender) and insect (receiver). More precisely, these type of colors should comply with 3 conditions to be considered as a signal: (a) effectively transmit information from the signaler to the receiver, (b) have evolved for this particular purpose, and (c) both parties should benefit from producing and monitoring these colors (Smith and Harper 2003; Bradbury and Vohrencamp 2011). Visual traits producing stimuli that do not meet the fore mentioned 3 criteria may be defined as a cue (Bradbury and Vohrencamp 2011). Unlike signals, cues have not specifically evolved for communication purposes and may be produced as a secondary effect or by-product of inherent anatomical characteristics to the emitter (Bradbury and Vohrencamp 2011).

In contrast to structural colors produced by flower petals, angle dependent colors produced by animals, as for example bird feathers (Finger et al. 1992), are known to be effectively used as signals for visual communication. For example, female peacocks use the color produced by the iridescent plumage of males to detect and visit mates (Loyau et al. 2007), the quality of the structural color in house sparrows is correlated with the nutritional condition of the bird (McGraw et al. 2002), plumage structural coloration of eastern bluebirds acts as honest signal of male quality and females mating with the most colorful males receive benefits from their mates (Siefferman and Hill 2003); and female starlings use structural coloration to rank male attractiveness (Bennett et al. 1997).

Flower colors produced by pigments can be classified as a visual signal (van der Kooi et al. 2018) as this type of color complies with the 3 requirements for effective communication between plant and insect. Flowers relying on hymenopterans to reproduce typically offer small nutritional rewards to their visitors (Goulson 1999) and have often evolved colors that maximizes their discriminability considering the visual system of important pollinators (Chittka and Menzel 1992; Dyer et al. 2012; Shrestha et al. 2013; Bukovac et al. 2016). Furthermore, insect pollinators like bees constantly visit conspecific rewarding flowers that are easily recognized to maximize their nutritional intake, and this flower constancy promotes the evolution of flower color signals that best correspond to the visual capabilities of important pollinators (Chittka et al. 1999; Burns and Dyer 2008; Shrestha et al. 2013). However evidence supporting the role of angle dependent colors in flowers as visual signals remain tenuous when considering natural environments (Morehouse and Rutowski 2009; van der Kooi et al. 2015, 2018).

A fundamental requirement for petal color to serve as a signal for visual communication is that this trait should unambiguously transmit information from the flower to the insect (Smith and

Harper 2003). Most pigment-based colors present in flower petals retain their chromatic appearance independently from viewing angle as they produce diffuse reflection (Lee 2005). This means that a pollinator approaching a flower from any direction will perceive the color independent of the angle of illumination. However, this may not be the case with angle dependent colors since by definition there can be significant changes in appearance depending on the direction of illumination and approach of a prospective pollinator (van der Kooi et al. 2015, 2018).

Let us consider the case of a hypothetical flower displaying a color pattern consisting of angle dependent color patches produced by 2 different phenomena, plus a diffuse, angle independent color produced by pigment (Figure 1). In our example, as in naturally occurring flowers, a pollinator may approach from any inclination angle (φ) along the vertical axis (red arrow in Figure 1), and from any orientation angle (θ) along the horizontal plane (green arrow in Figure 1). Furthermore, one of the angle dependent colors may result from Fraunhofer Diffraction produced by a grating as reported for *Hibiscus trionum* (Whitney et al. 2009b), where the intensity of the reflected radiation varies with viewing angle (Hecht 2002). The second angle dependent color may be the result of mirror-like reflectance as the type of angle dependent coloration observed in several species of the genus *Ranunculus* (family Ranunculaceae) (Galsterer et al. 1999; van der Kooi et al. 2017). In both cases, as in many examples of angle dependent colors, a pollinator would only see the angle dependent colors when approaching the flower at those specific angles where the petal microstructure allows for constructive interference of the radiation reflected by the petals (Hecht 2002; van der Kooi et al. 2016). For this reason it is of value to assess potential changes in the visual appearance of a flower by collecting information from different angles as those used by a free flying pollinator (van der Kooi et al. 2015), using calibrated digital images that allow to recover measurements of total reflectance from digital values (Garcia et al. 2014).

To communicate information that can drive an insect pollinator behavior as expected from a signal (Smith and Harper 2003; Bradbury and Vohrencamp 2011), the petal appearance resulting from the joint effect of the structural and pigment coloration should remain constant across all directions used by an insect to approach a flower. Alternatively, the insect has to be able to detect and identify a flower independently from changes in its appearance resulting from approaching the target from different directions (Figure 1). The latter condition implies that the pollinator has to use the overall change in appearance induced by the angle dependent colors as information for identifying the flower sending the signal (de Premorel et al. 2017). Laboratory measurements of the optical properties of various petals showing angle dependent colorations suggest that the former hypothesis does not hold true for several species. Some color effects produced by nano and ultra-structures such as iridescence (Whitney et al. 2009b) and specular reflection (Vignolini et al. 2012) are only visible at specific angles. However, studies considering changes in petal appearance due to angle dependent coloration under natural-like illuminations (van der Kooi et al. 2015; Vignolini et al. 2015) have not formally tested for the potential correlation between angle and changes in the visual appearance of the petals as perceived by potential pollinators.

Whether hymenopteran insect pollinators use visual information produced by the structural coloration to drive decisions remains a topic for debate (van der Kooi et al. 2018). Some authors have addressed this question through the use of a discrimination paradigm where bumblebees (*Bombus terrestris*) were trained to

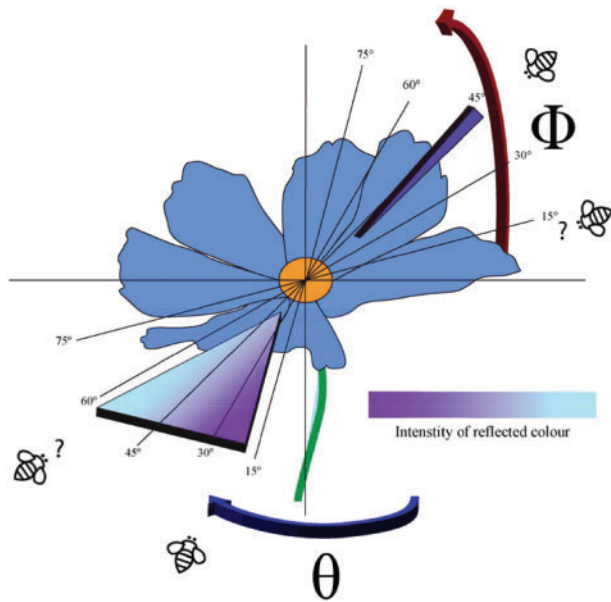


Figure 1. Diagram depicting a hypothetical flower whose color is the result of angle dependent and independent colors. The perceived appearance of the angle dependent colors depends on the optical phenomena producing them and view angle which is a combination of the inclination angle along the y-axis (φ , red arrow) and the orientation angle along the x-axis (θ , blue arrow). In this particular example, one of the angle dependent colors is a mirror-like reflectance only visible at a particular inclination angle (top right quadrant). The second angle dependent color is produced by a simple diffraction effect where the intensity of a given reflected color changes with angle (bottom left quadrant) here represented by the graded color. The third color is produced by radiation reflected by the pigment which produces a solid, diffuse color angle independent from view point (solid blue color). Depending on the particular approach angle, an insect pollinator will perceive different aspects of the angle dependent component of the color pattern (question marks). However, the appearance of the diffuse pigment color will remain the same independent from view point.

discriminate between angle dependent and diffuse colorations on artificial targets mimicking petal structural colors (Whitney et al. 2009b; de Premorel et al. 2017). In these experiments, bumblebees learned to discriminate between the angle dependent and angle independent colorations following an appetitive aversive differential conditioning which significantly improve learning in bees (Avarguès-Weber et al. 2010). Using this conditioning procedure each bee received a sucrose reward when choosing the iridescent targets, and was punished with a quinine solution when choosing the non-iridescent distractor. Although results from these experiments show that bumblebees can readily learn to discriminate angle dependent colorations from their angle independent counterparts after extensive conditioning, these experiments do not prove that under natural circumstances structural color are used as signals by insect pollinators.

In recent years it has become clear that understanding how a bee pollinator uses their color vision in a natural setting requires careful consideration of what motivates and modulates the attention of individuals (Dyer 2012). Testing on color vision in both honeybees (Giurfa 2004; Reser et al. 2012; Garcia et al. 2018) and bumblebees (Dyer and Chittka 2004; Garcia et al. 2018) shows that bees trained with absolute conditioning (i.e. target stimuli in isolation) only enables a relatively coarse level of discrimination. In contrast, bees trained with differential conditioning (i.e. rewarded target stimuli vs. non-rewarded and perceptually similar distractor stimuli) acquire fine color discrimination. Differential conditioning

results in the formation of a long-term memory (Dyer and Chittka 2004; Dyer and Garcia 2014), which has also been recently reported in other hymenopterans such as ants (Yilmaz et al. 2017). The use of appetitive-aversive conditioning, where choices for the correct distractor are punished with a bitter tasting quinine solution further improve color discrimination (Chittka et al. 2003), probably via modulation of attention (Avarguès-Weber et al. 2010). The question then becomes which type of condition is most appropriated for evaluating hypotheses about flower signal evolution. By comparing either absolute or differential conditioning functions for either honey or bumblebees to pigment-based flower color signals (Dyer et al. 2012; Garcia et al. 2018) or the flower constancy behavior of bees (Dyer 2006), it has been shown that for natural conditions absolute conditioning is the correct behavioral paradigm to use for understanding how bee pollinators use visual information in a way that might drive flower evolution. For example, color discrimination under absolute conditioning explains how insect pollinators may cope with the color variability observed in natural flowers to maintain flower constancy, and allow for “imperfect” camouflage in spiders preying on visiting honeybees (Garcia et al. 2018).

Here we address the important question of the reliability of structural color under simulated natural lighting conditions when considering both viewpoint and the spectral and spatial characteristics of the visual system of the honey bee (*Apis mellifera*). We used linearized digital images, which express total reflectance at each pixel location (Stevens et al. 2007; Garcia et al. 2013a), and a mechano-optical device which produces images with an spatial resolution close to that measured for honeybees (Knowles and Dartnall 1977; Williams and Dyer 2007). To fully understand the extent to which angle dependent colors are biologically relevant, we test free-flying honeybees, trained under absolute condition, on their capacity to use visual information from the different patterns produced by angle dependent patterns to drive decisions.

We specifically test the role of angle dependent coloration on a biologically relevant discrimination task as it is already known that honeybees (Giurfa et al. 1996; Dyer et al. 2008) and bumblebees (Spaethe et al. 2001; Dyer et al. 2008; predominantly use achromatic vision mediated by the long wavelength sensitive photoreceptor for flower detection. Therefore, color information is not used for flower detection in bees.

If a flower’s structural color does constitute a visual signal which provides information to the pollinator such that it may modify its behavior, one can hypothesize that: (i) color patches produced by angle dependent colors are perceivable when considering the visual acuity of a bee. (ii) it is robust enough as to enable flower identification independently from viewing angle and (iii) it is readily discriminable from pigment color. For the first hypothesis to be true, small color patches responsible for angle dependent colors in flowers should be easily discerned when observed through an optical device with the same resolution as that of the compound eye of a pollinator such as a honeybee. For the second hypothesis to hold true, the visual appearance of the color pattern of a flower should be independent from view point. Finally, for the third hypothesis, pollinators should be able to learn and recognize the pattern produced by a given angle dependent coloration when asked to choose between this option and a solid color whose appearance is independent from viewing angle. Altogether the null hypothesis framework is that angle dependent colors are only incidental effects.



Figure 2. RGB representation of flowers from the 6 plant species used for our experiments: (A) *Alyogyne huegelii*, (B) *Solanum laciniatum*, (C) *Lycianthes rantonnetii*, (D) *Tropaeolum majus*, (E) *Hibiscus heterophyllum*, and (F) *Pelargonium rodneyanum*.

Materials and Methods

Plant material

Flowers from 6 insect pollinated plant species: (a) *Alyogyne huegelii*, (b) *Solanum laciniatum*, (c) *Lycianthes rantonnetii* (previously *Solanum rantonnetii*), (d) *Tropaeolum majus*, (e) *Hibiscus heterophyllum*, and (f) *Pelargonium rodneyanum* (Figure 2) were collected from a botanical garden at Monash University, Clayton campus, Victoria, Australia during late Austral spring 2014 (September–November). Four native species to Australia: (a) *A. huegelii*, (b) *S. laciniatum*, (e) *H. heterophyllum*, and (f) *P. rodneyanum* were grown in the native plant section of the garden as an indigenous food plant; while the 2 naturalized species (*L. rantonnetii* and *T. majus*) were cultivated in a separate section of the garden. Flowers were placed inside a cooler at about 15°C and immediately brought to the lab for spectrophotometric measurement and photographic recording to ensure that petal microstructures potentially producing angle dependent coloration were preserved (Vignolini et al. 2015).

Our samples include 4 species from the closely related orders: Brassicales (*T. majus*), Geraniales (*P. rodneyanum*), and Malvales (*A. huegelii* and *H. heterophyllum*) (Wikström et al. 2001), whereas *S. laciniatum* and *L. rantonnetii* belong to the order Solanales. The 2 species of order Malvales were chosen to compare with *Hibiscus trionium*, the plant species for which angle dependent, iridescent colorations were first reported (Whitney et al. 2009b). The orders Brassicales and Geraniales are the closest to Malvales that serve as comparison between 2 groups whereas the remaining species of our sample, while *S. laciniatum* and *L. rantonnetii*, serve as a potential outgroup for comparison. These species were selected to compare within and outside the order to which *H. trionium* belongs to test if iridescence (Whitney et al. 2009) is a property observed in other plant groups. Moreover, these plants were also selected to understand plant–pollinator interactions in a broader phylogenetic scale.

Spectrophotometry

Spectra were measured from 300 to 700 nm using an Ocean Optics USB2000+ Spectrometer (Ocean Optics Inc., USA) equipped with quartz optics and connected to a PX-2 pulsed xenon light source (Ocean Optics Inc., USA, 2011). The spectrophotometer was controlled using the software package Spectra Suite (Ocean Optics, USA), and calibrated before each measurement to avoid drift from

electrical noise. Reflectance profiles were measured relative to a Lambertian, PTF WS-1 reflectance standard (Ocean Optics, USA). Mean reflectance spectrum for each species corresponds to multiple spectral measurements of 3 different flowers as described in Dyer et al. (2012) and Shrestha et al. (2013).

Scanning electron microscopy (SEM) imaging

We prepared replicas of the petal surfaces following methods described by van der Kooi et al. (2014). Briefly, sepals and petal were pressed into a dental impression material that solidifies within minutes. Positive surface replicates were subsequently generated by filling the mould with transparent nail polish, creating a cast. Casts were sputtered gold coated and images were acquired using a Scanning Electron Microscope (Philips XL30) at the RMIT Microscopy and Microanalysis Facility (RMMF), at RMIT University, Melbourne, Australia. We used 30 KV current with spot size 5 and magnification ranges 6,000 \times –1,800 \times with a 10 mm working distance from the sample to the current beam.

Photographic recording and image processing

Flower samples were located on a platform 55 cm high and inserted in a black cardboard shield to minimize potential reflection from background. To account for variations in the size and location of structural color patches arising from changes in viewing point, we recorded a total of 37 images for each flower within a hemisphere (dome) sampling grid centered at the flower sample. Sampling viewpoints were defined in terms of spherical coordinates using 3 parameters: (i) the angle on the x - y plane (azimuth, Θ) created from the x -axis to the camera's position, (ii) the angle between the x - y plane and the camera position (inclination, φ), and (iii) the distance (radius, r) between the center of the flower and the camera at each Θ , φ combination. These viewpoints represented typical approach angles observed for several bee species (*Apis* sp., *Bombus* sp., *Trigona* sp.) foraging in natural environments (Garcia et al. 2018; Dyer AG, Shrestha M, personal observation); refer to van der Kooi et al. (2015) for discussion.

Sampled azimuth angles ranged from 0° to 315° at 45° intervals. Five different inclination angles ($\varphi = 15^\circ, 30^\circ, 45^\circ, 60^\circ, \text{ and } 75^\circ$) plus the zenith ($\varphi = 90^\circ$) position were sampled for each orientation position excepting for $\Theta = 45^\circ$. At this azimuth angle, only the inclination $\varphi = 45^\circ$ was sampled to prevent shadowing the

illumination produced by the main light source. The light source consisted of a bare bulb (uncoated) Broncolor Pulso F2 flash lamp (Bron Elektronik, Switzerland) connected to a Broncolor Graffit A2 power pack (Bron Elektronik, Switzerland) raised 2.00 m from the floor and aimed perpendicular to a white ceiling of 4.10 m height. This arrangement simulated a lighting condition typical of open environments where the light reaching the target is made up by the mixture of the direct light emitted by a point source (the Sun) and the indirect light reflected or refracted by the sky, and resulted in a realistic environmental illumination difference ratio of about 8:1 (3 photographic stops) (Salvaggio 2009). For each flower, an additional image was recorded at the zenith of the sample.

Images were recorded with a calibrated Canon EOS 40D digital camera (Canon Inc., Japan) equipped with a 100-mm electro focused macro lens (Canon Inc., Japan). Images were stored as native RAW files and encoded into 8-bit, Adobe 1998 color space (Adobe Systems Incorporated 2005) TIFF files using the Adobe Camera Raw v.7.3 plug-in available as part of the Adobe Design and Web Premium Suite CS6 (Adobe Corp., USA). TIFF files were linearized to recover values equivalent to the total number of photons captured by each of the color channels, analogous to *P*-values *sensu* (Wyszecki and Stiles 1982; Chittka 1992) making up the RGB image at each pixel location. Linearization was carried out using look up tables (LUTs) specifically constructed for our imaging device (Garcia et al. 2013a, 2014). Recovered *P*-values were subsequently transformed into physiological receptor excitations, or *E*-values (Chittka 1992), by applying the Naka and Rushton transformation to accurately model pollinator color perception (Naka and Rushton 1966; Chittka 1992; Spaethe et al. 2001; Dyer et al. 2007; Whitney et al. 2009b).

Spectral threshold

Since ultraviolet (UV) reflecting patches are present in several flower species (Chittka et al. 1994; Kevan et al. 2001), we assessed our sample of flower species to evaluate if any flower reflected sufficient UV radiation to be perceivable when considering the spectral sensitivity of typical hymenopteran pollinators (Peitsch et al. 1992). In the current study, we considered flowers with apparent structural colors, but no modulation of the UV-sensitive photoreceptors of a bee (Figure 3 and Supporting Information S-1).

We employed the hexagon color space (Chittka 1992; Whitney et al. 2009b; Dyer et al. 2012; Garcia et al. 2017) to (a) model the chromatic appearance of the pigment coloration of each species and (b) to identify the color difference required by an angle dependent color patch to be distinguishable from its pigment background. We set a color distance of 0.04 hexagon units (hu) as the color discrimination threshold required by a honeybee to discriminate between angle dependent (structural) and angle independent (diffuse) colors. “Blue” color stimuli differing by 0.04 hu can be discriminated by a honeybee trained under an appetitive aversive conditioning about 96% of the time as predicted by the color discrimination function for this species modeled from behavioral data (Dyer and Neumeyer 2005; Garcia et al. 2017). Therefore, for each of the flower species used for our experiment, we represented the color discrimination threshold as a circle with a radius of 0.04 hexagon units centered at the *x*–*y* coordinates corresponding to the color produced by the pigment reflectance spectrum for each species (Figure 4).

We then established spectral discrimination threshold values for the 405–505 nm and 450–600 nm spectral intervals corresponding to the regions sensed by the respective “blue” and “green” channels of our camera system (Garcia et al. 2014) for each one of the flower

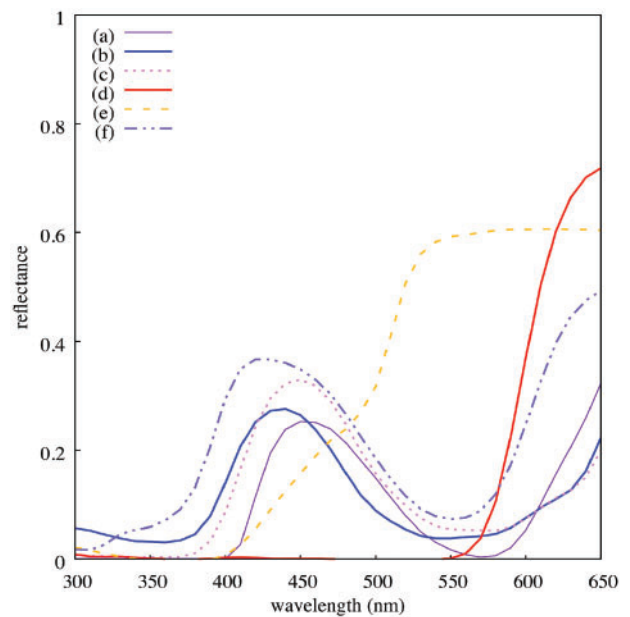


Figure 3. Reflectance spectra from the 6 plant species sampled for the study: (a) *A. heugelii*, (b) *S. laciniatum*, (c) *L. rantonnetii*, (d) *T. majus*, (e) *H. heterophyllum*, and (f) *P. rodneyanum*. Excepting from *L. rantonnetii*, selected species did not modulate the UV photoreceptor of the honeybee. As our imaging system had no sensitivity to this spectral interval, this species was excluded from subsequent analyses.

sample species. Spectral threshold values were obtained from a pair of *E*-vectors (i.e. modulation of color space excitation values), which systematically increased in the number of photon catches for the blue and green photoreceptors from those corresponding to the measured spectral reflectance for each flower and represented as the origin of the discrimination circle (blue and green arrows in Figure 4). The intersection point between each of the respective vectors and their corresponding color discrimination circle was then established as a threshold value for identifying petal regions where the structural color was perceptually different from the pigment-based hue in the linearized images. The result of the threshold operation consisted on a set of binary masks incorporating white for selected pixels, but black otherwise, representing petal regions with angle dependent color patches perceivable as being different from the pigment background for either the “blue” or the “green” channels of the linearized images.

Spatial threshold

The size of the lens and diameter of the rhabdoms making up most insect’s compound eyes limit their spatial resolution (resolving power) to less than about 1 cycle per degree of visual angle (cpd) (Land 1997). Large, simple lenses such as those present in vertebrate eyes and photographic optics typically have a minimum resolving power well above this limit (Kirschfeld 1976; Land 1997; Williams and Dyer 2007). This means that structural coloration patches, although potentially perceivable as being of different color on a photographic image, may not necessarily be resolved by an insect eye (van der Kooij et al. 2015). To account for this potential limitation, we recorded images corresponding to the threshold, binary masks using a mechano-optical device constructed on the optical principle of ray selectors (Knowles and Dartnall 1977; Williams and Dyer 2007). The device consisted on an array of about 4,500 black

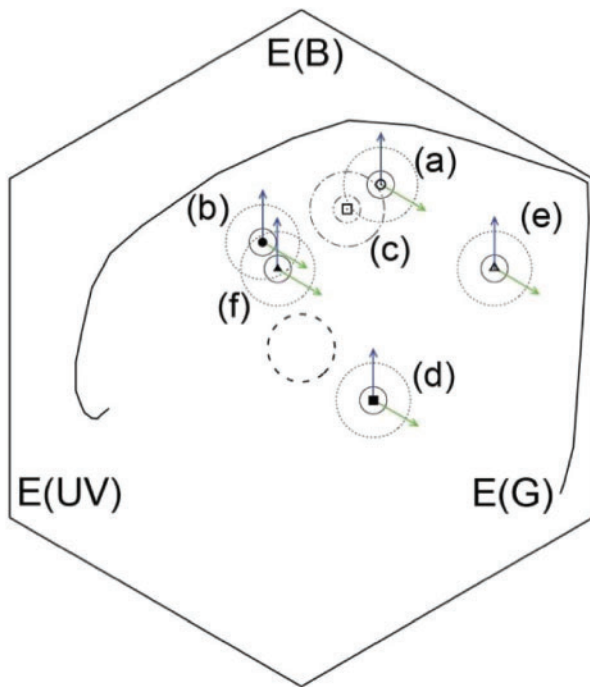


Figure 4. Representation of the petal colors corresponding to the reflectance spectra in Figure 3 in the hexagon color space (Chittka 1992): (a) *A. huegelii* (open circle), (b) *S. laciniatum* (solid circle), (c) *L. rantonnetii* (open square), (d) *T. majus* (closed square), (e) *H. heterophyllum* (open triangle), and (f) *P. rodneyanum* (closed triangle). Circles surrounding the markers indicating each flower species represent the discrimination threshold for a typical hymenopteran pollinator trained with differential conditioning when discrimination color differences of 0.04 hu (solid circle) and 0.11 hu (dashed circle). Arrows represent the shift in color space expected from increasing the photoreceptor excitation values (E-vectors) by either the medium (E(B), solid blue arrow) or long (E(G), solid green arrow) photoreceptors here modeled by the transformed linear response of the green and blue color channels of a characterized digital camera (García et al. 2013a, 2014). Photoreceptor excitation values corresponding to the point of intersection between the vector and the discrimination threshold are considered as being perceptually different from the pigment-produced color, and thus used as threshold values for differentiating structural from pigment colouration (refer to text for details).

tubes, 31 cm long with a diameter of 3 mm stacked in a 36 × 38 cm wooden frame which projected a single image on a piece of architecture tracing paper of the same dimensions. This arrangement produced images with a spatial resolution of about 0.24 cpd (Williams and Dyer 2007), very close to the 0.23 cpd corner resolution limit behaviorally determined for free flying honeybees (Srinivasan and Lehrer 1988). This visual acuity principle also approximately fits with how other bees like bumblebees use visual information to find flowers in complex-type environments (Spaethe et al. 2001; Dyer et al. 2008, 2016).

Binary mask images and their corresponding non-linear RGB representations were displayed on an LED 27" Thunderbolt Display (Apple Corp., USA) with a resolution of 2,560 × 1,440 pixels. Images were resized such that when projected through the mechano-optical device at a distance of 0.3 cm produced an image of sufficient size to cover a piece of architectural drafting paper attached to the device's wooden frame. This set-up replicated how a bee's compound eye may resolve the flower at close range (Williams and Dyer 2007). Images projected on the tracing paper corresponding to the different azimuth and inclination angles for each species were

photographed using the same Canon 40D camera used for recording the flower samples.

TIFF images containing the mechano-optical representation of the spectral threshold masks and their corresponding non-linear RGB images were then segmented following protocols for measuring and analyzing color patterns (García et al. 2013b) to identify and measure: (a) the petal's area corresponding to the angle dependent coloration and (b) the total visible area. These 2 variables were subsequently used for calculating the ratio of petal area occupied by angle dependent color patches (RAD) on each image by applying Equation (1).

$$\text{RAD} = \left(\frac{\text{petal area covered by angle dependent colors (mm}^2\text{)}}{\text{total petal area (mm}^2\text{)}} \right) \times 100. \quad (1)$$

Statistical analysis of images

To test the reliability of the color signal produced by the structural color component of the color pattern, we measured the correlation between RAD and azimuth and inclination angles using a measure of linear–circular association (Pewsey et al. 2013). We calculated the Mardia's Rank correlation coefficient for linear–circular association between the linear variable RAD, and the circular variables azimuth and inclination independently. In both cases, we tested for the null hypothesis of independence (Mardia 1976). Statistical analyses were performed using code by Pewsey et al. (2013) written for the statistical package R v.3.2.1 (Core Team R 2015).

Behavioral testing and statistical analysis

To test if honeybees could use angle dependent colorations as a signal we conducted behavioral experiments using the images of *S. laciniatum* as stimuli (Figure 5, third column, panels I–L) as this flower presents the highest proportion of angle dependent color patches relative to the entire petal surface (RAD) when considering free-flying bee vision (see the “Results” section).

Eight images representing the different azimuth angles (θ) measured at $\varphi = 45^\circ$ were used for the behavioral experiment. Stimuli were created from the RGB images recorded for measuring the RAD of *S. laciniatum* and divided into different sets (Figure 6). Set A consisted of 8 images of this species viewed at different θ angles but without any indication of the presence of angle dependent coloration (Figure 6 panels A–H). Set B contained the same images, but additionally included the pattern created by the patches produced by the angle dependent color at each viewpoint (Figure 6 panels I–P). Patches in this image set corresponded to colors which are potentially perceivable by a honeybee as predicted by our color modeling (see spectral and spatial threshold subsections above). The pattern produced by patches of angle dependent colors at the different inclination and orientation angles considered were indicated with a strong “yellow” color as this promotes the most rapid learning of spatial stimuli by honeybees (Morawetz et al. 2013). Set C (Figure 6 panels Q–X) displayed such patterns in insolation on a sample of the petal pigment color to control for potential innate color preference effects (Morawetz et al. 2013).

The use of the multiple stimuli sets allowed behavioral testing to determine if the patterns produced by angle dependent coloration could influence bee choices in a way that would be consistent with the definition of a signal. For the signal hypothesis to be true, a bee would need to reliably identify a stimulus by the pattern produced by the angle dependent patches, independent from the azimuth position.

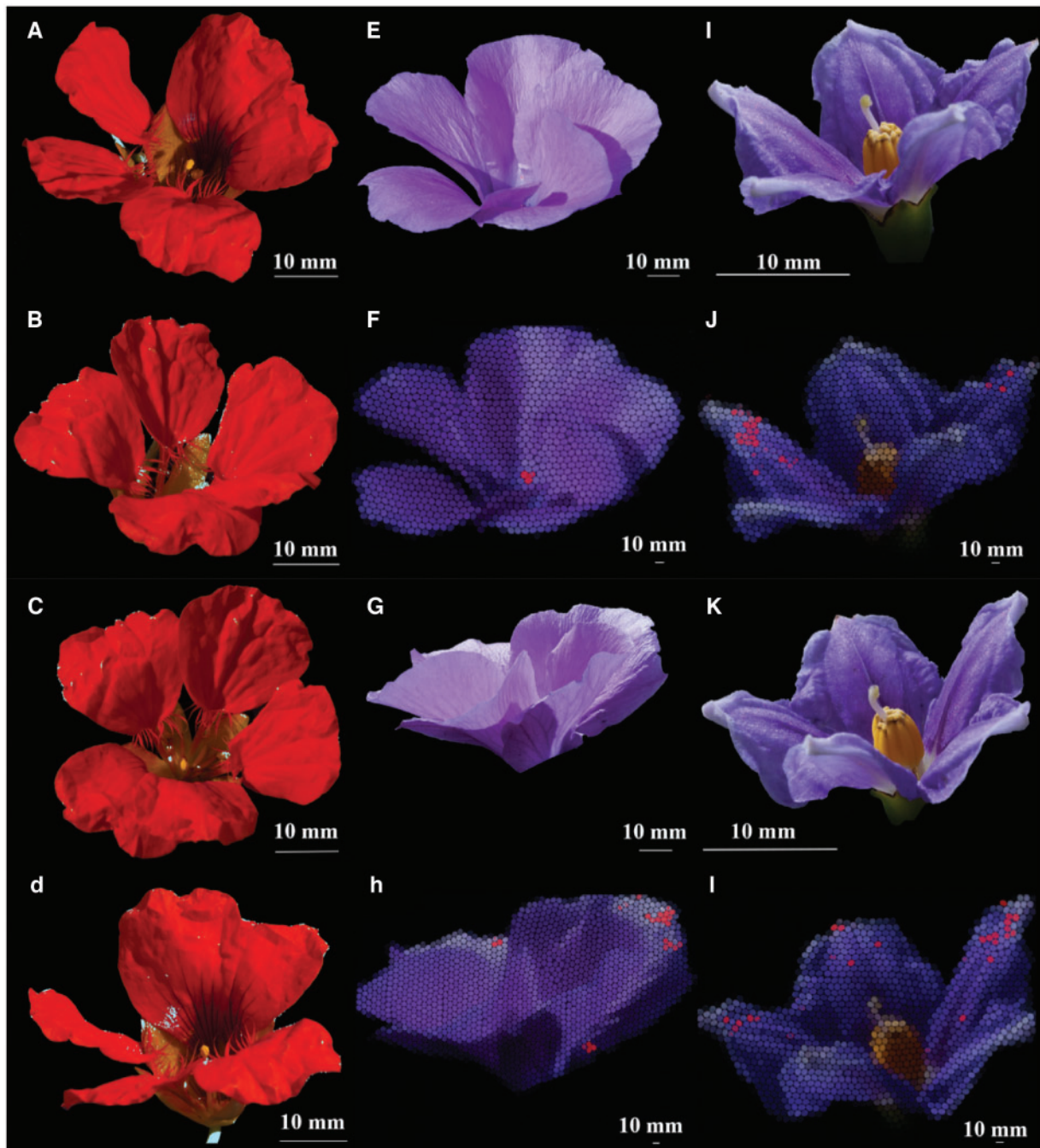


Figure 5. Composite images indicating regions of perceivable structural color in *T. majus* (first column, panels A–D) *A. huegelii* (second column, panels E–H), and *S. laciniatum* (third column, panels I–L). Areas of structural coloration potentially perceivable to a honeybee are indicated with cyan color if not present on the petal area or red color otherwise. Panels E, G, I, and K depict RGB representations of *A. huegelii* (panels E and G) and *S. laciniatum* (panels I and K) at 1:5 and 1:3 magnification ratios. Panels F, H, J, and L correspond to the same RGB images after being projected by a mechano-optical device: *A. huegelii* (panels F and H) and *S. laciniatum* (panels J and L). In panels F, H, I, and J, the red color indicates potentially perceivable structural color regions when considering both spectral and spatial threshold values set by the properties of the honeybee’s visual system. Scale bars on panels A–E, G, I, and K represent the flower’s size; on panels F, H, J, and L, scale represent the size of the projected image. *T. majus* images represent viewing angles: $\theta = 0^\circ$, $\varphi = 75^\circ$ (panel A); $\theta = 90^\circ$, $\varphi = 30^\circ$ (panel B); $\theta = 135^\circ$, $\varphi = 60^\circ$ (panel C); and $\theta = 315^\circ$, $\varphi = 60^\circ$ (panel D). Images corresponding to *A. huegelii* (second column) and *S. laciniatum* (third column) represent viewing points at which the percentage of structural to visible color area (RAD) were maximal for each species: (E, F) $\theta = 90^\circ$, $\varphi = 30^\circ$; (G, H) $\theta = 225^\circ$, $\varphi = 15^\circ$; (I, G) $\theta = 180^\circ$, $\varphi = 15^\circ$; and (K, L) $\theta = 270^\circ$, $\varphi = 30^\circ$.

The alternative hypothesis would suggest that angle dependent colors are a cue that bees may only use in limited circumstances.

We individually trained marked honeybees ($n = 13$) using absolute conditioning to 4 of the 8 stimuli presenting patches produced

by the angle dependent coloration (Supporting Information S-3 panel a). The 4 stimuli were randomly selected for each bee from the 8 different azimuth positions. For any testing run, all 4 training stimuli were simultaneously presented on a rotating screen which

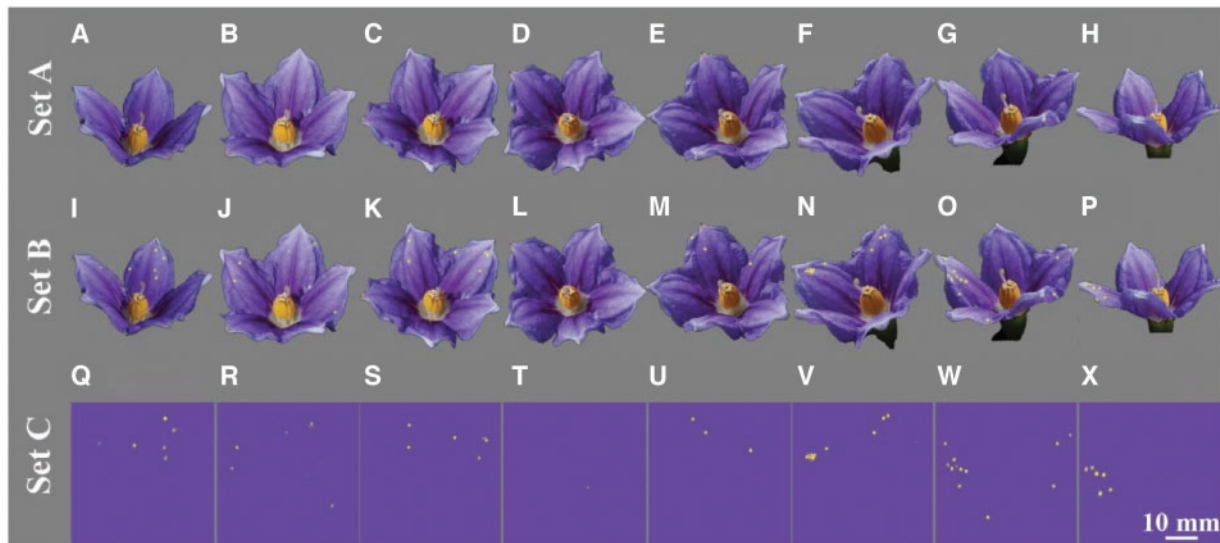


Figure 6. Images of the 8 stimuli triplets used for the behavioral experiments. Each column represents an image of *S. laciniatum* at an inclination angle (ϕ)=15° and various orientation (θ) angles: (A, I, Q) 0°; (B, J, R) 45°; (C, K, S) 90°; (D, L, T) 135°; (E, M, U) 180°; (F, N, V) 225°; (G, O, W) 270°; and (H, P, X) 315°. Images on Set A (first row) represent flowers of *Solanum* at different θ angles but without indication of angle dependent color effects. Set B represents the same θ angles as in Set A, but the pattern produced by the perceived angle dependent color patches at each viewpoint is indicated with a “yellow” color which is easily discriminated from the pigment color by a bee. Set C represents the same angle dependent color patterns as in Set B, but excludes visual information about flower morphology. The violet color making the background of images in Set C correspond to a printer ink interpretation of the petal color of images in Sets A and B (see details in Supporting Information S-2).

enabled realistic testing of honeybees using ecologically relevant stimuli (Stejskal et al. 2015). The absolute conditioning phase length was 30 choices (landing and drinking of sucrose) which is twice as long as bees typically take to learn color signals considering absolute conditioning (Giurfa 2004; Dyer 2012). This training regime mimics a potential signal that a bee would likely encounter to identify a flower in natural settings. Bees were rewarded with 15 μ L drops of 50% sucrose and allowed to return to the hive if satiated.

A learning test of 20 unconditioned choices was conducted after the absolute conditioning phase. Each bee was given a non-rewarded learning test where 2 of the 4 angle dependent color patches used as training stimuli were presented against 2 gray stimuli (Supporting Information S-3 panel b). Following the learning test, a transfer test and conflict test were conducted in pseudo-random order using the same protocol as the learning test with 4–8 refresher choices conducted between each test to maintain bee motivation. The transfer test presented bees with 2 of the 4 stimuli not used during training (Set A vs. matched Set B in Figure 6, Supporting Information S-3 panel c) to determine whether bees would prefer flowers presenting the angle-dependant coloration information. The conflict test presented bees with the remaining stimuli not used during the transfer test (Set A vs. Set C in Figure 6, Supporting Information S-3 panel d). The conflict test would determine whether bees prefer to visit flowers with no angle-dependant coloration information or a colored stimulus with angle-dependant color information presented.

To determine whether bees had learned to associate flowers of *S. laciniatum* presenting angle dependent colorations with a reward of sucrose, the “proportion of correct choices” data from all 3 tests were estimated by means of 3 independent generalized linear mixed models (GLMMs) assuming a binomial distribution for the binary response, and bee ID number as random effect to account for the repeated measurements (Zuur et al. 2009). The models only included the intercept term as fixed factor allowing for testing if the

observed proportion of choices was different from chance expectation (H_0 : proportion of choices for choices for target =0.5). Models were fitted using the routine glmer available as part of the package lme4 (Bates et al. 2015) for the R statistical language and programming environment. Overdispersion and residual plots were constructed for each model to validate the GLMM assumptions.

Results

Scanning electron microscope imaging

Scanning electron microscope images reveal 3 different cell types on the petals of our flower sample (Figure 7): (i) tabular rugose-striated cells in *A. huegelii*, (ii) conically shaped cells in *H. heterophyllum* and *T. majus*, (iii) flat, hexagonal cells in *P. rodneyanum*, and (iv) papillate cells in *L. rantonnetii* and *S. laciniatum*. *Alyogyne huegelii* presents quasi-parallel striations with separations smaller than 1 μ m (Figure 7 panel A), while epidermal cells of *T. majus* and *P. rodneyanum* show distinctive radially striated crests with separations smaller than 1 μ m (Figure 7 panels D and F).

Imaging experiments

We evaluated the reliability of a signal produced by angle dependent colors within the 405–505 nm (“blue”) and 450–600 nm (“green”) spectral intervals for 5 plant species: *A. huegelii*, *S. laciniatum*, *T. majus*, *H. heterophyllum*, and *P. rodneyanum*; when considering viewing angle, the spectral characteristics of the visual system of the honeybee and a color discrimination threshold value of 0.04 hexagon units (Table 1). Two of the species, *H. heterophyllum* and *P. rodneyanum*, did not present angle dependent color patches which could be discriminated from the pigment background about 95% of the time as being different from the pigment background in either the “green” or “blue” spectral intervals (Table 1). Most of the angle dependent color patches in *T. majus* corresponded to the “blue”

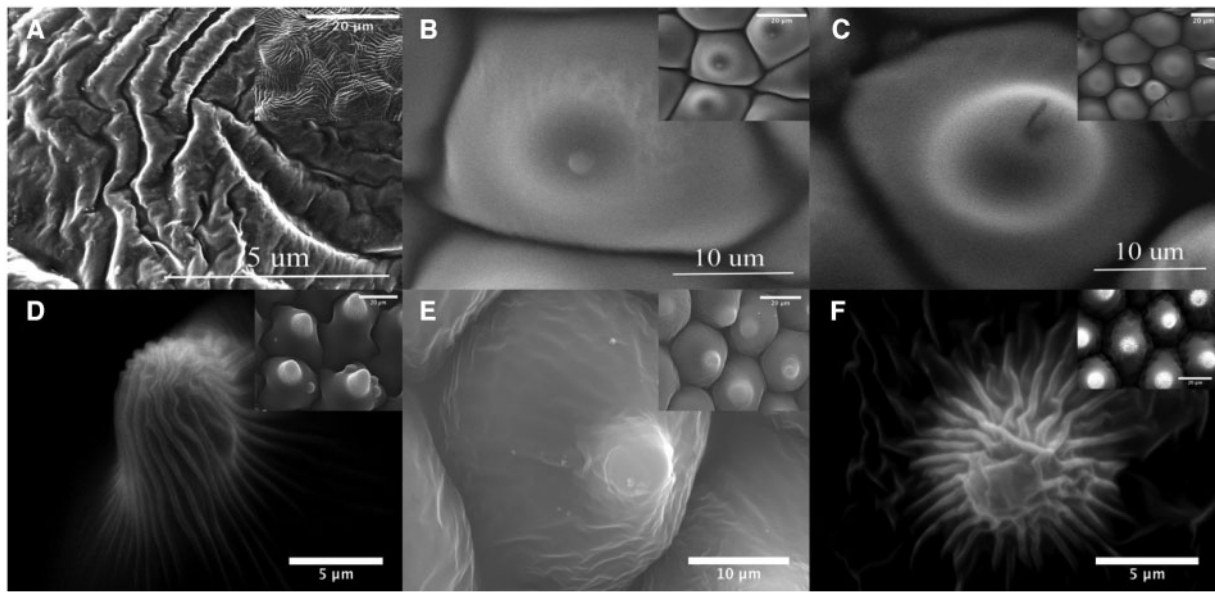


Figure 7. Scanning electron microscope images showing details of petal features present on the adaxial surfaces of the 6 species used for our study at various magnifications to accommodate for differences in feature size: (A) *A. huegelii* (6,000×), (B) *S. laciniatum* (3,383×), (C) *L. rantonnetii* (3,294×), (D) *T. majus* (3,406×), (E) *Hibiscus heterophyllum* (3,159×), and (F) *P. rodneyanum* (3,228×). Insets on each panel depict a less augmented version of each image. In all insets the scale bar represents 20 μm. All SEM images were acquired using a Philips XL30 SEM microscope.

Table 1. Threshold *E*-values for the “blue” and “green” spectral regions for the 5 plant species showing no modulation in the UV region when considering green adaptation background and a color discrimination threshold value of 0.04 hexagon units (second column)

Species\spectral parameters	Threshold <i>E</i> -value		Mean maximum <i>E</i> -value		Is iridescence perceivable?	
	“Green”	“Blue”	“Green”	“Blue”	“Green”	“Blue”
<i>Alyogyne huegelii</i>	0.417	0.764	0.412±0.09	0.472±0.05	Yes	No
<i>Solanum laciniatum</i>	0.406	0.786	0.477±0.04	0.497±0.02	Yes	No
<i>Lycianthes rantonnetii</i> ^a	NI	NI	NI	NI	NI	NI
<i>Tropaeolum majus</i>	0.328	0.050	0.018±0.002	0.196±0.05	No	Yes ^b
<i>Hibiscus heterophyllum</i>	0.804	0.709	0.493±0.01	0.378±0.08	No	No
<i>Pelargonium rodneyanum</i>	0.572	0.847	0.350±0.11	0.474±0.05	No	No

Mean *E*-values and standard deviations corresponding to the maximum *E*-value obtained on each of the $n = 37$ linearized images representing various viewing points recorded for each species, ^a*Solanum rantonnetii* potentially modulates the UV-sensitive photoreceptor in the honeybee; however, as this spectral region is beyond our current system capability, this species was not included in the reported results. NI, not included, ^bPerceivable iridescence mainly corresponds to flower regions not involved in sexual reproduction (Figure 5).

spectral region and were found the calyx region which is not involved in plant sexual reproduction (first column Figure 5A–D); for this reason, data corresponding to this species were excluded from subsequent analyses. *Alyogyne huegelii* (second column Figure 4e–h) and *S. laciniatum* (third column Figure 4i–l) only presented angle dependent coloration perceivable as being different from the pigment background in the “green” spectral region.

Threshold binary masks corresponding to *A. huegelii* and *S. laciniatum* were subsequently imaged with the mechano-optical device to obtain spatial measurements of optically resolvable angle dependent color patches by a honeybee. Examples of some of the resulting images are presented in Figure 5F, H, J, L.

We found a significant negative correlation between the area of the petal occupied by angle dependent color patches and the total area petal for the 2 species [Kendall’s tau (τ)_{*A. huegelii*} = −0.312, $P = 0.011$; τ _{*S. laciniatum*} = −0.335, $P = 0.004$]. For this reason, RAD values were used for the remaining analyses.

The RAD values significantly varied across the measured orientation and inclination angles, and in different ways for either the *A. huegelii* and *S. laciniatum* flowers (Figure 8, Supplementary Information videos V1 and V2, respectively). *Alyogyne huegelii* presented a larger RAD area than *S. laciniatum*, but the latter was characterized by having a greater number of RAD areas than its counterpart (Figure 8). Linear–circular correlation analysis and tests for independence evidenced different relationships between the size of the petal area displaying a perceptually different structural color, and the viewing angle in either *A. huegelii* or *S. laciniatum*. While structural coloration in *A. huegelii* was independent from azimuth angle [Mardia’s rank correlation coefficient (U) _{θ *A. huegelii*} = 777.0, $P = 0.825$] it was dependent on inclination (U _{ϕ *A. huegelii*} = 1.96×10^4 , $P = 0.008$). Structural coloration in *S. laciniatum* was dependent on azimuth (U _{θ *S. laciniatum*} = 1.43×10^4 , $P = 0.026$) but independent from inclination (U _{ϕ *S. laciniatum*} = 9.81×10^3 , $P = 0.098$).

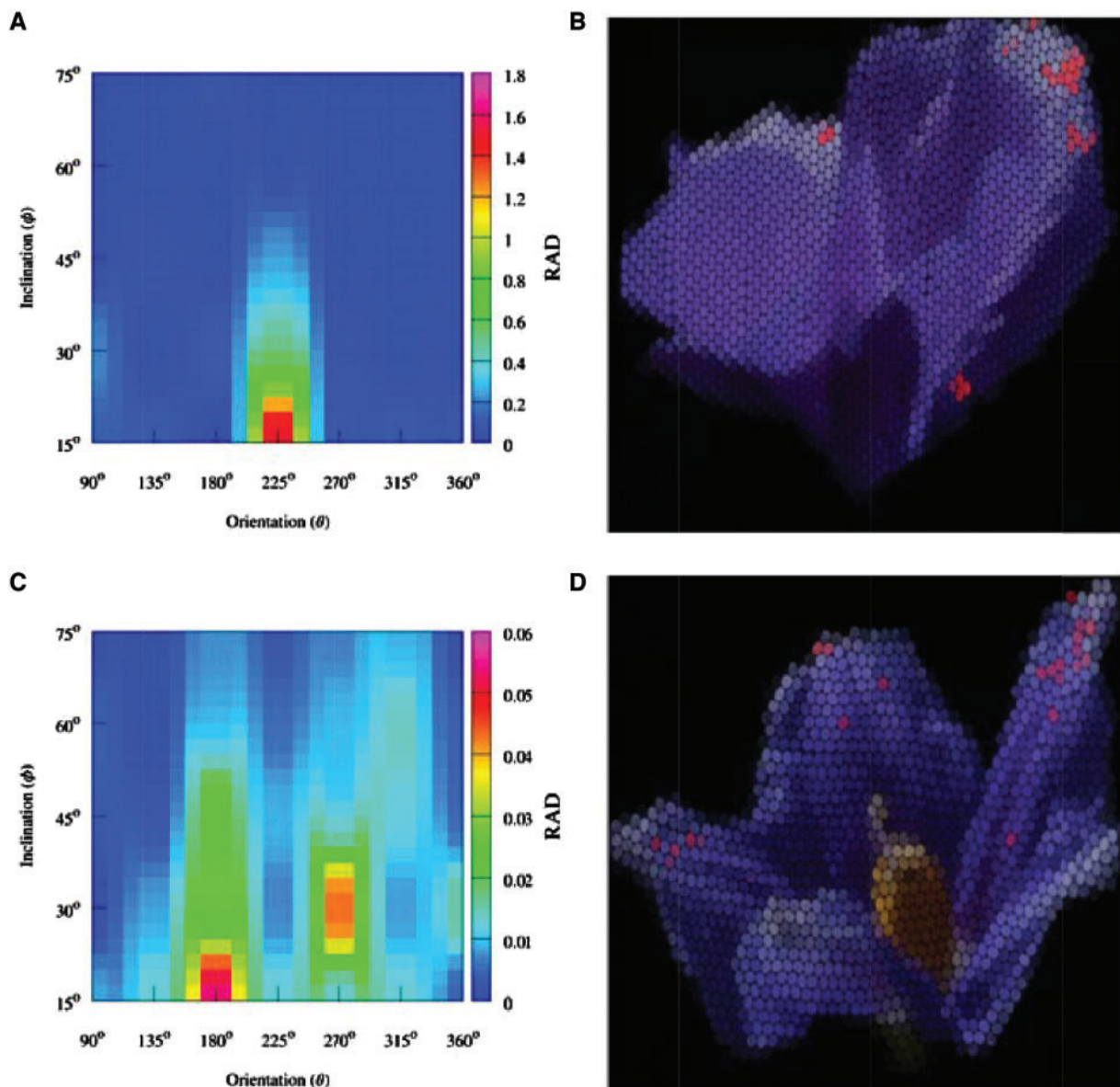


Figure 8. Color map representing the ratio of angle dependent color areas [indicated as red dots on panels (B) and (D)] to total visible area (RAD) as a function of orientation (x -axis) and inclination (y -axis) for *A. huegelli* [panel (A)] and *S. laciniatum* [panel (C)]. Panels (B) and (D) show and RGB representation of *A. huegelli* and *S. laciniatum*, respectively, as produced by the mechano-optical device used to simulate the image produced by the honeybee compound eye (Knowles and Dartnall 1977; Williams and Dyer 2007), at the orientation and inclination position showing the largest area of angle dependent coloration for each species. On panels (B) and (D) image regions where angle dependent coloration is discriminable from the pigment background 95 % of the time are indicated by a red color to aid visual interpretation by human observers.

Behavioral experiments

Figure 9 summarized the results of the 3 behavioral tests carried out. In the learning test, honeybees chose the target displaying the angle dependent coloration significantly more times than the gray stimulus (mean proportion of correct choices for angle dependent coloration [$\mu_{\text{adc}} = 0.737$ (0.679, 0.792 95% CI), $z = 7.04$, $P < 0.001$], thus demonstrating they had associated the images of *S. laciniatum* with a reward of sucrose.

Two further tests were conducted following the learning test: a transfer test (Set A vs. matched Set B in Figure 6, Supporting Information S-3 panel c) and a conflict test (Set A vs. matched Set C in Figure 6, Supporting Information S-3 panel d). For the transfer test, bees were presented with 2 of the 4 stimuli not used during

initial training against the matched versions of these stimuli that lacked angle dependent colors. If the signal hypothesis holds true, then bees must be able to perform this task above chance expectation (50%). Between each of the 3 tests, 4–8 refresher landings were presented to ensure motivation.

In the transfer test, where images representing novel azimuth angles plus angle dependent color marks were presented against the images recorded at the same angles but without presenting the pattern produced by the angle dependent coloration, bees did not show a preference for images displaying flowers with angle dependent coloration [$\mu_{\text{adc}} = 0.559$ (0.480, 0.639 95% CI), $z = 1.58$, $P = 0.114$], thus suggesting that honeybees did not use these patches to inform their choices.

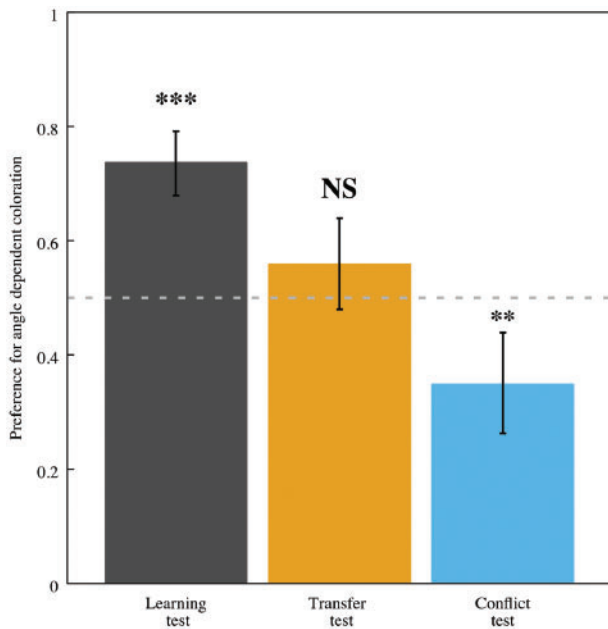


Figure 9. Mean proportion of honeybee choices for angle dependent stimuli when presented against different alternative stimuli: an achromatic, gray target without angle dependent or independent color (learning test), flowers with patterns produced by angle dependent colors at different orientation and inclination positions against the same flowers without the angle dependent patterns (transfer test); and, flowers at different orientation and inclination angles without the corresponding angle dependent color patterns against stimuli showing the respective angle dependent patterns on a uniform background with the same color displayed by the petals.

*** $P < 0.001$, ** $P < 0.01$, NS non-significant at $\alpha = 0.05$.

Finally, we performed a conflict test—bees were presented with the remaining stimuli not used for the transfer test against the pattern produced by the angle dependent coloration for the selected viewpoint on a square displaying the same color as *S. laciniatum*. Three outcomes were possible from the conflict test: (i) bees prefer the original flower even without angle dependent colors, OR (ii) there is a conflict caused by the angle dependent patches and flower information being presented; if we see chance performance in this test. Alternatively (iii), if bees have learnt to use the angle dependent color pattern as a signal, they would significantly prefer to choose the patterns produced by angle dependent colors on the purple squares. If bees could do the learning test, but not the transfer test, and in the conflict test they preferred the flower, then there is no evidence supporting the hypothesis of bees using angle dependent color signals in the presence of a strong pigment color signal. However, we can only consider angle dependent colors as being a signal if bees perform significantly above chance expectation in the learning and transfer tests, and do not prefer the flower in the conflict test. Bees did not show a significant preference for the colored squares containing the respective angle dependent color patches [$\mu_{\text{adc}} = 0.349$ (0.263, 0.439 95% CI)], but instead preferred the flower images which did not present angle dependent colors ($z = -3.40$, $P < 0.001$) thus suggesting that the signal hypothesis does not hold true in the context of our experiments.

Discussion

Insect pollination is essential for a large number of plant species, and for many flowering plants there is evidence that specific floral

traits enhance successful repeat visits from flower constant pollinators (Waser 1986; Fenster et al. 2004; Sargent and Ackery 2008; Schiestl and Johnson 2013; Ohashi et al. 2015). Recent reports that bumblebees can be trained in lab conditions with appetitive-aversive differential conditioning to learn iridescent colors (Whitney et al. 2009b, 2016; Moyroud et al. 2017; de Premorel et al. 2017) have raised the interesting possibility that structural coloration may have evolved in evolutionary distantly related flower species to serve as a signal to enhance plant–pollinator visual communication. However, several studies have questioned this interpretation because flowers are typically viewed by potential insect pollinators in complex environments where structural color is unlikely to be a robust source of information for a free-flying insect (Morehouse and Rutowski 2009; van der Kooi et al. 2014, 2015). Furthermore, the possibility that such stimuli may often be beyond the resolution of an insect compound eye has also been raised (van der Kooi et al. 2015). In the current study, we were able to employ recent advances in our understanding of how to model bee pollinator color (Garcia et al. 2017, 2018) and spatial vision (Dyer and Williams 2005; Howard et al. 2018) to formally test the potential role of angle dependent colors as potential signals for bees. Furthermore, we tested the hypothesis derived from image analysis regarding the potential use of angle dependent colors as visual signals by pollinating insects using free-flying honeybees.

The precise role of structural colors for plant–pollinator visual communication has largely remained unresolved, probably due to the synonymous use of the words *cue* and *signal* in the literature. However, these 2 words have different meanings in the context of biological communication (Smith and Harper 2003; Bradbury and Vehrencamp 2011) and would imply different evolutionary and behavioral relationships between flowers and their pollinators. For angle dependent colors to be considered salient visual signals for communicating with pollinators, as previously proposed for target flower discrimination (Whitney et al. 2009; Moyroud et al. 2017), it is necessary that these colors transfer meaningful information to a bee such that it can reliably identify a flower irrespective of viewing angle, and that it has evolved specifically for this purpose. Our results, however, indicate that this is not the case. Interestingly, bird predators in natural conditions cannot use angle dependent, iridescent colors reflected by the ventral wing of *Battus philenor* butterflies for prey identification (Pegram et al. 2015), also suggesting structural colors may be of limited value for visual signaling when viewing angle is variable. In one of the species (*T. majus*), angle dependent colors potentially discriminable from the pigment background color were found on the calyx, a flower part different from the petals (Figure 5a–c) and thus would likely serve no value in communicating with a potential pollinator. Similar optical effects have also been reported for other plant parts not involved with pollination such as fruits (Lee 1991), and on the leaves of non-flowering plants such as the red alga *Chondrus crispus* (Chandler et al. 2015) and the fern *Selaginella* (Héban and Lee 1984).

Finally, angle dependent color patches in *A. huegelii* and *S. laciniatum* fail to unambiguously transfer information to a bee pollinator due to: (a) the significant correlation between size of the petal area displaying such colors with viewing angle (*Spectral limitations*) and (b) the difficulty of resolving these patches by the insect compound eye (*Spatial limitations*).

Our behavioral experiments formally tested the hypotheses arising from the imaging results (Figure 9). When required to learn angle dependent color information from a variety of biologically plausible azimuth positions, bees subsequently showed no

preference for flowers images containing angle dependent color patterns when presented against flower images without such information in a transfer test. This was despite the fact that bees had learnt the flower image as rewarding in the learning tests. To be classified as signal particular visual information must allow for the unique identification of individual flowers, but this was not the case for angle dependent colors perceived by bee pollinator for our biologically plausible scenario. Specifically, in the transfer test bees were unable to use angle dependent patterns to identify a target flower and in the conflict test bees actually chose to prefer solid, flower colors rather than angle dependent color patterns (Figure 9). This means that angle dependent colors as those produced by ultra-structures are very unlikely to be a robust signal in complex natural conditions.

Spectral limitations

The correlation of viewing angle with the size of the area presenting perceivable structural coloration means that a bee could only uniquely identify a flower when approaching at a specific set of angles. If the angle dependent coloration serves as a signal for communication, the information transmitted by these colors would be unreliable for a free flying bee in a natural environment unless individual bees always approached different flowers from exactly the same viewpoint (Figure 1); and unlikely scenario in complex and competitive environments (Garcia et al. 2018). In contrast, pigment coloration transmits color information independently from angle due to its diffuse nature (Lee 2005), thus effectively reducing the ambiguity introduced by chromatic variation produced by changes in view point typical of structural colors (Doucet and Meadows 2009; de Premorel et al. 2017).

The correlation between view point and the size of the petal area displaying angle dependent colorations perceptually discriminable from the pigment background also limits the usefulness of structural colors as a mechanism to boost pigment color in flowers as it has also been proposed (Glover and Whitney 2010). Although optical and physiological properties of plants such as ultrastructure and heliotropism have been shown to significantly increase the temperature of internal flower parts, potentially increasing pollen growth and accelerate ovule fertilization in some species (van der Kooi et al. 2017; Wilts et al. 2018), its effect on pollinator attraction remains inconclusive (Totland 1996). Even though it is possible that under specific illumination conditions heliotropism and or ultrastructures may increase the effect of angle dependent coloration in certain species (Figures 5, 7), the production of such a coloration seems to be incidental rather than evolved as expected from a signal. For example, Totland (1996) showed that insect visitation was not affected by the alignment of *Ranunculus acris*, an heliotropic genus known to present angle dependent coloration (van der Kooi et al. 2017), relative to the sun.

Spatial limitations

Another important aspect to consider is the small size of the patches produced by structural colors (van der Kooi et al. 2015) observed in the 2 sampled species presenting potentially perceivable structural colors. Most of the angle dependent patches in *A. huegelii* and *S. laciniatum* occupy an area of less than 1% of the petal visible area (Figure 8), which can only be resolved by an hymenopteran at close range due to the optical properties of the compound eye (Kirschfeld 1976; Srinivasan and Lehrer 1988; Land 1997). Although it remains to be specifically tested if small patches of angle dependent

colorations might improve the efficiency of, or act as, “nectar guides,” such a possibility would not necessarily imply that angle dependent colors act as a signal for visual communication.

Firstly, petal marks are very likely to be resolved well after an insect pollinator has made the decision to land onto a petal; therefore, nectar guides are unlikely to serve for unambiguously identifying a flower from afar by an approaching insect as expected from a salient signal evolved for visual communication between plant (emitter) and insect pollinator (receiver). Both optical modeling and experimental behavioral data suggest that bee-sized insect pollinators cannot perceive such markings over long distances, in particular if they reflect short wavelength radiation as the L (long wavelength) photoreceptor is responsible for detecting small objects in honeybees and bumblebees through achromatic vision (Giurfa 1996; Hempel de Ibarra et al. 2009, 2015), while the hypothesis of a signaling role of structural colors is formulated in the context of color discrimination (Whitney et al. 2009, 2016). Secondly, the presence of petal marks does not seem to increase the number of pollinator visits as evidenced both by bumblebees (Manning 1956) or specialized pollinating flies (Hansen et al. 2012). Therefore, angle dependent colors present in nectar guides, if any, are more likely to act as an orientation cue rather than as a salient signal for visual communication between plant and insect, or as a signal for plant identification as previously hypothesized (Whitney et al. 2009; Moyroud et al. 2017). However, this does not exclude the possibility of structural colors present in nectar guides, if any, could serve as short distance visual signals for improving flower handling after landing.

It is possible that large bees like bumblebees that have chromatic processing channels with equivalent resolution to achromatic spatial channels (Dyer et al. 2008) may in some cases be able to resolve angle dependent color patches from our flower samples as suggested by experiments using artificial targets (Whitney et al. 2009b, 2016; Moyroud et al. 2017; de Premorel et al. 2017). However, behavioral testing of bumblebees detecting either wild-type or mixta-mutant flowers suggests that changes in petal structure have no significant effect on the efficiency of bees detecting flowers (Dyer et al. 2007). For honeybees, chromatic processing is coarser than the achromatic channel (Giurfa et al. 1996; Dyer et al. 2008) and so it is unlikely that honeybees, or smaller bees, could ever see patches of angle dependent coloration as a chromatic source of information unless the bee has practically already landed on the flower. The relatively small size of the patches observed in flowers reported as presenting angle dependent colorations (Whitney et al. 2009b; Vignolini et al. 2015; Moyroud et al. 2017, Figure 5 this study) may explain how bumblebees could slowly learn angle dependent colorations such as iridescence using ideal iridescent targets in controlled lab conditions. Indeed, when trained with appetitive-aversive differential conditioning, bumblebees took about 80 choices to achieve an accuracy of about 75% when discriminating artificial, iridescent stimuli (Whitney et al. 2009b), compared with a discrimination task between 2 dissimilar pigment colors where bumblebees took 20 choices to achieve a success rate of more than 90% (Dyer and Chittka 2004). Honeybees are known to be able to use salient small local cues to make decisions if specifically trained to do so (Avargues-Weber et al. 2015), but the results of our behavioral experiments using free-flying individuals show that in the presence of an angle independent color as that produced by the pigment background, bees did not use angle dependent color to make reliable repeat decisions in natural environments. The results obtained from our behavioral experiments are very likely to apply to a wide range of angle dependent colorations independently from the specific optical phenomena.

Structural colors act as cues in flowers

How can we then classify the role of patches produced by structural coloration in pollination? We agree that angle dependent colors could be treated as a *cue* in the context that the structural color can be correlated with a particular physical trait of the plant (Bradbury and Vohrencamp 2011), for example the particular texture of a flower's epidermal cells (Whitney et al. 2009b; Vignolini et al. 2012; van der Kooi et al. 2017; Moyroud et al. 2017). However from the classic definition of signals for communication, this does not imply that structural coloration has indeed evolved to transmit useful information to the observer as expected from a signal (Smith and Harper 2003), a proposed explanation for the presence of micro and ultrastructures on the petals of plants distantly related (Moyroud et al. 2017; Wilts et al. 2018). The fact that a pollinator can positively identify angle dependent colors displayed by artificial targets from that produced by a pigment in laboratory conditions under carefully specified lighting conditions is thus insufficient evidence for regarding iridescence and other structural colors as being visual signals as evidenced by the results of our behavioral experiments. Furthermore, the increasing number of plants species reported to display structural coloration by organs not related with pollination such as leaves and fruits (Héban and Lee 1984; Lee 1991; Chandler et al. 2015) and the calyx (Figure 5a–c) strongly suggests that the structures producing angle dependent colors may likely serve the plant for functions other than visual communication such as an aid to increase photosynthetic activity (Héban and Lee 1984) or increasing the temperature of specific areas of a flower (Wilts et al. 2018).

Before we can classify structural coloration in plants as an example of a visual signaling comparable to that observed in some animal species, attention must be given to answer the 3 important questions that differentiate a signal from a cue in this specific context: (i) what is the possible information potentially transmitted from the plant to the pollinator by angle dependent coloration? (ii) Is there a mutual gain by the production and monitoring of these colors? and (iii) How feasible is the perception of angle dependent color patches when considering the physiological characteristics of the pollinators and ecological setting where pollination takes place?

One possible avenue for further exploration is whether iridescence or other forms of angle dependent colors may provide useful information in low light or forest environments which have very different lighting conditions (Endler 1993) to what was tested in the current study; or if the micro and nano structures responsible for angle dependent colorations have evolved for a different purpose such as water repellence (Koch et al. 2009; Whitney et al. 2011), facilitate pollinator manipulation (Whitney et al. 2009a), temperature modulation (Koch et al. 2009; Wilts et al. 2018), or to facilitate detection in specific illumination and viewing conditions. However, we encourage future work to engage the formal framework of signaling for possible plant–pollinator iterations as here presented, and ideally mapping the complexity of the UV + B + G photoreceptor modulation.

Author Contributions

J.E.G., M.S., S.R.H., and A.G.D. designed the experiments, collected and analyzed data, and wrote the manuscript. P.P. designed the experiment, collected data, and wrote the manuscript.

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Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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Article

Fluorescent Pan Traps Affect the Capture Rate of Insect Orders in Different Ways

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Abstract: To monitor and quantify the changes in pollinator communities over time, it is important to have robust survey techniques of insect populations. Pan traps allow for the assessment of the relative insect abundance in an environment and have been promoted by the Food and Agricultural Organization (FAO) as an efficient data collection methodology. It has been proposed that fluorescent pan traps are particularly useful, as it has been suggested that they capture high numbers of insects in an unbiased fashion. We use a simultaneous presentation of fluorescent and non-fluorescent pan trap colours to assess how flower-visiting insects of different orders respond to visual stimuli and reveal a significant interaction between trap fluorescence and captured insect type. In particular, Coleoptera (beetles) and Lepidoptera (butterflies and moths) were captured significantly more frequently by fluorescent traps, whilst Dipterans (flies) were captured significantly less frequently by this type of pan trap. Hymenopterans (bees and wasps) showed no significant difference in their preference for fluorescent or non-fluorescent traps. Our results reveal that the use of fluorescent pan traps may differently bias insect capture rates when compared to the typical experience of colour flower-visiting insects in natural environments. Correction factors may, therefore, be required for interpreting insect pan trap data collected with different methodologies.

Keywords: urban environment; pan traps; native insects; habitat fragmentation

1. Introduction

Several studies have reported that global declines in insect pollinator populations are linked to phenomena including habitat fragmentation, pathogens, invasive species, climate change, and/or the widespread use of agricultural insecticides [1–9]. Understanding the different contributing factors to these potential mechanisms is valuable since about 35% of food production for human consumption relies on insect pollination [1,10–12], with an estimated value in the range of 235–577 billion US\$/year [12]. It is, thus, important to reliably quantify the relative abundance of potential flower-visiting insects in different environments to assist our understanding of changes in plant pollinator interactions, especially when considering habitat fragmentation caused by urbanization and agricultural intensification [1,5,7].

Studies and surveys of potential pollinating insects have employed pan traps (also called bowl traps) to estimate flower visitor numbers in a variety of different habitats. Pan traps have been proposed as an efficient method to collect insects from within a habitat with minimum sampling biases [13–24]. Different insect species, however, may present preferences in their perception of different colours [25–30]. For example, bees have trichromatic colour perception with ultraviolet-, blue-, and green-sensitive photoreceptors [31]. Some ants appear to perceive input from two different photoreceptor classes for colour perception [32]. Flies have four colour receptors that are further spectrally tuned with screening pigments [33,34]. Butterflies may have four or five photoreceptors that can also be spectrally tuned [33,35–37]. The dimensions of colour vision can dramatically influence how colour choices are made by these different animals [27,38]. To attempt to control for potential colour preference biases, previous studies have used differently coloured pan traps such as white, yellow, and blue, as perceived by human colour vision, to quantify the broad insect diversity potentially encountered in ecological settings [14,16,21,23]. These studies have typically used UV-fluorescent pan traps based on anecdotal evidence [39,40] that such stimuli collect more insects, although this factor of UV-fluorescence on insect capture rate has, only recently, been subject to formal testing [41].

Fluorescence is the phenomenon by which short wavelength radiation is absorbed by a material and re-emitted as longer wavelength radiation [42]. Materials with fluorescent properties may be of either biological [42] or non-biological origin (e.g., plastics or paints) [43]. For example, the effect is observed when we use UV-black-lights in nightclubs to produce UV-fluorescence from clothing, fluorescent highlighting pens, or Post-It notes (or sticky notes) [42]. Whilst fluorescence may enhance the intensity of a signal from a particular region of the spectrum, its effect typically results in an overall reduction of the total number of photons coming from a surface due to the conversion efficiency of the fluorescent material. Thus, to perceive and potentially benefit from fluorescence, the visual system of the receiver needs to be spectrally tuned to the wavelengths at which the fluorescence is produced [29,42]. Hence, it is reasonable to assume that insects with different visual systems may perceive fluorescent stimuli differently. This could induce sampling bias when using differently coloured pan traps, including the use of fluorescence, to attract insects.

Here, we address the question of whether pan traps displaying fluorescent properties may capture higher numbers of flower-visiting insects than non-fluorescent pan traps. Since Araneae (spiders) were also collected using our method and there is some evidence that spiders can visit flowers to prey on insect pollinators [44,45] or collect nectar [46], and that they are therefore potentially part of an extended pollination network, we also report the capture rate of these arthropods. Further, Orthopterans (crickets) are also included in our current analysis as these insects are pollinators of some flowering plants [47]. In our approach, we used typical colours employed previously for surveying insect populations for ecological studies [14,16,18,21]. We aim to test whether fluorescent pan traps catch more or less individuals of different orders of insects in comparison to non-fluorescent traps to inform us about the most effective way to survey potential insect flower visitors while minimising sampling bias.

2. Materials and Methods

2.1. Study Area

This study was conducted within the grounds of Monash University's Clayton campus in Melbourne, Australia. The university grounds include large areas of remnant native bushland, as well as extensive gardens providing abundant resources to flower-visiting insects. The grounds are located in the temperate zone (37°53' S–37°55' S, 145°06' E–145°08' E) (Figure 1). In this study, we established five study sites and sampled insects during the Australian summer (January to May 2016) with a temperature range from 17–42 °C. Detailed temperatures for specific dates are available in Table S1.

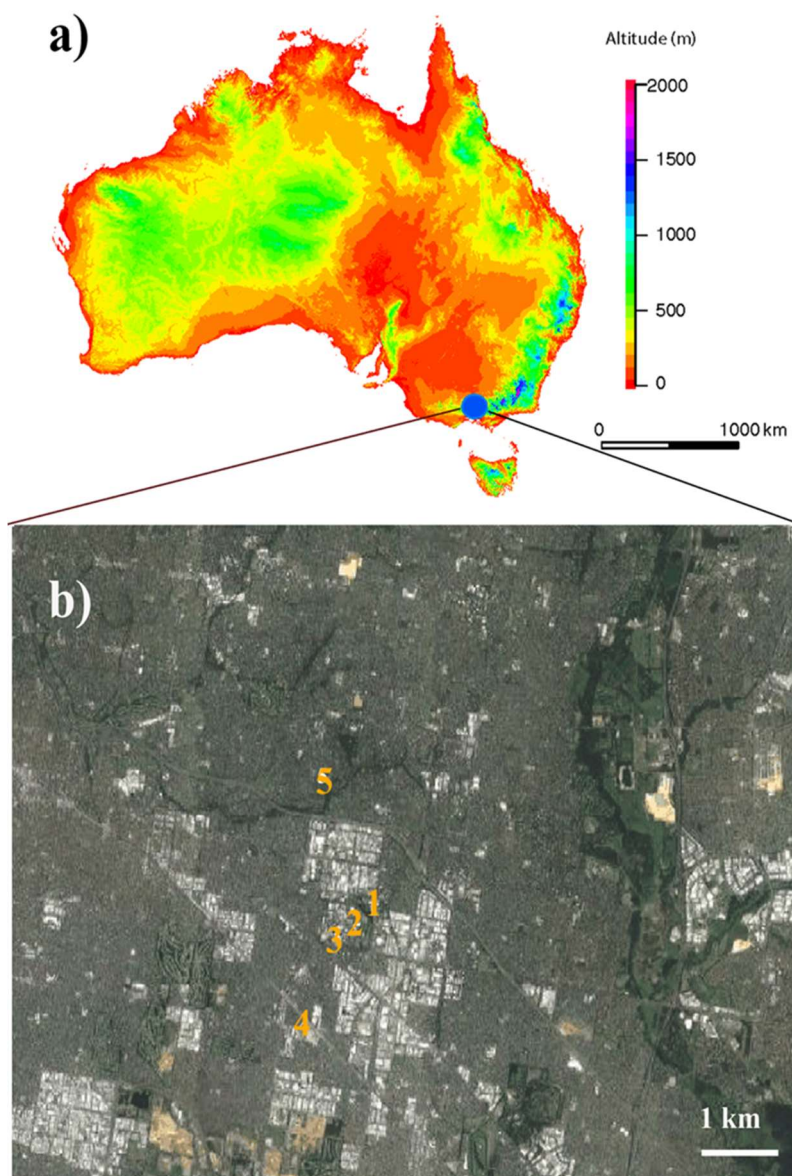


Figure 1. A map of the study area: (a) Map of Australia showing the geographical location of the study sites (blue solid circle) and (b) the numbers on the map show the sampling locations at Monash University, Clayton Campus and its surroundings of Melbourne, Australia. The map was prepared in R version 3.5.1 using packages “maps”, “dismo”, and “raster” [48] (R core Team 2018).

2.2. Data Collection

We installed eight differently coloured pan traps at each site to sample the different groups of potential flower-visiting insects. Pan traps were separated by approximately 25 cm (Figure 2A) which ensured that successive traps were viewed using colour processing by free flying bees [31]. We used pan trap colours perceived as white, blue, yellow, and green to human vision (Figure 2A). As it is well established that insect colour vision is different to human colour vision, we provide details for each colour stimulus in Table 1 including their spectral reflectance (Figure 2B). Each pan trap cluster ($n = 7$ clusters of pan trap bowls at 5 sites) contained eight plastic soup bowls (ca. 500 mL max. vol., diameter 14 cm, depth 4.8 cm) coloured with the different paints (Figure 2A). The standard 500 mL polypropylene soup bowls (Pro-Pac, Vechta, Germany) used as pan traps were painted with fluorescent or non-fluorescent blue, white, and yellow spray paints (Sparvar Leuchtfarbe, Spray-Color GmbH, Merzenich, Germany) following the protocol of by Reference [21] and dried over several weeks to remove any residual paint smell. Each pan trap was subsequently filled with about

400 mL of water. A few drops of odourless, liquid dishwashing detergent were added to break the surface tension of the water to increase insect capture [21].

Both fluorescent and non-fluorescent pan traps were simultaneously arranged in a circle, and the location of each individual pan trap within the circle was randomly allocated per set-up and site.. The pan traps were placed on the ground for 48 h (Figure 2C,D) following standard procedures [14,16,21,23]. Sampling was repeated every two weeks at each site for four repetitions. We stored the collected insects temporarily in 70% ethanol and/or freezers before they were pinned for taxonomic identification.

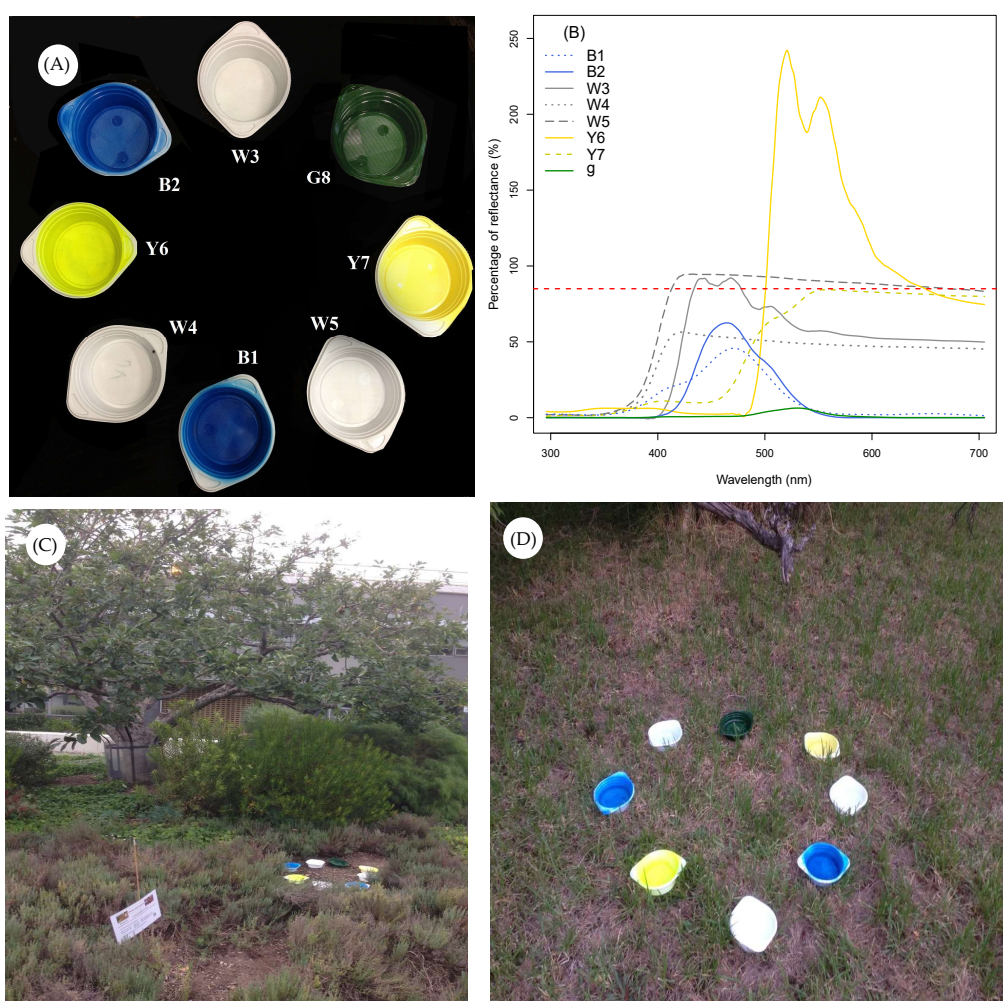


Figure 2. The pan trap experiment: (A) The pan trap bowls with eight different human perceived “colours”, (B) the percentage of reflected radiation plotted against wavelength for each pan trap “colour” type (See Table 1 for details and the different treatments involved for each pan trap type). The red dotted line shows the 90% threshold for categorisation as fluorescent or non-fluorescent stimuli, (C and D) the pan traps in the sample field settings.

2.3. Pan Trap Spectral Characterisation

We measured the reflectance spectrum of each pan trap colour with a spectrophotometer fitted with quartz optics and a PX-2 pulsed xenon UV-visible radiation source (USB 2000+, Ocean Optics, Dunedin, FL, USA) that closely matches the spectral profile of typical daylight illumination [49]. The spectrophotometer was attached to a computer running SPECTRA SUITE software 2011 (see References [50,51] for additional details of spectral recording methods and procedures). The reflectance spectra of the eight different pan trap types are shown in Figure 2B. Pan traps reflecting more than 90% of incident radiation at any point across the spectrum were categorized as fluorescent since very few artificial [43] or natural flower surfaces typically reflect radiation above this level

[29,52]. Whilst fluorescence may work in a variety of ways and produce weak changes in colour signalling [42], we use this definition in the current study to understand what pan trap features might influence the choices of insects.

2.4. Insect Identification

We identified all the collected insect specimens to the order level and some specimens to the genus level, using established protocols [53–58].

Table 1. List of the pan trap colours used in our data collection. The pan trap group (last column) in the table is categorized based on the spectral reflectance properties of each pan trap (Figure 2B).

Pan Trap Description				
Stimuli	Treatments Involved	Paint	Colour (Human Perception)	Pan Trap Group
B1	Blue paint	Blue	Blue	Non-Fluorescent
B2	Blue UV reflectance reduced	Blue “UV” fluorescent	Blue	Non-Fluorescent
W3	White UV reflectance reduced	White “UV” fluorescent	White	Fluorescent
W4	White paint	White	White	Fluorescent
W5	White	White bowl without paint	White	Non-Fluorescent
Y6	Yellow UV reflectance reduced	Yellow “UV” fluorescent	Yellow	Fluorescent
Y7	Yellow paint	Yellow	Yellow	Non-Fluorescent
g8	Green paint	Green	Green	Non-Fluorescent

2.5. Data Analyses

The data were recorded during the Australian summer to autumn 2016. The sampling periods spanned 48 h.

We arranged the data in a 2 × 2 contingency table to test for a potential interaction between pan trap type, i.e., fluorescent or non-fluorescent, and the order of the insects captured using a Pearson chi-square test for independence. As part of the analyses we also calculated the standardised residuals for each entry of the contingency table [59]. All analyses were performed using the package “gmodels” [60] in the R programming language version 3.4.1 [61].

3. Results

When we considered the main research question, we found a significant interaction between the type of trap, either fluorescent or non-fluorescent, and the order of insect captured ($\chi^2 = 27.374$, d.f. = 5, $p < 0.001$) (Figure 3). The main analysis was then followed by a residual analysis to identify those insect orders presenting significantly more or less captures than what is expected by chance (Table 2). This analysis revealed that Coleopterans (beetles) and Lepidopterans (butterflies or moths) were captured more frequently on fluorescent pan traps, whilst Dipterans (flies) were captured significantly less frequently in this type of pan trap (Figure 3, Table 2). Results are graphically summarised in Figure 3.

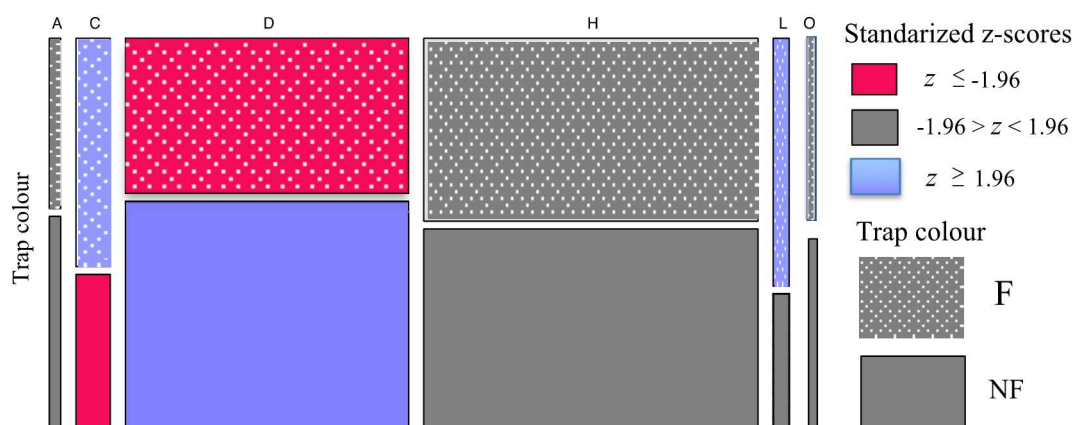


Figure 3. The mosaic plot shows the frequency of captures of the different insect types/order (coded with the following alphabetic letters for each group. H: Hymenoptera, D: Diptera, L: Lepidoptera, C: Coleoptera, O: Orthoptera, and A: Araneae. See Table 3 for details) for pan trap types classified as either fluorescent or non-fluorescent. The box width represents the proportion of captures for each insect order, whilst the box height is an indicator of the proportions of capture by the fluorescent pan traps (upper row dashed pattern) and the non-fluorescent traps (lower row solid pattern). The colour indicates the z-values for the respective standardized residuals (Table 2): blue indicates a significant preference for stimuli, red indicates that the stimuli collected significantly less individuals, and grey indicates the capture rate was not significantly different to the chance expectation (null condition). F = Fluorescent, NF = Non-Fluorescent.

Table 2. Summary of the z-scores and p-values for each insect order provided in Figure 3 for fluorescent and non-fluorescent pan traps. * indicates significant p-values at $\alpha = 0.05$. z-scores < 0 indicate a lower frequency of choices than those expected by chance. z-scores ≥ 0 indicate a frequency of choices higher than expected by chance.

Insect Order	Pan Trap Type	
	Fluorescent Pan Traps	Non-Fluorescent Pan Traps
	z (p-value)	z (p-value)
Hymenoptera	0.981 (0.327)	−0.900 (0.368)
Diptera	−2.285 (0.022) *	2.097 (0.036) *
Lepidoptera	2.007 (0.045) *	−1.842 (0.065)
Coleoptera	2.151 (0.031) *	−1.973 (0.048) *
Orthoptera	0.107 (0.915)	−0.098 (0.922)
Araneae	−0.112 (0.911)	0.103 (0.918)

4. Discussion

Pan traps are a conventional way of assessing insect–flower visitor distributions [14–24]. Several studies advocate the use of fluorescent stimuli due to the assumed higher rates of insect captures [14,17,21,62]. Although pan trapping with non-fluorescent traps has been used in several studies [16,41], it has rarely been considered whether the type of pan trap may bias the data collection of different insect orders due to the differences in colour processing among groups. We employed a combination of fluorescent and non-fluorescent pan trap stimuli and found that Hymenopteran insects have no significant preference for either the fluorescent or non-fluorescent pan traps. Other insect orders such as Coleoptera and Lepidoptera do show a preference for fluorescent pan traps (Figure 3, Table 2). In contrast, Dipterans (flies) demonstrated a preference for non-fluorescent stimuli. Our pan traps also collected some spiders (Araneae) and Orthopterans, although in relatively low numbers (Figure 3). Whilst these orders may have been an incidental by-catch, especially Orthoptera that may jump into the pan traps, these data were included in analyses as there is some evidence that Araneae and Orthoptera might participate in, or affect, pollination networks [46,47]. Neither of these orders showed any significant preference for pan traps. The evidence that Hymenopteran insects did not show a preference fits with the established literature that honeybees do not process stimulus intensity differences as a dimension of colour perception when making colour choices [63–67].

Currently, relatively little is known about the colour processing mechanisms of beetles, butterflies, and flies, but the spectral tuning of vision in insects of these orders is known [33–37] and might facilitate a capacity to process fluorescent signals [42]. Our data do suggest that such a possibility is worth exploring in detail with individual species from these insect orders. Such testing would also be of value with model bee species to validate whether indeed their visual system is insensitive to fluorescent signals as suggested by the current results.

To enable efficient censuses of insect pollinators in different environments, it is important to have a robust data collection method, and the use of fluorescent pan traps has been proposed to result in higher insect capture rate [14,17,21,62]. Our observations of nearby insects visiting flowering plants confirmed that many insects captured by our pan traps were also visiting flowers in the nearby plant communities and so may be potential pollinators (Figures 4 and 5) (personal observations by M.S., A.D., and A.G.D.).

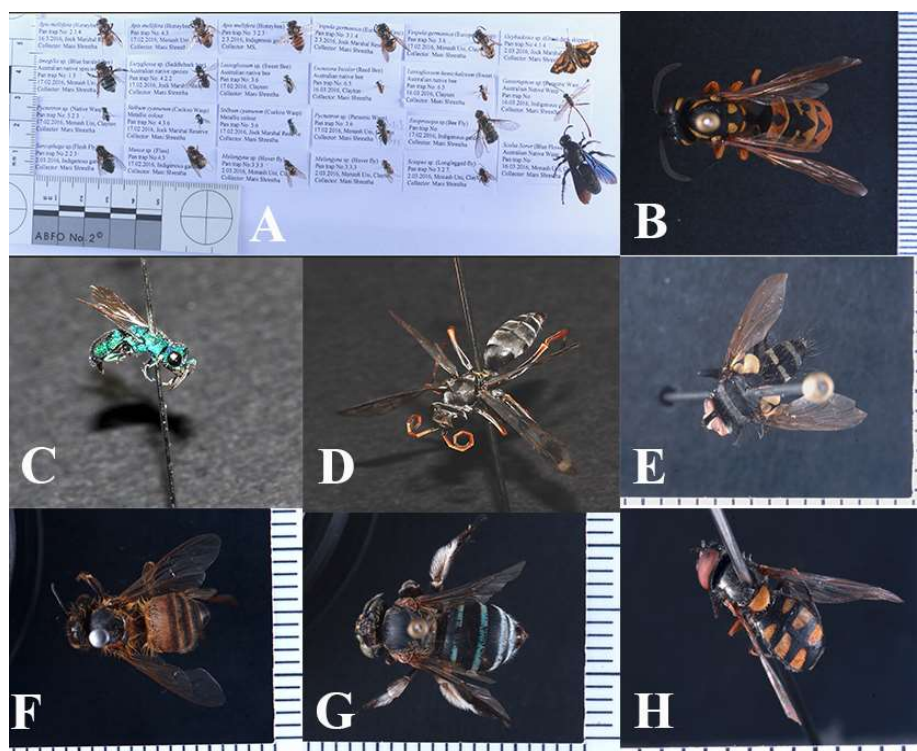


Figure 4. Sample insects captured by the pan traps: (A) Array of pinned insect samples, (B) European wasp (*Vespula germanica*), (C) cuckoo wasp (*Stibum cyanurum*), (D) male winged ant (*Myrmecia urens*), (E) long-legged fly (*Sciapus* sp.), (F) honey bee (*Apis mellifera*), (G) blue-banded bee (*Amegilla* sp.), and (H) hoverfly (*Melagyna* sp.). Images© Copyright M.S. and J.H.C.H.

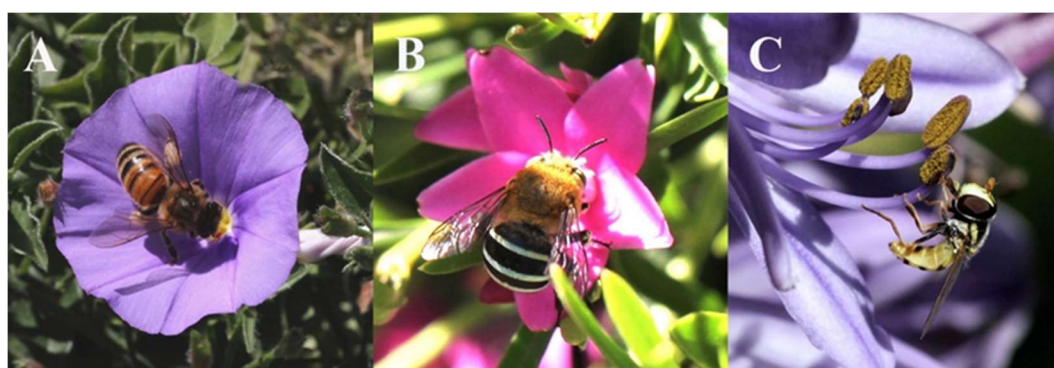


Figure 5. Colour photographs of flower visiting insects taken nearby the pan trap locations: (A) honey bee (*Apis mellifera*), (B) blue-banded bee (*Amegilla* sp.), (C) hoverfly (*Melagyna* sp.). Images Copyright© M.S.

The insects we captured included trichromatic native bees and introduced honeybees [68,69], hoverflies that are thought to have a four-colour visual system [34,70,71], native wasps that could potentially be trichromatic or tetrachromatic [68,69], and beetles that currently have a poorly understood colour visual system [72–74]. Our data on insect capture rates with either fluorescent or non-fluorescent stimuli shows that the choice of respective stimuli may result in a biased distribution

(Figure 3) of the relative abundances of different pollinator groups [29], although true bias is difficult to assess in outdoor experiments with free-flying insects where overall densities are typically unknown. Future work should dissect how the spectral profiles of coloured pan trap stimuli (Figure 2B) may be perceived by different insects and how the observed preferences might influence which flower colours are pollinated [28–30,75–77]. Corrections could then be estimated from the relative ratio of fluorescent and non-fluorescent capture rates as those shown in Figure 3, although preference effects may potentially vary between species within the insect orders (Table S1,S2) and so corrections would benefit longer term through validation testing with individual species. We acknowledge this is very difficult: so far, colour preference testing has been successfully performed with very few species [26–32,75–77].

5. Conclusions

We tested if fluorescent or non-fluorescent pan trap colours captured potential flower-visiting insects in a way that might be biased due to differences in how particular insect orders may process spectral information. Whilst for Hymenopteran species there was no significant difference in the frequency of individuals caught, flower-visiting flies were preferentially captured in non-fluorescent pan traps. In contrast, fluorescent pan traps captured significantly higher rates of beetles and Lepidopterans than non-fluorescent traps, suggesting that a fundamental difference in spectral processing may have influenced the insect capture by a particular pan trap. We, thus, suggest that to survey insect populations, care in interpretation is required in the selection of pan traps colours and that corrections should be considered when conducting meta-analyses on studies with different pan trap colours.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Insect capture using pan traps, Table S2: A complete list of insect species sampled in this study, with numbers of individuals collected with pan traps (PT).

Author Contributions: A.N., T.T., M.S., and A.G.D. designed the experiment; M.S., A.D., S.R.H., and A.G.D. collected the data; M.S. and J.H.J.C. imaged the insects; J.E.G., A.G.D., and M.S. analysed the data; all the authors edited and revised the manuscript and approved for final submission.

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Conflicts of Interest: The authors declare that they have no conflict of interest involving the work reported here.

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