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Brain miniaturization and its implications for cognition: evidence from Salticidae and Hymenoptera

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To my grandparents

Carla, Ernesto, Ester, Nicola

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ABSTRACT

English abstract

Scientists have always been fascinated and puzzled by the marvel that is the human brain. This organ seems to be of an exaggerated size for our body, demanding an enormous amount of energy and resources to be maintained. For evolution to favour such an enormous expenditure of energy, the benefits must outweigh the cost. During the history of neuroscience different authors have attempted to correlate the size of a species nervous system with its cognitive abilities, in the search for an explanation on why we have such a big brain: if possessing an oversized brain is how a species can produce outstanding cognitive abilities, then the survival advantage outweighs the costs. However, brain size correlates first of all with body size, having nothing to do with the cognitive capabilities of a species. To solve this problem, different measures have been adopted, like brain-to-body weight ratio, encephalization quotient, the raw number of neurons in the cortical areas. When confronted with empirical evidence, however, all these measures fail to predict the presence of complex cognition, especially for species phylogenetically distant from us. In particular, miniaturized organisms, like insects or spiders, exhibit outstanding behaviours, products of complex cognition, with brains multiple orders of magnitude smaller than ours. It has been proposed that our premise is misguided. Cognition does not need a big brain to manifest, quite the opposite: a higher number of neurons increase the memory buffer and becomes more robust against noise, while cognitive processes only require a handful of cells well organized in complex circuits. The process of brain miniaturization during evolution should have favoured the birth of small but complex neural circuits, capable of dealing with multiple situations. In this framework, in this thesis, I have presented some of the studies carried out during my PhD project on miniature organisms.

Firstly, the ants are described. As these insects are phylogenetically similar to bees and bumblebees, which have been extensively studied in the last three decades and have been found capable of outstanding cognitive processes, they represent the first candidate to understand if complex cognition is widespread in invertebrates With two different studies, we tested the ability of ants to perceive and register information from the environment. It appears that the process of miniaturization during evolution has favoured the development of clever circuitry, that let the ants process a great variety of information with only a handful of neurons, and register those with a load-independent memory process, suggesting the presence of complex cognitive abilities.

Secondly, as the main topic of my project, the jumping spiders are presented. These arachnids have recently caught the interest of scientists for their unique hunting strategies, that involve detouring,

perspective taking, categorization and other cognitive skills. I have tested their visual perception to understand if it is guided by the same rules that govern the human's one (e.g., Gestalt principles). However, I failed to design a methodology capable to consistently train the spiders, and as such the results were inconclusive. To overcome this problem, I designed an automated training system. This proved to be an effective way to train jumping spiders, opening future possibilities for the study of this species' cognitive abilities.

Italian abstract

Gli scienziati sono sempre stati affascinati e incuriositi dalla meraviglia che è il cervello umano. Quest'organo sembra essere di dimensioni esagerate rispetto al nostro corpo, richiedendo di conseguenza un enorme quantità di energie e risorse per funzionare. Perché l'evoluzione possa favorire un così drastico consumo di energia, i benefici devono superare i costi. Nella storia delle neuroscienze, diversi autori hanno tentato di metterei in correlazione la dimensione del cervello di una particolare specie con le sue capacità cognitive, alla ricerca di una spiegazione per un siffatto cervello: se un cervello sovradimensionato è necessario per produrre comportamenti complessi, il beneficio da essi garantito supera i costi. Ciononostante, la dimensione del cervello di una specie correla prima di tutto con la dimensione del suo corpo, avendo niente a che vedere con le sue capacità. Sono state proposte diverse misure, come il quoziente tra peso del cervello e peso del corpo, il quoziente di encefalizzazione, o il numero grezzo di neuroni presenti nella corteccia. Quando confrontate con le evidenze empiriche, tutte queste misure purtroppo sembrano inadatte a predire la presenza di capacità cognitive complesse, specialmente nelle specie più filogeneticamente distanti da noi. Nello specifico, organismi in miniatura, come insetti e ragni, mostrano comportamenti complessi, frutto di processi cognitivi, pur avendo cervelli di diversi ordini di grandezza più piccoli dei nostri. È possibile che la nostra premessa sia infondata. Non c'è bisogno di un grande cervello per produrre cognizione: un più alto numero di neuroni può aumentare le capacità mnemoniche e capace di ridurre il rumore di fondo, mentre processi cognitivi richiedono solo una piccola quantità di cellule organizzate in circuiti complessi. Il processo di miniaturizzazione durante l'evoluzione dovrebbe aver favorito lo sviluppo di piccoli ma complessi circuiti neurali, capaci di gestire più situazioni contemporaneamente. All'interno di questo contesto, in questa tesi, ho presentato alcuni esperimenti svolti durante il mio progetto di dottorato sugli organismi in miniatura.

Inizialmente, sono descritte le formiche. Questi animali, in quanto filogeneticamente vicini ad api e bombi, rappresentano il primo candidato per comprendere quanto e se la cognizione complessa sia comune negli invertebrati. Queste ultime due specie infatti sono state molto studiate negli ultimi trent'anni, e sono state trovate capaci di sorprendenti processi cognitivi. Attraverso due diversi esperimenti, abbiamo testato la capacità delle formiche di percepire e registrare informazioni dall'ambiente. Sembra che il processo di miniaturizzazione durante l'evoluzione abbia favorito lo sviluppo di circuiti ingegnosi, che consentono alla formica di processare una grande varietà di

informazioni con una manciata di neuroni, e di registrarle tramite un processo indipendente dalla quantità di dati, suggerendo la presenza di capacità cognitive complesse.

Successivamente, come tema principale del mio progetto, vengono presentati i ragni saltatori. Questi aracnidi hanno recentemente catturato l'interesse degli scienziati grazie alle loro uniche strategie di caccia, che richiedono la capacità di *detouring*, *perspective taking*, categorizzazione e altre capacità cognitive. Ho testato la loro percezione visiva per comprendere se essa sia guidata dalle stesse regole che guidano la nostra (ovvero, i principi della Gestalt). Purtroppo però, non sono riuscito ad individuare una metodologia capace di addestrare i ragni saltatori, e per questo motivo i risultati sono stati inconcludenti. Per superare questo ostacolo, ho disegnato un sistema di addestramento automatico. Questo sistema si è rivelato un metodo efficace per l'addestramento dei ragni saltatori, capace di aprire possibilità future per lo studio delle capacità cognitive di questa specie.

SECTION 1 – INTRODUCTION

Brain size as a predictor of behavioural complexity

> ‘primate (n.): Meaning “animal of the biological order including monkeys and humans” is attested from 1876, from Modern Latin Primates (*Linnæus*), from plural of Latin *primas*, “of the first rank, chief, principal”, from *primus* “first”, so called from supposedly being the “highest” order of mammals’ [adapted from 1]

Why do we, humans, have such a big brain? This question has puzzled scientists for centuries. Our brains are quite costly to maintain [2–6], and to accommodate their size we were forced to modify our body [5] and behaviour [7]. For evolution to favour such an enormous expenditure of energy, the benefits of a large brain must outweigh the costs. Many theories have been proposed to describe why we evolved with a bigger brain: to cope with a more complex social environment [8], through sexual selection [9] and more. These hypotheses all start from an underlying, fairly obvious assumption: brain size correlates directly with our ability to produce complex, intelligent behaviours and, particularly, our unique cognitive abilities. Under this reasoning, a bigger brain is evolved when the environment increases in complexity and the animal needs to cope with it, thus counterbalancing the cost of maintenance with an enormous advantage in fitness. In fact, among humans, individuals with bigger brains seem to possess a higher level of general intelligence [10], suggesting that this relationship may also hold true across species. Until the seventeenth century we believed that we had the biggest brain in the animal kingdom [11], and, as such, that we would be the most intelligent creature, which in turn made us able to become the dominant species of the planet. Unfortunately, we quickly learned that both elephants [12] and sperm whales [13] have a brain far bigger than ours. However, this discovery did not cause scientists to cast any doubt on the humans’ primacy, nor made them reconsider the supposed level of intelligence produced by these other massive brains.

Indeed, there is an undoubted evidence that brain weight correlates positively with total body weight [14,15]. Following this reasoning, the brain-to-body weight ratio should be used as a predictor for the presence of high cognitive abilities [16]. However, this measure does not work well with extreme cases, as Haller’s rule predicts that smaller animals tend to have bigger relative brain sizes [17], putting the tiny shrew on top of every other mammal, with a 3.33 ratio (humans have a 2.33 ratio) [11]. Instead of using a raw brain-to-body weight ratio, researchers have proposed using a relative measure: how much any given species’ brain size deviates from the expected one for the corresponding body weight. This measure has been named Encephalization Quotient (EQ)

[18,19], finally defining the human species as the pinnacle of brain evolution, with an EQ of 5.72 [20], representing an unprecedented deviation from the whole animal kingdom (figure 1.1). However, even from the onset of this theory, it generated strong critiques [21]: EQ fails to account for evolutionary constraints on body size that can make EQ change drastically, not because of differences in the brain, but solely because of body size differences. This may also explain why the highest EQ is observed in species belonging to the orders with the highest variability in body weight [20]. Moreover, allometry methodologies can introduce systematic errors when used to compare species of orders or even families too different from each others [21]. More recently some evidences have surfaced, that is indeed not a ratio between brain and body mass that predicts cognitive abilities in primates but brain weight alone [22].

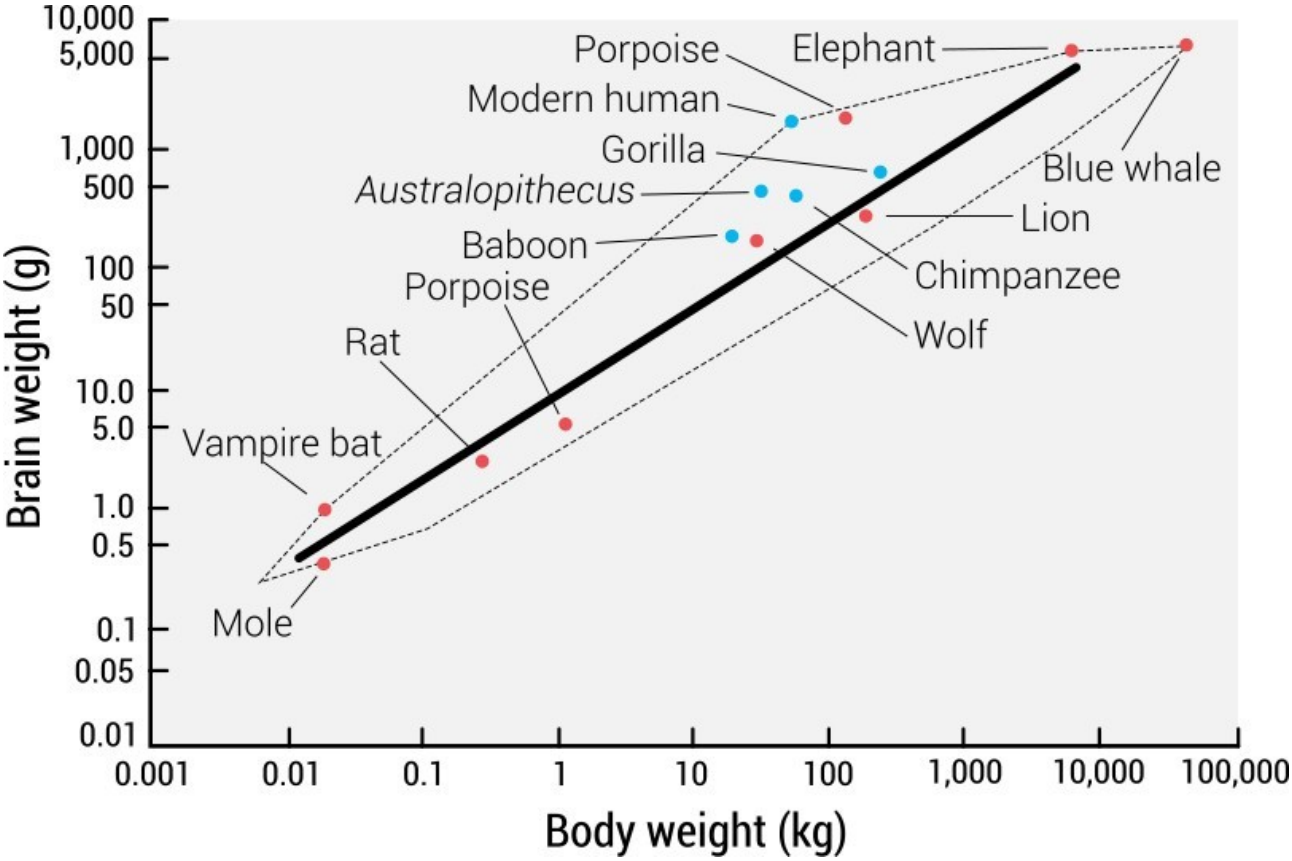

Figure 1.1 – Mammals regression line describing the expected brain weight (y axis) for a given body weight (x axis). Light blue dots are primates. The distance between any given point and the regression line is that species' Encephalization Quotient (EQ). Note how the modern human point is the furthest from the regression line in respect to all other mammals. On the other hand the elephant and the blue whale, that had a brain to body weight ratio greater than humans, are actually near the regression line. Adapted from [11].

It has been proposed that any measure based on brain weight suffers from the incorrect assumption that bigger brains possess more neurons. If the functioning unit of the brain is, in fact, the neuron, it

is the number of those that may predict the level of cognition, and not brain size in itself. However, the number of neurons in two similarly weighted brains can be profoundly different [23,24]. Also, when excluding the neurons dedicated to motor and body control, body size should not influence the processing power, as all other neurons are dedicated to it [25]. As such, the number of neurons seems to be the best candidate to predict cognition complexity in a species [24–26]. This measure also solves the apparent deviation that EQ shows and no longer describes the human being as an outlier in the nonetheless consistent brain-to-body weight ratio regression line. When considering the number of neurons, humans fit the evolutionary expectations [26], but still remaining on top of every other animal species, both for number of neurons and, consequently, cognitive complexity (figure 1.2).

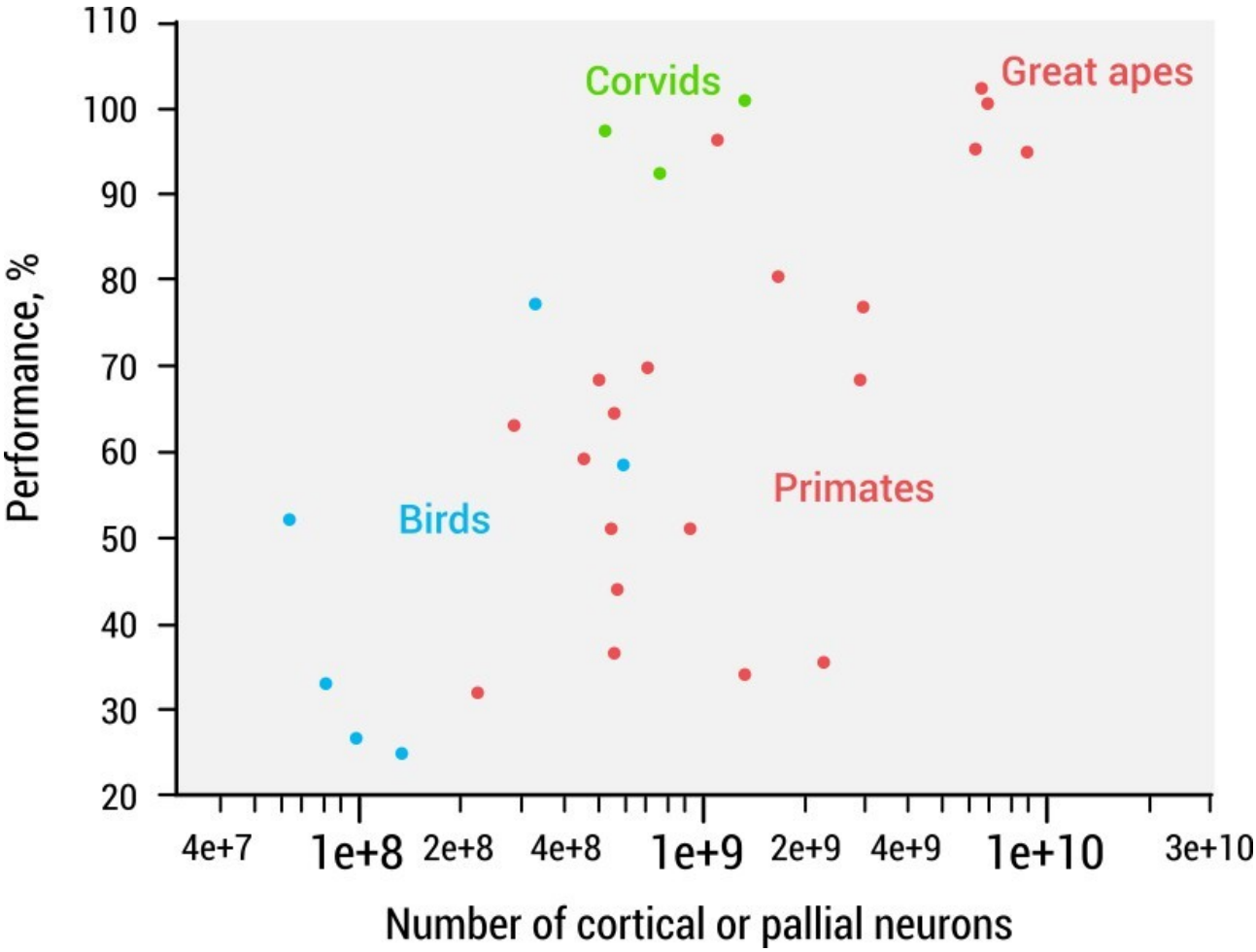

Figure 1.2 – Performances obtained in a cognitive task (y axis, from [27]) by different animal species, as a function of the number of neurons in their cortex or neopallium (x axis, from [24,28]). Note the correlation present between the two. Adapted from [25].

In my opinion, all of these measures, from the most basic to the most sophisticated, suffer from the same problems. What exactly are we correlating brain size/number of neurons with? How do we

measure cognition? Behavioural complexity stretches to such a vast amount of abilities that it is almost impossible to define it with a unique and measurable variable [15,29,30]. The studied correlation between brain size and cognitive complexity is profoundly dependent on the definition of cognition used, or on the test employed (even when looking only amongst humans [31] instead of across species). Defining cognition itself is not easy [32]. The word itself comes form the 15th century: '*cognicioun*, "ability to comprehend, mental act or process of knowing", from Latin *cognitionem* (nominative *cognitio*) "a getting to know, acquaintance, knowledge", noun of action from past participle stem of *cognoscere* "to get to know, recognize", from assimilated form of *com* "together" (see *co*-) + *gnoscere* "to know", from PIE root *gno- "to know." In 17c. the meaning was extended to include perception and sensation.' [33]. However this definition cannot be operationalized: the internal experience of "knowing" about the environment is impossible to demonstrate experimentally: when producing any behaviour, does an animal actually "know" what it is doing? A different, operationalizable definition is required to fruitfully continue this discussion on the brain-cognition correlation. For the purpose of this thesis, I will follow the definition given by Shettleworth [34], for whom: '[cognition is] the mechanisms by which animals acquire, process, store, and act on information from the environment'. With this definition we could theoretically encompass every single behaviour [32], except perhaps reflexes. However, we can distinguish between various levels of complexity in cognition itself based on the amount of processing that takes place between a stimulus and a response. In other words, it depends on the amount of information received and the amount of processing before a behaviour is produced. However, as the reader will discover in the next paragraph, perhaps our judgment on which mental processes are complex and which are not may be very far from the truth.

Even if we were able to operationalize cognitive complexity in a consistent manner, we would still lack experimental evidence on what species can produce what complex cognitive behaviour: any measure of cognitive performance suffers greatly from an anthropocentric bias. Research on comparative cognition today still focuses mainly on primates, followed by rats, pigeons, other birds, dogs and then all others [35]. The more we study these species, the more we discover impressive feats that their brains allow them to perform, the more we believe them to be cognitively capable. 'Absence of evidence is not evidence of absence' [36], and yet we systematically consider an animal incapable of producing a specific complex behaviour that has never been tested or when enough convincing evidence is lacking. On the other hand, the study of cognition suffers inevitably from the positive publication bias [37]: overestimating cognitive abilities of the species that are

tested more frequently, since publications about unsuccessful tests are incredibly rare. Most likely, some complex abilities are indeed possessed only by some species, which in turn may be considered more cognitively advanced, but until definitive proof is available, we should abstain from any consideration about species that have not been directly tested. Indeed, some studies have used a comparative approach to test different species on the same cognitive skill in order to understand differences and similarities. However, those studies are narrow in their definitions (as they should be), focusing only on a particular ability congruent with cognitive complexity, and they are focused only on some species: mostly primates [22,27], and corvids [38] (note that the latter seem to outperform great apes, even with a brain of multiple orders of magnitude smaller and with less cortical/neopallium neurons [25]). Ultimately, all the predictors of cognitive complexity based on brain size presented in this paragraph seem to have a narrow explanatory power and are based on measures that by their nature do not permit a wide comparison across species, remaining valid only amongst mammals, if even so.

Why do we, humans, have such a big brain? To this day the scientific community is unable to provide a unique, evidence-supported answer. Evolution would not have favoured the development of an organ so costly if it were not useful [15], but we will never discover it if we focus solely on justifying our presumptuous cognitive primacy. Our definition of cognition is so broad, and our understanding of the brain is so narrow, that every attempt in finding a correlation between any measure of the two is destined to fail [15,29,30]. The inadequacy of brain size as a predictor for cognitive complexity becomes even more evident when considering that animals possessing a brain immensely smaller than ours, can still produce outstanding behaviour, unsettlingly similar to the finest cognitive processes of humans [14]. In this thesis, I propose that to unveil the secret of our expensive brain, we should not focus on demonstrating that we are different and unique. Instead, we should direct our attention to what a miniature brain (thus possessing a supposedly low computational power) can do.

Solutions to being small

‘It is certain that there may be extraordinary activity with an extremely small absolute mass of nervous matter; thus the wonderfully diversified instincts, mental powers, and affections of ants are notorious, yet their cerebral ganglia are not so large as the quarter of a small pin's head. Under this point of view, the brain of an ant is one of the most marvellous atoms of matter in the world, perhaps more so than the brain of man.’

Charles Darwin, The Descent of Men, 1872 [39]

Arthropoda is one of the most diverse phylum, containing 80% of all animal species [40]. Moreover, it makes up 50% of the total Animalia kingdom biomass [41]. According to these numbers, evolutionary speaking, being an arthropod must be successful. At the start of their evolutionary history, arthropods did not face any limitations in what size they could reach. Some were already small, while others were massive, with some insects reaching a 70cm wingspan [42]. From the Jurassic period onward however, mainly due to the decrease in oxygen concentration in the atmosphere, they underwent a massive decrease in maximum size. This caused a general miniaturization of the body that forced many species to find a solution on how to fit their brain in a smaller space without having to renounce too many of its functions. The term “miniaturization” is generally used in a relative sense, not based on the objective size of an animal species, but on its relative dimensions with respect to phylogenetic relatives [43]. However, the overall size of an animal has an effect in itself [44]: if as discussed above the functional unit of the brain is the neuron [26], the space available will pose an objective limitation in the maximum number of cells that can fit inside the body. Nonetheless, arthropods still have a functioning brain and are able to act and react to their environment successfully. As Charles Darwin noticed [39], evolution must have come up with ingenious ways to fit a miniaturized, perfectly functioning brain inside these small bodies. Three different theories have been proposed to explain how a brain can function when undergoing a miniaturization [15]. These three hypotheses are not mutually exclusive, as different animals may employ different strategies, or even multiple strategies can be present in the same species.

Size limitation strategy

The first strategy that can be employed with a reduced cranial capacity is pretty simple: renounce some skills and computational strategies in order to reduce the needed brain power (and number of

neurons). This idea would progress in the same direction of the correlations between brain size and complexity presented in the previous paragraph. As already hinted, this strategy finds little experimental support when cognitive abilities are directly compared between two different-sized animals (i.e. corvids and primates [38]). Also amongst arthropods, specifically arachnids, the employment of this strategy struggles to find empirical support. Adult and juvenile spiders of the same species show no significant difference in complexity when building webs [45]. Also, different sized species orb-weaver exhibit the same absence of difference [46]. Moreover, juveniles jumping spiders possess a comparable visual performance to adults, despite the major size difference [47]. It seems that behavioural complexity is too valuable for survival that it may never be beneficial to forfeit it. One may argue that in order to retain high capacity in web building or vision, these tiny spiders may give up completely other processes that are not species-specific, such as rule learning: enlarging the "orb-weaving" brain area and shrinking the "rule-learning" one. This may be a plausible explanation, but as it will be described in the next paragraph and in the studies reported in this thesis, at least some arthropod species lack these predicted limitations in complex behaviour.

Over-sized brain strategy

A highly functioning brain may be so valuable that no amount of constraints can stop evolution from investing resources in neural circuits [48]. For this reason, the brain of miniature animals will increase in size as much as it is physically possible. This can be done using various mechanisms. One possibility is to expand the neural tissue to occupy the highest possible portion of the body. This pattern can clearly be observed in insects [49–52] and spiders [53–57], where the brain makes up a massive portion of the entire body weight. In some extremes, the brain even extends over its designed container, the cranial chamber, and expands into the limbs. This pattern is not unique to arthropods as already hinted in the introduction: the "Haller's rule" [17] predicts that smaller animals will have a relatively enlarged brain, thus testifying to an increased investment in the neural substrate.

A second alternative (often employed in conjunction with the first) is to decrease the size of each neuron in order to maximize their total number. This pattern has already been described in birds [25] and remains true for arthropods [49,51,53], where neurons are not only smaller but also more densely packed.

However this strategy does not come without costs and limitations. Even if the brain is expanded to its full potential, it remains massively smaller than the one possessed by bigger animals: some

neurons must be forfeited. Moreover, if the neurons are more densely packed, the energy density requirement also increases [49], posing another limit to how much neurons can fit inside a miniature organism. Lastly, neurons are inherently noisy, producing sometimes random action potential [58]. Smaller, more packed neurons produce even more noise [58–62], posing yet another limitation to how small each neuron can get and how many can fit in a given amount of space. Due to all these presented limitations the over-sized brain strategy can limit the amount of neurons lost in miniaturization but can never produce a miniaturized brain with a number of neurons comparable to the one of bigger species. Species that employ this strategy must also employ either the size limitation one (i.e. renouncing to at least some processing power), or the economy of design one, (i.e. redesigning the structure of the brain).

Economy of design strategy

The third option that miniature brains can embrace is to just become better. Having a large amount of neurons is not the only way to produce complex behaviours. In fact, neural circuits can be designed in a variety of ways, with some being more economic and conservative than others. As an example, animals' sensory system registers a variety of useless information that is then discarded when arriving in the central nervous system that retains and analyses only biologically relevant information [63,64]. The presence of this discrepancy can be the target of simplifying mechanisms, where the superfluous information can be selected and discarded before reaching the central nervous system, in turn decreasing the load and the number of cells needed. Moreover, the circuits in the central nervous system itself can be improved and fine-tuned in order to produce behaviours comparable to the ones of bigger animals [65]. We may be tempted to perceive the economy of design strategy as just another simplification method, where more complex processes are abandoned in favour of peripheral reflexes and where every process is just the bare bones of its high neurons number counterpart. However, this is far from the whole story. The central nervous system of arthropods can be surprisingly complex: what it lacks in neuron count it makes up for in the number of connections (see for example the description of a single neuron in the bee brain [66]).

It is possible that having less neurons does not have effect on the processes available. However, it has been proposed that a high number of neurons only increases the processing speed and precision, enabling parallel coding due to the presence of multiple copies of the same circuit [14,61]. Each circuit responsible for complex cognitive ability may actually need only a handful of neurons, according to neural network studies. Tasks such as visual categorization [67], selective attention [68], spatial learning [69,70], sequence learning [71], numerical discrimination [72] or even route

planning and anticipation [73] could be designed with numbers that can easily fit inside a miniature brain. These neural network designs cannot tell us anything about how miniature brains are designed and function, but definitely demonstrate that these systems are possible. Counter-intuitively, it seems that cognitive processes can be produced by networks with only tens of neurons, while thousands of them are only useful to reduce noise, as well as increase speed and precision. The difference between a big and a small brain may be quantitative, not qualitative.

When asked to remember various visual stimuli, honeybees can recall just up to six of them [74], in contrast to the virtually infinite amount that humans can register [75]. This discrepancy fits with the idea that more neurons can increase precision and speed as well as create parallel circuits, effectively increasing the amount of memory at disposal. Insects have indeed been shown to learn fast and effectively, but this ability deteriorates as a function of information amount [76,77]. However, the quantity of retained information can be increased drastically if a generalizing mechanism is employed instead: object categorization for example may be the bees' system to learn a rule about the world, instead of having to learn every single object in itself [14,78]. The same may be true for other complex cognitive abilities, which have already been described in Hymenoptera, like conceptual learning [79]. In this sense, cognitive processes are beneficial to miniscule brains, since a small circuit can this way cope with a massive amount of information, a quantity that could not be reached by simpler mechanisms.

All these evidences combined suggest that a miniature organism may be favoured in building circuits characterized by a low number of neurons capable of carrying out complex cognitive processes, in order to cope with the inability to form multiple parallel systems. As an example, being able to recognize the cause-effect relationship between events (a feat generally considered to be a complex cognitive ability) can be used to interpret a wide variety of situations, instead of being forced to individually learn each association between event pairs, task that requires a great number of neurons. Cognition may be favoured in small brains, and may also be the solution to the problem of miniaturization. This idea completely contradicts the proposed brain/behaviour correlation, showing that cognitive complexity is independent from brain size. As per the quote by Darwin at the start of this paragraph, miniature brains are a magnificent example of adaptation and economy, where the size constraints induce an increase in complexity and efficiency.

Aim of the study

With this thesis, I propose that to really understand if and how our brain is unique, we first need to describe if and how different brains can produce the analogous behaviours. This will let us work by exclusion, understanding what can be accomplished with different neural circuits, as well as highlighting the true, and not supposed, differences between big and small brains. As described in the previous paragraph, arthropods represent an outstanding model: they are generally modest in size and have small brains. Nonetheless, multiple experiments have demonstrated that they possess outstanding cognitive abilities. There is, however, a significant limitation in the literature about arthropods' cognition: most of the studies focused on bees and bumblebees (as will be further described in the next section), leading us to consider the hypothesis that these hymenoptera are just a flux of evolution, especially selected to produce complex cognition, without it being the elective solution to the problem of miniaturization. For this reason, with my PhD project I focused on two different arthropod species.

First, I will report the experiments carried out on ants. I have studied this species during a 6-month period in Regensburg, Germany, under the supervision of Dr Tomer Czaczkes. Even though they have not been the main focus of my PhD project, I discuss them first in this thesis for the sake of argument. These Hymenoptera in fact are phylogenetically similar to bees and bumblebees and possess a comparable central nervous system. However, even if a vast literature about their cognitive abilities exists, there are still many feats that have been tested only on bees and bumblebees. As such, expanding our knowledge on the cognitive abilities of ants would let us understand if those abilities are widespread in all the Hymenoptera families, or if they are only a flux of evolution. According to the hypothesis presented above, cognition should be beneficial for miniature brains, and as such the same cognitive ability useful for bees and bumblebees should be found in ants.

Secondly, I will present the experiments carried out on jumping spiders. These have instead been the main focus of my PhD project. Through collaboration with my supervisor Prof. Regolin and Dr. Enzo Moretto, I have founded a new laboratory on the study of this model Family. As they are arachnids, this Family is phylogenetically very far from Hymenoptera. Nonetheless, in the last three decades, some papers have reported outstanding behaviours that they can produce (see section 3 of this thesis). However, most of the literature still lacks direct controls aimed at understanding the underlying processes of these behaviours: are they the outcome of cognitive processes or are they the effect of preprogrammed routines? These spiders represent a promising model for the study of

miniature cognition, as they would demonstrate that cognition is widespread amongst the entire arthropod spectrum.

SECTION 2 – COGNITION IN ANTS

The ant as a model species for cognition and simplification

‘*Propter parvitatem autem sui capitis habet oculos sitos super quaedam additamenta, quae per modum duorum pilorum egrediuntur de capite suo : cuius signum est, quia quando illa amputantur, tunc vadit errando nesciens quo vadat, et tunc quamcumque apprehenderit aliarum formicarum, illam fortissime tenet, ut per ipsam ad casam revertatur, nec facile se ab ipsa permittit separari.’*

Alberti Magni, De Animalibus libri VIII [80]

‘Because of the small size of their [ants’] heads, the eyes are placed on top of two hair-like appendices : proof of this lies in the fact that when those are amputated, then [the ant] wonders without knowing where it is going, and will attach to any other ant, strongly holding to it in order to go back home thanks to her, and will not easily allow to be separated.’ (English translation)

It is often difficult to enter the world of species so alien to us. We generally look at animals’ behaviour with our lenses, attributing human-like reasoning to some behaviours and disregarding others that do not fit in our repertoire. As stated in the quotation above, Saint Alberti Magni believed that the eyes of the ant are on top of its antennae, because the animal is lost without them: the concept that vision may not be the primary sense for ants was completely disregarded. In the same way, when we look at the behaviours of animals so alien to us, we fail to find a direct explanation for the individuals’ behaviour. Each ant seems to wander around mechanically; however we recognize a final goal in the group behaviour, as the colony appears to be ultimately organized and well functioning. From this perspective, there is no wonder that, for the general public, individuals in a social insect’s colony are considered simple miniature automata: the complexity only emerges from their collective behaviour [81]. This is far from the whole story. Social insects show decentralized organization [82]; there is no leader in a beehive or ant colony. Instead, every individual autonomously takes its own decisions based on its own experience and innate predispositions. Those decisions are then often communicated to others [83–87]. Consequently, members of a group can compare their own memory and experience to the shared information provided by the other colony members, and make strategic decisions about the source of information onto which is best to rely [88–90]. Such individual decision-making based on decentralized information sharing is not so different from how we use the Internet reviews of

restaurants or other services to make our own best possible choice with limited direct experience. Indeed, rather than suppressing individuality, the efficiency of a social insect colony depends on and benefits from the cognitive abilities present at the individual level [91]. Thanks to the increased understanding and appreciation for the complexity of hymenoptera behaviour, a massive amount of literature about their cognitive abilities has been flourishing. Most of it has focused on bees and bumblebees, which have been found to produce virtually every behaviour they have ever been tested in [76,92–97].

Ants themselves posses a plethora of cognitive abilities. Each worker can form complex memories [98,99] and even show disappointment if their expectations are not met [100]. However, most of the studies on ant cognition focus on their navigation abilities [101,102]. Nonetheless, there is still a great variety of abilities that has never been observed nor looked for in ants, that we may erroneously assume they are present because they have been demonstrated to exist in other Hymenoptera. Experiments on ant cognition are crucial, as they can serve as comparative studies to understand how much specific cognitive abilities are widespread amongst the same Family and the same class [93,97,101].

In the following paragraph I will describe the structure of the ant brain, with particular attention to the mushroom bodies (MBs), which is the area that have been associated with learning, memory and other forms of cognition. Then, I will present two studies.

The brain of Hymenoptera

Thanks to the involvement of multiple scientists around the world in the past two decades, there is now a vast and ever-growing literature about how the brains of insects, Hymenoptera in particular, are structured and function. Covering all the literature would require a dissertation in itself, and is beyond the purpose of this thesis. In the following paragraph I will report only the most salient information for the study of cognition and a description of the gross anatomy.

The insect's body is divided into 3 parts: the head, the thorax and the abdomen. In most species of insects, each section has its relatively autonomous neural circuit, as the brain is generally organized in separated ganglia [103]. The head contains the eyes, the antennae, the ocelli, the mouth part and, crucially, the most developed part of the nervous system: the brain.

The bee brain contains approximately 960,000 neurons and has a volume of 1mm ^{3} [104], and it contains 7 main neuropils responsible for sensory decoding and information integration [103,105–

107]. Two main sensory systems are located in the brain: the visual system and the olfactory system.

The visual system starts from the two compound eyes. These are composed of multiple *ommatidia*, each surrounded by pigment cells. Each ommatidium is composed of an external lens (thick and chitinous), followed by a crystalline and, lastly, a rhabdome surrounded by retinal cells [108]. The retinal cells project neural fibres towards the brain and the start of the *lamina* (i.e., the first neuropil of the hymenoptera visual system). This in turn projects its neurons to a second neuropil, the *medulla*, which connects both to a third-order visual processing centre, the *lobula*, and to the central brain section. These first three neuropils are probably responsible for the decoding of simple visual features, such as colour, motion, and orientation [14,109,110] in a segregated manner. The information is integrated successively by the central areas. The lobula itself connects to the central brain, mainly to the central complex (CX) (figure 2.1) (but also to the MBs, which will be described afterwards) [111]. The CX consists of two deeply interconnected sections: the protocerebral bridge, situated in the posterior brain; and, more anteriorly, the central body. The latter is divided into an upper region (i.e., the fan-shaped body) and a lower one (i.e., the ellipsoid body) [112]. For a long time, researchers have believed that the CX is only a visual decoding area, as it only possesses direct connections to the other visual areas. However, in the last years, it has received increasing interest, as it has been identified as having a key role in many crucial tasks, such as locomotor control [113,114], spatial orientation [113,115–118] and visual memory [117,119,120] (note that most of these findings do not come directly from Hymenoptera, but are instead generalized from other insects, mostly *Drosophila*). Because of its function and evolutionary history, the CX has been compared to the human cerebellum [121,122] and basal ganglia [123]. It is important to point out that an area homologous to the CX exists also in the spider's brain – that is, the arcuate body (see next section).

The olfactory system has been extensively studied in Hymenoptera, as it is the main sense for these animals. The literature is especially rich on the role of olfaction in relation to the reward system in the bee brain: Most of the experiments administered a sucrose reward concurrently with an olfactory stimulus. Odours are detected by olfactory receptor neurons present in the antennae that subsequently project their connection to the corresponding antennal lobe (one for each antenna). The antennal lobes project both to a third-order olfactory neuropil, the lateral horn, and to the MBs [104,124,125]. These are not only olfactory neuropil: In the brain of Hymenoptera, they receive projections from many other areas, functioning as an integration centre [126]. In fact, many studies

have focused on the implication of the MBs in learning and memory [127–131], and are now considered the functional equivalent of the human hippocampus [132–135]. The MBs input section is the Calyx (figure 2.1). This contains segregated inputs for olfaction, vision and mechanoception [14,104,136]. and are composed of Kenyon cells [137]. These are neurons characterized by a substantial arborization that composes the chalice such as the structure of the calyx. The axon-like branch divides again, forming a bundle of connections named pedunculus, and then it terminates into two different areas: the α and β lobes. These two last areas act as an output for the hymenoptera MBs connecting to other brain areas [14]. It is important to point out that also for the MBs there is a homologous and homonymous area in the spider brain (see next section).

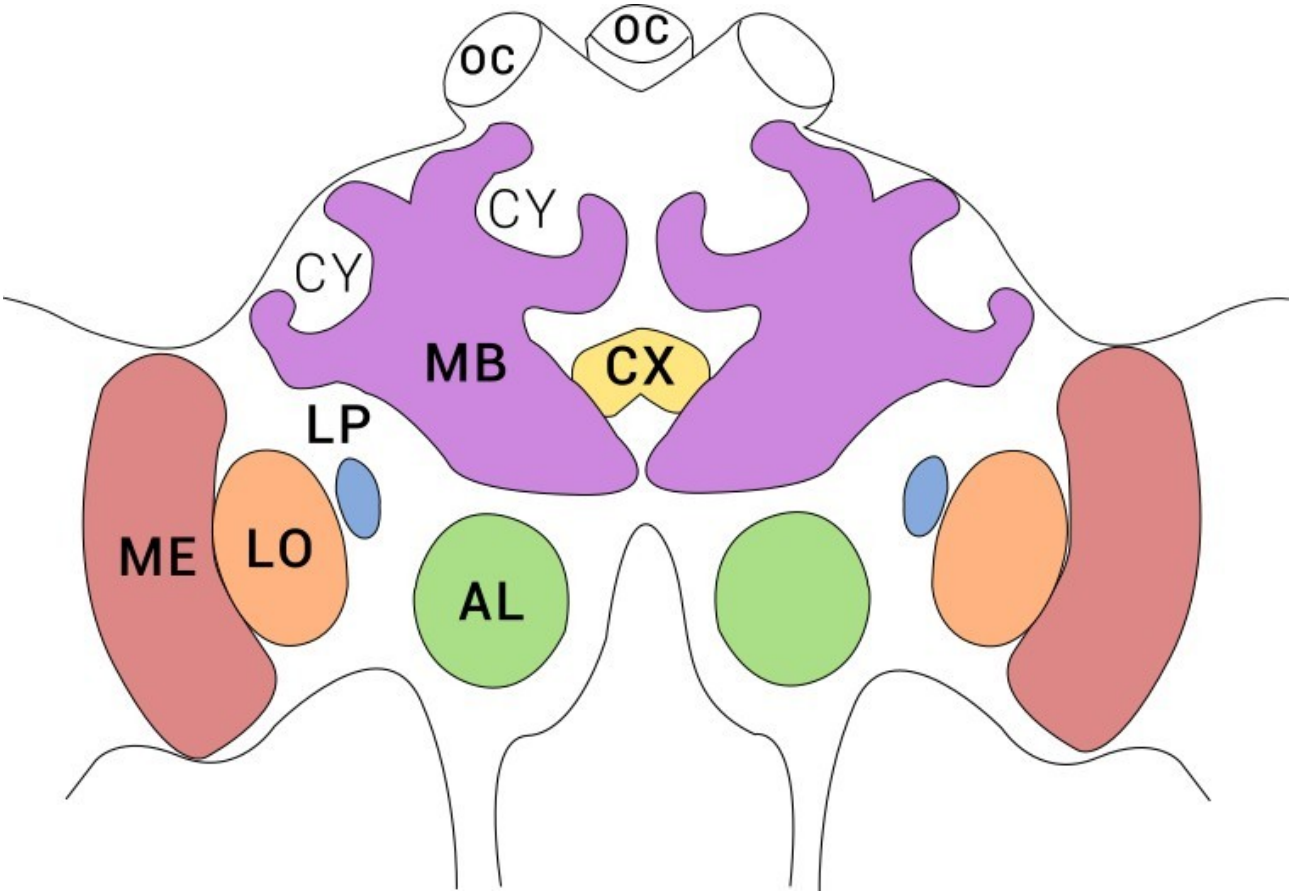

Figure 2.1 – Main areas of the Hymenoptera brain. ME = medulla; LO = lobula; AL = antennal lobe; LP = lateral protocerebrum, third olfactory neuropil; MB = mushroom body; CY = calyx; CX = central complex; OC = ocelli. Adapted from [104].

To conclude, we have a wide literature that describes the Hymenoptera brain, the function of many areas and especially the proof that some of them are involved in complex behaviour and cognition. Also, we know a lot about the individual cognition of some social insects, such as bees and bumblebees. Lastly, we have a vast literature on ants behaviour, especially on their navigational

ability. However, we still lack many direct experiments to test the presence of specific cognitive abilities in ants, too often given for granted as they have been demonstrated on phylogenetically similar species. In the context of the hypothesis that cognition may be widespread in miniature brains as an effective solution to the small number of neurons, it is crucial to test if cognitive abilities are indeed widespread, and used over mechanistic systems. In the following paragraphs two studies will be presented. The first will focus on the ant's perceptual mechanisms, and how they may facilitate complex comparisons between multiple, different-valued food sources thanks to perceptual rules. The second will focus on the ability of ants to record multiple information from different sensory sources, testing if the memorization is in fact dependent from the amount of the information (and so limited by neuron numbers) or based on a different process.

Study 1: Support for the perceptual basis of irrational risk aversion in ants

This study has been submitted and is available as a preprint at [138].

Introduction

Finding a good meal is not easy: the environment provides a broad variety of food sources, but individuals are not necessarily able to explore all of them before committing to one [139]. The food sources the organism inspects will often have different attributes, and options can be compared to choose the best one. This economic decision process is so crucial for organisms that the ability to compare options is found not only in animals, but even in non-neuronal organisms such as plants and slime-moulds [140–142]. This clearly suggests that a big brain is not needed in order to compare values of food, as it can be done with no brain at all. However, this is puzzling: to compare different values, the animal needs to register all of them, in turn increasing the information load which has been described in the introduction to be the Achilles' heel of having few neurons.

Traditionally, organisms were assumed to maximize energetic gains while minimizing costs on the basis that evolution should drive animals to have optimal behavioural strategies. However, the optimal foraging theory framework [143] fails to fully describe behaviour – organisms do not always behave optimally. These violations of optimality may shed light on the mechanism that these miniscule brains use to register and compare many "value" information. Extensive examples of violation of optimality in animal species can be found, for example, in the literature about risk sensitivity. For the purpose of this study, I define risk as a situation in which the probabilities associated with an option (e.g. food source) are known, but its exact value of is not. Conversely, "uncertainty" is when not even the probabilities of the various possible pay-offs are known.

Risk sensitivity theories – the budget rule

Caraco et al. [144] effectively inaugurated risk sensitivity studies, by studying the preference of yellow-eyed juncos for different amount of seeds: one of the two alternatives available to the birds was stable, presenting always the same, medium amount of food (safe feeder), while the other one fluctuated in value, but had the same mean pay-out as the safe feeder (risky feeder). The authors then, based on the preference of the animals, designed a utility function [145], computing the perceived value (utility) for each number of seeds for the animals. Yellow-eyed juncos presented a concave utility function (and so were risk averse) when in a high energy budget, whereas their

utility function was convex (and so they were risk prone) when in a low energy budget. This behaviour was soon formalized as the Energy Budget Rule [146]. However, a growing body of work on risk sensitivity failed to provide consistent empirical support for the budget rule [147,148]. For this reason Lim et. al. [149] recently reformulated the budget rule. They argued that the classical budget rule is often misused in its binomial interpretation: animals are either risk prone (when in a low energy budget) or risk averse (when in a high energy budget). However, the optimum risk sensitivity in a given situation lies on a continuum, depending on the remaining energy budget of the animal, even arriving at extreme conditions (very low energy budget and very high energy budget) in which risk indifference arises again. Such a continuous interpretation of the budget rule may accommodate results considered to be inconsistent in the classical budget rule hypothesis (e.g., [150]).

Risk-sensitivity theories – Scalar Utility Theory

An alternative to prescriptive theories (based on optimality modelling) are descriptive theories, which explain behaviours in terms of proximate mechanisms. If risk sensitivity arises as a side-effect of the neural or cognitive architecture of an animal, or due to evolutionary constraints, one need not attempt to fit this behaviour into fitness benefits. A striking pattern in risk preference studies is that animals are often risk averse when risking amounts, but risk seeking when risking delays [147]. Animals (and humans) are also generally risk averse for potential gains, but risk prone for potential losses [151]. These patterns are elegantly explained by an understanding of how animals perceive the world, as described by Psychophysics [152–154]. Stimulus strength has a logarithmic relationship with perception, as formalized by the Weber-Fechner law [155]. Thus, a constant feeder that always presents 5 seeds and a variable feeders presenting alternatively 1 or 9 seeds have the same average; however, 5 seeds are perceived as 5 times more than 1 on a logarithmic curve, while 9 is not even twice as good as 5. Hence, while the mathematical average, and thus the true energetic value, of the variable feeder is the same as that of the safe feeder, its geometric average is lower. In logarithmic distributions, such as the Weber-Fechner law by which animals perceive the world, the median is coincident with the geometrical average, and is the measure that describes the overall perceived value of an option, as it is the middle point between the two alternatives. Based on these insights, Kacelnik & El Mouden [148] developed Scalar Utility Theory (SUT) to describe risk aversion behaviour. They point out that, based on the Weber-Fechner law, the variance of the memory representation of a food value increases as the value itself increases. For this reason, two options with identical mathematical average (means) but different

variances will have different medians, with the more variable option having a lower one (see figure 6 from [148] for a complete explanation) However, support for this descriptive theory is also mixed: Lim et al. [149] argued that SUT has even weaker support than the budget rule, with only 8 of the 35 studies reviewed by Kacelnic & Bateson [147] finding complete risk aversion when risking potential resource gains. Shafir [156] argued that it is the strength of risk preference that is driven by perceptual mechanisms, while the direction is driven by budget considerations, and could thus accommodate both risk seeking and risk aversion in a manner consistent with logarithmic perception. However, Shafir's model can only account for alternatives with the same mean value. Whether risk sensitivity is best understood in terms of adaptation or constraints on perceptual mechanisms is thus still under debate.

Ants as a model for risk sensitivity

Risk sensitivity has been studied in a great variety of animals [for a review, see 148]. Amongst those, nectarivores have received particular scrutiny [156,157]. The majority of studies on nectarivores have been carried out on bees. Results have, however, been unclear: bees have been observed to be risk indifferent [157–159], to be risk averse [160,161], to follow the budget rule [162,163], or a mixture of those depending on risk variability [156,164–166]. Bees and other eusocial insects represent a special case for risk sensitivity. For eusocial insects with non-reproductive workers, the colony is the main unit of selection and a colony can be considered a superorganism [81,167]. As such, the foraging successes of the individual workers are pooled. This buffers colonies against short-term (negative) fluctuation coming from risky choices made by individual foragers. Colonies can also visit multiple food sources simultaneously, allowing them to more efficiently exploit their environment [168,169]. Lastly, many eusocial insects can make collective foraging decisions using recruitment mechanisms to channel workers towards certain resources in the environment [170,171].

While research on risk preference and collective decision-making is extensive, these have rarely been combined. Collective risk sensitivity has been explicitly studied in ants: Burns et al. [172] presented colonies of rock ants (*Temnothorax albipennis*) a fixed-quality mediocre nest and a variable quality nest. Ants were allowed to explore (and hence evaluate) each nest and then recruited nest-mates, and colonies were found to be risk prone. On the other hand, Hübner & Czaczkes [173] tested the risk sensitivity of black garden ant (*Lasius niger*) colonies to food values. Each colony was presented with two feeders: a stable one, always presenting the same medium

quality sucrose solution (0.55M), and a variable one, presenting (changing every 3 minutes) either a low or high quality sucrose solution alternatively (0.1M – 1.0M). Almost all trials showed a clear collective decision for one of the two feeders (as is expected due to symmetry breaking in ants collective decisions, see [83,174–176]), but overall colonies were risk-indifferent: half the colonies chose the safe feeder, and half chose the risky one.

This work aimed to explore individual risk preference in individual *Lasius niger* ant foragers. Although their collective behaviour appears to be rational, individual workers may not be [177]. They could be subjects to the same perceptual constraints discussed above and could be strongly influenced by expectations, causing disappointment for some food alternatives and triggering risk sensitivity.

Materials and Methods

Subjects

Twenty-two queenless *Lasius niger* colony fragments of around 1,000 ants were used in the study. Each fragment was collected from a different wild colony on the University of Regensburg campus. Colonies fragments forage, deposit pheromone and learn well [99,178]. Each fragment was housed in a transparent plastic box (30×20x40cm), with a layer of plaster on the bottom. A circular plaster nest, 14cm in diameter and 2-cm thick, was also provided. The colonies were kept at room temperature (21-25 c°) and humidity (45-55%), on 12:12 light:dark cycle.

Each colony was fed 0.5mol sucrose solution *ad libitum*, and was deprived of food 4 days prior to each test. Water was provided *ad libitum* and was always present.

Experiment 1 – Risk preference between options of equal absolute value

The aim of this experiment was to assess the preference of individual ants between two food sources which provide, on average, an equal amount of sucrose: one feeder provided a stable moderate value (0.55M sucrose, the ‘safe’ option) and one provided a fluctuating value, either high or low (0.1M or 1.0M, the ‘risky’ option). This was achieved by teaching each individual ant to associate each feeder type (risky or safe) with a different odour, and then testing their preference in a Y-maze. Preliminary tests (see Appendix 1) and previous work [98,179] show that *L. niger* foragers learn quickly (within 3 visits to each odour) and reliably to associate odours with feeders of different types. In total we tested 64 ants from 4 colonies. Each condition (scent association, feeder

order, risky feeder order, scent side on the Y-maze) was balanced and equally distributed among colonies.

Training

To begin each trial ants were allowed onto a 15cm long, 1cm wide runway, with a drop of sucrose at the end. The first ant to encounter the sucrose was marked with a dot of paint, and all other ants were returned to the nest. The marked ant was allowed to drink to satiety and then return to the nest to unload the collected sugar. She was then allowed to make 7 further training visits to the runway and feeder. If in any of the visit the ant did not drink the reward in 5 minutes, she was let back to the nest and recollected for the following visit. In each visit we recorded the number of pheromone depositions (counted by observing the movement of the worker's abdomen) performed on the runway towards the feeder and towards the nest after foraging. Over the 8 visits the quality and odour of the feeder varied systematically so that the ant alternately encountered a moderate quality drop of sucrose solution (0.55M, 'safe') scented with one odour, or either a low (0.1M) or high (1.0M)('risky') drop of sucrose scented with another odour. These values are clearly distinguishable by the ants [180] and correspond to moderate, low, and high value food sources for *L. niger* [181]. Note that the average of the low and high quality solutions equals that of the moderate quality. The solutions were scented using either rosemary or lemon essential oils (0.05 µl per ml). The runway leading to the feeder was covered with a paper overlay scented identically to the sucrose solution being offered. Overlays were scented by storing them in a sealed box containing cotton soaked in essential oil. Overlays were discarded after each return to the nest, to ensure fresh odour and to prevent a build-up of trail pheromone from occurring.

Testing

After the 8 training visits, the runway was replaced with a Y-maze (arm length 10cm, bifurcation angle 120°). The stem of the Y-maze was overlaid with unscented paper, whereas the two other arms were covered with scented overlays – one bearing the 'risky' associated scent, and the other the 'safe' associated scent. The maze tapered at the bifurcation to ensure that the ant perceives both scented arms at the same time [following 182]. No sucrose was present on the Y-maze. We recorded the ants' initial arm decision, defined by the ants' antennae crossing a line 2cm from the bifurcation point. We also recorded the ants' final decision, defined by the ant crossing a line 8cm from the bifurcation point. However, the initial and final decisions of the ants were almost always the same, and analysis of either choice provides the same results (see Appendix 2). For brevity we henceforth

discuss only the initial decision data. On reaching the end of an arm the ant was allowed to walk onto a piece of paper and brought back to the start of the Y-maze stem, to be retested. The Y-maze test was thus repeated 3 times, to assess reliability of the ant choice. However, this handling may have caused some disruption (see Appendix 2) and repeated unrewarded trials affect motivation, so we conservatively analysed only the first Y-maze test. After testing, the ant was permanently removed from the colony.

For each tested ant, one odour corresponded to the "risky" feeder and one to the "safe" feeder. The association between odour and feeder type, the initial feeder type encountered, the initial value of the 'risky' feeder, the side on which the risky or safe associated odours were presented on the Y-maze test, and the scents associated with the risky and safe options were all balanced between ants. Performing treatments blind was attempted, but due to the clear negative contrast effects shown by ants upon encountering a low quality food source after better ones [180], true blinding was not possible.

Experiment 2 – Risk preference between options of different absolute value

Experiment 1 demonstrated very strong risk aversion in individual ant foragers. Experiment 2 was designed to test whether risk aversion would be maintained "irrationally", that is, when the risky feeder had a higher average quality than the safe feeder.

As in experiment 1, the safe feeder always presented a medium quality drop (0.55M, indistinguishable for the ants from the solution provided ad libitum to the colony). However, the risky feeder alternated between a low quality reward (0.1M) and a very high quality reward (1.5M). The average molarity of the risky feeder (0.8M) was thus higher than the average molarity of the safe one. *L. niger* foragers can distinguish between the three presented molarities [100]. Moreover, in a pilot experiment, we observed that when presented with three different molarities ants do learn all three molarities and their associated odours (see Appendix 1). Each ant was tested on the Y-maze 5 times, but as in experiment 1, only data from the first test was ultimately used (see Appendix 2). In total we tested 64 ants from 8 colonies. Each condition (scent association, feeder order, risky feeder order, scent side on the Y-maze) was balanced and equally distributed among colonies.

Experiment 3 – Risk preference between psychophysically balanced options

One hypothesis explaining the widespread risk aversion found in animals towards reward quantities arises from the psychophysics of perception: intensity is generally perceived logarithmically (see

introduction, [147,148]). It is thus the geometrical average between the two risky alternatives that may describe the perceived value. This hypothesis predicts that animals should be indifferent between a safe and a risky option, if the risky option balances the logarithmic differences between the low and high quality reward. In experiment 2, these were not balanced: the geometrical average of the risky feeder ($\sqrt{0.1 \times 1.5} = 0.387$) was still lower than the one of the safe feeder ($\sqrt[1]{0.55} = 0.55$), thus the risky option may still have been perceived as worse than the safe option. In this experiment we set out to offer a risky option in which the perceived qualities of the low and high reward were balanced relative to the moderate reward. We chose a moderate reward of 0.3M, and a low and high reward of 0.1M and 0.9M respectively. The geometrical average of the risky option ($\sqrt{0.1 \times 0.9} = 0.3$) was now equal to the one of the safe option. We thus hypothesised that ants would be indifferent between these two options. Each ant was tested on the Y-maze 5 times, but again only data from the first test was used (see Appendix 2). In total we tested 40 ants from 10 different colonies. Each experiment (scent association, feeder order, risky feeder order, scent side in the Y-maze) was balanced and equally distributed amongst colonies.

Statistical analysis

Statistical analyses were carried out in R 3.3.3 [183]. Following Forstmeier and Schielzeth [184], we included in the models only factors and interactions for which we had *a priori* reasons for including. We employed generalized linear mixed effect models (GLMMs) using the package lme4 [185], with colonies as a random effect. Y-maze choice data was modelled using a binomial distribution and logit link function. We used the following model:

Initial decision =

*first presented feeder(risky-safe)**

first presented risky alternative (good-bad) +

random effect (colony)

We then used the package car [186] to test which factors of the model had a significant effect on the dependent variable. Subsequently, we carried out post-hoc analysis with Bonferroni correction using the package emmeans [187] both for the general preference of the ants for either the safe or the risky feeder (safe choice probability against random probability), and for the factors with a significant effect to analyse the direction of the difference. Plots were generated using the package ggplot2 [188].

Pheromone deposition count was modelled using a poisson distribution and logit link function. Good model fit was confirmed using the DHARMa package [189], and the pscl package [190,191] was used to produce the zero-inflated poisson models when needed. Pheromone deposition was not the focus of the current study, but we include it as descriptive data since it may shed light on how individual perception can shape group choice. We modelled pheromone deposited towards the nest and pheromone deposited on the way back separately, since these are conceptually very different: depositions towards the food reflect the ants' expectation, and depositions on the return to the nest reflect the ants' perception. The models used were the following:

Pheromone towards the drop =

*visit (2-8)**

value (molarity)+

random effect (ant nested in colony)

Pheromone back to the nest =

*visit (1-8)**

value (molarity)+

random effect (ant nested in colony)

Pheromone deposition data from each of the three experiments were analysed separately, as they were taken by three separate experimenters, and so could not reliably be compared between experiments. Path decisions allow much less observer error, so Y-maze data can be pooled between experiments.

Only main results are reported below. For the full analysis see Appendix 2.

Results

Experiment 1 – Risk preference between options of equal absolute value

Y-maze choice tests

Ants were strongly risk averse, with 91% (58/64) ants initially choosing the safe option (figure 2.2) (GLMM post-hoc with estimated means, probability=0.911, SE=0.36, z=5.142, $p<0.0001$). We

found no effect of the first presented feeder (GLMM Analysis of Deviance, chi-square=0.709, DF =1, p=0.3), nor of the first presented risky alternative (χ2=0, DF=1, p=1), nor of the interaction between those two factors (χ2=0, DF=1, p=1).

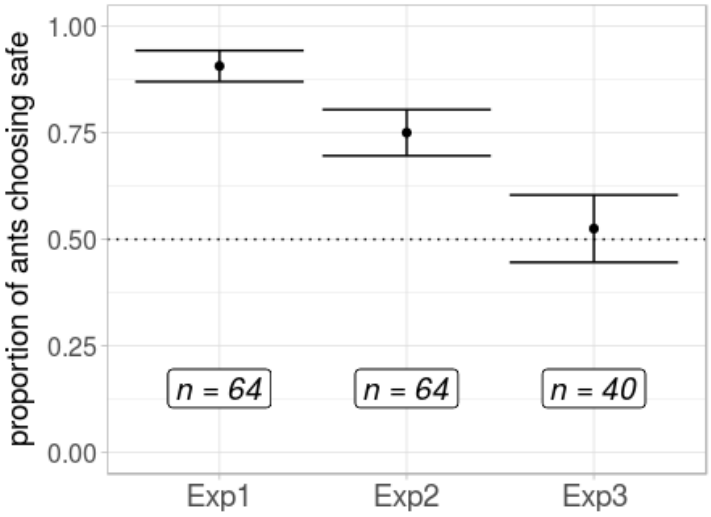

Figure 2.2 - Proportion of ants choosing the safe feeder. Ants prefer the safe feeder experiment 1 (prob.=0.911, SE=0.36, z ratio=5.142, p-value<0.0001), where it always has a value of 0.55M, while the risky option can be either 0.1M or 1.0M. They sill prefer the safe food in experiment 2 (prob.=0.792, SE= 0.068, z ratio = 3.248, p-value =0.001), where it always has a value of 0.55M while the risky one changes between 0.1M and 1.5M. There is instead no preference in experiment 3 (prob.=0.535, SE=0.086, z ratio=0.403, p-value=0.687), where the safe feeder has a value of 0.3M and the risky one fluctuate between 0.1M and 0.9M.

Pheromone deposition

Considering pheromone deposition towards the feeder, we found an effect of molarity (GLMM Analysis of Deviance, χ2=12.992, DF=2, p=0.001) and an effect of the interaction between molarity and visit number (GLMM Analysis of Deviance, χ2=14.469, DF=2, p=0.0007). Specifically, we found that the ants deposited overall more pheromone when going towards the 0.55M drop in comparison to the 1.0M drop (figure 2.3A, GLMM post-hoc with estimated means, estimate=0.657, SE=0.227, z=2.891, p=0.015). Note that the ant may be expecting to find the 0.1M drop when going towards the 1.0M, because it last experienced the low value associated with that scent. We found no differences in pheromone deposition between the other molarities. Overall, the ants deposited more pheromone on the way to the safe feeder relative to the risky one (GLMM post-hoc with estimated means, estimate=0.498, SE=0.19, z=2.616, p=0.036).

Considering pheromone deposited when returning to the nest, we found an effect of molarity (GLMM Analysis of Deviance, χ2=85.97, DF=2, p<0.0001), an effect of visit (GLMM Analysis of

Deviance, $\chi 2=5.11$, DF=1, p=0.024), but no effect of their interaction. Specifically, we found that the ants deposited overall less pheromone when going back from the 0.1M drop in comparison to the 0.55M drop (figure 2.3D, GLMM post-hoc with estimated means, estimate=-2.67, SE=0.154, z=-17.352, p<0.0001) and from the 0.1M drop in comparison to the 1.0M drop (GLMM post-hoc with estimated means, estimate=-2.78, SE=0.194, z=-14.308, p<0.0001). However, there was no difference between the 0.55M drop and the 1.0M drop. Overall the ants deposited more pheromone on the way back from the safe feeder relative to the risky one (GLMM post-hoc with estimated means, estimate=1.28, SE=0.14, z=9.149, p<0.0001).

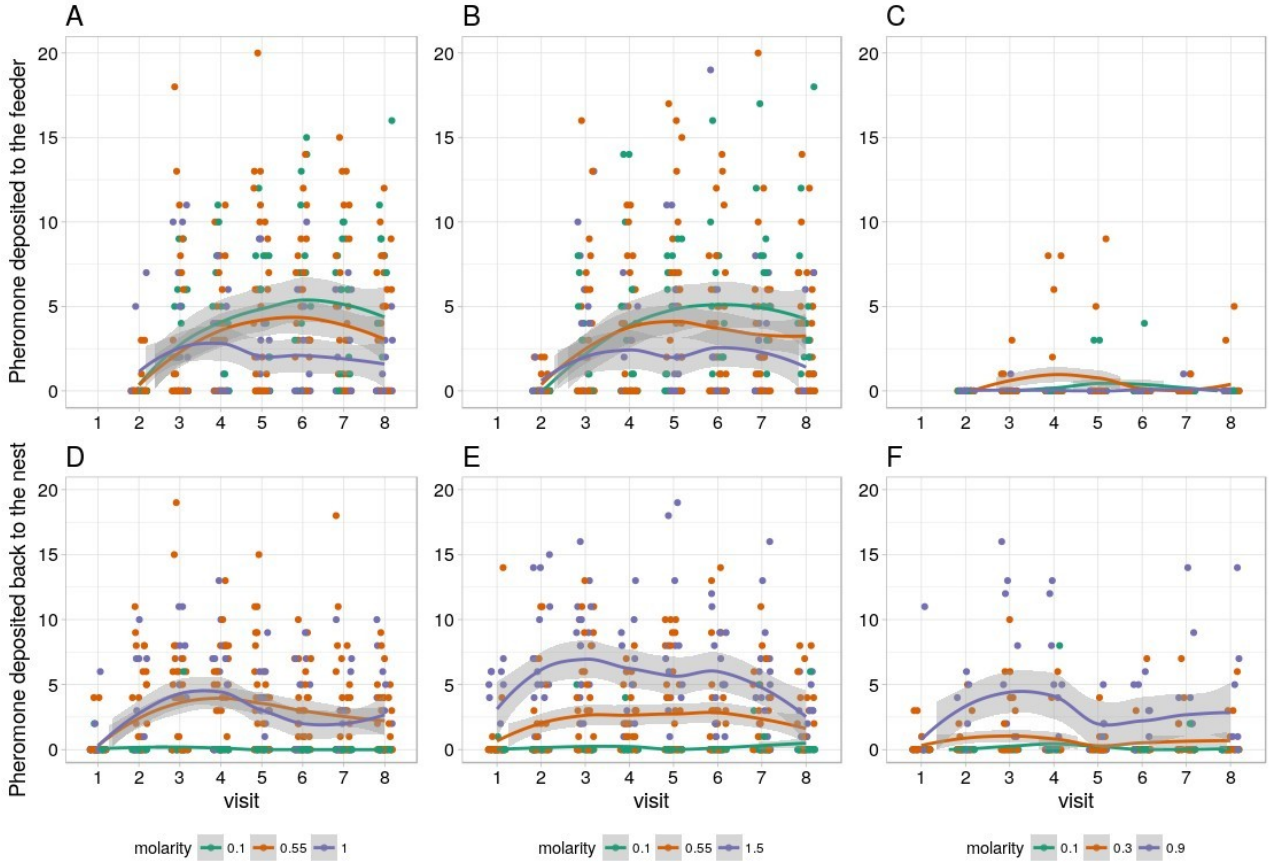

Figure 2.3 – Amount of pheromone deposited by the ants going to the drop and back to the nest across visits in the three experiments. Considering the pheromone deposited on the way to the drop, we found a higher deposition rate for the safe feeder in experiment 1 (A) and in experiment 3 (C) but not in experiment 2 (B). Considering the pheromone deposited on the way back to the nest, we found a higher deposition rate for the safe alternative in experiment 1 (D) and experiment 2 (E), but not in experiment 3 (F).

Experiment 2 – Risk preference between options of different absolute value

Y-maze choice tests

Ants were again strongly risk averse, with 75% (48 / 64) ants initially choosing the safe option (figure 2.2) (GLMM post-hoc with estimated means, probability=0.792, SE=0.068, z=3.248, p=0.001). We found no effect of the first presented feeder (GLMM Analysis of Deviance, $\chi2$=2.015, DF=1, p=0.156), nor of the first presented risky alternative ($\chi2$=0.197, DF=1, p=0.657), nor of the interaction between those two factors ($\chi2$=1.807, DF=1, p=0.179).

Pheromone deposition

The data for the pheromone deposition are summarized in figure 2.3B and 2.3E.

Considering pheromone deposited towards the drop, we found an effect of the molarity (figure 2.3B, GLMM Analysis of Deviance, $\chi2$=7.489, DF=2, p=0.024). However, post-hoc analysis revealed no difference between any of the molarities: the differences were probably so small that Bonferroni correction in the post-hoc analysis brought them above significance.

Considering the pheromone deposited back to the nest, we found an effect of molarity (GLMM Analysis of Deviance, $\chi2$=133.424, DF=1, p<0.0001), an effect of visit (GLMM, chi-square=10.249, DF=1, p=0.001), and an effect of their interaction (GLMM, $\chi2$=11.339, DF=2, p=0.003). Ants deposited less pheromone for the 0.1M drop in comparison to the 0.55M drop (figure 2E, GLMM post-hoc with estimated means, estimate=-2.683, SE=0.17, z=-15.742, p<0.0001), less pheromone for the 0.1M in comparison to the 1.5M (GLMM post-hoc with estimated means, estimate=-3.474, SE=0.204, z=-17, p<0.0001) and less for the 0.55M in comparison to the 1.5M (GLMM post-hoc with estimated means, estimate=-0.79, SE=0.19, z=-4.144, p=0.0001). Overall the ants deposited more pheromone on the way back from the safe feeder relative to the risky one (GLMM post-hoc with estimated means, estimate=0.946, SE=0.14, z=6.341, p<0.0001).

Experiment 3 – Risk preference between psychophysically-balanced options

Y-maze choice tests

53% (21/40) of ants chose the safe option (figure 2.2), a proportion not different from chance (GLMM post-hoc with estimated means, probability=0.535, SE= 0.086, z=0.403, p=0.687).

We found an effect of the first presented feeder (GLMM Analysis of Deviance, $\chi 2=4.424$, DF=1, p=0.0354). Specifically, 71% of the ants presented with the safe feeder in visit 1 choose the safe smell during testing, while 35% of the ones initially presented with the risky feeder did.

Pheromone deposition

Considering pheromone depositions towards the feeder, we found an effect of molarity (GLMM, chi-square=16.133, DF=2, p=0.0003). Ants deposited more pheromone when going towards the 0.3M drop in comparison to the 0.9M drop (figure 2.3C, GLMM post-hoc with estimated means, estimate=10.444, SE=1.751, z=3.769, p=0.0007), while we found no difference between 0.1M and 0.3M (GLMM post-hoc with estimated means, estimate=0.477, SE=0.174, z=-2.032, p=0.169) and between 0.1M and 0.9M (GLMM post-hoc with estimated means, estimate=4.981, SE=3.452, z=2.317, p=0.082). Overall, ants deposited more pheromone for the safe feeder (GLMM post-hoc with estimated means, estimate=4.679, SE=1.751, z=4.124, p=0.0001).

Considering the pheromone deposition back to the nest, we found an effect of molarity (GLMM, $\chi 2=47.083$, DF=2, $p<0.0001$). Ants deposited less pheromone when returning from the 0.1M drop in comparison to the 0.3M one (figure 2.3F, GLMM post-hoc with estimated means, estimate=-882, SE=0.143, z=-6144, $p<0.0001$), less for the 0.1M in comparison to the 0.9M (GLMM post-hoc with estimated means, estimate=-1.479, SE=0.18, z=-8193, $p<0.0001$) and less for the 0.3M in comparison to the 0.9M (GLMM post-hoc with estimated means, estimate=-0.597, SE=0.165, z=-2.615, p=0.001). Overall the ants deposited the same amount of pheromone on the way back from the safe feeder relative to the risky one (GLMM post-hoc with estimated means, estimate=0.142, SE=0.126, z=1.134, p=1).

Discussion

Ants show strong risk aversion given equal average pay-offs between the risky and safe options (0.1/1.0M vs. 0.55M, experiment 1). Even if the risky option offers 45% higher mean pay-offs than the safe reward (0.1M/1.5M vs. 0.55M), ants still show strong risk aversion (experiment 2). We predicted, based on psychophysical principles, that logarithmically-balanced rewards should be perceived as having equal value. We tested this in a situation where the risky reward offered 66% higher pay-offs than the safe reward (0.1/0.9M vs 0.3M) and observed, as predicted, indifference between the two options.

Support for the perceptual basis of risk sensitivity

Our demonstration of risk aversion in resource amounts strongly supports the perceptual, descriptive theory of risk sensitivity proposed by Kacelnik & Bateson [147] and developed by Kacelnik & El Mouden [148]. Specifically, our data suggest functional risk aversion arising from risk neutrality filtered through logarithmic perception. Budget Rule theories [146] would also predict risk aversion in our context, since the ants are on a positive energy budget – *Lasius niger* would survive for over a week without feeding. However, our ability to accurately predict an indifference point based on logarithmic perception strongly implies that perceptual mechanisms are driving risk aversion in this species. Alternatively, we may have by chance chosen the precise point where logarithmic balancing matches the balance point between improved average gains from a risky option and the premium garnered by a safe bet according to the budget rule. However, this seems unlikely.

The ants in our experiment never showed a preference for the risky alternative. This may seem to imply that the ants were failing to learn the risky option, and associate it with an odour. However, this hypothesis can be ruled out, as it cannot account for the results of experiment 3, where neither food sources were preferred. If the ants were unable to learn the risky option, the only other explanation for experiment 3 would be that a 0.3M is not preferred over complete uncertainty. This can be ruled out, however, as ants clearly preferred 0.3M over 0.1M (Appendix 1).

The Budget Rule is neither supported nor refuted

Budget Rule theories [146] would also predict risk aversion in our context, since the ants are on a positive energy budget – *Lasius niger* would survive for over a week without feeding. However, our ability to accurately predict an indifference point based on logarithmic perception strongly implies that perceptual mechanisms are driving risk aversion in this species. Our data neither supports nor refutes the Budget Rule [144,146,149]: we tested all ants after exactly 4 days of starvation, so we cannot know how ants would have behaved on a different energy budget. Lim et al. [149] strongly critiqued the Scalar Utility Theory, since it predicts suboptimal behaviour, which should be selected against. Logarithmic perception, however, is a widespread phenomenon in the animal kingdom, from roundworms [192] to humans [155], and is argued that the logarithmic scale is the best possible neural representation of magnitudes amongst other biologically feasible scales [193]. A more precise food evaluation may require more energy than the energy gained from the additional precision, and may not be able to fit inside a miniature brain. However, this has never been tested in

the context of risk sensitivity [149]. Even if the benefits accrued from a more linear perception of value would outweigh the costs, developmental constraints or pleiotropy may prevent such perception from evolving.

Lack of support for Prospect Theory

Other theories of risk sensitivity based on perceptual mechanisms exist. Prospect Theory [151], a hugely influential economic theory of decision-making under risk in humans, predicts that an individual should be risk averse in the context of gains but risk prone in the context of losses. This again derives from logarithmic perception of cumulative gains and losses. However, in Prospect Theory the dividing point between gains and losses is not necessarily at zero. Rather, gains and losses are defined relative to a reference point, which is usually the expected pay-off, but may be socially induced (e.g. by comparing ones own salary to that of ones colleagues). Anything above the reference point is perceived as a gain and anything below the reference point is a loss. Disappointment for a lower value after a reference has been established has already been demonstrated in the honeybee [194] and ants [180], and suggested in bumblebees [195]. The reference point for our colonies might have been 0.5M: the solution that the ants are regularly fed on. If this were the case, in experiment 1 the true choice would be between an always neutral value (0.55M, safe), and a risk between a gain (1.0M) and a loss (0.1M). This hypothesis is also supported by the fact that almost no pheromone was deposited for the 0.1M drop. In this case Prospect Theory would still predict risk aversion, as losses are assumed to be perceived more strongly than gains. To test this hypothesis we repeated experiment 1, but with colonies that had been fed ad libitum 1.5M sucrose 1 month prior testing (data and procedure can be found in ESM1). If the ants were taking their standard feeding solution as a reference point, every presented solution in this experiment should have been perceived as a loss, and so the ants should have showed risk-seeking. However, we observed the same preference that we saw in the main first experiment – strong risk aversion. Either the ants behaviour is poorly described by Prospect Theory, or the normal feeding solution does not set the reference point. Another possibility is that the reference point is not set by the normal feeding solution, as the four-day food deprivation period may erase the ants memory of the feeding solution. Instead, the reference point could be the most common solution in the current context. In experiment 1 this would be 0.55M, maintaining the same situation of one neutral vs. a loss or a gain, and so predicting the same outcome under Prospect Theory. This hypothesis, however, does not fit the result obtained in experiment 3: if the 0.3M would have been taken as a reference, we should still have observed a preference for the safe option. Either Prospect Theory

does not well describe the behaviour of ants, or their reference point remains at 0 in every situation, with every reward being a gain: in the domain of gains Prospect Theory predicts simple logarithmic value perception.

Risk neutrality at the colony level

Does our understanding of individual behaviour in a risk-choice situation help explain the risk indifference of ants at a colony level [173]? Pheromone deposition rates of individual foragers vary hugely between individuals, even when presented with identical food sources. This is to be expected, given the fact that individual variability may aid collective decisions [196,197] . However, the appropriate measure of pheromone for colony-level decisions is total pheromone deposited. Examining the mean deposition rates for both feeders in experiment 1, we see that ants, on average, deposited more pheromone to the safe feeder (5.5 dots per ant) than the risky feeder (3.9 dots per ants). In Hübner & Czaczkes [173] each ant made only one or two visits to the feeder, but even when considering only the first two visits ants made more pheromone depositions to the safe (1.5 dots per ant) than to the risky (0.89 dots per ant) feeder. The finding of risk neutrality at the colony level is, thus, still a puzzle. However, the two experiments are not directly comparable. Firstly, in the current experiment pheromone was removed from the trail after every visit. Pheromone presence is known to reduce further pheromone deposition [198], perhaps damping out the differences between the two feeders. Secondly, the presence of odours on a path affects pheromone deposition: while pheromone deposition on odourless paths is usually higher on the nest-ward journey [174,198–200], pheromone deposition is higher on outward journeys on scented paths [this study,98,179]. Finally, it should be noted that perception of pheromone, much like perception of quality, is also logarithmic [201], thus emphasising initial differences in pheromone concentration but damping out differences between strong trails. Nevertheless, it seems that colony-level decision-making effectively filters out the ants individual perceptual constrains [this study, 177], but the mechanism used to achieve this is still unknown.

In this study, we found that ants demonstrated risk aversion due to a logarithmic perception of food value. Individual risk preference does not predict colony behaviour, which seems able to filter out perceptual biases.

Study 2: Multi-modal cues integration in the black garden ant

This study is under second review [202]

Introduction

As discussed above, cognition may be beneficial for minutes brain, as a small, complex network can use a rule or a mechanism to categorize a massive amount of stimuli that may could not be individually registered otherwise. This may be of even a greater importance in tasks that require learning: the information overload can quickly become too much to bear for a small brain. Even though which information is processed and how it is remembered can vary greatly, remembering the past is an ability of great importance for many animals. One of the most widespread and most well-understood mechanisms is to establish associations between a reward (e.g., food) and an event in the environment occurring at a defined time interval (e.g., a bell ring) (Classical conditioning [203]). This learning mechanism is dependent on the amount of information: an animal needs to associate the unconditioned stimulus to the conditioned one. This can be done for many different stimuli, but each one of them needs to be independently associated to a reward. Apart from learning and remembering relationships between stimuli or between behaviour and its outcome (Operant conditioning [204]), humans possess autobiographical memories of episodes of their own lives. Such episodic memory (EM) is defined as the subjective consciousness of a self-experience in terms of what (first school day), where (school) and when (in the morning) something happened to the individual [205,206]. These episodes contain a multitude of information coming from many different sensory origins. However, they are not registered one by one, since the episode is a cohesive unique memory [207].

For a long time, EM was considered a human prerogative [208]. This is not surprising, because only humans can self-report their knowledge of past events (in its what-when-where components) in a first-person perspective. For this reason, it is impossible to test the presence of EM in other animals using paradigms of human studies, as we can only infer animals' internal states indirectly from their behavioural responses. However, an animal's incapability to provide verbal reports does not necessarily imply the absence of egocentric memories. To overcome such restrictions, animal studies focus on the presence of three defined EM components (what-where-when) to infer the presence of EM. Researchers adopted the term episodic-like memory (ELM) when referring to non-human evidence of EM to point out this fundamental difference [209]. Remembering an individual past event can, in fact, be very useful as the recognition of similar situations allows to adjust

behaviours to a predictable future (e.g. by recognizing a situations' where-when components to predict the concurrent What).

In a pioneering study, Clayton and Dickinson [207] showed that Western scrub-jays recovered perishable worms, or non-perishable peanuts (what) from their caches (where) depending on how long the food was hidden (when). These results were successfully replicated [210] and adapted to other animal models (i.e., pidgeons [211], mice [212], dogs [213], bees [214], cuttlefish [215]). All these studies support the idea that ELM is not uniquely human. In the last decade, it has been suggested to further conceptualize animal ELM by using the components of what-where-which, assuming that the context information (which) is ecologically more relevant for animals with respect to the exact point in time (when) [216].

Regarding potential benefits from an ability to recall a single past event with all its characteristics, the ant represent a promising organism. Individual foragers make long foraging bouts during which they memorise multiple cues from different information modalities [217,218]. These different cues can be interpreted as what-where-which components.

Concerning the "which" component, ants often rely on visual cues during navigation using a mechanism called snapshot matching [218–220], where they compare the current view to snapshots memorised from the surrounding landscape in previous visits. The discrepancies between the current view and the saved snapshot allows the ant to orient itself in space. The wide-field surrounding and context (panorama) strongly influences recall in ants [221,222]. Relying on panoramas instead of distinct visual landmarks also accounts for the often low spatial resolution of ants' eyes [222]. In other words, ants tend to memorise the overall visual context rather than specific landmarks in the environment. This information is consistent with the "which" component of an episode. Apart from the panorama, ants are also able to perceive colours and associate these with rewards [223,224; Appendix 3].

Olfactory cues are heavily used in ant navigation, often in the form of trail-pheromone [83,225], and in species that navigate without trail pheromones [226–230]. Characteristic olfactory landmarks help to locate food or the nest, and odours can help to recall abundant food sites [231] or induce active searches for the sites [232]. Moreover, ants are able to learn different odours [228–231] and to obtain odour-reward associations even after a single exposure [233]. Such use of odour cues as landmarks could correspond to the ELM "what" component.

It is crucial to point out that the multi-modal nature of the different cues used in insect navigation [218,228,234] amplify the expected correctness over a single-cue system. Ants can in fact extract and learn bimodal cues (visual and olfactory) simultaneously, and their combined presence enhances each other's conspicuousness, thus favouring the learning process [228]. Such synergism is indicative of a neural integratory system of information from different modalities [217]. It has been proposed, that multiple cues are registered according to their expected predictive power of the presence of a reward. These are then compared and combined in order to accurately pinpoint the nest or food sources, averaging the different cues into a cohesive decision [219,220,235,236].

However, to our knowledge, ants have never been tested in a task where the predictive power of every cue is dependent on the presence or absence of another in a conditional manner: an olfactory cue may not be able to predict the presence of a reward, unless a contextual cues is concurrently presented. In this situation, it will not be sufficient for the cues to be compared for their predictive power, but they will need to be integrated in a cohesive, single memory linked with another.

In this paper, we aim to establish ants as model organisms for the study of ELM in an invertebrates. We first tested *Lasius niger* ants in a Y-maze in which they had to integrate olfactory (what), contextual (which) and spatial (where) cues across 12 training trials to obtain a reward. With this first experiment we tested the ability of these animals to register and integrate all three components together. As described above, EM is defined as the memory of a single past event, not as a formed association between multiple conditional stimuli. Therefore, we designed a second experiment, where we presented the ants with the what-where-which situation in a single unexpected event at the end of a training procedure.

Methods

Subjects

We used 4 queenless *Lasius niger* colony fragments collected from different colonies on the University of Regensburg campus consisting of ~1000 workers each. Queenless colonies behave normally and are often used in foraging experiments [237,238]. Each fragment was kept in a plastic box (30×20×40cm) with a floor of plaster and a circular plaster nest (14 cm in diameter and 2 cm thick). Temperature (21-25°C) and humidity (45-55%) were kept constant, and colonies were kept in a 12:12 light:dark cycle. Each colony was fed 0.5M sucrose solution ad libitum and was deprived of food 4 days prior to each test. Water was provided ad libitum.

Experiment 1 – Information integration

The aim of this experiment was to discover if ants are able to learn a visual context (which), a side (where) and a scent (what) simultaneously. While the scent was always predictive of the location of a reward, side and context were predictive only when considered together, not by themselves. To this end, ants were trained on a Y-maze to associate a scent (what, either lemon or rosemary) presented on the maze arm to a 1.0M sucrose (Merck KGaA, Darmstadt, Germany) solution drop. The side of the rewarded arm alternated side (where, left or right) consistently with the colour of the background (which, e.g. when the Y-maze had a blue background, reward was on the left, when the background was yellow, reward was on the right). During the test phase, the rewarded scent, which represented the only reliable information per se, was applied on both arms and thus became uninformative. To locate the reward, ants therefore had to integrate background colour with side in order to find the reward. A schematic representation of the procedure is available in figure 2.4.

Training

In the training phase, ants were allowed on a 15-cm long, 1-cm wide runway (referred to as entering runway) that led to a Y-maze (arm length 10 cm, bifurcation angle 120°). Both the stem of the Y maze and the entering runway were covered with unscented paper overlays. The two arms were covered with paper overlays of a different scent each. The scented runways were prepared by placing the paper overlays in an enclosed box containing 100µl of either rosemary or lemon essential oil. Ants have been shown to not have any innate preferences for either [239]. The paper overlays were left in the box for at least two hours before being used. The maze was tapered at the bifurcation to ensure that ants perceived both scented arms at the same time (following [182]). One of the two arms led to a drop of 1.0M sucrose solution, corresponding to a high-value reward for *Lasius niger* [181]. The other arm led to a drop of water, visually similar but bearing no reward. Around the Y-maze, a 5cm tall wall was placed. The wall surface could either be blue or yellow. In a pilot experiment, we demonstrated that ants can clearly distinguish between these two colours (see Appendix 3). The first ant that reached the sucrose drop and started drinking was marked with a dot of paint and allowed to drink until satiation while all other ants were put back into the nest. After drinking fully, it was allowed back to the nest to unload the sucrose to nest-mates via trophallaxis (mouth-to-mouth feeding [81]).

After unloading, only the marked ant was selectively allowed onto the setup using a movable bridge 11 more times, resulting in a total of 12 training visits. On each visit, the position of the rewarded

scent was changed, so that both sides were rewarded in alternation across visits. The wall colour was changed accordingly, in order to always have the same colour associated to the same side. For each visit, we recorded (i) pheromone depositions on the way towards the drop and on the way back to the nest, both were recorded only on the scented part of the setup (pheromone deposition is a stereotyped behaviour in *L. niger* and can be quantified by eye [225]); (ii) the ant's initial decision, scored when the ant crossed a decision line 2-cm inwards of a Y-maze arm; and (iii) the final decision, scored when the ant crossed a decision line 8cm inwards of an arm. For each ant the rewarded scent was kept constant, but we randomized the rewarded scent, background colour at start, rewarded side at start and colour-side associations across ants.

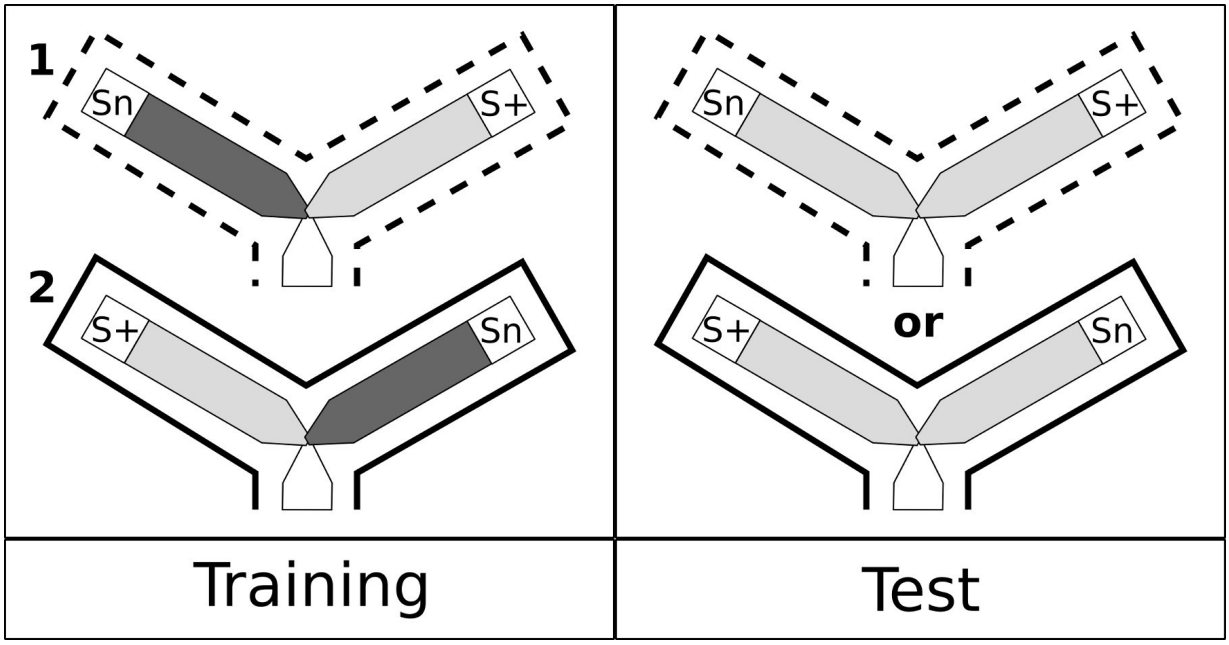

Figure 2.4 – Schematic representation of the procedure for experiment 1. During training, the ants were let onto a Y-maze. During a first visit (number 1 in the picture) one of the arms of the Y-maze led to a was coated with lemon scented paper (light grey) and led to a 1.0M sucrose solution (S+). The other arm was coated with rosemary scented paper (dark grey) and led to a water drop (Sn). The walls around the Y-maze could either be yellow or blue (dashed or solid line around the maze). The colour of the walls and the position of the rewarded scent (left or right) were always associated (e.g. blue walls, lemon on right, yellow walls, lemon on left). After the first visit the ant was let back to the nest, the position of the scent was switched as well as the wall colour (as number 2 in the picture) Across the total of 12 training trials that were performed wall colour and consequently the position of the lemon scent were alternated. In the testing phase, we removed the scented paper predicting Sn (rosemary) and left the rewarded scent on both arms. At this point the scent information became uninformative. To still be able to locate the reward, the ants needed to remember that when the walls are blue, reward could have been found on the left, and vice-versa with the yellow walls, effectively demonstrating the ability to integrate side (where) and colour (which) information. Rewarded scent (lemon or rosemary) and colour-side association were balanced across individuals.

Test phase

Ants were tested on the 13th visit to the Y-maze. No sucrose was present in the test visit. The background colour was either blue or yellow. The rewarded scent, however, was now placed on both arms of the maze and thus made uninformative. Now, ants were only able to choose the "correct" arm (consistent with the colour-side association) if they concurrently learned the association between background colour and side during training. After the tested ant reached the end of either arm, it was allowed on a piece of paper and gently placed back to the Y-maze stem, in order to repeat the test. This way, each ant made 5 decisions during the test phase, providing an estimate of choice reliability and drop-out probability.

Experiment 2 – Information integration in an episode

Experiment 1 demonstrated that ants learn the association between background colour and side despite the presence of scent as sufficient predictor for reward during training (see results). In this second experiment, we tested if ants were still able to succeed in the testing phase after experiencing the colour-side combination only once. This would require the ability to retrieve a single event and its features and is consistent with the definition of episodic memory (see introduction), as opposed to associative learning, which strengthen incrementally over visits. A schematic representation of the procedure is available in figure 2.5.

Training

In this experiment, the training setup was a 10-cm long and 1-cm wide runway instead of a Y-maze. This runway was scented and a drop of either water or 1.0M sucrose solution was placed at its end. The scent of the runway was consistent with the drop quality, in order to let the ant form an association between the scent and the reward. As before, multiple ants were allowed on the setup; the first ant that started drinking on the drop was marked and the others moved back to the nest. Only the marked ant, thereon, was allowed onto the setup for 5 further training visits, resulting in a total of 6 visits (including the first). Each visit alternated between sucrose solution (visits 1,3,5) or a drop of water (visits 2,4,6) and the overlay scent was alternated accordingly, so that one scent always predicted a reward while the other always predicted a water drop. The rewarded scent was balanced between ants. For each visit we recorded pheromone deposited both on the way to the drop and on the way back on the 10cm long scented overlay.

On the 7th visit, ants were confronted with a Y-maze identical to the training setup in the first experiment. The two arms presented the two scents (lemon and rosemary), and the walls were either

be blue or yellow. At the end of one arm, a 1.0M sucrose solution was placed and a water drop was placed on the other, according to the scent-reward association established in visits 1-6. The 8 th visit was identical to the 7 th, but both the rewarded side and the colour of the wall were switched. For both of those visits, we recorded the number of pheromone depositions on the scented portion (on the way to the drop and back) and the side choice, as described for the first experiment. The latter in particular was used to assess whether the ants had learned the scent-reward association. These two visits were used to make the ants experience the association between spatial (where, side) and contextual (which, wall colour) information, other than the conditioned stimulus (what, the scent). In both visit 7 and 8 the ants could have integrated together all the information to form a cohesive memory of an episode. We needed to present an event for each colour and side to prevent the ant from exclusively relying on either the side or the background colour as predictor for food presence.

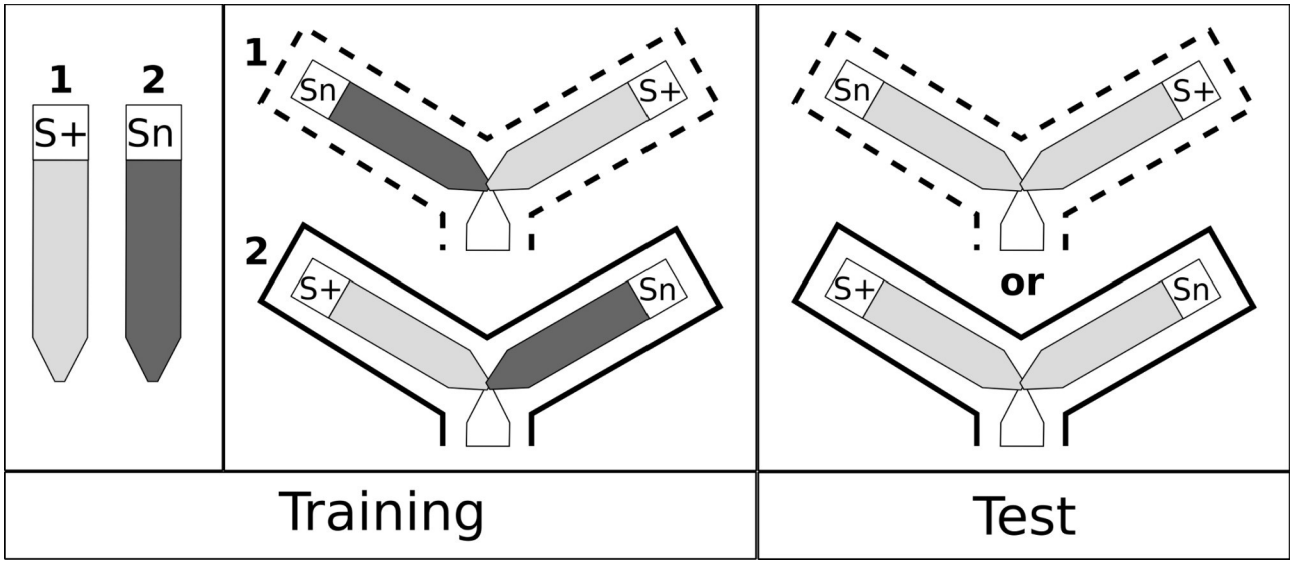

Figure 2.5 – Schematic representation of the procedure in experiment 2. In the training phase the ant was initially let onto a straight runway. This could have been covered with lemon scented paper (light grey) and leading to a drop of 1.0M sucrose solution (S+), or with rosemary scented paper (dark grey) and leading to a drop of water (Sn). In this experiment the runway were surrounded by white walls. Across the 6 trials we alternated the two scents and consequently the reward with the neutral. After the first 6 training trials the ant was let onto a Y-maze, with the exact same setup of the training phase in the first experiment. The Y-maze training was repeated for only two trials, to let the ant experience each combination (e.g. blue walls, lemon on the left, yellow walls, lemon on the right) once. After these last two training trials, the ants were tested with the same procedure used for experiment 1. Here, to still predict the reward position, the ants not only needed to integrate all the available information during training, but also had to be able to do so remembering only a single event.

Test phase

The test phase was identical to experiment 1: the rewarded scent was presented on both arms of the Y-maze and thus made uninformative. No water or sucrose solution was presented on either side. The wall colour was either blue or yellow. If ants remembered an episode in which they experienced the rewarded scent being on one particular side with one particular coloured background (visit 7 & 8), they should be able to choose the "correct" arm. Colour of background, colour-side association and correct scent were balanced between ants, as well as background colour order in visits 7 and 8. During the test, we recorded the initial and final decision of ants, as described in experiment 1. After the tested ant reached the end of one arm, it was allowed onto a piece of paper and was gently placed back on the Y-maze stem to repeat the test. As in experiment 1, each ant was tested 5 times to assess choice reliability as well as dropout probability.

Statistical analysis

Statistical analyses were carried out in R 3.3.3 [183]. Following Forstmeier and Schielzeth [240], we only added factors in the models for which we had a priori reasons for including, namely correct scent (lemon or rosemary), correct side (left or right) and wall colour (blue or yellow). Our primary dependent variable was the binomial arm choice of the ants. We also include analysis on the pheromone deposition in Appendix 4 (we decided to not include it in the main paper as pheromone is often interpreted as a measure of relative preference. Since in all our tests the ants had to choose between either a reward or nothing, therefore the relativeness was less crucial). As we found no difference between initial and final decisions, only the initial decision was used in the analysis (see Appendix 4 for the supporting analysis).

To see in which of the trials the ants learned the association between the scent and reward, we looked at ant choice during training visits. Given the fact that we had multiple observations of each individual, and that some individuals were from the same colony, we employed generalized linear mixed effect models using the package lme4 [185], with ants nested in colonies as a random intercept effect. Y-maze choice data was coded as a binomial data (1 for choosing rewarded and 0 for choosing unrewarded scent) and so were modelled using a binomial distribution with a logit link function. We then carried out a post-hoc analysis with Bonferroni correction using the package emmeans [187] to test each visit probability against chance level.

Subsequently, we analysed ant choice during the test phase. We only included the first testing trial of each ant (see Appendix 4 for the full analysis regarding testing repeated measures), and

accordingly added colony as a random intercept effect. We then used the package car [186] to test which factors of the model had a significant effect on the dependent variable.

We tested model fit using the DHARMa package [189]. When needed, we used a zero-inflated model with the pscl package [190,241]. Plots were generated using the packages ggplot2 [242] and cowplot [243].

Results

Only the main results are reported below. For the full analysis see Appendix 4.

Experiment 1 – Information integration

During training, in the second visit 62.5% (20/32) of the ants choose the correct scent (GLMM post-hoc with estimated means, probability=0.683, SE=0.102, z=1.627, p=1). Already in the third visit the percentage rose to 75% (24/32) (GLMM post-hoc with estimated means, probability=0.831, SE=0.077, z=2.915, p=0.039), reaching 90% in the fourth visit (29/32) (GLMM post-hoc with estimated means, probability=0.977, SE=0.025, z=3.443, p=0.002) and remaining more or less stable across all other visits. In the test trial, 87.5% (28/32) of the ants correctly chose the side that was associated with the background colour (GLMM post-hoc with estimated means, probability=0.875, SE=0.058, z=3.64, p=0.0003) (figure 2.6A). We found no effect of any of the modelled predictors.

Experiment 2 – Information integration in an episode

In both trial 7 and 8, 96.9% (31/32) ants choose the correct scent (GLMM post-hoc with estimated means, probability=0.969, SE=0.031, z=3.38, p=0.001). However, in the test phase, the percentage of ants correctly choosing the side associated to the presented background colour dropped to 65.6% (21/32) (GLMM post-hoc with estimated means, probability=0.656, SE=0.084, z=1.737, p=0.0823) (figure 2.6B). We found no effect of any of the modelled predictors.

Discussion

In the first experiment, *Lasius niger* ants were provided with an olfactory cue that fully predicted the location of a reward in a Y-maze along with contextual cues (maze wall colour and arm side). Yet, once the olfactory cue was made uninformative, 87% of ants were still able to integrate side and colour cues to successfully locate the correct arm of the maze. These results clearly demonstrate that foraging ants not only learn contextual cues in addition to the most predictive cue, but also integrate them to find a food source.

Recent research suggests that insects can combine cues weighed by their uncertainty in the current context [220,244–246] rather than creating information hierarchies in which one cue reliably dominates over the others. Accordingly, hierarchical-like decisions, in which it appears as if animals only learned one cue in the environment could, in fact, be based on a very strong weight on one cue, but still involve processing of additional cues. While, for instance, Myrmica foragers were found to rely predominantly on visual cues in bright light but switch to olfactory cues when light intensity decreased [247,248], such behaviour does not imply an exclusive reliance and learning of either cue. In our study, ants clearly did not use information in a strictly hierarchical order: if ants exclusively relied on odour cues, they would have performed at chance level in the test.

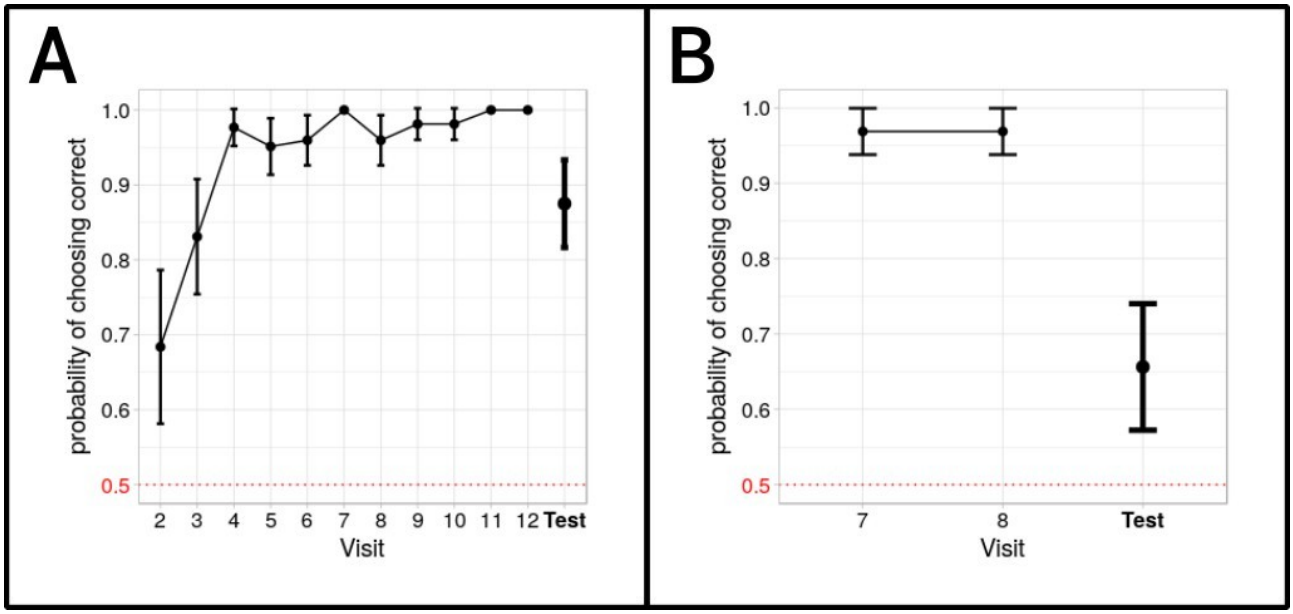

*Figure 2.6 – **A**: Probability of ants choosing the correct side during training visits and during test according to the GLMM model. Dots represent average probability, error bars are SE, dotted red line is chance level. Already in the second training trial performance was above chance level. In the test, the ants chose correctly even in the absence of odour cues (GLMM post-hoc with estimated means, probability=0.875, SE=0.058, z=3.64, p=0.0003). **B**: Probability of ants choosing the correct side during training visits with colour background and during test according to the GLMM model. Dots represent mean probability, error bars are SE, dotted red line is chance level. For both visit 7 and 8 performance is at 96%. In the testing trial, however, the probability of choosing correctly is not different from chance level (GLMM post-hoc with estimated means, probability=0.656, SE=0.084, z=1.737, p=0.0823).*

However, weighing of cues according to their uncertainty would also not lead to success unless the cues are not additively, but conditionally integrated. Weighed-cue integration combines multiple information sources by their level of certainty (i.e., predictive power). While such weighing can create a compromised prediction as a result of an additive integration process, the different cues do not influence each other, nor does the weight of one change the other. In our study, scents predicted

the presence of the reward with 100% certainty, while colour and side had only a 50% certainty by themselves, as each colour and each side were rewarded equally. The predictive power of colour with side becomes 100% only when considered conditionally: if colour A then side B, thus switching from an additive weighed process to a conditional one. Such learning of additional, seemingly redundant, information about the environment might initially demand higher costs, but can greatly decrease susceptibility to environmental perturbations and risk of disorientation when foraging [228,245,249,250], and, thus, it is worthy for the animal to invest energy.

It has already been demonstrated that ants can acquire information through very few or sometimes even single expositions. Studies demonstrated that ~70% of *L. niger* ants can learn a feeder location after only one visit [99,198,251], as it seemed to happen in our first experiment, where 68% of ants chose the correct odour on the second trial, ~80% of ants made a significant choice towards the correct side at the third visit and reached almost 100% at only the fourth visit in experiment 1. The 66% of correct choices we observed in the test of experiment 2 seems to suggest the same single trial learning effect, although our sample size was not sufficient to find significance. We are however aware that changing the sample size after having collected and analysed the data can often lead to type II errors, so we decided to discuss and present the data as it is. Nonetheless, trial 2 of experiment 1 and the test of experiment 2 vary fundamentally in their levels of complexity: even though all three components are presented in both cases, in experiment 1 the scent alone had 100% predictive power. The 68% ants that could solve the task might have been the ones more likely to learn scents, or better associative learning altogether. In contrast, visit 7 of experiment 2 confronted ants with two novel cues, not predictive in themselves, while a perfectly reliable odour cue was present simultaneously. In an associative learning context, we should have observed an effect of blocking [252]. Moreover, the amount of cues in this case that needed to be registered in a singe trial is double: to be able to find the reward in the following test the ant did not need only scent, as in the second trial of experiment 1, but it required both colour and side combined. The fact that the same percentage of ants chose correctly in a situation where one cue would have sufficed (scent cue, experiment 1) and in a situation where stimuli had to be combined (colour and side, experiment 2) suggests that the learning process employed by the ants is information amount independent, as would be expected of an ELM based mechanism.

To conclude, the results of our first experiment show that *Lasius niger* ants extract and readily combine contextual cues while foraging to locate a food source. This ability may rely on weighed cue integration with the addition of a sophisticated integration process on top of an additive one.

Moreover, we found a striking similarity in performances in the experiment 2 test phase and the second training trial of experiment 1. The similarity between these two suggest that information load had no effect on performance. This study gives the first evidence of information integration (what-where-which) ability on ants, surpassing the additive multi-modal recording. This ability constitutes the basis of ELM, and it might be active on single episodes.

Ants appear to be an optimal candidates for the investigation of ELM, especially considering their increases in navigation accuracy when using multisensory information instead of single elements [228,245,249,250]. Future studies should further explore the presence of episodic memory in ants, whose required building blocks have been presented in this study.

SECTION 3 – COGNITION IN JUMPING SPIDERS

The jumping spider as a model species for cognition

We, as humans, are often biased in the categorisation of animals. We tend to perceive some to be very different to each other, like a dog from a deer, while some others as very similar, like an insect and a spider, as also the common names suggest ("deer" is much more specific that "insect"). We are better able to categorise how different two species are depending on the familiarity we have with them: a same-race effect [253] extended for animal species. When discussing behaviour, brain and their evolution we have to be careful in avoiding this bias and always keep in mind the phylogenetic relationship between the species. Spiders and insects are related at the level of the phylum: Arthropoda. This means that they are even more different to each other than a chimpanzee is to a fish (related at the level of the subphylum: Vertebrata). As discussed in the previous section, we cannot generalize the abilities that we have observed in bees to the entirety of order Hymenoptera (as we would not assume cognitive abilities found in dolphins are also present in rodents), let alone to spiders.

For these reasons we extended our study onto a completely different species of invertebrate: the spider's Family Salticidae. As the name implies, this spider Family is unique in the fact that it actively stalks its preys thanks to their ability to jump, sometimes for distances of several body lengths. However, jump is far from being the only skill that these spiders possess: these animals are marvellous hunters that catch dangerous and bigger preys by outwitting them [254]. It is exactly because of their surprising hunting strategies that scientists, started looking into the neural and cognitive bases of these outstanding behaviours.

The first evidence came from the genus *Portia*, which has been observed in nature to take detours in order to reach its prey unseen [255] (but note that this behaviour has also been observed in other species [256]. Subsequent studies have investigated this ability thoroughly, describing how *Portia* can plan its movements in order to reach a target [257] thanks to a pattern of movement that lets them scan the environment [258–260]. Many other abilities have been studied by scientists, amongst which are numerical cognition [261], learning [262–265], problem solving [266–268], spatial cognition [269,270], attention [271,272] and planning [273,274]. Barrett [275], however, raised the point that these behaviours may be the result of automatic, preprogrammed processes, instead of cognition. It is indeed true that many studies lack direct control to discriminate between complex cognition and more simple preprogrammed patterns, focusing mostly on the behaviour itself rather than on the underlying processes. It is uncontested that this Family is a promising

model for the study of cognition. In the following paragraph I will describe the structure of its brain, with particular attention to the visual system.

Jumping spiders' neurobiology

Every spider's body is divided into two sections [276]. Starting from the back, the first section is called *opisthosoma*, or abdomen. This is presented as a soft and sack-like body, and it contains most of the internal organs (lungs, heart, intestines and ovaries in the females). At the back end of the opisthosoma the spinnerets are present, which are the appendices engaged with the silk production. On the opposite end the abdomen, the *prosoma* is connected to the frontal section trough a narrow stalk called *pedicel*. From the prosoma develop all the remaining appendages of the spider: the four pairs of legs, the palps pair and the chelicera. The prosoma also contains the 4 pairs of eyes. It is inside this anterior section that lays the spider's brain.

The central nervous system

Differently from what has been said for hymenoptera, the brain of spiders is far less understood and the function of many areas is still debated today. For this reason, this thesis focuses more on the description of the nervous system, highlighting the areas similar to the ones of Hymenoptera and describing the existing controversies. The first description of the spider nervous system has to be attributed to Saint-Remy [277], which was however strongly limited by the technology of his time, preventing any appreciation of fine anatomy. With the invention of Golgi's staining technique [278] new possibilities opened up. Hanstrom [279] layed the basis for all the subsequent work carried out in the 1970' and 1980' [e.g., 54,55,57], recognizing the homology between different areas of the spider brain with the ones of insects and crustaceans (that will be more in depth described below. Our current knowledge on the spider brain is mostly based on the study of *Cupiennus salei* (Family: Ctenidae) [55,280–283]. We also have some description of different species of Salticidae, including *Phidippus johnsoni* and *claurus* [57], *Phidippus carneus* [283], *Phidippus regius* [284], *Salticus scenicus* and *Habrocestum pulex* [285], and lastly by the very recent descriptions of *Marpissa muscosa*'s brain anatomy [56] and development [286].

The spider central nervous system consists in a fused mass of nervous tissue, comprised of multiple segmental ganglia [57,276], for this reason named *Synganglion* [56]. The brain of spiders can be described following different criteria of subdivision. For example, this mass can be divided horizontally from the point in which the oesophageal tube traverse it, into *supra-oesophageal* and *sub-oesophageal* ganglion. The division, however, is purely abstract because there is generally no

clearly discernable border between the two. For the purpose of this thesis, we will use the description used by Steinhoff et. al. [56], which divides the brain according to its evolutional history and, consequently, its functional properties. The spider nervous system is composed of different neuromeres: the "protocerebrum", "deutocerebrum", "tritocerebrum", and four neuromeres that project and receive axons from the walking legs. On the back, also a 5 th unpaired neuromer named *cauda equina* connects the brain to the opisthosoma [276]. The latter neuromeres are referred to as ventral nerve cord, and correspond to what we would consider a spinal cord in mammals. The first three, on the other hand, are referred to as "syncerebrum" or, more simply, brain (figure 3.1). The deutocerebrum is responsible for the control of the chelicera, while the tritocerebrum for the control of the pedipalps, which have a sensory functions. The protocerebrum contains the visual system of the spiders, of great importance especially in Salticidae, that possess the highest visual acuity amongst the entire arthropod phylum [287]. The protocerebrum is the most extended neuromer of the syncerebrum, being even more expanded in Salticidae in respect to other Families of spiders.

The eyes

Most spiders possess four pairs of eyes devoted to different tasks. Different families of spiders have eyes arranged into different positions and with different relative sizes, depending on the importance the eyes have for each species. In Salticidae, the eyes can be divided into two distinct categories: the principal eyes and the secondary eyes. Of course, the former name suggests their crucial role in the behaviour of Salticidae. These consist of a single pair of eyes called antero-median (AME). From the carapace surface of the spider, the AME present a cornea: a transparent, cuticular convex region; immediately below, a non chitinous lens; and behind, a cellular vitreous [288]. Subsequently, a long tubular structure extend inside the cephalothorax, culminating in the retinae and then the first optic tract [288]. A set of six antagonistic muscles surrounds the tubular eyes, moving and rotating the end of the tube and effectively reorienting the retinae [288]. The latter is composed of a long and narrow strip of receptors laying in a V-shaped section at the back of the tube. The receptors are not distributed on a straight line, instead they are bent in the "fovea" by 30°, generating a "boomerang-shaped" distribution. Because of the size of the strip of receptors, the visual field of each eye is narrow, around 5°, and the visual fields of the two eyes do not overlap. The resulting visual field of the two AMEs is "X" shaped, with a piece missing in the centre [260]. The receptors in the retinae are organized in 4 different layers, one on top of the other in respect to the light source. These layers are far enough from each others, that an object focused for one of the layers will be defocused on the other three [288]. For this reason some authors suggested that this

defocusing may very well be the mechanism by which the spiders judge distances [289], as they lack stereoscopic vision. The receptors in layers 1 and 2 (counting from the furthest point from the light) are well organized, tightly packed into a hexagonal lattice and green-light sensitive [288,290–292]. Layers 3 and 4 instead are composed of less organized, UV-sensitive cells [290–292]. Moreover, at least some species possess a layer of red filter pigments directly in front of some cells of layer one, enabling the vision for a third colour [292]. The presence of the red pigment in *Habronattus pyrrithrix* seems to be crucial during mating [293]. Moreover they have been shown to be innately able to use aposematic prey colouration to avoid unpalatable ones [265]. It is reasonable to believe that this structure exists in other species of jumping spiders, as many of them possess red colouration on the body and may benefit from being able to perceive such colour for the same reasons.

There are 3 pairs of secondary eyes: antero-lateral (ALE), posterior-medial (PME) and posterior-lateral (PLE). In contrast with the AME, these are immobile, but they possess a wide visual field, which totally cover almost 360° around the spider, with a 25° of overlap of the two ALE directly in front of the animal [288]. The spatial sensitivity of the secondary eyes is also far less than that of the principal, other than not granting colour vision. What they lack in spatial sensitivity however, they make up in temporal sensitivity, functioning as motion detectors [294], directing the attention of the spider's AME. Land [260] originally described in great detail how jumping spiders analyse visual stimuli. When no stimulus is detected, the frontal eyes move freely right and left spontaneously. Once a stimulus is detected by the secondary eyes, the spider turns its body in order to face the object with its frontal eyes. At this point, the eyes perform saccades in order to fixate the object at the centre of the visual field. Once locked, the stimulus can be tracked by the eyes without being lost again. Perhaps the most important movement is the one named "scanning", where the spider moves its visual field across the object in a repetitive pattern, in a manner almost unique in the whole animal kingdom. It has been suggested that scanning is involved in object recognition, as the spider observes its target in all its components [260]. The secondary eyes, however, may be more than just motion detectors. In Salticidae, the ALE are forward facing – as the AME are – and are the second biggest pair. Moreover, the fact that their visual fields overlap suggests that they may also have other functions. It seems, for example, that a first, simple categorisation of objects can already be operated by the ALE [295,296], eliciting the fixation with the AME only if the stimulus is already recognized as a prey. The function of the ALE may have been underestimated until now, and it may deserve more attention in the future.

The visual system

Especially when describing the structure and organization of the spider protocerebrum, it is crucial to make a distinction between spider Families. Both the number and connections (and possibly the function) of the different neuropils changes greatly. It is especially important to point out this difference, since until very recently most of the knowledge about the jumping spiders' visual system was derived from the analysis of the wandering spider *Cupiennus salei* [281,282], which led to some incorrect assumptions about how the Salticidae visual system is structured. What remains true for all spiders, is that the inputs coming from the principal and secondary eyes are directed to two completely separate circuits [56,57,276,281,282].

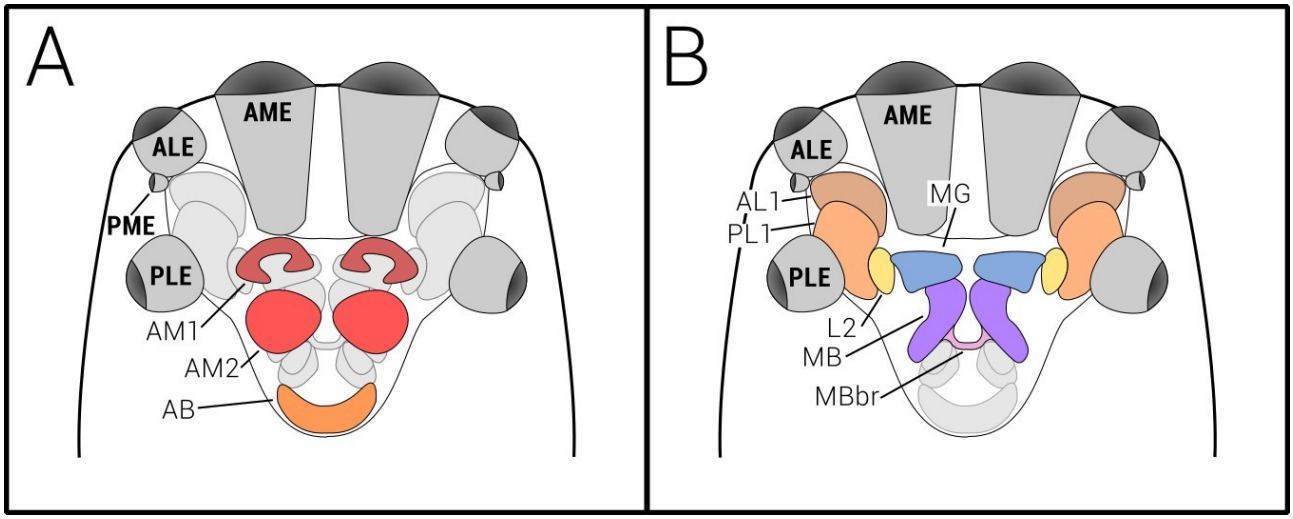

Figure 3.1 – Schematic representation of the jumping spider syncerebrum and the 8 eyes: AME = Antero-Medial Eyes; ALE = Antero-Lateral Eyes; PME = Posterior-Medial Eyes; PLE = Posterior-Lateral Eyes. ***A****: Areas part of the principal eyes visual system are highlighted. AM1 = first order neuropil, other areas are greyed out; AM2 = second order neuropil; AB = arcuate body.* ***B****: Areas part of the secondary eyes visual system are highlighted, AM1 and AM2 are removed to show areas under them. All other areas are greyed out. AL1 and PL1 = first order neuropils for antero-lateral and posterior-lateral eyes respectively; L2 = second order neuropil; MG = microglomeruli; MBs = mushroom bodies; MBbr = mushroom bodies bridge.*

The principal eyes visual system

From the end of the AME tube, two optic tracts connect the outputs of the retinae to a first order neuropil (AM1) [56,57]. The AM1 is composed of two, concentric horseshoe-shaped glomerular strips [288] organized in a columnar structure [56]. Presumably, this neuropil operates a primary analysis of the visual input. However no functional study to this day has been carried out on these areas. AM1 project fibres directly to a second order neuropil (AM2) [57], which is oval shaped and larger than AM1 [56]. AM2 projects to the arcuate body [279] (AB, also named central body by some authors), an unpaired neuropil situated at the back of the syncerebrum, which is considered to

be homologous to the insect central complex [56,279,286,297]. The AB has been described as having a retinotopical organization in *Cupiennus salei* [282], and, as such, has been interpreted as a third order visual neuropil [298]. In other families, however, this area has been associated with motor coordination, as it is enlarged in orb weaver spiders [299]. In Salticidae, the AB is composed of two separate units: a smaller, dorsal unit (ABd) and a bigger ventral one (ABv). The former is layered in multiple subunits, and may be responsible for handling sensory informations [57]. The ventral lobe sends multiple connections to the ventral nerve cord [57], a fact that further supports its implication in motor control and suggests the AB may be responsible for visual-motor coordination (this hypothesis was instead rejected by Strausfeld & Barth [281] in their study of *Cupiennus salei)*. Barth [298] claimed the AB may be an integration centre of the spider brain, based on its interconnections with many other regions of the CNS. Recently, a group of researchers were able to perform the first electrophysiological studies on jumping spiders, demonstrating that neurons in the AB indeed respond differently to different meaningful categories of visual stimuli [300], but also to vibration and sound [301]. The presence of a higher integration centre inside a sensory system may be a clever solution to the miniaturization problem: vision is so crucial for these animals that it cannot be forfeited in favour of cognitive processes, but can indeed coexist and function in the same area. This may be facilitated by the presence of two consecutive visual neuropils (AM1 and AM2), that probably analyse and select visual information, sending an already simplified and meaningful output to the AB.

The secondary eyes visual system

Each one of the secondary eyes is associated with a different first order neuropil (anterior-lateral first neuropil, AL1; posterior-medial neuropil, PM1; posterior-lateral neuropil, PL1). AL1 and PL1 present a highly convoluted surface [57], while PM1 is so small that originally, Hanstrom [279] was unable to locate it, leading to the conclusion that these eyes may be vestigial [294]. Hill [57], on the other hand, suggests that these eyes might have the same function as the insect ocelli. AL1 and PL1 are both connected to the same secondary visual neuropil (L2), which probably integrates the information coming from both eyes [56]. This structure is profoundly different from what is found in other families of spiders, where each first order neuropil of the secondary eyes projects to micro-glomeruli, organized in three separate second order visual neuropils [281,282]. These micro-glomeruli are indeed present in the jumping spider brain, but are not connected to AL1 or PL1. Instead they lie on top of the spider mushroom bodies (MBs, see below), to which they may be connected, however this has to be demonstrated. L2 and PM1 project connections to the MBs [56].

These are the most prominent neuropils in the spider brain, and they are homologous to the same-named structure in the insect brain [302]. They serve as a third order neuropil in *Cupiennus salei* [281], but at least in Salticidae they are responsible for much more. They are divided into two or three sections (depending on the inclusion or exclusion of the micro-glomeruli), and the two symmetrical parts of the two hemispheres are connected with the mushroom bodies bridge (MBbr) [56]. The presence of connections to multiple parts of the brain shows that MBs are higher order processing and integratory centres in the spider brain, possibly responsible for learning and cognitive processes [56,57]. The MB also project connections to the ventral nerve cord, suggesting an involvement in motor control.

Overall, the jumping spider brain, and especially the visual system, provide an interesting model for the study of cognition. Despite the profound differences between arachnids and insects, some homologous structures are maintained. In particular, the MBs, which are the areas associated with cognition and memory in insects, are present and highly developed in spiders, are linked to the visual system. Moreover, the linkage between visual system, motor control, and integration, as well as the presence of different orders of neuropils, suggests that these miniaturized brains have been modified by evolution to optimize information processing and to reduce the load on integrative centres thanks to early selection of meaningful stimuli. This might also be the case for the scanning process, which gives the spider the possibility to engage with his high principal eyes' visual system only with stimuli that have already been selected by the secondary system. The structure of the Salticidae brain describes a Family capable of complex cognition, with a primary focus on vision. For this reason, we carried out a study on visual cognition with the jumping spider *Phidippus regius*, to describe how visual information is registered and if it follows the same principles ultimately described in humans.

Study 1: Visual discrimination learning and amodal completion in a jumping spider

Data from this study have been published as [303][1].

Introduction

We know from previous literature that jumping spiders can distinguish and categorize visual stimuli [300], but the underlying process is still unknown. Land [260] stated that "An important task that the retinae are performing during scanning is therefore going to be the detection of lines or contours with particular orientations and in appropriate positions". This suggests that the local features of stimuli are crucial for object recognition, in particular, in the task of categorizing prey. Dolev and Nelson [304] tested *Evarcha culicivora* (Family: Salticidae) to assess which elements of a figure are necessary for the spider to trigger a stalking reaction. The authors identified some local features crucial for responding to a mosquito (preferred prey of *Evarcha*). In a more recent study, Dolev and Nelson [305] further analysed the attention of *Evarcha* to those local features, testing the preference of the animals between simple stimuli containing features of preferred prey (mosquito) and a realistic representation of other, non-preferred prey (flies). Evarcha showed a clear preference for the first class of stimuli and chose the simple features independent of their global configuration (but also showing selective attention to the relative orientation of the elements present in each stimulus). On the contrary, *Hypoblemum albovittatum*, a generalist predator, preferred the realistic representations to the simpler stimuli in all instances. Dolev and Nelson [305] suggested that the privileged use of local features could represent an adaptation for a specialist predator to locate more effectively its preferred prey. This would not be the case for a generalist predator, which could instead benefit from using the general configuration of the image to identify its prey. However, in the study by Dolev and Nelson [305], realistic (and perceptually richer) pictures were contrasted with much simpler shapes (made of lines and circles). Stimuli were not paired so as to test for the use of the actual configuration (as would be obtained when globally different but locally identical stimuli are contrasted), except for one comparison (conditions A and F of their study). No conclusions could be drawn as both species performed at random (i.e., they did not preferentially stalk any of the two stimuli presented).

1 From the original publication, the data has been re-analized and a new improved criterion of inclusion has been chosen.

The studies mentioned above [260,304,305] focused on the cues that the spiders used to distinguish prey from non-prey, and they demonstrated that local features can be crucial for triggering the spider's stalking response. But what about the general mechanisms underlying the perception of stimuli which are not necessarily prey? Would the local features still be crucial, or would the spider in this case disregard the local features and instead respond to the whole configuration (i.e., to a particular arrangement of the local features)? Many animal species were shown to preferentially respond to the global configuration in object recognition, and often times this was tested by exploiting a mechanism called “amodal completion”. Through amodal completion, an animal can perceive a figure as a whole even if another object conceals a portion of it [306]. Mammals [307,308], birds [309], fish [310] and even invertebrates [311,312] use this mechanism just as humans do [313].

We investigated jumping spiders’ ability to discriminate between two abstract, geometrical shapes through associative learning, using the model species *Phidippus regius* (Koch, 1846). A previous study [314] tested the ability of jumping spiders to discriminate between the moving image of a cricket (meaningful) and a moving rectangle (abstract), but the animals seemed unable to learn to discriminate between the two. However, the focus of the study was the detection of the motion of stimuli, rather than their shape. In our study, once the spiders had undergone the training phase, we assessed their free choice in unrewarded test trials to assess discrimination learning. If *Phidippus regius* was able to learn to associate a neutral visual stimulus (an abstract shape) with a reward, its performance was expected to be higher than chance level (i.e., the spider was expected to approach the previously positively rewarded shape and disregard the previously negatively rewarded shape). Successively, we tested spiders' ability to generalize the learned response to a partly concealed version of the stimulus reinforced during training. If this species could perform amodal completion similarly to humans (and also to many other species) when confronted with an occluded version of the previously positively rewarded shape, the spiders were expected to choose to approach such a stimulus and not its “broken”, non-occluded version. Both stimuli in this comparison would present identical local features, but only the occluded stimulus would trigger shape completion to the human eye. If, on the other hand, the spider’s vision relied on local features, we expected a random choice between the two stimuli presented in this comparison.

Methods

Subjects

Eighteen adult female spiders (instars 8 – 9) were used for the experiment. Five of them came from one egg-sac and 13 from another one. The egg-sacs were laid by two different spiders. The subjects were housed individually in test tubes upon emerging from the mothers' nest. Then, after the first moult, the spiders were individually placed in plastic boxes (each with dimensions of 7 x 16 x 6 cm), with each featuring a portion of a cardboard egg container to provide shelter and a walking surface, as well as a wet sponge to provide humidity and drinking water. The dimensions and contents of boxes were chosen according to the findings of Carducci and Jakob [315] given the fact that jumping spiders show better performance when raised in larger, enriched environments. The box walls were transparent and with holes on the sides to allow airflow. Light was shed on the boxes via neon lamps with natural light (5000 kelvin colour temperature, 36 watts, 3350 lumens). The light cycle was set at 12 hr light and 12 hr dark. We fed the spiders one to three *Drosophila melanogaster* every three days between the second and third instars. After this, the spiders were fed *Tenebrio molitor* larvae (one each week) that were similar in size to the spiders. The larvae were bred in the laboratory and fed a specific diet to ensure their best development. Prior to the experiment, the spiders had no interactions amongst one another (apart from the days spent inside their nests). Moreover, they had no previous experience with the types of stimuli presented during the experiment, either unconditioned or conditioned (e.g., drop and shape as described below).

Apparatus

The experimental apparatus consisted of a box of the same size as the housing ones. The walls of the box were covered with white paper to insulate the animal from external cues (Figure 3.2A). The lid of the box remained transparent to allow lighting and to allow for recording. The spiders were presented with two red-coloured drops. One of the drops contained sugar (20% weight by volume solution), and the other contained citric acid (25% weight by volume solution). The drops appeared visually identical to the human eye. However, because sugar absorbs ultraviolet light, the two drops could have appeared different to the spiders [291]. I chose to use these unconditioned stimuli based on the experiment by Liedtke and Schneider [263], in which they appeared to be good rewards for training jumping spiders. Moreover, jumping spiders have been observed multiple times consuming nectar in nature [316], and as such we argue that nectar is a natural food for these animals. Each drop (approximately 40 μl) was placed on a white plastic square (Figure 3.2B). A vertical piece of

plastic was placed on one of the four sides to hide the drop from the spider's visual perspective. On both sides of the vertical wall, a black shape was glued, either an ‘X’ or an ‘O’. Both shapes were matched for total area (7 mm2), made via a cutting plotter (Signpal PUMA II) on adhesive plastic (approximately 0.1 mm thick).

Experimental design and procedure

Prior to the testing period, the spiders underwent seven days of fasting to ensure a high level of motivation. The day before the testing period, the subjects were placed in an empty testing chamber, in which they remained until the end of the experiment. Each spider underwent a training and a testing phase.

Training Phase

The training phase lasted for seven days. Each day, three trials were performed for each spider, except for the fourth day, during which no test was performed, to allow the spider to regain motivation for the reward. In total, each spider underwent 18 training trials. For each trial, the subject was positioned in the centre of the box and covered with a small opaque screen. Then, the two platforms with the two drops, one with citric acid and one with sugar, were placed at the two ends of the box. Finally, the spider was released. Each trial lasted for approximately 45 min, during which the animal was free to move, look at and taste both drops before the trial ended. The timing of the trials was chosen because in pilot trials, the spiders showed a long latency prior to choosing a stimulus. Between trials, a period of approximately 60 min elapsed to avoid placing excessive stress on the animals. The training shape was randomly assigned to each spider so that half of the subjects were trained on one and the other half on the other. To prevent the spider from using external cues (i.e., the position of the light) to locate the drop of sugar water the box was rotated after each trial. Moreover, the position (left or right) of the correct stimulus was changed randomly from trial to trial. Between each trial, the plastic squares were thoroughly cleaned with alcohol to remove any possible trace of sugar or citric acid. Moreover, the stimuli were placed in the box using new latex gloves every time to exclude any possible chemical cues (other than the drop itself).

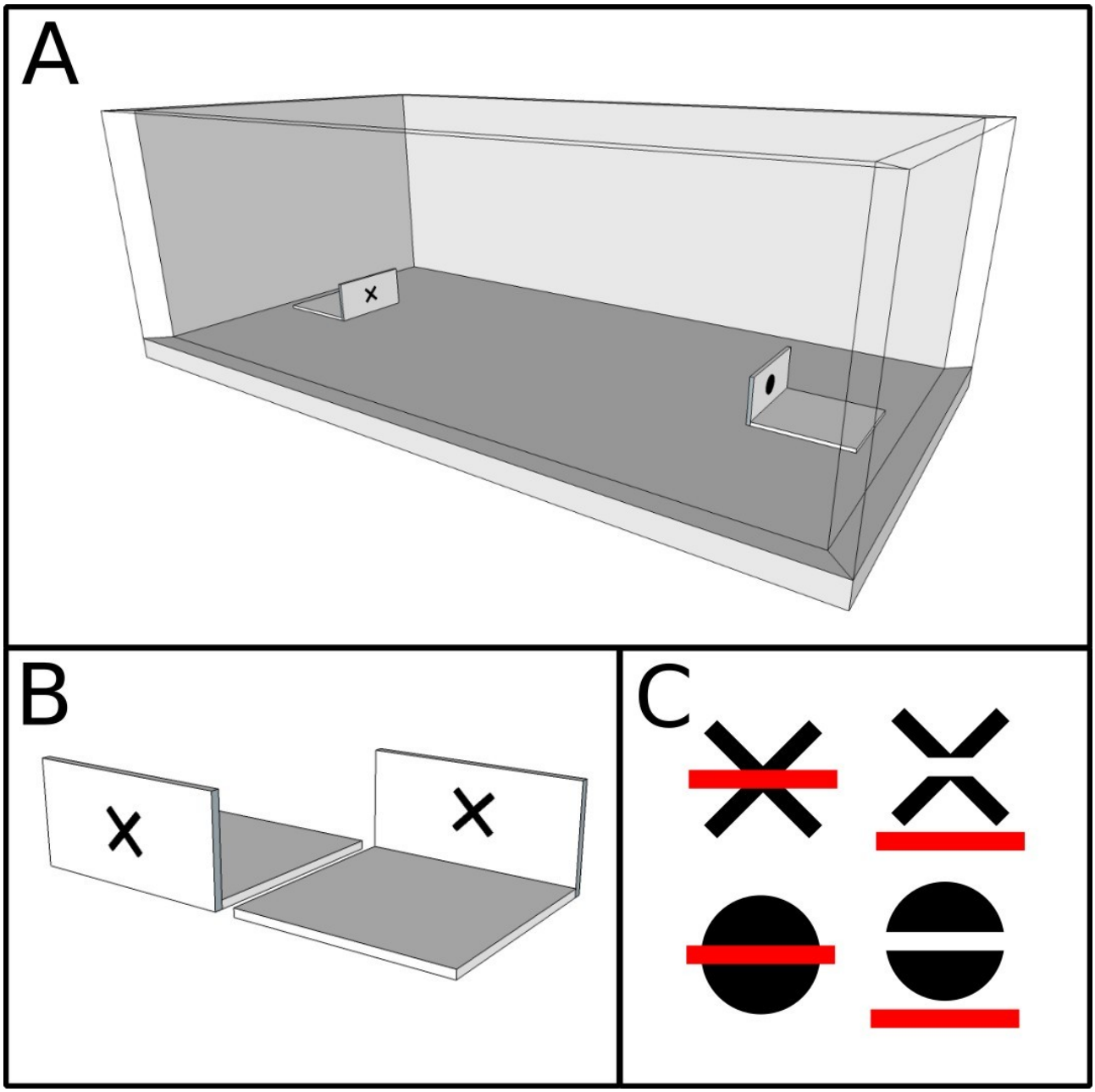

*Figure 3.2 – **A**: Experimental apparatus with the two platforms ("X" and, "O"). The spider's starting position was located in between and exactly at the same distance from either of the two platforms. **B**: "X" platform, front and back views. The drop is placed in the centre of the base. The base is 20mmx20mm, 0.8mm thick. The vertical wall is 10mmx20mm, 0.8mm thick. **C**: Stimuli presented to the spiders in the "unrewarded-illusion" condition. Spiders trained on the "X" shape were exposed to the 2 stimuli at the top, spiders trained on the "O" shape were exposed to the ones at the bottom. The geometrical features of the two stimuli presented to each spiders are identical (local features), but they differ in their global configuration if the observer can perform amodal completion. An animal capable of completing occluded objects should in fact perceive as whole only the "occluded" stimulus hence recognising this as more similar to the shape associated with the reward during training.*

Testing Phase

After the training phase, a two-day pause took place, during which a damp sponge and small Tenebrio molitor larvae were placed in the box. This was necessary because the sugar water, even though it represented a suitable reward for the animals, was not sufficiently nutrient rich. The testing phase lasted between one and three weeks. The number of sets that a spider underwent depended on its age and level of stress. Younger spiders were considered able to handle a higher amount of stress. Moreover, some spiders died of old age after the first or second week, so they underwent fewer tests. Lastly, if a spider started to lose strength and decrease its activity level due to the lack of nutrient-rich food, the test was stopped, then started again after a week's pause with a retraining. Each spider underwent three trials per day. Every three days, a 24-hr interval was observed. As in the training phase, each spider performed a total of 18 trials.

Two-thirds of the trials were completely identical to the training phase: we still presented the spiders with the two different drops with sugar and citric acid (rewarded trials). Those trials were useful for maintaining the learned association between shape and reward. A third of the trials were unrewarded testing trials, during which only rose-coloured water (with neither sugar nor citric acid) was presented. Removing the reward and the punishment was necessary to control for any cues, such as odour, colour and taste. Two different types of testing trials were conducted. In the first type, the shapes presented were the same as those used in training ('unrewarded-shape trials'). They were used to assess the outcome of the training phase: If a spider learned the association between the shape and the reward, it was expected to choose the drop behind the stimulus that had been associated with the sugar water. In the second type of testing trial ('unrewarded-illusion'), the spiders were presented with novel stimuli: An occluded shape (either the 'X' or the 'O') and a cut version of the same shape (Figure 3.2C). For each spider, the shape used was the one that had been associated with the sugar water during its training phase. The occluder in the occluded stimulus was red in colour and of a different luminance to the shape to be clearly distinguishable from the actual shape. The local features (i.e., the visible parts of the shape) were identical in the two stimuli, apart from the position of the occluder, so that to distinguish the two stimuli, the animals had to use a global type of visual processing. If a spider relied on amodal completion, it was expected to choose the occluded version of the shape, which would be perceived as the whole shape behind a red bar, whereas the cut stimulus was expected to be seen as a non-complete shape. If the spiders relied on local visual processing, and thus focused on the separate features to identify the shape, they were expected to choose randomly in this condition given the fact that the two stimuli presented identical

features. For the 18 testing trials, we used a random procedure to determine which trial was rewarded, unrewarded-shape or unrewarded-illusion, maintaining the ratio of two-thirds rewarded trials and one-third unrewarded trials (half with training shapes, and half with illusory vs. cut stimuli). In addition, in the testing phase, the boxes were rotated, and the respective positions of the correct and incorrect drops were randomly determined.

We used four cameras to record the behaviour of the spiders. Each camera could record four boxes at the same time. For this reason, a maximum of 16 spiders were tested at the same time. From the videos, it was impossible to tell which spider was trained on the 'X' and which on the 'O' because a letter code was used to identify each animal. For this reason, the observer was blind to the experimental conditions. We used the software BORIS [317] to code the different behaviours of the spiders during the 45 min of exposure. We measured the total time, start and end of each event for the following behaviours:

- *Stasis* – The spider does not move at all.
- *Behind* – The spider is positioned behind the wall with the shape, in contact with the plastic platform, with at least one limb.
- *Drinking* – The spider drinks either drop. It is not sufficient to see the spider on top of the drop to code this behaviour given the fact that often spiders walk on top of the drop without actually tasting it. When the animal actually drinks, it stops with the chelicera on top of the drop for several seconds and opens its palps wide.
- *See* – We also registered the latency to first detection of each drop. Note that the screen in which the shapes are glued covers the drop, so the spider has to actually move toward the drop to see it. The "drinking" behaviour was used as the choice indicator. It was, in fact, a sign of a clear preference of the spider, in addition to being an indicator of motivation (the spider drank only when actually hungry).

Results

A total of 864 trials (576 rewarded, 144 unrewarded-shape, 144 unrewarded-illusion) were performed in the testing phase. A total of 802 trials (538 rewarded, 131 unrewarded-shape, 133 unrewarded-illusion) were successfully recorded and analysed. Unfortunately, due to technical problems, 62 trials were not recorded (most of them from the same day of testing).

Of the trials we analysed, the spiders drank either drop in 171 trials (106 rewarded, 38 unrewarded-shape, 27 unrewarded-illusion), whereas only in three trials did the subjects drink from both drops. The performance in these three trials was discarded from the final analysis because it indicated lack of choice, just like in the trials in which the spiders did not drink from any drop. The analysis was performed on 168 observations (106 rewarded, 35 unrewarded-shape, 27 unrewarded-illusion) (Table 1).

	R	**US**	**UI**	**Total**
Trials performed	576	144	144	864
Trials recorded	538	131	133	802
Trials recorded (% on performed)	93.40%	90.97%	92.36%	92.82%
Trials answered	106	38	27	171
Trials answered (% on recorded)	19.70%	29.01%	20.30%	21.32%
Trials analysed	106	35	27	168

Note. The difference between percentages of answered trials among groups (19.70%, 29.01%, 20.30%) is not statistically significant (proportional 3 sample test – $\chi^2(2) = 5.5364$, $p = 0.063$).

Table 1 – Number of trials performed, successfully recorded, answered and analysed, and relative percentages, divided for conditions (R=Rewarded, US=Unrewarded-shape, UI=Unrewarded-illusion).

Due to the non-independence of the repeated-measures procedure, the individual heterogeneity and the variable individual number of observations, a mixed generalized linear model (GLMM) with binomial distribution was employed, considering the individual subjects as a random effect for all three conditions. Subsequently, we did a post-hoc analysis with Bonferroni correction to determine what values were significantly different from chance level. All of the analyses were performed using the software R (v.3.2.3) [183] with the packages lme4 [185], car [186], emmeans [187], DHARMa [189], MASS [318], ggsignif [319] and ggplot2 [242]. Only the main results are reported here. For the complete analysis refer to Appendix 5.

We used “drinking” behaviour as the dependent variable. We found a difference between the conditions in the probability of drinking the correct drop (GLMM analysis of deviance, chi-square = 27.427, p-value < 0.0001). A post-hoc analysis revealed that the performance was above chance level for the rewarded condition (GLMM post-hoc, estimate = -0.962, SE = 0.187, z-ratio = -6.354, p-value < 0.0001), but not for the unrewarded-shape (GLMM post-hoc, estimate = 0.689, SE =

0.078, z-ratio = 2.143, p-value = 0.0964) nor for the unrewarded-illusion (GLMM post-hoc, estimate = 0.481, SE = 0.096, z-ratio = -0.192, p-value = 1) conditions (figure 3.3).

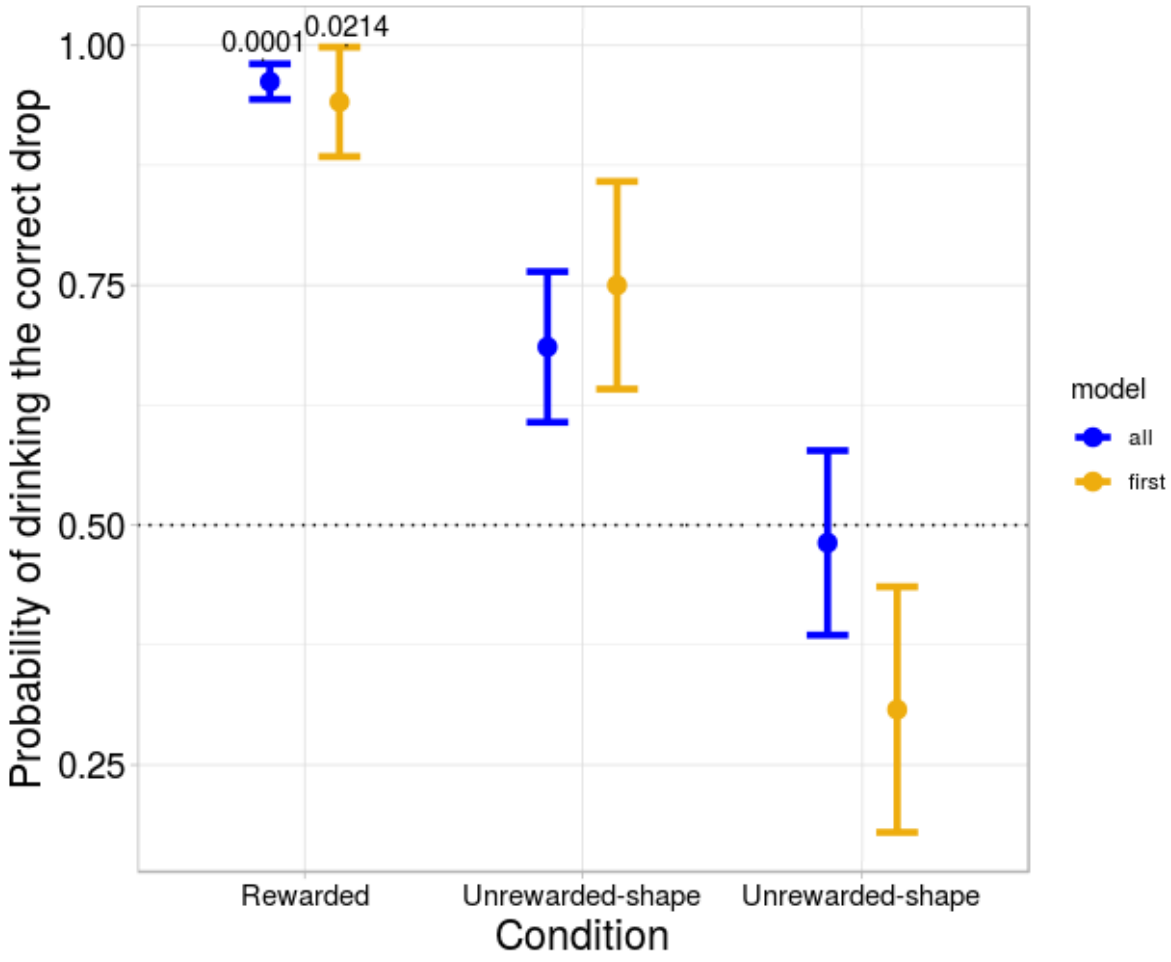

Figure 3.3 – Correct performance (coloured dots) of the spiders on the rewarded, unrewarded-shape and unrewarded-illusion trials, with standard errors. The two models (considering all trials or only the first trial for each spider) are represented in different colours. 0.5 is the expected level of chance.

To exclude the possibility that the learned tasks were extinguished after the animals were faced with unrewarded trials, we considered only the very first trial for each condition. The probability of choosing the correct stimulus in the rewarded condition remained significantly above chance level (GLMM post-hoc, estimate = 0.941, SE = 0.0571, z-ratio = 2.69, p-value = 0.021). The performance recorded in the unrewarded-shape condition raised to 0.75, but was still unable to reach significance (GLMM post-hoc, estimate = 0.75, SE = 0.108, z-ratio = 1.903, p-value = 0.171). In the unrewarded-illusion condition the performance remained at chance level, with a slight, non significant preference for the 'wrong' shape (the broken X or O) (GLMM post-hoc, estimate = 0.308, SE = 0.128, z-ratio = -1.349, p-value = 0.5316)

Discussion

In the unrewarded-shape condition, we presented the spiders with the two training shapes but in the absence of any reward (sugar water or citric acid water). Even though the performance appeared high, even more so when considering only the first trial (0.75 probability of choosing correctly), it was not enough to reach significance. This was probably due to the low amount of subjects that this method permitted to reach. As discussed above, out of the many hours of tests performed and recorded, only a handful of data could be registered. The percentage of correct choices is still promising, and we believe this is because the procedure presented some advantages. The modality of the presentation of the stimuli granted the spiders the possibility to freely inspect them instead of forcing a choice at every trial. In fact, in both the training and the testing phase, the animals wandered in the experimental apparatus looking at both shapes and both drops multiple times prior to making their choices. They inspected the images from different distances and even touched the screens. This observation aligns with the idea of a 'visual inspection', as Cross and Jackson [320] proposed and as described in some Spartanae species prior to engaging in detour behaviours [257–259]. We think that this active and prolonged scanning behaviour is fundamental for the process of learning novel visual stimuli, as well as in novel detours. In the study by Bednarski et al. [314], described in the introduction, the spiders had no possibility of engaging in this visual inspection because the stimuli were moving. This procedure seems to be a promising method for training spiders, a task that is known to be far from simple [321].

In the illusory condition, the spiders were presented with an occluded version of the previously rewarded stimulus and a broken version of the same stimulus. The two stimuli were identical in their local features but differed in their global configurations (which, to the human eye, could be perceived only in the occluded stimulus). Because we have no clear indication that the spider learned to discriminate the training stimulus, no real conclusion can be drawn from the illusory condition. If we were to discuss the 0.75 performance observed in the unrewarded-shape condition as an indication of learning, the spiders' performance at the chance level may indicate that the spiders were not using a global visual strategy, or the amodal completion mechanism, to identify the correct stimulus. Although preferential processing of local features has been described in various animal species [322], the mechanisms underlying such strategies are not fully understood. Spiders could provide a further and valuable model for their investigation. The use of a local strategy (i.e., basing the choice on the local features, which were identical in the two stimuli presented) is, however, not the only possible explanation. One alternative possibility is that the spiders had

previously learned avoidance of the punished shape rather than association with and approach of the positively rewarded one. When the spiders were presented with two versions of the positively rewarded shape in the illusory condition, neither stimulus could trigger avoidance (or the choice of the other stimulus), in spite of the use of a global visual strategy. However, further experiments are needed to test both hypotheses.

Unfortunately, we cannot draw any information from the learning trials in which sugar water and citric acid water were present (training phase and rewarded condition of the testing phase). Almost no spiders drank the citric acid drop because, we assume, they were able to easily recognize the taste as unpleasant beforehand with a simple touch. We considered using a different indicator of choice, such as the first drop approached, although we realized that approach behaviour does not constitute reliable information (see the “Results” section). Detecting and scoring from the video recordings any simple touch of the drops was impossible. For this reason, we lacked a measure of the training progression. A feasible solution would be to implement an automated system both for scoring and stimulus presentation. Such a method should optimize the objectivity and reliability of the behavioural measures [323]. In fact, scoring is time consuming and relies heavily on the expertise of the scorers. In a few trials (10 out of the 802 recorded trials), we had to analyse the recordings multiple times and compare the opinions of all scorers because the animal’s position or orientation made it hard to judge whether it was drinking or not.

In conclusion, although promising, this methodology is not efficient to train jumping spiders. Despite (or even because of) the freedom given to the spider to choose only in some trials, greatly reducing the number of choices made with low motivation, the experiment was too time consuming and granted too few data points. For this reason, I designed an automated training paradigm, which would greatly decrease the time cost and human error.

Study 2: Design and validation of an open source “Skinner-box” system for the study of land arthropods

This study is in preparation [324].

Introduction

As discussed in the study above, the inadequacy of the procedure was, in my opinion, the main reason we were unable to find any significant effect. The manual scoring and carrying out of the experiment posed some clear limitations in the training methodology. Most of the time, experiments carried out in the lab rely on human intervention, introducing possible confounding factors arising from the experimenters’ manipulation [325] or even their mere presence [326]. Also, scoring procedures are generally carried out manually: this may not be a problem for simple binomial measures (behavioural response A vs behavioural response B), but becomes increasingly complex when the scored behaviour is not immediately evident (i.e. [303]), relying mostly on the unconsciously biased experimenter. To solve this problem, double-blind procedures [327] can be implemented. However, these can be complex, and extremely costly in terms of time and resources to reach affordable results.

A possible solution to this problem is the automation of both experimental procedures and data collection [323]. The invention of the first operant conditioning chamber [204], the Skinner-box system, was an enormous scientific breakthrough in the study of learning and conditioning. Today, Skinner-box like systems are used throughout most animal research, especially for classical model species such as rodents, birds and monkeys. More recently many automated systems have been developed for the study of Hymenoptera, for tasks ranging from training [328,329] to automated tracking [330,331]. These methodologies did not only permit higher objectivity but also greatly expanded the range of possibilities regarding what can be discovered through behavioural research (i.e. 24/24hr recording of individual ant behaviour and interactions between colony members [332], impossible to achieve with manual techniques). Machines can collect a massive amount of data, not comparable with what is achievable through manual procedures. Moreover, precision is improved, granting the exact reward contingencies and timing during training.

Training jumping spider, however, remains one of the biggest challenges to overcome when studying this Family [321]. The reason so many methodologies fail to find significant results is the animals’ lack of motivation. Spiders can survive for multiple weeks after having consumed a single

prey (personal observation, but see also [333]: in nature, *Cyrba algerina* had a prey in its chelicera in only 2.7% of all the sightings); moreover, they have a very brief period of activity during the day [257]. The low energetic needs of jumping spiders prevent long-lasting procedures, forcing research to focus on simple S+/S- associations, that require shorter, simpler training procedures. Alternatively, longer training can be carried out, but with immense expenditure of time and resources upfront of an underwhelmingly low amount of data [303]. An automated training system would solve most of these problems, working continuously to catch the period of activity of the animals, being able to sustain prolonged training to cope with the low motivation of the animals. However, to my knowledge no automated training procedure has ever been designed to test jumping spiders. To date, most experiments focusing on jumping spiders cognitive abilities have been carried out with spontaneous choice procedures, since our lack of reliable training methodologies prevented testing them outside of the natural domain.

In this section, I present the SPiDbox: a Skinner-box system based on the Raspberry Pi. This system was intended to solve all of the above-mentioned problems, increasing reward contingencies precision and decreasing training times, while also requiring fewer human resources.

Design, software and electronics

The SPiDbox was designed with four main requirements: it had to be easy to produce, easy to use, low cost, and open source. All the components are cheap and readily available, or even 3D printable. The total cost of the system is around €100-150 ($115-170). Moreover, once built, the machine can be operated by anybody, and the software can be easily modified to fit different reward routines. Source code, circuit design and 3d models will be available in a continuously update repository on git-hub at the time of the submission in peer reviewed journal.

General structure

The SPiDbox (figure 3.4) is divided into three sections. The bodies of the sections were all designed in openSCAD (version 2018.01.06) [334] and 3D printed in PLA plastic with a Creality CR-10.

Starting from the bottom, the first section is constituted by a rectangular box (11×11×15cm), with a window on the front to fit an OLED screen (part of the user interface, see below). Inside the plastic box, all the circuits and electronic components are placed. This section also provides the needed space below the experimental box to fit cables and tubing that reach the components above.

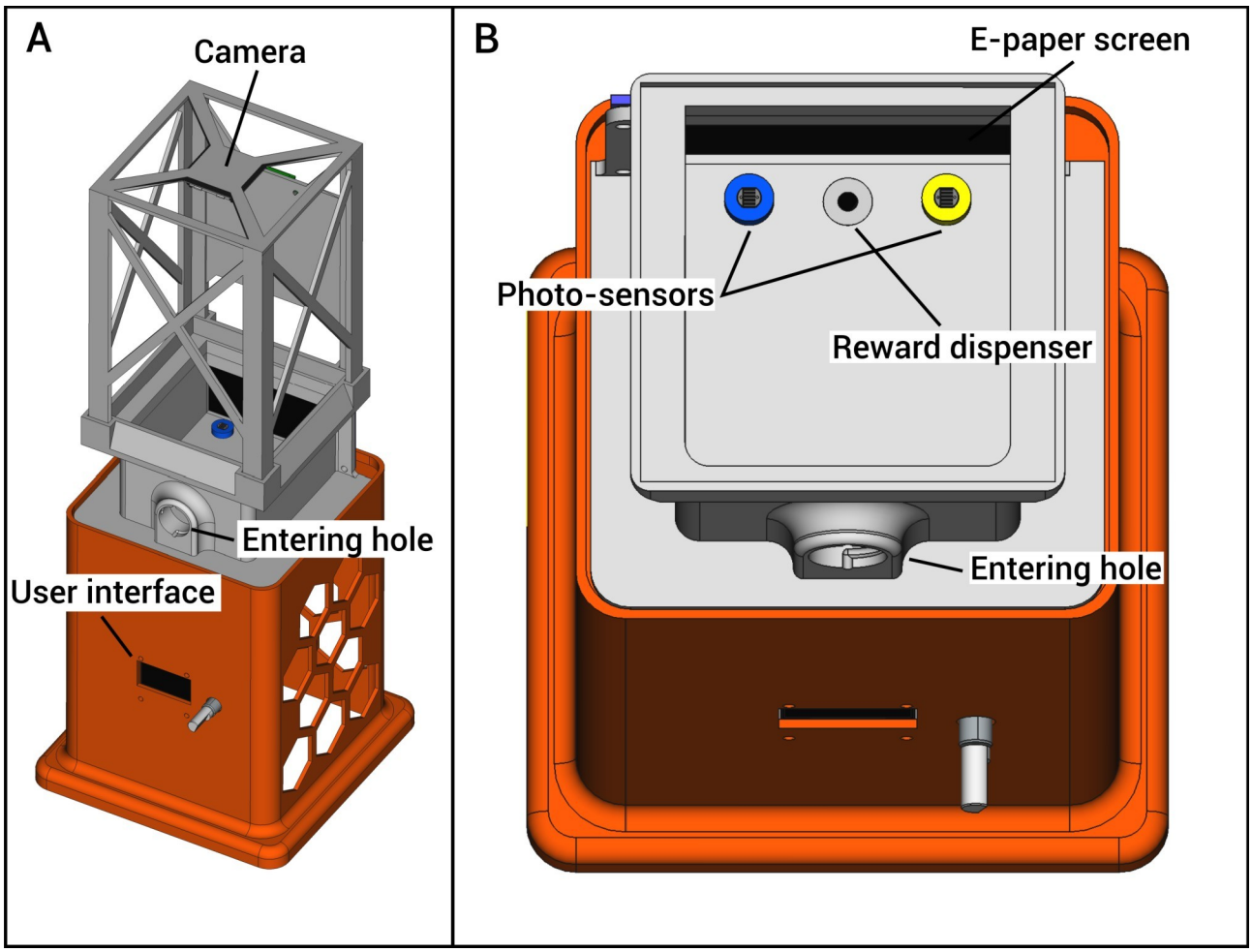

Figure 3.4 – (A) Full view of the SPiDbox system. (B) Top view of the experimental chamber. The two photo-sensors were respectively blue and yellow in colour.

The experimental chamber lies on top of the base box (figure 3.4). It is constituted by a white box (7x8x4cm). On the front wall, a hole (3 cm in diameter) permits the insertion of the subject. The box floor presents three holes, positioned near the back wall. The centre hole is used to fit the reward dispenser, and the other two are used to fit the photo-sensors (see below). The back wall of the box is constituted of an e-paper screen (2.9-inch e-paper module, Waveshare) that can be used to project stimuli for the study of the animal’s visual abilities. I chose to use an e-paper instead of any other alternative because it does not produce any backlighting, not disrupting the functioning of the photo-sensors and avoiding any interference with the animal’s visual system (many arthropods show a phototactic response, moving towards light and possibly ignoring the task). The ceiling of the box is open to enable recording of the spider and sealed with a transparent plastic lid. On top, a

second plastic frame was placed to lock the lid in place. This top frame presented all-around a 12V LED string to provide constant lighting in the box.

Lastly, on top of the experimental apparatus, a 13cm-tall frame was placed, with a pi-camera attached at the top to record the experiment. The Raspberry Pi Zero was secured to the back of this frame.
In total, the three components occupy an area of 11×11x35cm. The two pumps needed to provide and remove the reward were placed outside of the box, on both sides.

Reward dispenser
To provide a liquid reward (in this case a sucrose solution; see the experimental validation), the SPiDbox employs a peristaltic pump based on a Nema 17 motor stepper motor, with 200 steps per rotation. The motor is controlled by a Polulu A4998 driver breakout board (Sparkfun). The peristaltic pump contains silicone tubing with an internal diameter of 2 mm. The peristaltic pump head has a total diameter of 3.4 cm and contains 6 rotors, each with a diameter of 0.7 cm. With these specifications, one full rotation of the motor dispenses 0.2 ml of liquid. Theoretically, the pump can deliver a liquid amount as low as 1µl (0.2 ml/200 steps). One end of the peristaltic pump tubing is placed inside a sucrose solution reservoir, and the other is attached through a coupler to a drop dispenser: a small plastic piece with two couplers at the bottom and a conical hole at the top. This drop dispenser is attached to the experimental box, flush with the floor. This way, when the peristaltic pump is activated, a drop of sucrose solution appears on the floor of the experimental box.
After a set amount of time, the reward is removed by a DC motor-based peristaltic pump. One end of the tubing of this second peristaltic pump is attached to the drop dispenser, and the other is inserted into an empty cup, acting as a discard reservoir.

To provide the same amount of sucrose solution every time, I designed a motion routine for the stepper motor peristaltic pump. At the start of every experimental session, the pump is primed: The motor moves continuously to collect the liquid inside the tubing and send it up to the experimental box. The motor stops as soon as the drop is seen coming out of the drop dispenser. After that, the motor is rotated until it reaches a predetermined position. This is needed to know at every step where each rotor is located. In a peristaltic pump, not every step of the rotation pushes the same amount of liquid: at certain angles, the rotors engage the tubing at the entrance of the pump, and successively, at a second angle, rotors disengage from it at the exit. While the tube is engaged, the

liquid gets pushed onwards, and when any rotor disengages the tube, the amount of liquid that it displaced is sucked back into the tubing (figure 3.5). Setting a point 0 for the motor, in which the position of all the rotors is known, enables us to predict which steps will be ‘push’ steps and which will be ‘retract’ steps. After the motor reaches its starting position, the excess solution still in the drop dispenser is removed and the experiment can be started. Given the ‘push-retract’ pattern of the pump and the volume of the food dispenser, 1/6 of rotation is needed to fill it, or 33 steps. After dispensing the reward twice, the third movement consists of 34 steps, to arrive at half rotation with exactly 100 steps. The same routine is repeated for the second half of the rotation.

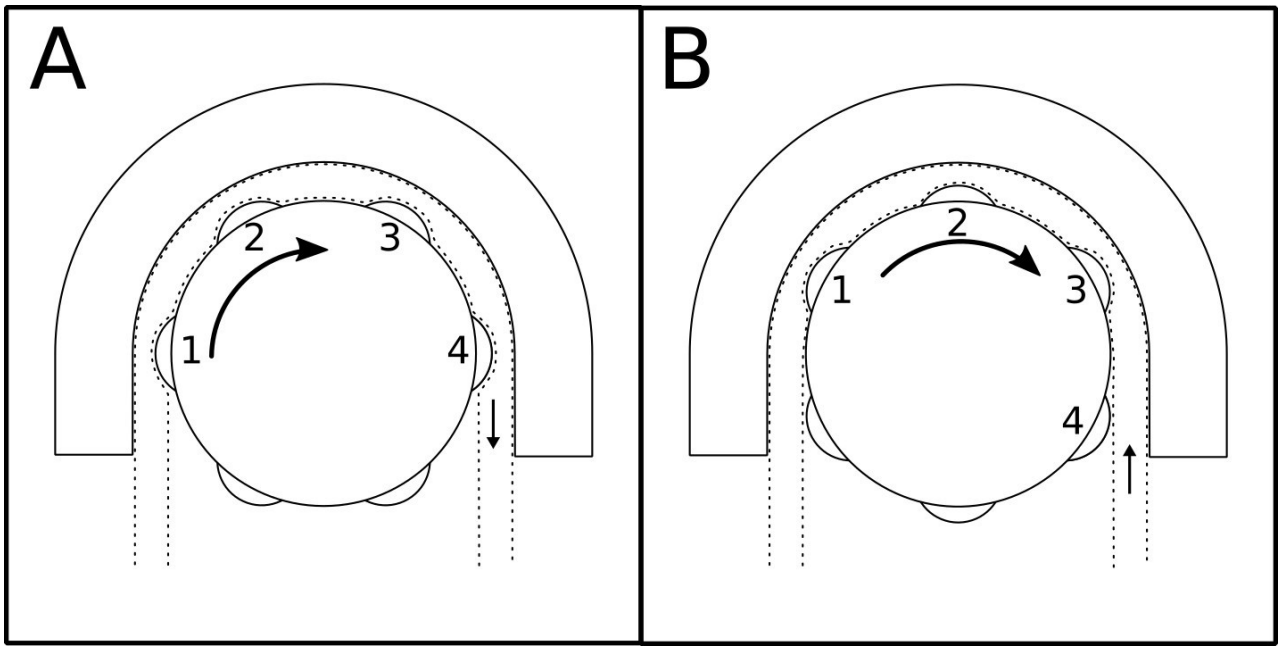

*Figure 3.5 – **A**: Top view of the stepper peristaltic pump. The centre circle is the rotor shaft, with attached 6 different rotors. Transparent tubing depicted with dotted lines. The tube is placed between the rotor shaft and an outside wall. In four different points (indicated with numbers 1-4) the tubing is squeezed by the rotors against the external wall. The liquid gets trapped between each squeeze point and is transported from the left to the right tube end, while the rotor shaft rotates clockwise. **B**: The rotor shaft have been rotated clockwise 30°. The rotor number 4 has now disengaged the tubing and relaxed the contraction. This in turn increased the space available for the liquid, sucking back a small amount. The rotor will need to be rotated more to compensate for this retraction. Since the shaft has 6 rotors, a complete cycle of “push-retract-push” is achieved with a rotation of 60° (or 33.3 steps), with which each rotor end in the position that the next rotor occupied before.*

The machine can never provide multiple drops at the same time. Before being able to dispense a second, the first has to be removed by the DC motor peristaltic pump. This way, the drop dispenser can either be full or empty, never changing the amount of sucrose solution that it contains. During experiments, the drop was dispensed only when the spider touched a particular object,

acting as the unconditioned stimulus. This object was based on a photo-resistor to detect the spider's presence.

Data registration

In the two lateral holes of the experimental box, I fitted two different plastic pieces with a photo-resistor attached in the middle. The two objects can be the same or of different colours, at floor level or raised, etc. The photo-resistors were subsequently attached to a voltage divider, feeding its output to a comparator circuit. The other end of the comparator was attached to the output of a trimmer. The photo-resistor had a variable resistance depending on the light hitting it; when the spider passed on top of the photo-resistor, it decreased the amount of light reaching the surface and increased its resistance. Regulating the trimmer, a resistance threshold could be set, at which the comparator switched from a 0 (photo-resistor not covered) to a 1 (photo-resistor covered). I regulated each threshold for the comparator to change state when at least 50% of the photo-resistor surface was covered. Each activation of the photo-resistor was registered in a CSV file, along with the time of the day and duration.

The activation of one photo-resistor works as an input for the machine, which in turn dispenses a drop of sucrose solution. Having only one photo-resistor, however, would not have been enough for me to measure learning rate: the change in the amount of activation of just one button can not be the only sign of learning by the animal, as an increase in general activity would give the same results. To solve this problem, I used two photo-resistors: one able to release the reward and one that did absolutely nothing. This way, I could compare the activation of both "buttons", enabling discrimination of a general increase or decrease in activity from the effect of a learned association.

User interface

For the machine to be used even by people not accustomed to the Python script, I decided to design a user interface from which the experimenter can select training routines, define subjects, and prepare experiments. I added to the box a rotary encoder, used to navigate to a menu projected onto an OLED SSD1306 (driven with the library luma.oled [335]). At start-up, the user is prompted with two options to choose from: launch and settings.

Selecting "launch" prompts the opening of a sub-menu, containing all defined training routines to choose from. New training routines can be programmed in Python and then added to this list to be used. After selecting any training routine, the user is prompted to input the subject ID and the trial

number. During the course of any experiment, the screen shows the subject ID and trial number, as well as elapsed time and the amount of each photo-resistor's activation.

In the “settings” sub-menu, I defined some routines to test, prepare, and clean each element of the machine. The user can prime the peristaltic pump, as described above, and clean it after the machine has been used, letting clear water run through the tubing. Moreover, from this section, new subject IDs can be defined. Lastly, the photo-resistor can be tested, prompting the sensor state on the OLED screen.

Experiment presentation

With all the components interacting as described, I designed a full training procedure. At first, the animal was inserted into the experimental box through the entrance hole. Inside, it could interact with two buttons, a rewarding one and an inert one. In this experiment, I used sensors of different colours (see experimental validation). Perhaps more interestingly, the button could have been programmed to be rewarding or not depending on what stimuli were presented on the e-paper screen, such as different shapes. The subject would stay in the box for a fixed amount of time, free to press any sensor any number of times. The procedure was then repeated on subsequent days as needed. The different number of activations between sensors across trials was used to assess learning.

Experimental validation: Colour discrimination in the jumping spider *Phidippus regius*

To test the efficacy of the SPiDbox I designed a simple colour discrimination task. Note that the following experiment is not intended to be a demonstration of the presence of any ability in the jumping spider. In fact, it has already been demonstrated that jumping spiders can see a wide colour spectrum [265,292,336,337], and can be trained to discriminate between blue and yellow [263], the two colours employed in this validation. The following experiment is solely intended as a demonstration that this methodology is effective in training jumping spider. I chose a yellow/blue colour discrimination task because it has already been demonstrated in jumping spiders. This way, a negative result would be fully imputable to a fault in the methodology, and not to the inability of the animals to perform the task.

Subjects

I employed 30 *Phidippus regius* in the experiment. Due to subject availability, at the time of testing 2 of those were adult females, 8 were adult males and the remaining 20 were juveniles. All the spiders were born in the laboratory, from the same breeding couple. Upon emergence from an egg-sac, the animals were kept together in the same box (39×28x27cm) and fed *ad libitum Drosophila melanogaster* once every two days. Upon reaching the 4th instar, the spiders were separated and housed in individual transparent boxes (17x9x6cm), which contained a cardboard egg holder cut-out, to provide shelter and enrichment, as well as a florist sponge, functioning as a water and humidity source. Box size was chosen according to Carducci and Jackob [338]. All the individuals were kept at a temperature between 27 and 29 degrees Celsius, and with a light:dark cycle of 12:12 hours. Spiders were starved a week prior to starting the first trial and were never fed outside the SPiDbox until the end of the full procedure. None of the spiders that underwent the experiment had been fed with sucrose solution before the test.

Procedure

The experiment lasted a total of three weeks for each individual and it was divided into three sections.

The first section was a habituation phase. Each spider was inserted into the experimental chamber through the entrance hole. Here, no photo-sensor was present: instead, the drop dispenser was programmed to activate at random time intervals, between 30 and 90 seconds. The peristaltic pump was loaded with a blue-coloured, 0.6M sucrose solution. It has been demonstrated that many jumping spider species feed on flower nectar in nature [316], and this reward has been reported in literature as an appropriate reward for training [263]. After being dispensed, the drop remained for 60 seconds before being removed by the second pump. The spider was free to explore the apparatus and drink from the drop for a total of two hours. After this period of time, the subject was removed from the apparatus, and the latter was cleaned to start a trial for a second spider. I repeated the habituation trial five times, once each day. This section had two main goals: first, I wanted to habituate the spider to a new type of food (the sucrose solution drop) and to the reinforce contingencies (the box is rewarding and the drop comes from that specific spot); second, I wanted to start an association between the colour blue and a reward.

The second section consisted of the actual training. I added the two photo-sensors to the experimental box. One of those was fitted into a blue casing, and the other one into a yellow casing.

The two photo-sensors were then placed on each side of the drop dispenser and remained in the same position through all the phases (however, the sides were balanced between spiders). At this point, the drop was dispensed only when the spider covered more than 50% of the blue-cased photo-sensor surface. I chose the blue photo-sensor as the correct one to exploit the positive association formed with the colour blue in the habituation phase. After being dispensed, the drop (still blue-coloured 0.6-M sucrose solution) remained available for 60 seconds, then was removed. Covering the yellow photo-sensor had generally no effect on the experiment, as I added it only as a control to check if the number of activations of the correct one changed (see the previous section). However, if the yellow sensor was activated during the 60 seconds of drop presence, the latter was immediately removed. This was done to avoid inadvertently training the spider in an unwanted sequence of activation combining both sensors. Each trial lasted a total of two hours, following the same schedule as the first one, and was repeated 5 times, once each day.

The third section was identical to the second, which I decided to repeat to test for improvement between no training and 5 days of training.

I implemented a two-day pause between each section, to account for satiation.

Results

Performance and errors

Before proceeding with the main analysis, I fully reviewed the video recording of the experiment and compared it with the automated data collection of the machine. This process was, in theory, superfluous. However, because this was the first experiment ever carried out on this system, I needed to assess its reliability and correctness.

In two out of the 300 total training trials, the system registered a total of 200 activations of the wrong sensor, whereas reviewing the video showed clearly that the subject never touched either sensor. This error was probably due to an imprecise setting of the trimmer controlling the threshold level of the aforementioned sensor. In fact, the two trials were of two consecutive days from the same machine. The two trials were excluded from the analysis.

Analysis procedure

Analyses were carried out with the statistical software R 3.3.3 [183]. Only the main analysis is reported here, for the full script, see Appendix 6. As suggested by Forstmeier and Schielzeth [240], I

included in the models only factors that I had an a-priori reason for including. I employed a generalized linear mixed effect model, with subject as a random effect, using the package lme4 [185] with a Poisson error structure, since the dependent variable (the number of activations) was a count data. The model was the following:

Number of activations =
*sensor (blue/correct or yellow/wrong) **
*test block (1 or 2) **
test number (1 to 5 of the test block) +
random effect (subjects)

The goodness of the fit was checked with the package DHARMa [189], I had to ascertain a case of zero inflation. Accordingly, the data were remodeled with the package pscl [190,241]. Subsequently, the significance of the model predictors was calculated with an analysis of deviance carried out with the package car [186]. Afterwards, a post-hoc Bonferroni corrected analysis was run on the factor that showed to have an effect on the dependent variables with the package emmeans [187]. Lastly, the plots were generated trough the package ggplot2 [242].

Experiment results

The results are summarized in figure 3.6. From the model emerged a significant difference between sensors (correct or wrong) (GLMM analysis of deviance, chi-square = 45.297, p-value < 0.0001) and a significant different between blocks (GLMM analysis of deviance, chi-square = 12.6204, p-value = 0.0004) but no effect of the test number (GLMM analysis of deviance, chi-square = 2.4558, p-value = 0.117) nor of any of the interactions. The post-hoc analysis revealed that overall the spiders activated more the correct sensor in respect to the wrong sensor (GLMM post-hoc, estimate = 0.893, SE = 0.132, z-ratio = 6.751, p-value < 0.0001) and, in general, activated sensors less, regardless of their value (correct or wrong), in the first test block over the second one (GLMM post-hoc, estimate = -0.449, SE = 0.128, z-ratio = -3.494, p-value = 0.0029). More specifically, the spiders preferred the correct sensor over the wrong sensor both in the first (GLMM post-hoc, estimate = 0.732, SE = 0.177, z-ratio = 4.127, p-value = 0.0002) and in the second block (GLMM post-hoc, estimate = 1.053, SE = 0.189, z-ratio = 5.56, p-value < 0.0001). Moreover, the spider activated more times the correct sensor in the second block over the first block (GLMM post-hoc, estimate = 0.610, SE = 0.189, z-ratio = 3.233, p-value = 0.0074). However, there was no difference

in the number of activations for the wrong sensor between blocks (GLMM post-hoc, estimate = -0.288, SE = 0.173, z-ratio = -1.669, p-value = 0.5708).

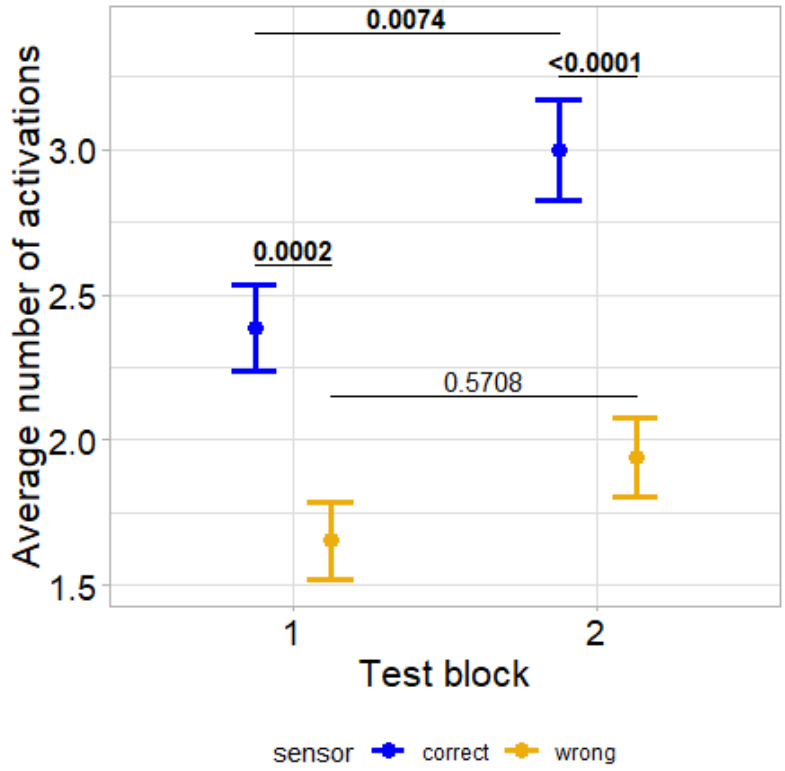

Figure 3.6 – Average number of activations (dots) for each sensor (blue/correct or yellow/wrong), with standard errors. On the X axis the two different blocks. Both in the first and in the second block the spider activated the correct sensor more than the wrong one. Between block one and block two there is an increase in the amount of the correct sensor activations, while there is no increase in the wrong one.

Results Discussion

The training procedure appeared to be successful since the spider increased its number of visits towards the correct, blue sensor over test blocks while maintaining the same amount of visits to the wrong, yellow sensor. Note that I did not expect a decrease in the number of visits to the yellow sensor since it presents no negative effect for the spiders. Accordingly, the spider continued to visit, most likely randomly, the wrong sensor. On the other hand, the increase in the number of activations of the blue sensor attests that the spiders were increasingly attracted to it over test blocks, suggesting that they learned the association between the sensor and the reward.

It is worth discussing that in the first test block, I observed a higher number of visits to the correct sensor over the wrong sensor. There are two possible explanations for this effect. This early

preference could be an effect of a pre-existing innate preference of the spiders for the blue over the yellow colour. This explanation is indeed likely, because blue is a primary colour for these species, whereas yellow is not [292] (note, however, that jumping spiders should be able to perceive yellow, because its wavelength is perceived by the green photoreceptors). It is however important to point out that Liedtke & Schneider [263] tested *Marpissa muscosa* (Family: Salticidae) in two colour-discrimination tasks. In the first, the spiders were asked to learn to discriminate between a yellow and a blue drop, one of which contained sugar and the other citric acid. The authors did not find any difference in the leaning speed for either colour, nor did they observe any innate preference. In the second task, they tested reversal learning abilities of the aforementioned species, which were required to walk behind a blue or a yellow wall to find the sucrose drop. Again, the authors did not find any effect of the colour of the rewarded wall on learning speed and reversal efficiency. These results cannot be simply generalized to our model experiment, as *Marpissa muscosa* and *Phidippus regius* are two very different species of jumping spiders. However, these findings can still provide insight on the plausibility of an innate preference.

The early preference could also be an effect of the habituation phase: in fact, I used a blue-coloured sucrose solution to form an early association between the colour and the reward. If this is the case, it suggests that the spiders may be able generalize a characteristic of the reward (the colour) to new objects, an impressive ability that will require future studies to be more deeply understood. In the future, the two hypotheses about the origin of the preference in the first block could be disentangled, replicating the experiment using yellow-coloured sucrose solution and having the yellow, not the blue, sensor as the correct one. Because this experiment was intended only as a validation of the system, rather than an inquiry on jumping spiders' visual discrimination, the origin of the preference in the first block is of marginal importance. Even in the highly unlikely hypothesis that the spider only perceived the blue sensor, the increased activation for the latter between blocks clearly shows a direct intention towards it, because a general random activity increase would have been registered by both sensors.

In this experiment, no effect of the training day was found. The lack of a clear learning pattern seems counter-intuitive, as there is an overwhelming amount of literature showing that learning process happens over time, with a day-by-day improvement [339]. It has to be considered, however, that this system does not solve the inherent problems of training jumping spiders but just bypasses them. These animals are still hardly motivated, and will, during many trials, not activate any sensor, as demonstrated by the zero-inflation of the model distribution. The Skinner-box permits testing a

high number of subjects for a prolonged time, so the random unmotivated behaviours are eventually filtered out in the test blocks, letting the learning effect rise above significance. However, with this sample size, a trial-by-trial effect cannot be observed. Anecdotally, in Appendix 6, a day-by-day graph is provided. No learning trend is apparent; however, in each block (but especially in the first), an overall decrease of activity can be appreciated, probably due to the drop in motivation. Future studies may focus on the learning pattern and timing, increasing both the sample size and the number of consecutive trials.

Overall, the SPiDbox was shown to be a reliable and effective way of training jumping spiders, as well as being able to provide useful insight into how learning takes place.

Flawed registration of the sensors

A few words are worth spending over the limitation of the system, especially regarding the sensor errors that I observed after data collection.

The comparator-based activation of the sensor is based on a fixed threshold, provided by the manual adjustment of a trimmer. As such, it could not account for the natural fluctuations of the ambient light, forcing the experimenter to set the threshold as low as possible to avoid false positives. This, in turn, caused the problem of fast activation and deactivation while the spider moved on top of the sensor, as described in the results section: because the threshold was set so low, minimal movement of the animal could cause the reading to bounce above and below the set level. Moreover, this system relied too much on human judgement, because the trimmer had to be manually set by the experimenter. This did not cause problems most of the time but might have still caused misreadings, as happened in two trials (see results sections). Both these problems were solved easily during data analysis, but an improvement to the system is still needed.

For this purpose, I decided to design a new reading system, switching from the comparator, threshold-based digital reading (0 and 1) to an analogical reading. Both the comparator and the trimmer were removed, feeding the output of the voltage divider directly to an analogical to digital converter (ADS1116, breakout board from Adafruit). In this way, the Raspberry Pi can read the exact resistance of both sensors in each iteration, calculating two separate moving averages with the following formula:

$$\bar{x} = (c \cdot x) + [(1 - c) \cdot \bar{x}_1]$$

where $\bar{x}$ is the calculated average, x is the reading for the current cycle, $\bar{x}_1$ is the calculated average of the previous cycle, and c is a constant that determines the level of smoothing applied to the resulting variable. Note that the reading x is already being smoothed, averaging the last three raw readings x_r together, in order to remove small random fluctuations. To detect an activation of the sensor, instead of using a threshold value as before, a Δ value was calculated for each raw reading, defined as the difference between x (the current read value) and $\bar{x}$ (the moving average). An activation was registered when Δ exceeded a predetermined value t. When an activation was detected, the moving average is locked on the last calculated value. The activation was considered over when the value of Δ became inferior than $\frac{t}{2}$, preventing unwanted deactivations due to random fluctuations of Δ around the value t. When a deactivation occurs, the moving average is then calculated again as normally (figure 3.7).

Future directions and alternative usages

It is worth noting that this system can not only be used to train jumping spiders but could be extended to many similarly sized, solitary arthropods. The consumption of sucrose solution is widespread amongst many invertebrates, and as such, I expect it to be a suitable reward for many species. However, the biology and ethology of each species should be thoroughly understood before designing the training procedure. The system could also be used for social arthropods, but it would require some modifications: The experimental box should be connected to the colony, and locomotor systems should be taken into account (for a flying insect, a complete restructuring of the system would be needed. However, for bees and bumblebees, other alternatives already exist [329]).

The SPiDbox is an effective system for the training of jumping spiders. Due to the easy accessibility and low cost of the components, as well as the open-source nature of the software and design, it could provide scientists with the needed instruments to study this fascinating arthropod family. As per its intended purpose, this system can be used to carry out training for complex stimuli and behaviours, increasing the number of sensors, changing their positions, inserting complex patterns of activations, and overall expanding the possibilities to study skills that were up to now out of reach of existing experimental methodologies.

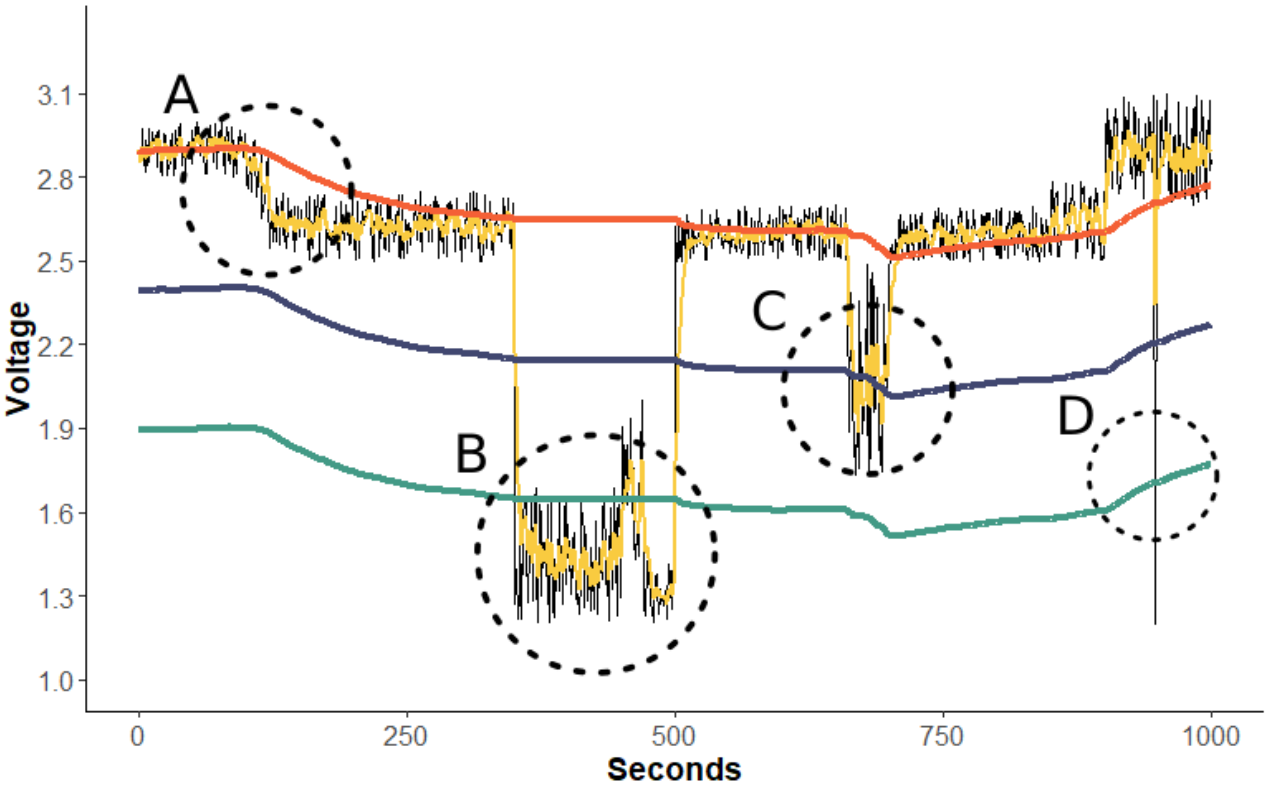

Figure 3.7 – Simulated data describing how the moving average system works. Y axis = Voltage readings from the analogue to digital converter; X axis = Time (seconds). Black line represent raw readings (x_r); yellow line represent the smoothed reading x, averaging the last three x_r; red line represent the moving average $\bar{x}$; light blue line represent the moving average $\bar{x}$ minus the defined value t; dark blue line represent the moving average minus $\frac{t}{2}$. Three sections of the graph have been highlighted to point out how this system functions. ***A****: the voltage readings drop slightly, as can happen in case of a general change of light in the environment. In this occasion, the moving average can adjusts itself.* ***B****: example of a contact of the spider with the sensor. The readings suddenly drop and their difference to the moving average (Δ) drops below t. In this case a contact is registered by the program, and the moving average is locked on the last calculated value. Note how around 450 seconds in the graph the values briefly go above the $\bar{x}-t$ line. This can happen when the spider move while on top of the sensor, maybe uncovering some sections of it and letting more light pass before covering it again. The system does not register this event, as to be considered the end of the contact the value has to raise above the $\bar{x}-\frac{t}{2}$ line.* ***C****: a sudden change in values, that can occur for example if the spider cast its shadow on the sensor while walking on the ceiling. This is however not registered as a contact, as the value does not go below $\bar{x}-t$.* ***D****: a random voltage fluctuation, or misreading by the machine, can cause the raw reading to drop significantly, even below $\bar{x}-t$. However, the first smoothing averaging the last three readings prevents the system from considering these events as contacts by the spiders.*

CONCLUSIONS

In this thesis, I presented the main studies carried out for my PhD project focused on the cognitive abilities that small brains display through their resulting complex behaviour. First, I reported the work done on ants. In the first study about value perception in a risk context, I found evidence in support of the idea that their decision process is based on a perceptual mechanism: the Weber-Fechner law. As already mentioned in the introduction section of the study, this system is very advantageous, and requires a very limited number of neurons that can evaluate and compare an otherwise immense amount of information. This fits in the broader context of simplification: a small network able to decode a wide variety of stimuli from the environment is advantageous with respect to associative mechanisms. In fact associative learning would require to register each individual value and compare it with others one-by-one. In the second study, I tested the ant memory using an information integration paradigm. The animals have shown to be able to retain and combine information of multiple sensory modalities in order to locate the reward, suggesting an integration skill far deeper than we expected. Perhaps more interestingly, the ants seemed able to perform this task in just a single trial, suggesting that their learning speed is independent of the amount of information presented. Unfortunately, the results of this second condition were not conclusive. Yet, these results inspire some further discussion how this information load-independent mechanism may be beneficial for the ants. With a small brain, the amount of data that can be recorded drops significantly, while a more complex mechanism, such as episodic-like memory, could solve this problem with a lower number of neurons. To conclude, both studies carried out on ants support the "economy of design" brain strategy.

Subsequently, as for the main topic of my PhD project, I reported the studies performed with Salticidae. In the first study I tested the visual system of *Phidippus regius*, to inquire if they employ the Gestalt principles, as a way to categorize, discriminate and interpret visual stimuli with few perceptual rules instead of a more demanding "pixel by pixel" system. Unfortunately, the results were inconclusive, since the training procedure may have not been successful. For this reason I designed and implemented an automated system, capable of training the spiders in a variety of associative tasks, allowing to investigate memory, vision, and a variety of cognitive processes. This open-source and inexpensive system will function as a tool supporting experimental endeavours aimed at deepening our understanding of how much cognition is truly spread in the whole animal kingdom.

The studies reported in this thesis join the ever-growing scientific literature about cognition in animals that we would have never thought capable of complex neural processes just 30 years ago. Nowadays, impressive feats are reported in more and more species, even outside the animal kingdom: a new field of psychology has been recently sprouting, focusing on the study of cognition in non-neuronal organisms. Plants, for example, can orient themselves in space [340] and perceive and interpret complex environmental stimuli such as those conveyed by light wavelengths or air vibrations [341,342]. Even a unicellular organism, the slime mould, has been found capable of decision making [141,343], learning [344], spatial orientation [345] and many others [346]. It is crucial to remember that neurons themselves do not produce cognition, which is instead the outcome of the connection network. Brain cells, however, are not the only ones capable of transferring information between one another. The ability to produce signalling impulses pre-dates the evolution of neurons [347–350]. The fact that cognition may manifest both in miniature brains and in organisms that do not possess a brain at all, forces us to reconsider the need for a massive and expensive neuronal tissue. Why do we, humans, have such a big brain? The ability to produce complex behaviour is unlikely to be the cause. The struggle to find a correlation between the number of neurons in the cortex and cognitive performance (see the introduction, [25]), should probably be redimensioned as some forms of cognition may pre-exist the evolution of a brain itself. Today more and more scientists agree to the idea that cognition (graded forms of it) is indeed widespread in the whole animal kingdom [32], even in tiny animals [14,94].

From the birth of philosophy in the western world, we have been convinced that many abilities were unique to humans: cultural transmission, teaching, language and many others. Experimental evidence has been accumulating that these abilities are present in a variety of species, including miniature organisms [85,86,92,351,352]. In light of this evidence, we should be cautious with any future claim of uniqueness – of humans, mammals or vertebrates – as such claims may, in time, be proven false. It has been proposed that too often our inquiry is driven by our perspective [323]: we describe animals' abilities with definitions rooted in the human experience. Moreover, also our definition of complexity is based on our own perception (see the introduction: we consider rule learning to be more complex than associative learning, however miniature brains are more likely to produce rule learning circuits than associative ones [14,67–73,78]). These preconceptions inform our scientific process and our interpretation of results: the Ockham's razor [353] dictates to use the least amount of assumptions and the simplest explanation to interpret an event. When asked to

formulate a hypothesis on a newly observed behaviour, we should carefully consider that cognition may be in fact the simplest and most likely explanation.

Thanks to this promising and intriguing evidence, the field of studies interested in information processing in miniature brains is constantly growing. Yet, our understanding of how most of these animals function and thrive in their natural environment is still greatly limited. Henceforth, future studies on new and different species will shed more and more light on the evolution of cognition, its origin and its ultimate goal. I am convinced that a better understanding of ourselves among the other forms of life on earth shall come from understanding the creatures furthest from us.

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APPENDICES

Appendix 1 – pilot experiments: Support for the perceptual basis of irrational risk aversion in ants

Ant perception of 0.1, 0.3 and 0.9 sucrose molarities

In experiment 3, the ants were presented with feeders offering 0.1, 0.3 and 0.9 molar sucrose. Relative to experiments 1 and 2, the medium and the low quality drop had very similar molarities in absolute terms, so that we decided to run a pilot experiment to test whether the ant could discriminate, and subsequently choose reliably between, the three molarities.

We ran two testing blocks. In the first, the ants were presented with two drops of different molarities, 0.1 and 0.3, and were trained to associate each to a smell. We followed the methodology described for the main experiment (see methods section in the main paper), alternating the presentation of the low quality alternative and the high quality alternative in the 8 training visits. Afterwards we tested the ants in the Y-maze, repeating the test 5 times. The second experiment was identical to the first, but the ants were presented with 0.3 and 0.9 molarities. We did this last block just as a control, since as 0.3 and 0.9 are further apart than 0.55 and 1.0 we were confident that they could discriminate between the two. We tested 20 ants for each block (40 in total), stemming from 6 different colonies. First, we tested the robustness of the ants' choices, checking whether with subsequent visits the number of ants choosing the high value drop decreased. We modelled as follows:

High value choice (all tests) =
Testing visit (1-5)+
Contrast (0.1vs0.3 or 0.3vs0.9)
random effect (individual ant nested in colony)

We found that the ants did not change their preference over subsequent visits for either of the two contrasts (table S3). This could be because this task is easier than the risk vs safe evaluation, having to compare only two molarities in which one is definitely better than the other. For the subsequent analysis, we kept all 5 testing visits. We modelled the data as follows:

High value choice (all tests) =
Decision line+
Contrast (0.1vs0.3 or 0.3vs0.9)
random effect (individual ant nested in colony)

Then, we ran a post-hoc test to check which of the groups differed from chance level. We found that the ants significantly preferred 0.3 over 0.1 when considering both the first decision line and the second decision line. However, we found that the ants did not significantly preferred 0.9 over 0.3,

remaining at chance level (Table S4). This was surprising to us, as the contrast between 0.9 and 0.3 should be easier to sense, or at least equally difficult if the ants follow a logarithmic perception, and in both cases easier than the contrast between 0.55 and 1.0. We suspect that, due to the lower sample size in these experiments, we have experienced a type II error (false negative). However, we decided to present our data as it is, without a post-hoc increase in sample size, following good scientific practice.

Factor	Chi-square	Degrees of freedom	p-value
Contrast	3.5857	1	0.058
Testing visit	1.46	1	0.227
Contrast:Testing visit	0.024	1	0.876

Table S3 – Analysis of deviance (Type II chi-square test) of the model to check difference between testing visit. For this model we had to drop colony as random factor because the model did not converge otherwise. Note that both Testing visit and the interaction between Testing visit and the contrast are not significant. We can conclude that there is no difference between the test visits and there is no difference between the two contrast in testing visits change.

Contrast	Decision line	probability	SE	Z ratio	p-value
0.1 vs 0.3	First	0.86	0.057	3.844	0.0005
0.1 vs 0.3	Last	0.87	0.054	3.988	0.0003
0.3 vs 0.9	First	0.648	0.095	1.461	0.576
0.3 vs 0.9	Last	0.724	0.085	2.264	0.094

Table S4 – post-hoc analysis of the probability of ants choosing the high value alternative, bonferroni corrected.

Ant preference among 3 molarities

In the main experiment the ants were presented with three different food qualities, and were required to remember all three in order to make a choice between the two feeders. We decided to run a pilot experiment on order to test whether the ants could remember three molarities, rather than just the best one among others.

The ants performed 9 sequential visits to a runway, identical to the one of the main experiment. At the end of the runway the ant may find either a 1.5M drop, always unscented, a 1.0M drop, either rosemary or lemon scented, and a 0.25M drop, scented with the other odour (see table S5).

Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9
1.5M	**1.0M**	**0.25M**	1.5M	**1.0M**	**0.25M**	1.5M	**1.0M**	**0.25M**
1.5M	**0.25M**	**1.0M**	1.5M	**0.25M**	**1.0M**	1.5M	**0.25M**	**1.0M**

Table S5 – Training visit sequence for the three molarity experiment. Bold text represent scented visits. The same molarity always have the same scent.

Afterwards we tested the ant preference between the 1.0M scent and the 0.25M scent in the Y-maze, repeating the test 5 times. If the ants could only learn the best alternative among the presented ones, they should choose randomly between the second best and the worst. However, if the ants can remember and compare all three values, they should prefer the 1.0M.

We planned to test 32 ants coming from 8 different colonies. However, after having tested 15 ants coming from 5 different colonies we decided to stop the pilot, given the clear preference of the animals: On the first trial, both initial and final decision, 100% of the ants choose the scent associated with 1.0M. We observed a decrease on the ants performance in subsequent files, however the overall percentage remained at 92%. While we are aware that stopping an experiment prematurely when results are as expected can lead to type I errors (false positives), we felt that the unambiguous nature of these results warranted doing so here.

Risk preference in the context of losses (maintained on 1.5M sucrose)

Prospect Theory predicts that individuals should be risk averse in the context of gains and risk prone in the context of losses. The reference point from which we decide if something is a gain or a loss is not necessarily 0: we may take an expected value as a reference. For ants, this value it could be the feeding solution they are maintained on, normally 0.5M. We decided to replicate experiment 1 (see main paper) with 4 colonies that had been fed *ad libitum* 1.5M sucrose instead of the usual 0.5M for one month prior testing. 63 ants were tested in total. Training and testing procedure were identical to those described in the main paper. We found that 82% (52/63) of the ants preferred the safe alternative. All data and analysis are provided in Appendix 2.

Appendix 2 – Data Analysis of the study: Support for the perceptual basis of irrational risk aversion in ants

This supplement provides the entire R script and output of the statistical analysis we performed and figures produced, in their original form. It is presented in the spirit of open and transparent science, but has not been carefully curated.

Data analysis

first I load packages

```
library(lme4)
library(DHARMa)
library(car)
library(emmeans)
library(reshape2)
library(ggplot2)
library(knitr)
library(pscl)

set.seed(123)#set seed for replicability in random simulations
```

Binomial Choice

Preliminary questions

first, I want to know if initial and final choice differ

Cond 1

```
fsdiff<-melt(risksa, measure.vars = c("firstchoicesafe","endchoicesafe"))

mdiff<-glmer(value~variable+(1|colony/antID),data=fsdiff,family="binomial")
## boundary (singular) fit: see ?isSingular
Anova(mdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: value
##           Chisq Df Pr(>Chisq)
## variable 0.5111  1     0.4746
e<-emmeans(mdiff, ~variable, type="response")
pairs(e)
##  contrast                        odds.ratio    SE  df z.ratio p.value
##  firstchoicesafe / endchoicesafe      0.814 0.234 Inf -0.715  0.4746
##
## Tests are performed on the log odds ratio scale
```

there is no difference between initial and final choice, I will now on only use the initial for further analysis

Cond 2

```
fsdiff<-melt(riskirr, measure.vars = c("firstchoicesafe","endchoicesafe"))

mdiff<-glmer(value~variable+(1|colony/antID),data=fsdiff,family="binomial")
Anova(mdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: value
##           Chisq Df Pr(>Chisq)
## variable 0.2903  1       0.59
e<-emmeans(mdiff, ~variable, type="response")
pairs(e)
##  contrast                        odds.ratio    SE  df z.ratio p.value
##  firstchoicesafe / endchoicesafe        1.1 0.195 Inf 0.539   0.5900
##
## Tests are performed on the log odds ratio scale
```

there is no difference between initial and final choice, I will now on only use the initial for further analysis

Cond 3

```
fsdiff<-melt(riskgeo, measure.vars = c("firstchoicesafe","endchoicesafe"))

mdiff<-glmer(value~variable+(1|colony/antID),data=fsdiff,family="binomial")
## boundary (singular) fit: see ?isSingular
Anova(mdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: value
##           Chisq Df Pr(>Chisq)
## variable 0.1981  1     0.6563
e<-emmeans(mdiff, ~variable, type="response")
pairs(e)
##  contrast                        odds.ratio    SE  df z.ratio p.value
##  firstchoicesafe / endchoicesafe        1.1 0.243 Inf 0.445   0.6563
##
## Tests are performed on the log odds ratio scale
```

there is no difference between initial and final choice, I will now on only use the initial for further analysis

now, I want to know if the visits differ from one another

Cond 1

```
risksa$visit<-as.numeric(risksa$visit)
mvisdiff<-glmer(firstchoicesafe~visit+(1|colony/antID),data=risksa,family="bino
mial",
```

```
                glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
1000000000)))
## Warning in checkConv(attr(opt, "derivs"), opt$par, ctrl = control
## $checkConv, : Model failed to converge with max|grad| = 0.0157403 (tol =
## 0.001, component 1)
mvisdiff<-glmer(firstchoicesafe~visit+(1|antID),data=risksa,family="binomial",
                glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
1000000000)))
Anova(mvisdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: firstchoicesafe
##       Chisq Df Pr(>Chisq)
## visit 9.668  1   0.001875 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
summary(mvisdiff)
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: binomial  ( logit )
## Formula: firstchoicesafe ~ visit + (1 | antID)
##    Data: risksa
## Control:
## glmerControl(optimizer = "bobyqa", optCtrl = list(maxfun = 1e+09))
##
##      AIC      BIC   logLik deviance df.resid
##    196.7    206.4    -95.3    190.7      189
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -2.8804  0.3022  0.3472  0.5081  0.8520
##
## Random effects:
##  Groups Name        Variance Std.Dev.
##  antID  (Intercept) 0.3172   0.5632
## Number of obs: 192, groups:  antID, 64
##
## Fixed effects:
##             Estimate Std. Error z value Pr(>|z|)
## (Intercept)   9.0752     2.5512   3.557 0.000375 ***
## visit        -0.7616     0.2449  -3.109 0.001875 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##       (Intr)
## visit -0.996
```

the percentage of ants choosing safe decreases with successive visits. this means that more and more ants after not finding the sugar drop start doing a random search. I will from now on only observe the first visit, being it a clearer indication of choice

Cond 2

```
mvisdiff<-glmer(firstchoicesafe~visit+(1|colony/
antID),data=riskirr,family="binomial",
                glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
100000)))
Anova(mvisdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: firstchoicesafe
##        Chisq Df Pr(>Chisq)
## visit 5.8851  1    0.01527 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
summary(mvisdiff)
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: binomial  ( logit )
## Formula: firstchoicesafe ~ visit + (1 | colony/antID)
##    Data: riskirr
## Control:
## glmerControl(optimizer = "bobyqa", optCtrl = list(maxfun = 1e+05))
##
##      AIC      BIC   logLik deviance df.resid
##    415.4    430.4   -203.7    407.4      316
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.8473 -1.1416  0.6020  0.7331  1.0362
##
## Random effects:
##  Groups       Name        Variance Std.Dev.
##  antID:colony (Intercept) 0.14277  0.3778
##  colony       (Intercept) 0.03706  0.1925
## Number of obs: 320, groups:  antID:colony, 64; colony, 8
##
## Fixed effects:
##             Estimate Std. Error z value Pr(>|z|)
## (Intercept)  2.96566    0.97581   3.039  0.00237 **
## visit       -0.20978    0.08647  -2.426  0.01527 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##       (Intr)
## visit -0.988
```

the percentage of ants choosing safe decreases with successive visits. this means that more and more ants after not finding the sugar drop start doing a random search. I will from now on only observe the first visit, being it a clearer indication of choice

Cond 3

```
mvisdiff<-glmer(firstchoicesafe~visit+(1|colony/
antID),data=riskgeo,family="binomial",
                glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
100000)))
## boundary (singular) fit: see ?isSingular
Anova(mvisdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: firstchoicesafe
##        Chisq Df Pr(>Chisq)
## visit 0.5282  1     0.4674
summary(mvisdiff)
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: binomial  ( logit )
## Formula: firstchoicesafe ~ visit + (1 | colony/antID)
##    Data: riskgeo
## Control:
## glmerControl(optimizer = "bobyqa", optCtrl = list(maxfun = 1e+05))
##
##      AIC      BIC   logLik deviance df.resid
##    282.4    295.6   -137.2    274.4      196
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.1864 -0.9476  0.6974  0.9092  1.2280
##
## Random effects:
##  Groups       Name        Variance Std.Dev.
##  antID:colony (Intercept) 0.3117   0.5583
##  colony       (Intercept) 0.0000   0.0000
## Number of obs: 200, groups:  antID:colony, 40; colony, 10
##
## Fixed effects:
##             Estimate Std. Error z value Pr(>|z|)
## (Intercept)  0.94158    1.16051   0.811    0.417
## visit       -0.07576    0.10425  -0.727    0.467
##
## Correlation of Fixed Effects:
##       (Intr)
## visit -0.989
## convergence code: 0
## boundary (singular) fit: see ?isSingular
```

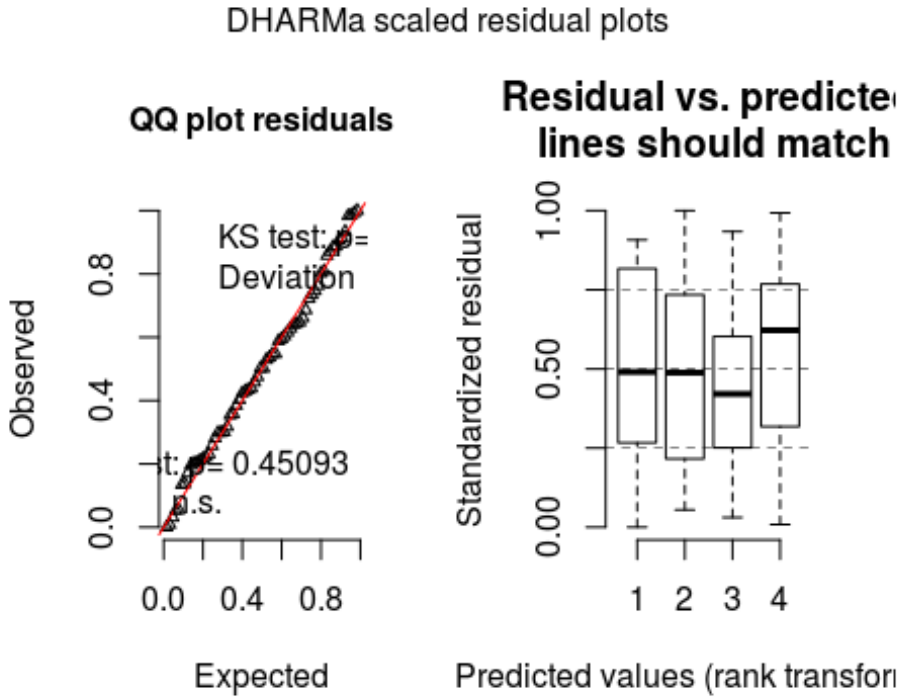
there is no difference between visits. I will use only first for consistency, but I expect random choice. in this case, it is clear why there is no decrease: if the choice is already random there is no room for reverting to random choice with subsequent visits.

Modeling

now to the actual model. I drop antID because I kept only one observation for each ant

Cond 1

```
risksasing<-subset(risksa,risksa$visit==9)

mExp1<-glmer(firstchoicesafe~firstfeed*firstrisk+(1|
colony),data=risksasing,family="binomial",
             glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
simres<-simulateResiduals(mExp1) #standard seed for random values is 123
plot(simres, asFactor=T)
```



model is good here

```
Anova(mExp1)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: firstchoicesafe
##                      Chisq Df Pr(>Chisq)
## firstfeed           0.7092  1     0.3997
## firstrisk           0.0000  1     1.0000
## firstfeed:firstrisk 0.0000  1     1.0000
```

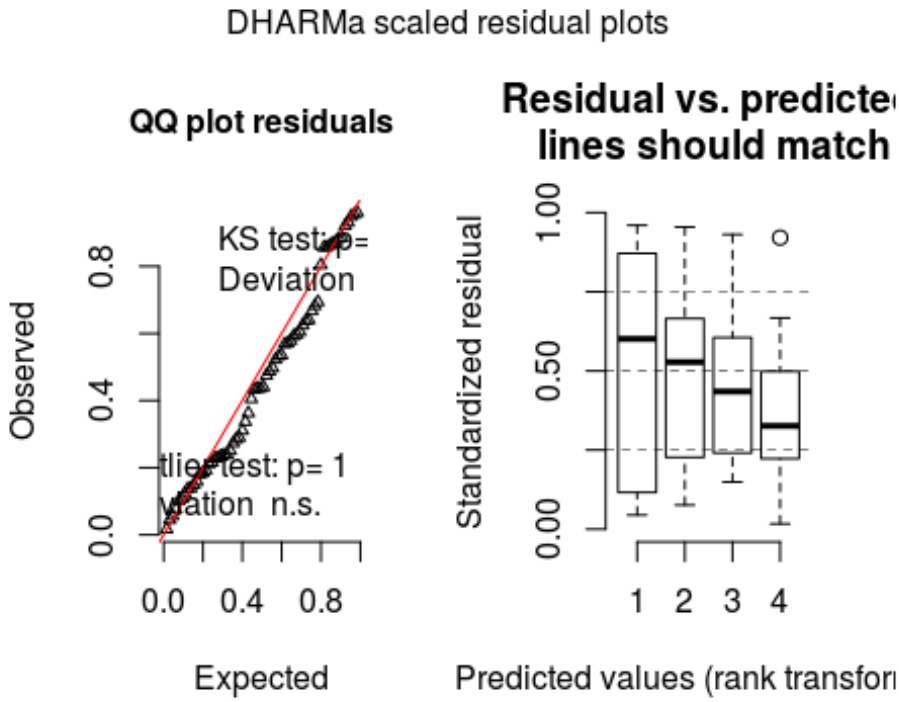
no effect of any of the factors. will just test overall preference

```
meanobj <- emmeans(mExp1,~1, type="response")
(test(meanobj))
##  1        prob     SE  df z.ratio p.value
##  overall 0.911 0.0367 Inf 5.142   <.0001
##
## Results are averaged over the levels of: firstfeed, firstrisk
## Tests are performed on the logit scale
```

ants prefer the safe **91%**

Cond 2

```
riskirrsing<-subset(riskirr,riskirr$visit==9)

mExp2<-glmer(firstchoicesafe~firstfeed*firstrisk+(1|
colony),data=riskirrsing,family="binomial",
             glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))

simres<-simulateResiduals(mExp2) #standard seed for random values is 123
plot(simres, asFactor=T)
```



good model also here

```
Anova(mExp2)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: firstchoicesafe
##                      Chisq Df Pr(>Chisq)
## firstfeed           2.0148  1     0.1558
```

```
## firstrisk           0.1969  1     0.6572
## firstfeed:firstrisk 1.8066  1     0.1789
```

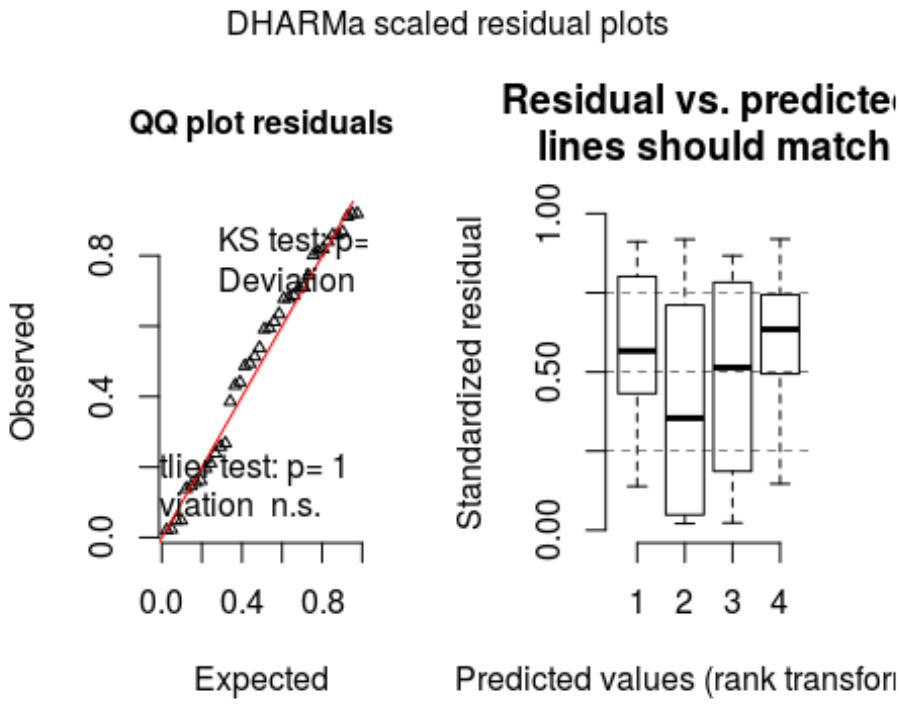
still, no effect of factors.

```
meanobj<-emmeans(mExp2,~1, type="response")
(test(meanobj))
##  1        prob     SE  df z.ratio p.value
##  overall 0.792 0.0678 Inf 3.248   0.0012
##
## Results are averaged over the levels of: firstfeed, firstrisk
## Tests are performed on the logit scale
```

ants prefer the safe **79%**

Cond 3

```
riskgeosing<-subset(riskgeo,riskgeo$visit==9)

mExp3<-glmer(firstchoicesafe~firstfeed*firstrisk+(1|
colony),data=riskgeosing,family="binomial",
             glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
simres<-simulateResiduals(mExp3) #standard seed for random values is 123
plot(simres, asFactor=T)
```



still, good model

```
Anova(mExp3)
```

```
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: firstchoicesafe
##                      Chisq Df Pr(>Chisq)
## firstfeed           4.4237  1    0.03544 *
## firstrisk           0.0146  1    0.90388
## firstfeed:firstrisk 0.6679  1    0.41377
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

there is an effect of the first presented feeder. first of all let's look at overall percentage

```
meanobj<-emmeans(mExp3,~1, type="response")
(test(meanobj))
##  1        prob     SE  df z.ratio p.value
##  overall 0.535 0.0864 Inf 0.403   0.6870
##
## Results are averaged over the levels of: firstfeed, firstrisk
## Tests are performed on the logit scale
```

ants prefer the safe **53%**.

```
meanobj<-emmeans(mExp3,~firstfeed, type="response")
## NOTE: Results may be misleading due to involvement in interactions
pairs(meanobj)
##  contrast     odds.ratio   SE  df z.ratio p.value
##  risky / safe      0.216 0.15 Inf -2.207  0.0273
##
## Results are averaged over the levels of: firstrisk
## Tests are performed on the log odds ratio scale
meanobj
##  firstfeed  prob    SE  df asymp.LCL asymp.UCL
##  risky     0.348 0.107 Inf     0.175     0.574
##  safe      0.712 0.104 Inf     0.477     0.870
##
## Results are averaged over the levels of: firstrisk
## Confidence level used: 0.95
## Intervals are back-transformed from the logit scale
```

more ants go to the safe when this is presented fist, more ants go to the risky when is presented first.

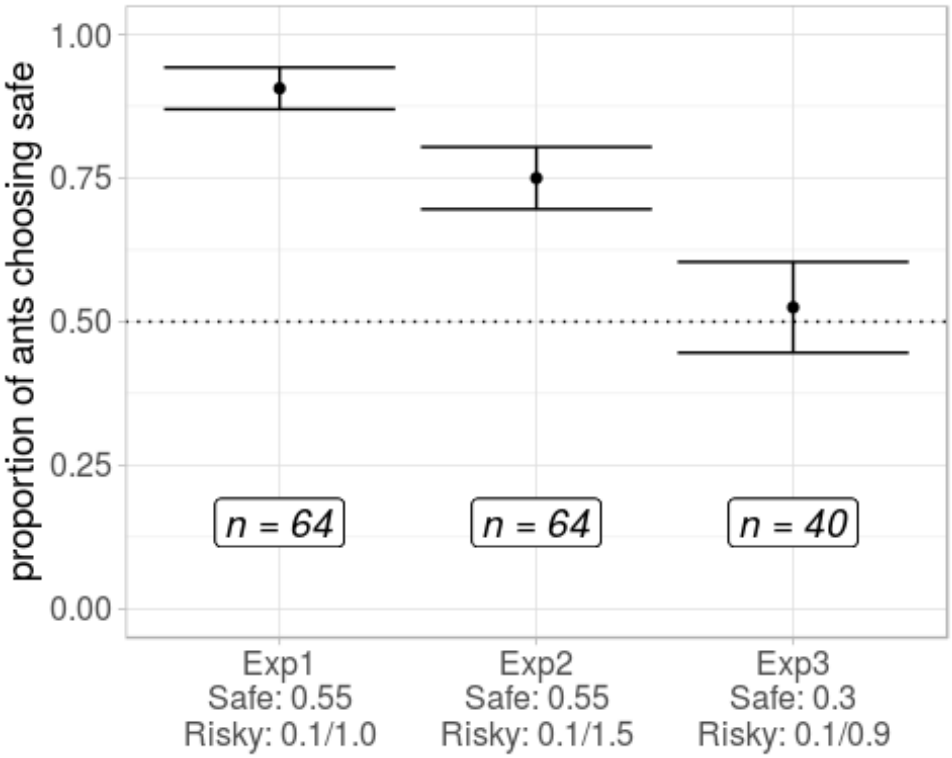
overall is random! probably in front of a random choice they just go for the first experienced.

Graph together

need to calculate a full model to get SE in order to plot.

```
risksing<-subset(risk,risk$visit==9)
library(plyr)
risksing$condition<-revalue(risksing$condition, c("GeomAvrg"="Exp3",
"Irrational"="Exp2","sameAvg"="Exp1"))

mTot <- glmer(firstchoicesafe~condition+(1|
colony),data=risksing,family="binomial",
```

```
              glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
100000)))
## boundary (singular) fit: see ?isSingular
Anova(mTot)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: firstchoicesafe
##             Chisq Df Pr(>Chisq)
## condition 16.918  2   0.000212 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
meanobj<-emmeans(mTot,~condition, type="response")
toplot1<-as.data.frame(meanobj)
(pairs(meanobj))
##  contrast    odds.ratio     SE  df z.ratio p.value
##  Exp3 / Exp2      0.368 0.1579 Inf -2.330  0.0517
##  Exp3 / Exp1      0.114 0.0609 Inf -4.068  0.0001
##  Exp2 / Exp1      0.310 0.1604 Inf -2.263  0.0611
##
## P value adjustment: tukey method for comparing a family of 3 estimates
## Tests are performed on the log odds ratio scale
ggplot(toplot1,aes(x=condition,y=prob))+
  ylab("proportion of ants choosing safe")+
  ylim(0,1)+
  scale_x_discrete(name= NULL,
                   limits=c("Exp1","Exp2","Exp3"),
                   labels=c("Exp1" = "Exp1\nSafe: 0.55\nRisky: 0.1/1.0",
                            "Exp2" = "Exp2\nSafe: 0.55\nRisky: 0.1/1.5",
                            "Exp3" = "Exp3\nSafe: 0.3\nRisky: 0.1/0.9"))+
  theme_light()+
  theme(axis.text.x = element_text(size=12),
        axis.text.y = element_text(size=12),
        axis.title.y = element_text(size=14))+
  geom_label(x=1, y=0.15, label="n = 64",size=5, aes(fontface=3))+
  geom_label(x=2, y=0.15, label="n = 64",size=5, aes(fontface=3))+
  geom_label(x=3, y=0.15, label="n = 40",size=5, aes(fontface=3))+
  geom_hline(yintercept = 0.5,linetype="dotted")+
  geom_point()+
  geom_errorbar(aes(ymin=prob-SE,ymax=prob+SE))
```



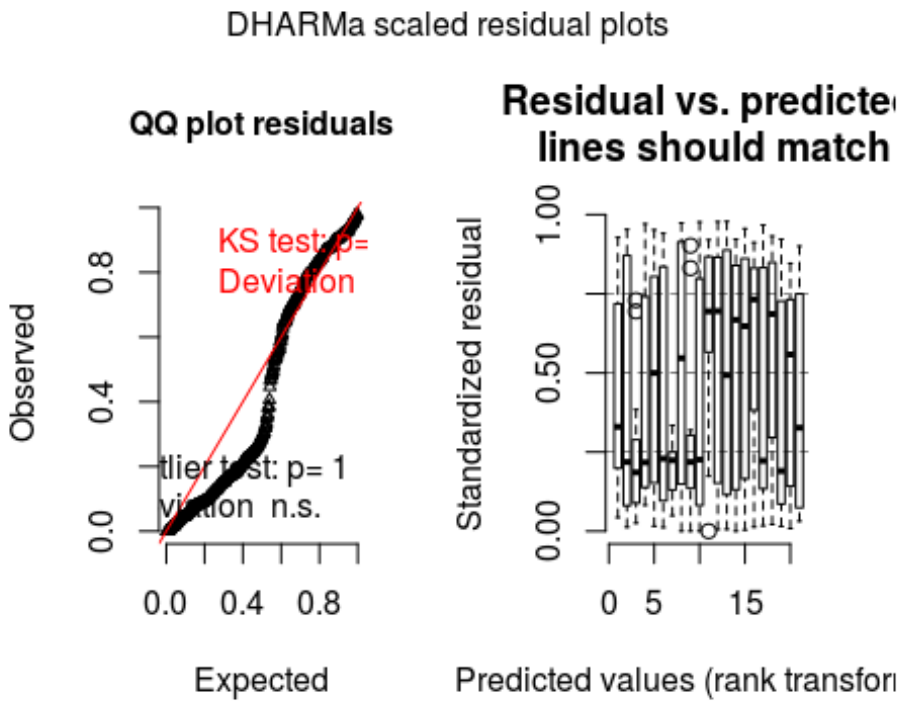
Pheromone deposition

I will look at the pheromone deposited on the way to the drop and back to the nest for each experiment across visits.

Cond1

To the drop

```
risksa$visit<-as.numeric(risksa$visit)

mpExp1<-glmer(phergo~visit*mol+(1|colony/antID),data=risksa,family="poisson",
              glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
100000)))
## boundary (singular) fit: see ?isSingular
simres<-simulateResiduals(mpExp1) #standard seed for random values is 123
plot(simres, asFactor=T)
```



the model is zero inflated. let's remodel

```
mpExp1 <- zeroinfl(phergo ~ visit*mol + 1 | colony/antID, data = risksa)
## Error in optim(fn = loglikfun, gr = gradfun, par = c(start$count,
start$zero, : valore non finito fornito da optim
```

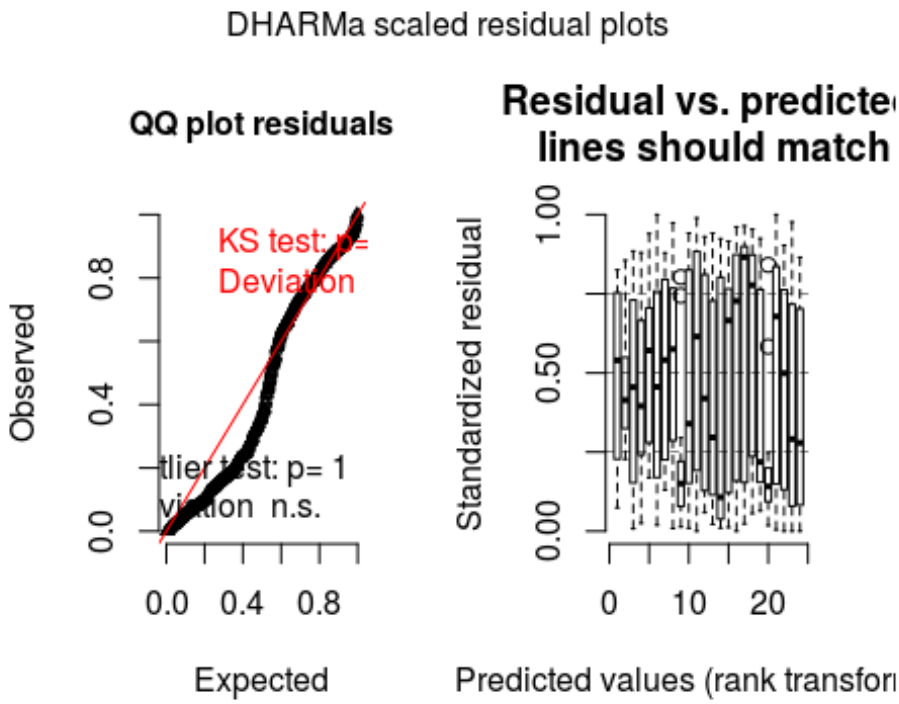
does not work. I will remove colony from random effect.

```
mpExp1 <- zeroinfl(phergo ~ visit*mol + 1 | antID, data = risksa)

Anova(mpExp1)
## Analysis of Deviance Table (Type II tests)
##
## Response: phergo
##           Df   Chisq Pr(>Chisq)
## visit      1  1.7587  0.1847906
## mol        2 12.9922  0.0015093 **
## visit:mol  2 14.4692  0.0007212 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
meanobj<-emmeans(mpExp1,~visit*mol,type="response")
contrast(meanobj,list(mol0.1vs0.55=c(1,-1,0),
                      mol0.1vs1.0=c(1,0,-1),
                      mol0.55vs1.0=c(0,1,-1),
                      SafeVsRisky=c(-0.5,1,-0.5)),
         adjust="bonferroni")
```

```
##  contrast     estimate    SE  df z.ratio p.value
##  mol0.1vs0.55   -0.339 0.224 Inf -1.508  0.5258
##  mol0.1vs1.0     0.319 0.243 Inf  1.311  0.7599
##  mol0.55vs1.0    0.657 0.227 Inf  2.891  0.0154
##  SafeVsRisky     0.498 0.190 Inf  2.616  0.0356
##
## Results are averaged over the levels of: antID
## P value adjustment: bonferroni method for 4 tests
risksap<-subset(risksa,risksa$visit<9) #just remove tests
risksap <- droplevels(risksap)

phg1<-ggplot(risksap,aes(x=visit,y=phergo, color=mol))+
  labs(title = "A")+
  scale_color_brewer(name="molarity", palette="Dark2")+
  ylab("Pheromone deposited to the feeder")+
  scale_x_discrete(limits=c(1,2,3,4,5,6,7,8))+
  ylim(0,20)+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black"),
        axis.text.y = element_text(size=12,colour="black"),
        axis.title.x = element_blank(),
        axis.title.y = element_text(size=14),
        plot.title = element_text(size=18),
        legend.position="none")+
  geom_jitter(width = 0.2,height=0)+
  geom_smooth()
```

Back to the nest

```
mpExp1<-glmer(pherbk~visit*mol+(1|colony/antID),data=risksa,family="poisson",
              glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
100000)))
simres<-simulateResiduals(mpExp1) #standard seed for random values is 123
plot(simres, asFactor=T)
```



the model is zero inflated. let's remodel

```
mpExp1 <- zeroinfl(pherbk ~ visit*mol + 1 | colony/antID, data = risksa)
## Error in optim(fn = loglikfun, gr = gradfun, par = c(start$count,
start$zero, : valore non finito fornito da optim
```

does not work. I will remove colony from random effect.

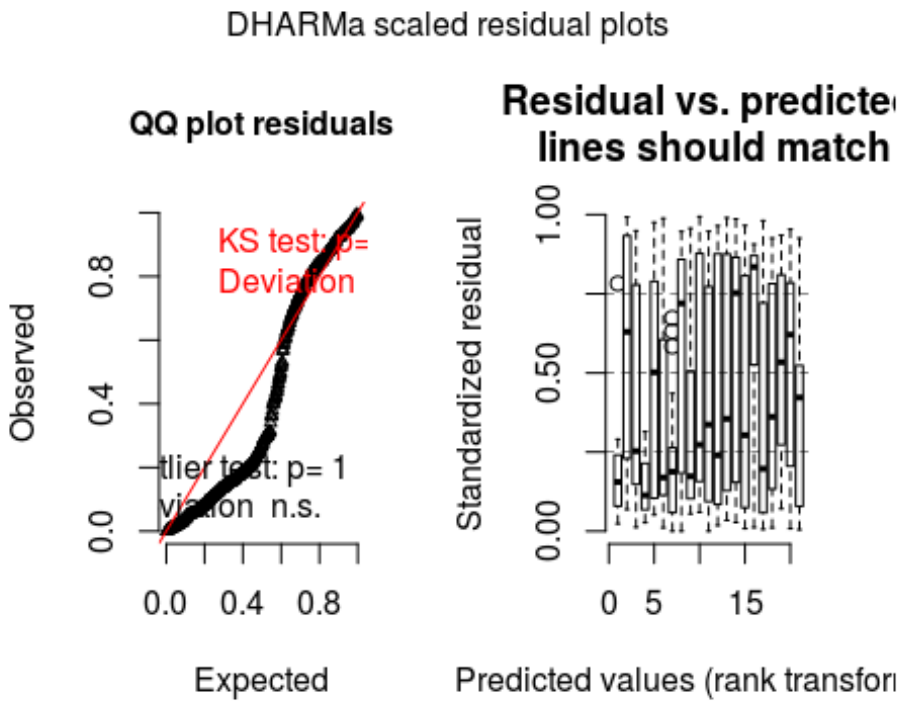
```
mpExp1 <- zeroinfl(pherbk ~ visit*mol + 1 | antID, data = risksa)

Anova(mpExp1)
## Analysis of Deviance Table (Type II tests)
##
## Response: pherbk
##           Df   Chisq Pr(>Chisq)
## visit      1  5.1128    0.02375 *
## mol        2 85.9726    < 2e-16 ***
## visit:mol  2  3.9549    0.13842
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
meanobj<-emmeans(mpExp1,~visit*mol,type="response")
contrast(meanobj,list(mol0.1vs0.55=c(1,-1,0),
                      mol0.1vs1.0=c(1,0,-1),
                      mol0.55vs1.0=c(0,1,-1),
                      SafeVsRisky=c(-0.5,1,-0.5)),
         adjust="bonferroni")
```

```
##  contrast     estimate    SE  df z.ratio p.value
##  mol0.1vs0.55   -2.670 0.154 Inf -17.352 <.0001
##  mol0.1vs1.0    -2.780 0.194 Inf -14.308 <.0001
##  mol0.55vs1.0   -0.111 0.185 Inf  -0.597 1.0000
##  SafeVsRisky     1.280 0.140 Inf   9.149 <.0001
##
## Results are averaged over the levels of: antID
## P value adjustment: bonferroni method for 4 tests
phb1<-ggplot(risksap,aes(x=visit,y=pherbk, color=mol))+
  labs(title = "D")+
  scale_color_brewer(name="molarity", palette="Dark2")+
  ylab("Pheromone deposited back to the nest")+
  scale_x_discrete(limits=c(1,2,3,4,5,6,7,8))+
  ylim(0,20)+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black"),
        axis.text.y = element_text(size=12,colour="black"),
        axis.title.x = element_text(size=14),
        axis.title.y = element_text(size=14),
        plot.title = element_text(size=18),
        legend.position="bottom")+
  geom_jitter(width = 0.2,height=0)+
  geom_smooth()
```

Cond2

To the drop

```
riskirr$visit<-as.numeric(riskirr$visit)

mpExp2<-glmer(phergo~visit*mol+(1|colony/antID),data=riskirr,family="poisson",
              glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
100000)))
simres<-simulateResiduals(mpExp2) #standard seed for random values is 123
plot(simres, asFactor=T)
```



the model is zero inflated. let's remodel

```
mpExp2 <- zeroinfl(phergo ~ visit*mol + 1 | colony/antID, data = riskirr)
## Error in optim(fn = loglikfun, gr = gradfun, par = c(start$count,
start$zero, : valore non finito fornito da optim
```

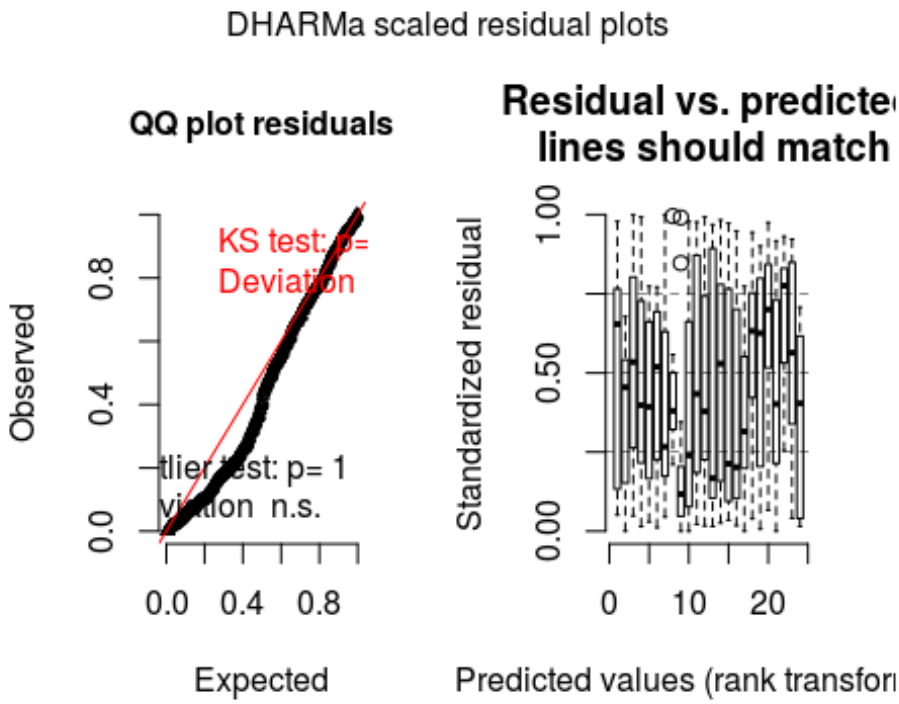
does not work. I will remove colony from random effect.

```
mpExp2 <- zeroinfl(phergo ~ visit*mol + 1 | antID, data = riskirr)

Anova(mpExp2)
## Analysis of Deviance Table (Type II tests)
##
## Response: phergo
##           Df  Chisq Pr(>Chisq)
## visit      1 0.2798    0.59680
## mol        2 7.4888    0.02365 *
## visit:mol  2 1.6650    0.43495
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
meanobj<-emmeans(mpExp2,~visit*mol,type="response")
contrast(meanobj,list(mol0.1vs0.55=c(1,-1,0),
                      mol0.1vs1.5=c(1,0,-1),
                      mol0.55vs1.5=c(0,1,-1),
                      SafeVsRisky=c(-0.5,1,-0.5)),
         adjust="bonferroni")
```

```
##  contrast     estimate    SE  df z.ratio p.value
##  mol0.1vs0.55   -0.174 0.229 Inf -0.760  1.0000
##  mol0.1vs1.5     0.323 0.265 Inf  1.217  0.8945
##  mol0.55vs1.5    0.497 0.233 Inf  2.131  0.1324
##  SafeVsRisky     0.336 0.189 Inf  1.771  0.3061
##
## Results are averaged over the levels of: antID
## P value adjustment: bonferroni method for 4 tests
riskirrp<-subset(riskirr,riskirr$visit<9) #just remove tests
riskirrp <- droplevels(riskirrp)

phg2<-ggplot(riskirrp,aes(x=visit,y=phergo, color=mol))+
  labs(title = "B")+
  scale_color_brewer(name="molarity", palette="Dark2")+
  ylab("Pheromone deposited to the feeder")+
  scale_x_discrete(limits=c(1,2,3,4,5,6,7,8))+
  ylim(0,20)+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black"),
        axis.text.y = element_text(size=12,colour="black"),
        axis.title.x = element_blank(),
        axis.title.y = element_blank(),
        plot.title = element_text(size=18),
        legend.position="none")+
  geom_jitter(width = 0.2,height=0)+
  geom_smooth()
```

Back to the nest

```
mpExp2<-glmer(pherbk~visit*mol+(1|colony/antID),data=riskirr,family="poisson",
              glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
100000)))
simres<-simulateResiduals(mpExp2) #standard seed for random values is 123
plot(simres, asFactor=T)
```



the model is zero inflated. let's remodel

```
mpExp2 <- zeroinfl(pherbk ~ visit*mol + 1 | colony/antID, data = riskirr)
## Error in optim(fn = loglikfun, gr = gradfun, par = c(start$count,
start$zero, : valore non finito fornito da optim
```

does not work. I will remove colony from random effect.

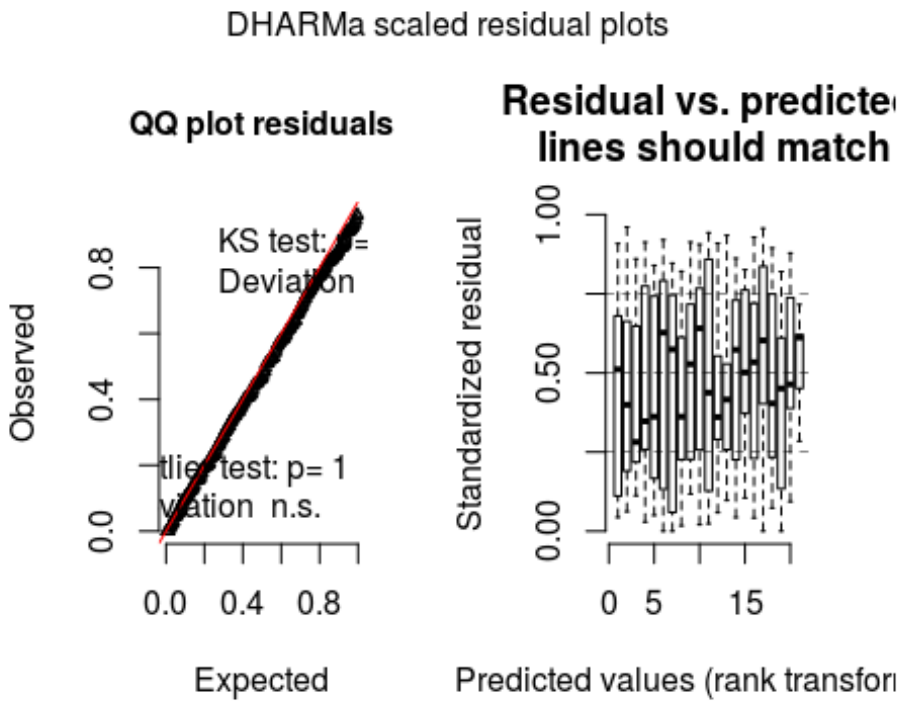
```
mpExp2 <- zeroinfl(pherbk ~ visit*mol + 1 | antID, data = riskirr)

Anova(mpExp2)
## Analysis of Deviance Table (Type II tests)
##
## Response: pherbk
##           Df   Chisq Pr(>Chisq)
## visit      1  10.249   0.001368 **
## mol        2 133.424  < 2.2e-16 ***
## visit:mol  2  11.339   0.003449 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
meanobj<-emmeans(mpExp2,~visit*mol,type="response")
contrast(meanobj,list(mol0.1vs0.55=c(1,-1,0),
                      mol0.1vs1.5=c(1,0,-1),
                      mol0.55vs1.5=c(0,1,-1),
                      SafeVsRisky=c(-0.5,1,-0.5)),
         adjust="bonferroni")
```

```
##  contrast     estimate    SE  df z.ratio p.value
##  mol0.1vs0.55   -2.684 0.170 Inf -15.742 <.0001
##  mol0.1vs1.5    -3.474 0.204 Inf -17.000 <.0001
##  mol0.55vs1.5   -0.790 0.191 Inf  -4.144 0.0001
##  SafeVsRisky     0.947 0.149 Inf   6.341 <.0001
##
## Results are averaged over the levels of: antID
## P value adjustment: bonferroni method for 4 tests
phb2<-ggplot(riskirrp,aes(x=visit,y=pherbk, color=mol ))+
  labs(title = "E")+
  scale_color_brewer(name="molarity", palette="Dark2")+
  ylab("Pheromone deposited back to the nest")+
  scale_x_discrete(limits=c(1,2,3,4,5,6,7,8))+
  ylim(0,20)+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black"),
        axis.text.y = element_text(size=12,colour="black"),
        axis.title.x = element_text(size=14),
        axis.title.y = element_blank(),
        plot.title = element_text(size=18),
        legend.position="bottom")+
  geom_jitter(width = 0.2,height=0)+
  geom_smooth()
```

Cond3

To the drop

```
riskgeo$visit<-as.numeric(riskgeo$visit)

mpExp3<-glmer(phergo~visit*mol+(1|colony/antID),data=riskgeo,family="poisson",
glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
simres<-simulateResiduals(mpExp3) #standard seed for random values is 123
plot(simres, asFactor=T)
```

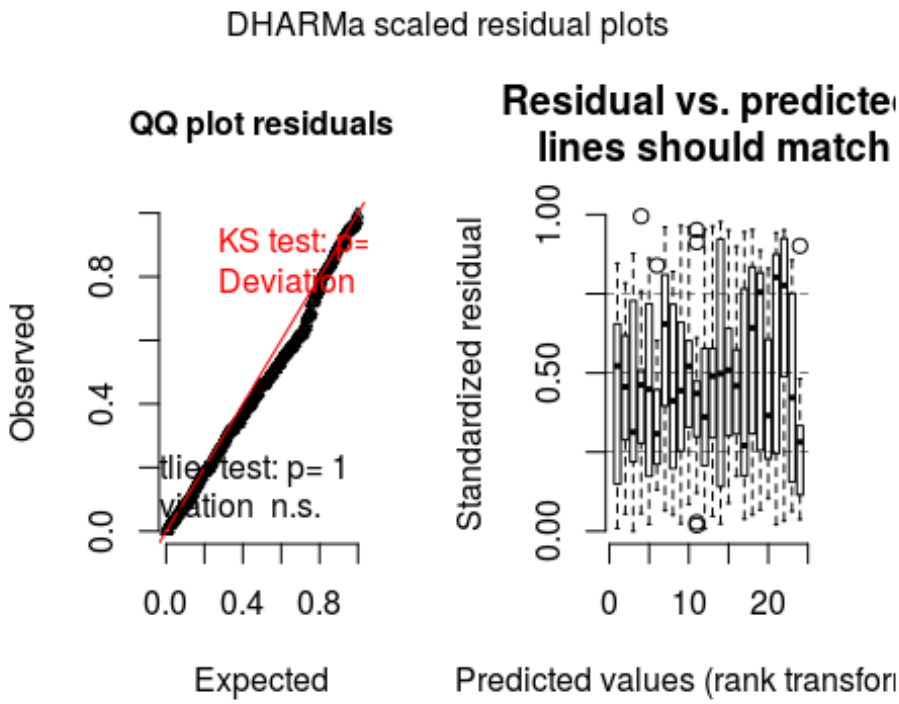



```
Anova(mpExp3)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: phergo
##             Chisq Df Pr(>Chisq)
## visit      0.2874  1  0.5918885
## mol       16.1336  2  0.0003138 ***
## visit:mol  3.7139  2  0.1561452
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
meanobj<-emmeans(mpExp3,~visit*mol,type="response")
contrast(meanobj,list(mol0.1vs0.3=c(1,-1,0),
                      mol0.1vs0.9=c(1,0,-1),
                      mol0.3vs0.9=c(0,1,-1),
                      SafeVsRisky=c(-0.5,1,-0.5)),
         adjust="bonferroni")
##  contrast     ratio    SE  df z.ratio p.value
##  mol0.1vs0.3  0.477 0.174 Inf -2.032  0.1687
##  mol0.1vs0.9  4.981 3.453 Inf  2.317  0.0821
##  mol0.3vs0.9 10.444 6.501 Inf  3.769  0.0007
##  SafeVsRisky  4.679 1.751 Inf  4.124  0.0001
##
## P value adjustment: bonferroni method for 4 tests
## Tests are performed on the log scale
riskgeop<-subset(riskgeo,riskgeo$visit<9) #just remove tests
riskgeop <- droplevels(riskgeop)
```

```
phg3<-ggplot(riskgeop,aes(x=visit,y=phergo, color=mol))+
  labs(title = "C")+
  scale_color_brewer(name="molarity", palette="Dark2")+
  ylab("Pheromone deposited to the feeder")+
  scale_x_discrete(limits=c(1,2,3,4,5,6,7,8))+
  ylim(0,20)+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black"),
        axis.text.y = element_text(size=12,colour="black"),
        axis.title.x = element_blank(),
        axis.title.y = element_blank(),
        plot.title = element_text(size=18),
        legend.position="none")+
  geom_jitter(width = 0.2,height=0)+
  geom_smooth()
```

Back to the nest

```
mpExp3<-glmer(pherbk~visit*mol+(1|colony/antID),data=riskgeo,family="poisson",
              glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
100000)))
simres<-simulateResiduals(mpExp3) #standard seed for random values is 123
plot(simres, asFactor=T)
```



the model is zero inflated. let's remodel

```
mpExp3 <- zeroinfl(pherbk ~ visit*mol + 1 | colony/antID, data = riskgeo)
## Error in optim(fn = loglikfun, gr = gradfun, par = c(start$count,
start$zero, : valore non finito fornito da optim
```

does not work. I will remove colony from random effect.

```
mpExp3 <- zeroinfl(pherbk ~ visit*mol + 1 |antID, data = riskgeo)

Anova(mpExp3)
## Analysis of Deviance Table (Type II tests)
##
## Response: pherbk
##           Df   Chisq Pr(>Chisq)
## visit      1  0.3297     0.5658
## mol        2 47.0827  5.972e-11 ***
## visit:mol  2  0.8738     0.6460
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
meanobj<-emmeans(mpExp3,~visit*mol,type="response")
contrast(meanobj,list(mol0.1vs0.3=c(1,-1,0),
                      mol0.1vs0.9=c(1,0,-1),
                      mol0.3vs0.9=c(0,1,-1),
                      SafeVsRisky=c(-0.5,1,-0.5)),
         adjust="bonferroni")
##  contrast    estimate    SE  df z.ratio p.value
##  mol0.1vs0.3   -0.882 0.144 Inf -6.144  <.0001
##  mol0.1vs0.9   -1.479 0.181 Inf -8.193  <.0001
##  mol0.3vs0.9   -0.597 0.165 Inf -3.615  0.0012
##  SafeVsRisky    0.142 0.126 Inf  1.134  1.0000
##
## Results are averaged over the levels of: antID
## P value adjustment: bonferroni method for 4 tests
phb3<-ggplot(riskgeop,aes(x=visit,y=pherbk, color=mol))+
  labs(title = "F")+
  scale_color_brewer(name="molarity", palette="Dark2")+
  ylab("Pheromone deposited back to the nest")+
  scale_x_discrete(limits=c(1,2,3,4,5,6,7,8))+
  ylim(0,20)+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black"),
        axis.text.y = element_text(size=12,colour="black"),
        axis.title.x = element_text(size=14),
        axis.title.y = element_blank(),
        plot.title = element_text(size=18),
        legend.position="bottom")+
  geom_jitter(width = 0.2,height=0)+
  geom_smooth(method="loess")
```

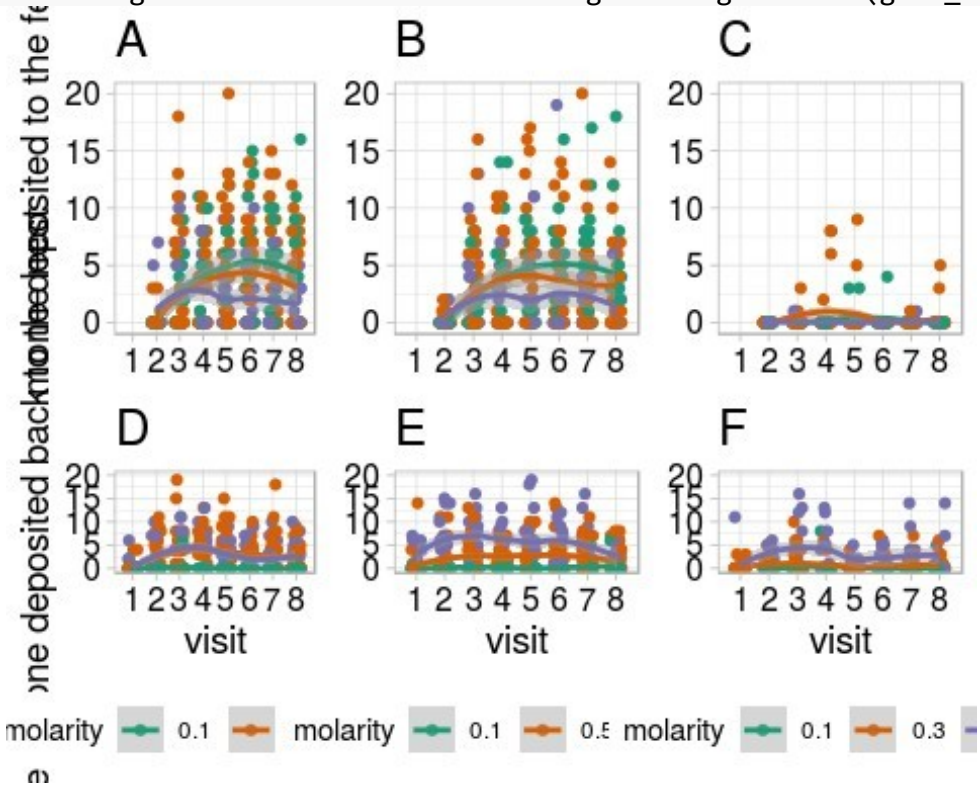
graph together

now I will plot the pheromone deposition all together for the three experiments

```
# Multiple plot function
#
# from: http://www.cookbook-r.com/Graphs/Multiple_graphs_on_one_page_(ggplot2)/
```

```
#
# ggplot objects can be passed in ..., or to plotlist (as a list of ggplot
objects)
# - cols:   Number of columns in layout
# - layout: A matrix specifying the layout. If present, 'cols' is ignored.
#
# If the layout is something like matrix(c(1,2,3,3), nrow=2, byrow=TRUE),
# then plot 1 will go in the upper left, 2 will go in the upper right, and
# 3 will go all the way across the bottom.
#
multiplot <- function(..., plotlist=NULL, file, cols=1, layout=NULL) {
  library(grid)

  # Make a list from the ... arguments and plotlist
  plots <- c(list(...), plotlist)

  numPlots = length(plots)

  # If layout is NULL, then use 'cols' to determine layout
  if (is.null(layout)) {
    # Make the panel
    # ncol: Number of columns of plots
    # nrow: Number of rows needed, calculated from # of cols
    layout <- matrix(seq(1, cols * ceiling(numPlots/cols)),
                     ncol = cols, nrow = ceiling(numPlots/cols))
  }

 if (numPlots==1) {
    print(plots[[1]])

  } else {
    # Set up the page
    grid.newpage()
    pushViewport(viewport(layout = grid.layout(nrow(layout), ncol(layout))))

    # Make each plot, in the correct location
    for (i in 1:numPlots) {
      # Get the i,j matrix positions of the regions that contain this subplot
      matchidx <- as.data.frame(which(layout == i, arr.ind = TRUE))

      print(plots[[i]], vp = viewport(layout.pos.row = matchidx$row,
                                      layout.pos.col = matchidx$col))
    }
  }
}

multiplot(phg1,phb1,phg2,phb2,phg3,phb3,cols=3)
## `geom_smooth()` using method = 'loess' and formula 'y ~ x'
## Warning: Removed 64 rows containing non-finite values (stat_smooth).
## Warning: Removed 64 rows containing missing values (geom_point).
## `geom_smooth()` using method = 'loess' and formula 'y ~ x'
```

```
## Warning: Removed 1 rows containing non-finite values (stat_smooth).
## Warning: Removed 1 rows containing missing values (geom_point).
## Warning: Removed 14 rows containing missing values (geom_smooth).
## `geom_smooth()` using method = 'loess' and formula 'y ~ x'
## Warning: Removed 69 rows containing non-finite values (stat_smooth).
## Warning: Removed 69 rows containing missing values (geom_point).
## Warning: Removed 1 rows containing missing values (geom_smooth).
## `geom_smooth()` using method = 'loess' and formula 'y ~ x'
## Warning: Removed 18 rows containing non-finite values (stat_smooth).
## Warning: Removed 18 rows containing missing values (geom_point).
## `geom_smooth()` using method = 'loess' and formula 'y ~ x'
## Warning: Removed 43 rows containing non-finite values (stat_smooth).
## Warning: Removed 43 rows containing missing values (geom_point).
## Warning: Removed 29 rows containing missing values (geom_smooth).
## Warning: Removed 5 rows containing non-finite values (stat_smooth).
## Warning: Removed 5 rows containing missing values (geom_point).
## Warning: Removed 16 rows containing missing values (geom_smooth).
```



Supplemental pilot experiment

Ant perception of 0.1,0.3,0.9

```
m0<-glmer(value~contrast*Visitnumber+(1|
AntID),data=ctrlmelted,family="binomial",
          glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000000)))
Anova(m0)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
```

```
## Response: value
##                       Chisq Df Pr(>Chisq)
## contrast             3.5857  1    0.05828 .
## Visitnumber          1.4604  1    0.22686
## contrast:Visitnumber 0.0242  1    0.87635
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

no difference between visits

```
m1<-glmer(value~contrast*variable+(1|Colony/
AntID),data=ctrlmelted,family="binomial",
          glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000000)))
## boundary (singular) fit: see ?isSingular
e<-emmeans(m1,~contrast*variable,type="response")
test(e,adjust="bonferroni")
##  contrast variable      prob     SE  df z.ratio p.value
##  0.1vs0.3 Firstchoice  0.860 0.0569 Inf 3.844   0.0005
##  0.3vs0.9 Firstchoice  0.648 0.0953 Inf 1.461   0.5763
##  0.1vs0.3 Secondchoice 0.870 0.0539 Inf 3.988   0.0003
##  0.3vs0.9 Secondchoice 0.724 0.0850 Inf 2.264   0.0942
##
## P value adjustment: bonferroni method for 4 tests
## Tests are performed on the logit scale
```

Discriminate three drops

```
m0<-glmer(value~Visitnumber+(1|Colony/AntID),data=melted,family="binomial",
          glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000000)))
Anova(m0)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: value
##              Chisq Df Pr(>Chisq)
## Visitnumber 4.5959  1    0.03205 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

difference between visits

```
m1<-glmer(value~variable+(1|Colony/AntID),data=melted,family="binomial",
          glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000000)))
Anova(m1)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: value
##           Chisq Df Pr(>Chisq)
## variable 0.1038  1     0.7473
```

no difference between first and last choice. I will just look at all the percentages together.

```
melted$Visitnumber<-as.factor(melted$Visitnumber)
m2<-glmer(value~variable*Visitnumber+(1|Colony/AntID),data=melted,family="binom
ial",
          glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000000)))
```

```
## Warning in checkConv(attr(opt, "derivs"), opt$par, ctrl = control$checkConv,
: Model is nearly unidentifiable: large eigenvalue ratio
##  - Rescale variables?
e<-emmeans(m2,~variable*Visitnumber,type="response")
e
##  variable     Visitnumber      prob         SE  df asymp.LCL asymp.UCL
##  Firstchoice  10          1.0000000 0.00000025 Inf 0.0000000 1.0000000
##  Secondchoice 10          1.0000000 0.00000048 Inf 0.0000000 1.0000000
##  Firstchoice  11          0.9580370 0.05009378 Inf 0.6650518 0.9962051
##  Secondchoice 11          0.9580370 0.05009390 Inf 0.6650502 0.9962052
##  Firstchoice  12          0.9580370 0.05009394 Inf 0.6650499 0.9962052
##  Secondchoice 12          0.9580370 0.05009397 Inf 0.6650495 0.9962052
##  Firstchoice  13          0.9580370 0.05009384 Inf 0.6650510 0.9962052
##  Secondchoice 13          0.9580370 0.05009396 Inf 0.6650496 0.9962052
##  Firstchoice  14          0.9074061 0.08321675 Inf 0.5844690 0.9855654
##  Secondchoice 14          0.8481657 0.11228549 Inf 0.5028421 0.9686049
##
## Confidence level used: 0.95
## Intervals are back-transformed from the logit scale
```

I have a 100% probability of choosing safe for the first trial, the percentage decrease with subsequent, but it remains very high.

fed on 1.5 risk in losses

Preliminary questions

first, I want to know if initial and final choice differ

```
fsdiff<-melt(risksa15, measure.vars = c("firstchoicesafe","endchoicesafe"))

mdiff<-glmer(value~variable+(1|colony/antID),data=fsdiff,family="binomial")
## boundary (singular) fit: see ?isSingular
Anova(mdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: value
##           Chisq Df Pr(>Chisq)
## variable 0.0952  1     0.7577
e<-emmeans(mdiff, ~variable, type="response")
pairs(e)
##  contrast                        odds.ratio    SE  df z.ratio p.value
##  firstchoicesafe / endchoicesafe        1.1 0.339 Inf 0.309   0.7577
##
## Tests are performed on the log odds ratio scale
```

there is no difference between primary and secondary choice, I will now on only use the primary for further analysis

now, I want to know if the visits differ from one another

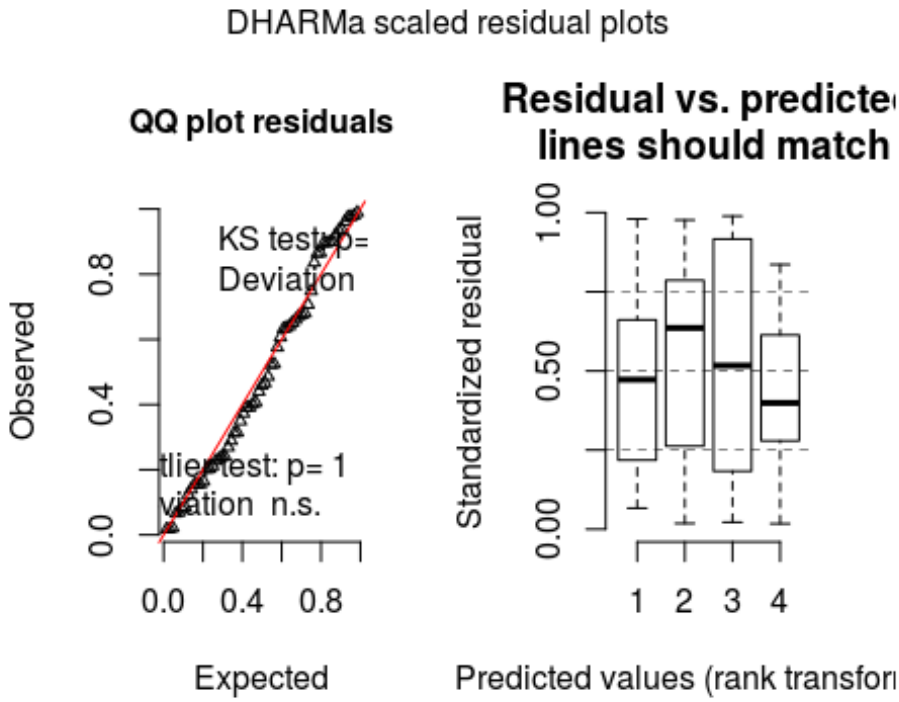
```
risksa15$visit<-as.numeric(risksa15$visit)
mvisdiff<-glmer(firstchoicesafe~visit+(1|colony/antID),data=risksa15,family="bi
nomial",
                glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
1000000000)))
## boundary (singular) fit: see ?isSingular
Anova(mvisdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: firstchoicesafe
##        Chisq Df Pr(>Chisq)
## visit 4.6318  1    0.03139 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
summary(mvisdiff)
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: binomial  ( logit )
## Formula: firstchoicesafe ~ visit + (1 | colony/antID)
##    Data: risksa15
## Control:
## glmerControl(optimizer = "bobyqa", optCtrl = list(maxfun = 1e+09))
##
##      AIC      BIC   logLik deviance df.resid
##    193.9    206.9    -93.0    185.9      185
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -2.4228  0.2194  0.2921  0.3888  0.8847
##
## Random effects:
##  Groups       Name        Variance Std.Dev.
##  antID:colony (Intercept) 2.385    1.544
##  colony       (Intercept) 0.000    0.000
## Number of obs: 189, groups:  antID:colony, 63; colony, 4
##
## Fixed effects:
##             Estimate Std. Error z value Pr(>|z|)
## (Intercept)   7.5727     2.7825   2.722   0.0065 **
## visit        -0.5722     0.2659  -2.152   0.0314 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##       (Intr)
## visit -0.991
## convergence code: 0
## boundary (singular) fit: see ?isSingular
```

the percentage of ants going for safe decreases with successive visits. this means that more and more ants after not finding the sugar drop start doing a random search. I will from now on only observe the first visit, being it a clearer indication of choice

modeling

now to the actual model. I drop antID because I kept only one observation for each ant

```
risksa15sing<-subset(risksa15,risksa15$visit==9)

mExp1<-glmer(firstchoicesafe~firstfeed*firstrisk+(1|
antID),data=risksa15sing,family="binomial",
             glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
1000000)))
## boundary (singular) fit: see ?isSingular
simres<-simulateResiduals(mExp1) #standard seed for random values is 123
plot(simres, asFactor=T)
```



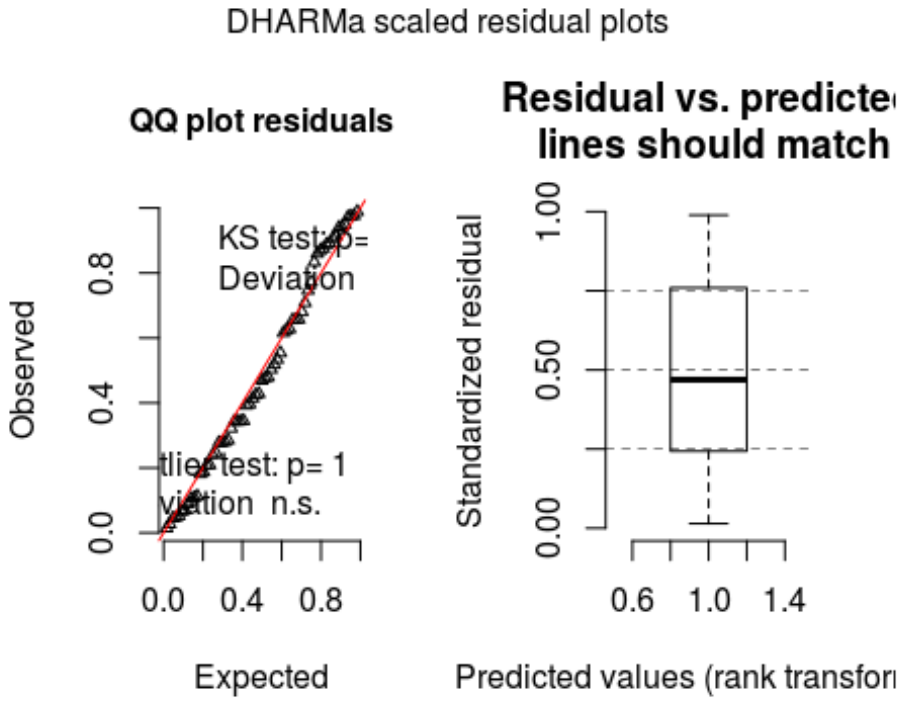
model is good here. it says nearly unidentifiable. probably I have complete separation of one data point, like 100% prob for one group. let's go on

```
Anova(mExp1)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: firstchoicesafe
##                       Chisq Df Pr(>Chisq)
```

```
## firstfeed           1.1779  1     0.2778
## firstrisk           0.9249  1     0.3362
## firstfeed:firstrisk 0.0010  1     0.9752
```

no effect of any of the factors, so I will redo the model without factors

```
mExp1<-glmer(firstchoicesafe~+(1|antID),data=risksa15sing,family="binomial",
             glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
1000000)))

simres<-simulateResiduals(mExp1) #standard seed for random values is 123
plot(simres, asFactor=T)
```

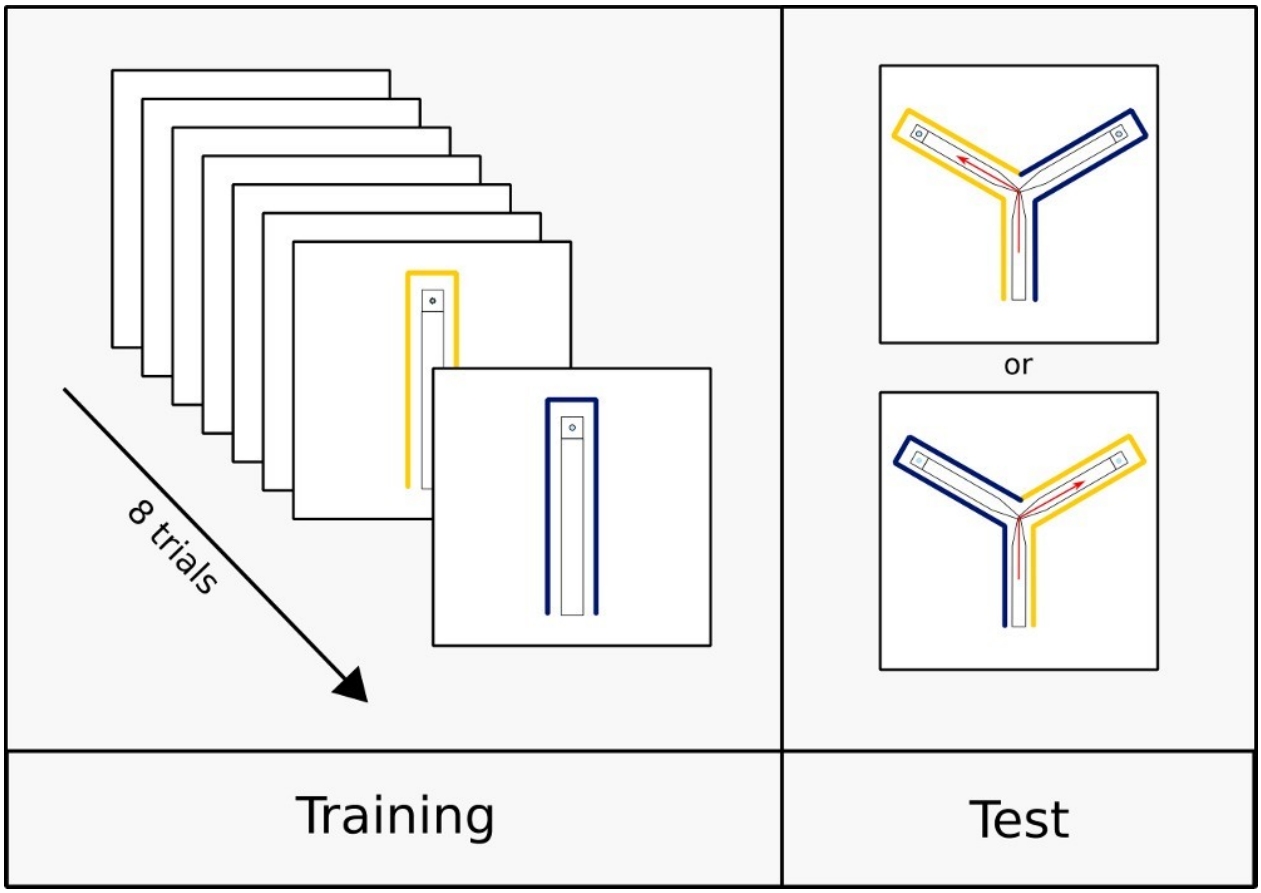



```
meanobj <- emmeans(mExp1,~1, type="response")
(test(meanobj))
##  1        prob    SE  df z.ratio p.value
##  overall 0.825 0.048 Inf 4.665   <.0001
##
## Tests are performed on the logit scale
```

ants prefer the safe **82%**.

Appendix 3 – Pilot experiment: colour discrimination learning in *Lasius niger*

Before we could test information integration in the main paper, we needed to test whether ants could learn to discriminate between two colours presented as background of the apparatus. In order to do this we designed a pilot experiment divided in a training phase and a test phase. We employed 16 ants coming from two different colonies. Marking procedure was identical to the one described in the main paper. A schematic representation of the experiment is available in the figure.



In the training phase each ant was let on a straight, 10cm long runway. The runway was surrounded by a coloured wall, that could either be blue or yellow. At the end of the runway we placed either a drop of 1.0M sucrose solution (S+) or a drop of water (Sn). For each ant, the association between reward and wall colour was constant (e.g. blue walls always predicted sucrose solution, yellow walls always water). We repeated the procedure for a total of 8 training visits.

To test the learned association, after the 8 training visits we let the ant onto a Y-maze. The walls around the stem of the maze had now two colours, blue on the left side and yellow on the right, while the walls around the left and right arm were now respectively completely blue and yellow (colour side was balanced between ants). If the ants had learned the colour-reward association we expected them to choose the arm with wall colour consistent with the rewarded one in the training phase.

81% (13/16) ants chose the correct arm as their first choice, a percentage significantly higher than chance level (GLMM post-hoc with estimated means, probability=0.8125, SE=0.098, z=2.289, p=0.022). For the complete analysis see Appendix 4.

Appendix 4 – Data analysis for the study: Multi-modal cues integration in the black garden ant

Setup

first I load packages

```
library(lme4)
## Loading required package: Matrix
library(DHARMa)
library(car)
## Loading required package: carData
library(emmeans)
library(reshape2)
library(ggplot2)
library(knitr)
library(pscl)
## Classes and Methods for R developed in the
## Political Science Computational Laboratory
## Department of Political Science
## Stanford University
## Simon Jackman
## hurdle and zeroinfl functions by Achim Zeileis
set.seed(123)#set seed for replicability in random simulations
```

Cond 1

Primary Choice

Preliminary questions

first, I want to know if primary and secondary choice differ I will ceck for both testing and training phases

```
fsdiff<-melt(EpisodicMemory, measure.vars = c("Firstbin","Lastbin"))

mdiff<-glmer(value~variable*VisitType+(1|Colony/antID),data=fsdiff,family="bino
mial", glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 1000000000)))
## boundary (singular) fit: see ?isSingular
Anova(mdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: value
##                      Chisq Df Pr(>Chisq)
## variable            0.1761  1     0.6747
## VisitType          44.5179  1   2.52e-11 ***
## variable:VisitType  0.3732  1     0.5413
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
e<-emmeans(mdiff, ~variable*VisitType, type="response")
pairs(e,simple="variable")
```

```
## VisitType = test:
##  contrast           odds.ratio    SE  df z.ratio p.value
##  Firstbin / Lastbin      1.227 0.347 Inf  0.725  0.4686
##
## VisitType = train:
##  contrast           odds.ratio    SE  df z.ratio p.value
##  Firstbin / Lastbin      0.955 0.285 Inf -0.155  0.8768
##
## Tests are performed on the log odds ratio scale
```

I find of course a difference between testing and training in the number of correct choices. I do not care about this for the moment, is the subsequent analysis. What is important is that for both training and testing primary and secondary choice do not differ. from now on I will use only primary choice.

now, I want to know if the visits differ from one another. here I will test separated testing and training. I could do a full model, but there is no reason to put everthing together here, it is just more complicated. Anyway I am sure that I will see an increase in correct choice for the training phase, as is of course expected. The one I am really interested in are the testing phase choices.

```
train<-subset(EpisodicMemory,EpisodicMemory$VisitType=="train")

mvisdiff<-glmer(Firstbin~VisitNumber+(1|Colony/antID),data=train,family="binomi
al", glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 1000000000)))
## boundary (singular) fit: see ?isSingular
Anova(mvisdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: Firstbin
##              Chisq Df Pr(>Chisq)
## VisitNumber 18.452  1  1.743e-05 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
summary(mvisdiff)
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: binomial  ( logit )
## Formula: Firstbin ~ VisitNumber + (1 | Colony/antID)
##    Data: train
## Control:
## glmerControl(optimizer = "bobyqa", optCtrl = list(maxfun = 1e+09))
##
##      AIC      BIC   logLik deviance df.resid
##    154.3    169.6    -73.2    146.3      333
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -4.3282  0.0677  0.1414  0.2733  1.2871
```

```
##
## Random effects:
##  Groups       Name        Variance Std.Dev.
##  antID:Colony (Intercept) 0.9787   0.9893
##  Colony       (Intercept) 0.0000   0.0000
## Number of obs: 337, groups:  antID:Colony, 31; Colony, 4
##
## Fixed effects:
##             Estimate Std. Error z value Pr(>|z|)
## (Intercept)   0.1844     0.5325   0.346    0.729
## VisitNumber   0.4907     0.1142   4.296 1.74e-05 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr)
## VisitNumber -0.771
## convergence code: 0
## boundary (singular) fit: see ?isSingular
```

As I expected, correct choices increase over time.

```
test<-subset(EpisodicMemory,EpisodicMemory$VisitType=="test")

mvisdiff<-glmer(Firstbin~scale(VisitNumber)+(1|Colony/antID),data=test,family="
binomial", glmerControl(optimizer="bobyqa", optCtrl = list(maxfun =
1000000000)))
## boundary (singular) fit: see ?isSingular
Anova(mvisdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: Firstbin
##                      Chisq Df Pr(>Chisq)
## scale(VisitNumber) 6.7707  1   0.009267 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
summary(mvisdiff)
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: binomial  ( logit )
## Formula: Firstbin ~ scale(VisitNumber) + (1 | Colony/antID)
##    Data: test
## Control:
## glmerControl(optimizer = "bobyqa", optCtrl = list(maxfun = 1e+09))
##
##      AIC      BIC   logLik deviance df.resid
##    146.0    158.3    -69.0    138.0      155
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.9304  0.1208  0.1981  0.3248  1.5876
##
```

```
## Random effects:
##  Groups       Name        Variance  Std.Dev.
##  antID:Colony (Intercept) 3.948e+00 1.987e+00
##  Colony       (Intercept) 8.236e-15 9.075e-08
## Number of obs: 159, groups:  antID:Colony, 32; Colony, 4
##
## Fixed effects:
##                     Estimate Std. Error z value Pr(>|z|)
## (Intercept)           2.3248     0.6011   3.867  0.00011 ***
## scale(VisitNumber)  -0.7018     0.2697  -2.602  0.00927 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr)
## scl(VstNmb) -0.281
## convergence code: 0
## boundary (singular) fit: see ?isSingular
```

I observe that the number of correct choices decreased over subsequent trials. This is probably due to the fact that over time more and more ants revert to random search, not finding food at the end of the chosen pole. From now on I will only keep first choice.

modeling

First of all want to see how many training trials the ants need to learn odour-reward association. I will have trial number as a factor in this case, because I am interested in every trial against chance level. This is because I want to know which is the earlyest trial in which the ants ants have learned.

```
train$VisitNumber<-as.factor(train$VisitNumber)

mExptrain<-glmer(Firstbin~VisitNumber+(1|Colony/antID),data=train,family="binom
ial", glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 1000000)))
## boundary (singular) fit: see ?isSingular
```

not converging. I will drop colony from random effects

```
mExptrain<-glmer(Firstbin~VisitNumber+(1|antID),data=train,family="binomial",
glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 1000000)))
Anova(mExptrain)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: Firstbin
##               Chisq Df Pr(>Chisq)
## VisitNumber 19.668 10    0.03255 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

there is indeed a difference between visits. let’s test each one against chance

```
meanobj <- emmeans(mExptrain,~VisitNumber, type="response")
(test(meanobj, adjust="bonferroni"))
##  VisitNumber      prob         SE  df z.ratio p.value
##  2           0.6839396 0.10256300 Inf 1.627   1.0000
##  3           0.8309868 0.07673042 Inf 2.915   0.0391
##  4           0.9767330 0.02466426 Inf 3.443   0.0063
##  5           0.9513316 0.03763521 Inf 3.657   0.0028
##  6           0.9596376 0.03345344 Inf 3.669   0.0027
##  7           1.0000000 0.00000040 Inf 0.119   1.0000
##  8           0.9596376 0.03345358 Inf 3.669   0.0027
##  9           0.9812597 0.02106635 Inf 3.455   0.0061
##  10          0.9812596 0.02106652 Inf 3.455   0.0061
##  11          1.0000000 0.00000044 Inf 0.105   1.0000
##  12          1.0000000 0.00000043 Inf 0.106   1.0000
##
## P value adjustment: bonferroni method for 11 tests
## Tests are performed on the logit scale
```

In the second trial already 68% of the ants choose correct. this is however not significant. At the third trial 83% choose correct and the level is maintained high trough all the experiment. In some trials the percentage of correct is even 100%.

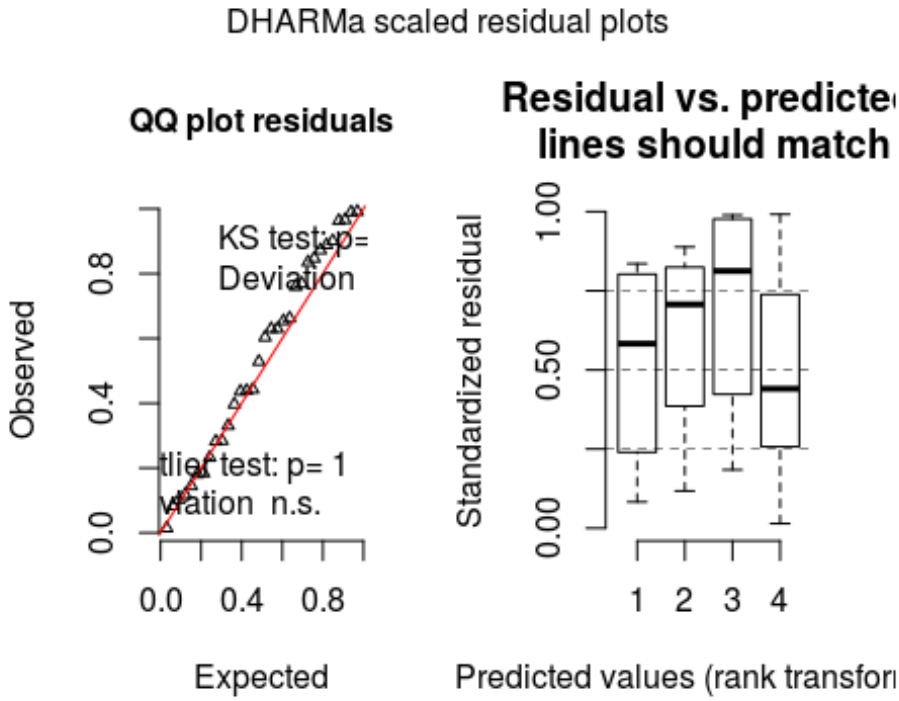
```
toplot1<-as.data.frame(meanobj) #I am just saving this for future plot
```

now to the actual model. I drop antID because I kept only one observation for each ant

```
sing<-subset(EpisodicMemory,EpisodicMemory$VisitNumber==13)

mExp<-glmer(Firstbin~VisitColor*CorrectSide*CorrectSmell+(1|
Colony),data=sing,family="binomial", glmerControl(optimizer="bobyqa", optCtrl =
list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
```

too many factors to use in the random model. I will try removing random effect just to see if there is any important effect to consider.

```
mExp<-
glm(Firstbin~VisitColor*CorrectSide*CorrectSmell,data=sing,family="binomial")
simres<-simulateResiduals(mExp) #standard seed for random values is 123
plot(simres, asFactor=T)
```



model is good here

```
Anova(mExp)
## Warning: glm.fit: fitted probabilities numerically 0 or 1 occurred

## Warning: glm.fit: fitted probabilities numerically 0 or 1 occurred

## Warning: glm.fit: fitted probabilities numerically 0 or 1 occurred
## Analysis of Deviance Table (Type II tests)
##
## Response: Firstbin
##                                    LR Chisq Df Pr(>Chisq)
## VisitColor                           0.0000  1    1.00000
## CorrectSide                          1.3814  1    0.23986
## CorrectSmell                         6.5115  1    0.01072 *
## VisitColor:CorrectSide               2.0708  1    0.15014
## VisitColor:CorrectSmell              0.0000  1    0.99997
## CorrectSide:CorrectSmell             0.0000  1    0.99995
## VisitColor:CorrectSide:CorrectSmell  0.0000  1    0.99989
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

no differences between colors or sides. I find a difference between the correct smells. Will redo the model inserting again the random effect.

```
mExp<-glmer(Firstbin~CorrectSmell+(1|Colony),data=sing,family="binomial",
glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
```

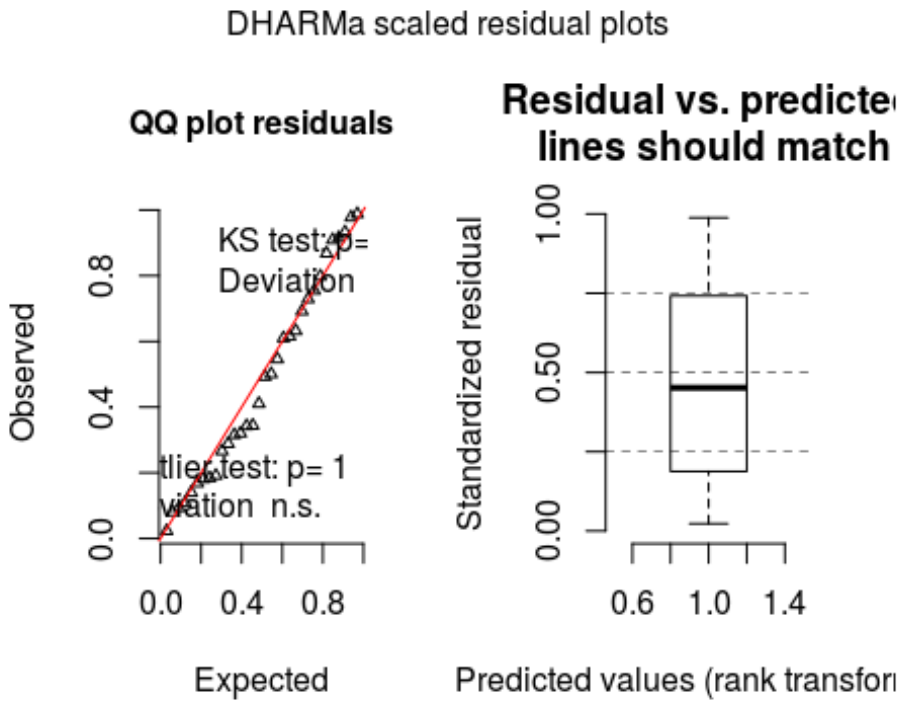
model is not working again. Hessian is numerically singular. this means that for one of the two scents I either have 100% correct or 0%correct.

Let's look at the data.

```
tapply(sing$Firstbin,sing$CorrectSmell,mean)
##    lemon rosemary
##     0.75     1.00
```

here is the problem. 100% of ants were correct with rosemary, 75% with lemon. now we have observed this difference, which however does not seem so crucial having just 16 subjects per smell. I will just do the model as a whole and test the overall performance against chance level.

```
mExp<-glmer(Firstbin~(1|Colony),data=sing,family="binomial",
glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
simres<-simulateResiduals(mExp) #standard seed for random values is 123
plot(simres, asFactor=T)
```



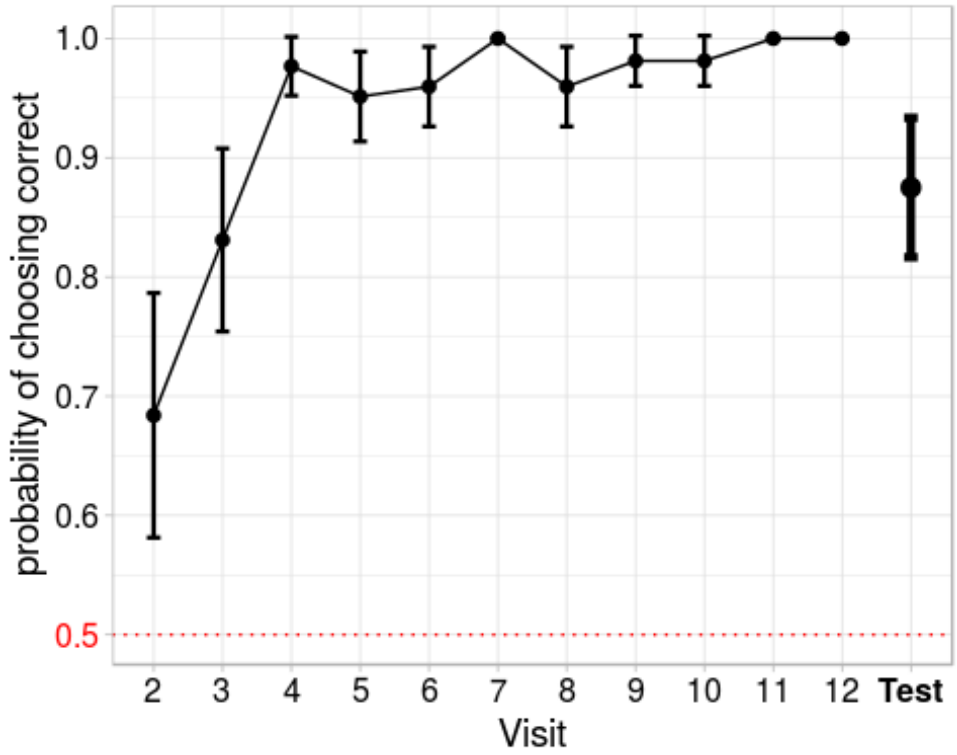

```
meanobj <- emmeans(mExp,~1, type="response")
toplot2<-as.data.frame(meanobj) #again as before, saving for plot
kable(test(meanobj))
```

87% of ants choose accordingly to background color*side when scent information is not available anymore.

Plotting

I want to plot probability of correct choice both for training and for testing

```
toplot <-data.frame(Visit=c(seq(2,12),"Test"),
                    group=c(rep("train",11),"test"),
                    prob=c(toplot1$prob,toplot2$prob),
                    SE=c(toplot1$SE,toplot2$SE))
toplot$Visit = factor(toplot$Visit,
levels=c("2","3","4","5","6","7","8","9","10","11","12","Test"))
ggplot(toplot,aes(x=Visit,y=prob,group=group))+
  geom_point(size=c(rep(2,11),3))+
  geom_line()+
  geom_errorbar(aes(ymin=prob-
SE,ymax=prob+SE),width=0.2,size=c(rep(0.8,11),1.5))+
  geom_hline(yintercept=0.5,linetype="dotted",color="red")+
  scale_y_continuous(breaks=seq(0.3,1.1,0.1))+
  ylab("probability of choosing correct")+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black",face=c(rep(1,11),2)),
        axis.text.y =
element_text(size=12,colour=c("red","black","black","black","black","black")),
        axis.title.x = element_text(size=14),
        axis.title.y = element_text(size=14),
        plot.title = element_text(size=18))
```

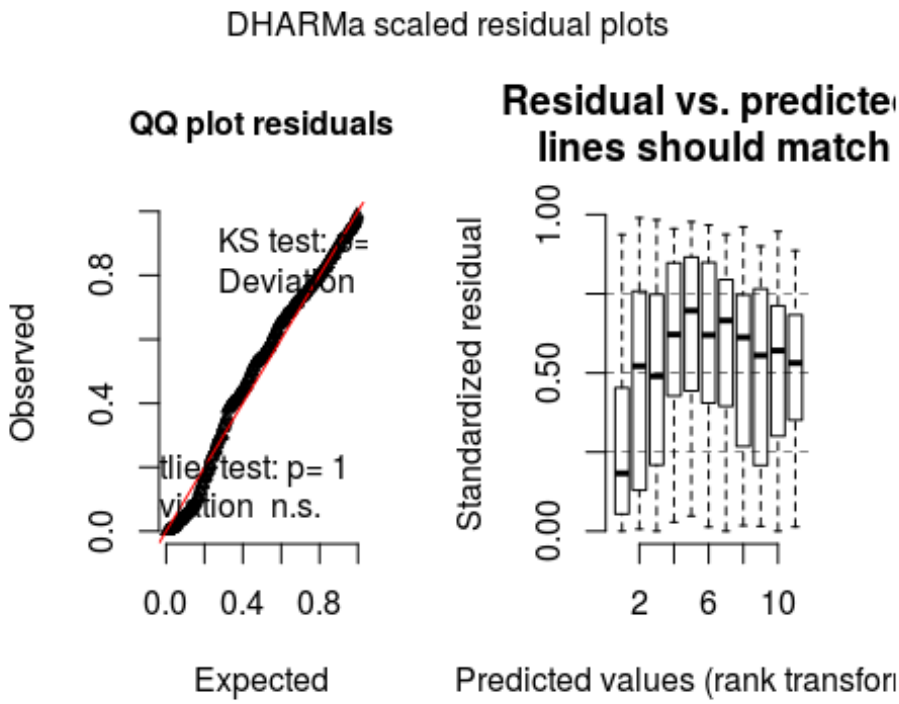


Pheromone deposition

I will look at the pheromone deposited on the way to the drop and back to the nest across visits.

To the drop

```
mpExp<-glmer(pherGo~scale(VisitNumber)+(1|Colony/
antID),data=EpisodicMemory,family="poisson", glmerControl(optimizer="bobyqa",
optCtrl = list(maxfun = 100000)))
simres<-simulateResiduals(mpExp) #standard seed for random values is 123
plot(simres, asFactor=T)
```



the model is fine

```
Anova(mpExp)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: pherGo
##                      Chisq Df Pr(>Chisq)
## scale(VisitNumber) 32.222  1  1.375e-08 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
summary(mpExp)
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula: pherGo ~ scale(VisitNumber) + (1 | Colony/antID)
##    Data: EpisodicMemory
## Control:
## glmerControl(optimizer = "bobyqa", optCtrl = list(maxfun = 1e+05))
##
##      AIC      BIC   logLik deviance df.resid
##   1424.0   1439.2   -708.0   1416.0      322
```

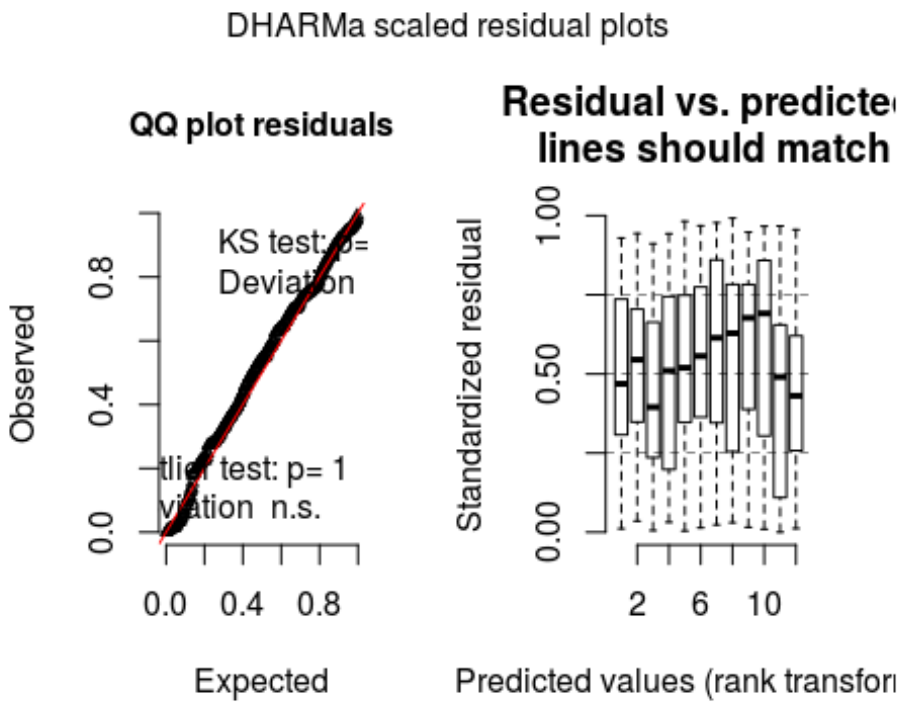
```
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.9952 -0.7407 -0.0352  0.6314  3.4016
##
## Random effects:
##  Groups       Name        Variance Std.Dev.
##  antID:Colony (Intercept) 0.25985  0.5098
##  Colony       (Intercept) 0.05697  0.2387
## Number of obs: 326, groups:  antID:Colony, 31; Colony, 4
##
## Fixed effects:
##                     Estimate Std. Error z value Pr(>|z|)
## (Intercept)          1.31446    0.15475   8.494  < 2e-16 ***
## scale(VisitNumber)  0.24849    0.04378   5.676 1.38e-08 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr)
## scl(VstNmb) 0.086
train$VisitNumber<-as.numeric(train$VisitNumber)

phg1<-ggplot(train,aes(x=VisitNumber,y=pherGo))+
  labs(title = "A")+
  ylab("Pheromone deposited to the drop")+
  scale_x_discrete(limits=c(1,2,3,4,5,6,7,8,9,10,11,12))+
  ylim(0,15)+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black"),
        axis.text.y = element_text(size=12,colour="black"),
        axis.title.x = element_text(size=14),
        axis.title.y = element_text(size=14),
        plot.title = element_text(size=18),
        legend.position="none")+
  geom_jitter(width = 0.2,height=0)+
  geom_smooth()
```

there is a general increase of pheromone deposited towards the drop across visits. this has to be expected, given the fact that ants learn the associations ad so are more able to predict the presence of a drop.

Back to the nest

```
mpExp<-glmer(pherBk~scale(VisitNumber)+(1|Colony/
antID),data=EpisodicMemory,family="poisson", glmerControl(optimizer="bobyqa",
optCtrl = list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
```

```
simres<-simulateResiduals(mpExp) #standard seed for random values is 123
plot(simres, asFactor=T)
```



the model is fine

```
Anova(mpExp)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: pherBk
##                     Chisq Df Pr(>Chisq)
## scale(VisitNumber) 10.54  1   0.001168 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
summary(mpExp)
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula: pherBk ~ scale(VisitNumber) + (1 | Colony/antID)
##    Data: EpisodicMemory
## Control:
## glmerControl(optimizer = "bobyqa", optCtrl = list(maxfun = 1e+05))
##
##      AIC      BIC   logLik deviance df.resid
##   1382.6   1397.6   -687.3   1374.6      309
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.32143 -0.73026 -0.04768  0.62410  2.90110
##
```

```
## Random effects:
##  Groups       Name        Variance Std.Dev.
##  antID:Colony (Intercept) 0.2033   0.4509
##  Colony       (Intercept) 0.0000   0.0000
## Number of obs: 313, groups:  antID:Colony, 31; Colony, 4
##
## Fixed effects:
##                     Estimate Std. Error z value Pr(>|z|)
## (Intercept)          1.24118    0.08977  13.826  < 2e-16 ***
## scale(VisitNumber) -0.13216    0.04071  -3.247  0.00117 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr)
## scl(VstNmb) 0.235
## convergence code: 0
## boundary (singular) fit: see ?isSingular
phb1<-ggplot(train,aes(x=VisitNumber,y=pherBk))+
  labs(title = "B")+
  ylab("Pheromone deposited back to the nest")+
  scale_x_discrete(limits=c(1,2,3,4,5,6,7,8,9,10,11,12))+
  ylim(0,15)+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black"),
        axis.text.y = element_text(size=12,colour="black"),
        axis.title.x = element_text(size=14),
        axis.title.y = element_text(size=14),
        plot.title = element_text(size=18),
        legend.position="bottom")+
  geom_jitter(width = 0.2,height=0)+
  geom_smooth()
```

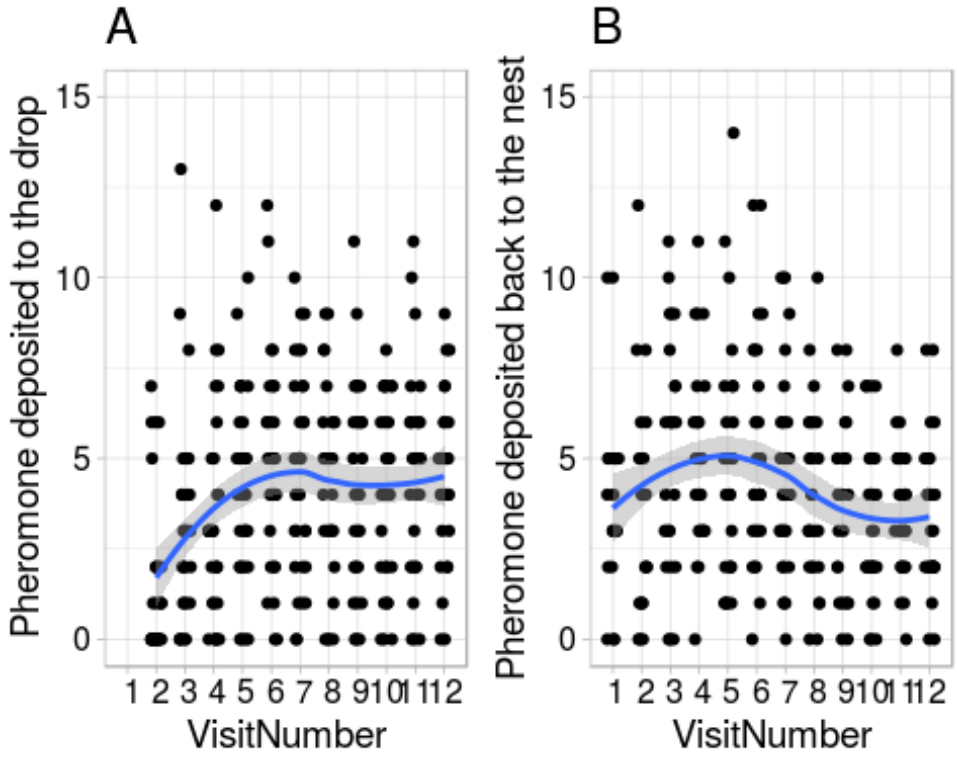
on the countrary, here I observe a general decrease in pheromone deposited back to the nest. This is probably due to a general habituation of the ants, that after having deposited much pheromone in subsequent vists decrease the general deposition.

I will plot the two together.

graph together

```
library(cowplot)
##
## Attaching package: 'cowplot'
## The following object is masked from 'package:ggplot2':
##
##     ggsave
plot_grid(phg1, phb1, nrow=1)
## `geom_smooth()` using method = 'loess' and formula 'y ~ x'
## Warning: Removed 58 rows containing non-finite values (stat_smooth).
## Warning: Removed 58 rows containing missing values (geom_point).
```

```
## `geom_smooth()` using method = 'loess' and formula 'y ~ x'
## Warning: Removed 71 rows containing non-finite values (stat_smooth).
## Warning: Removed 71 rows containing missing values (geom_point).
```



Cond 2

Primary Choice

Preliminary questions

first, I want to know if primary and secondary choice differ I will ceck for both testing and training phases

```
EpisodicMemoryPretraining<-read.csv("EpisodicMemoryPretraining.csv")
fsdiff<-melt(EpisodicMemoryPretraining, measure.vars = c("Firstbin","Lastbin"))

mdiff<-glmer(value~variable*VisitType+(1|Colony/antID),data=fsdiff,family="bino
mial", glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 1000000000)))
## boundary (singular) fit: see ?isSingular
Anova(mdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: value
##                       Chisq Df Pr(>Chisq)
## variable             0.1208  1     0.7282
## VisitType           25.1271  1  5.367e-07 ***
## variable:VisitType   0.0012  1     0.9718
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
e<-emmeans(mdiff, ~variable*VisitType, type="response")
pairs(e,simple="variable")
## VisitType = test:
##  contrast           odds.ratio       SE  df z.ratio p.value
##  Firstbin / Lastbin       1.13 3.93e-01 Inf  0.348  0.7282
##
## VisitType = Ytrain:
##  contrast           odds.ratio       SE  df z.ratio p.value
##  Firstbin / Lastbin       0.00 8.20e-06 Inf -0.035  0.9720
##
## Tests are performed on the log odds ratio scale
```

I find again a difference between testing and training in the number of correct choices. As before I ignore it.no difference between primary and secondary choice. from now on I will use only primary choice.

now, I want to know if the visits differ from one another I test separated testing and training. Be aware that this time training does not contain all the visits, but just 7 and 8, as only those two are Y mazes

```
train<-
subset(EpisodicMemoryPretraining,EpisodicMemoryPretraining$VisitType=="Ytrain")

mvisdiff<-glmer(Firstbin~VisitNumber+(1|Colony/antID),data=train,family="binomi
al", glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 1000000000)))
## boundary (singular) fit: see ?isSingular
Anova(mvisdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: Firstbin
##             Chisq Df Pr(>Chisq)
## VisitNumber     0  1          1
```

Absolutely no difference between visit 7 and 8. either they did not learned at all, or they learned and is very solid and stable. Will see in next analysis block.

```
test<-
subset(EpisodicMemoryPretraining,EpisodicMemoryPretraining$VisitType=="test")

mvisdiff<-glmer(Firstbin~VisitNumber+(1|Colony/antID),data=test,family="binomia
l", glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 1000000000)))
## boundary (singular) fit: see ?isSingular
Anova(mvisdiff)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: Firstbin
##              Chisq Df Pr(>Chisq)
## VisitNumber 0.0126  1     0.9106
```

Here i see no change across visits. this is a bit strange, because i should see a decrease in percentage if the ants have learned, given the fact that there is no reward provided. This may suggest a random choice, or on the other hand a very robust choice. I will still keep only first trial for consistency with the other experiment.

modeling

First, 2 Y maze training trials to assess if the ants have learned smell-food association

```
train$VisitNumber<-as.factor(train$VisitNumber)

mExptrain<-glmer(Firstbin~VisitNumber+(1|Colony/antID),data=train,family="binom
ial", glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
meanobj <- emmeans(mExptrain,~VisitNumber, type="response")
toplot1<-as.data.frame(meanobj)
(test(meanobj, adjust="bonferroni"))
##  VisitNumber  prob     SE  df z.ratio p.value
##  7           0.969 0.0308 Inf 3.380   0.0015
##  8           0.969 0.0308 Inf 3.380   0.0015
##
## P value adjustment: bonferroni method for 2 tests
## Tests are performed on the logit scale
```

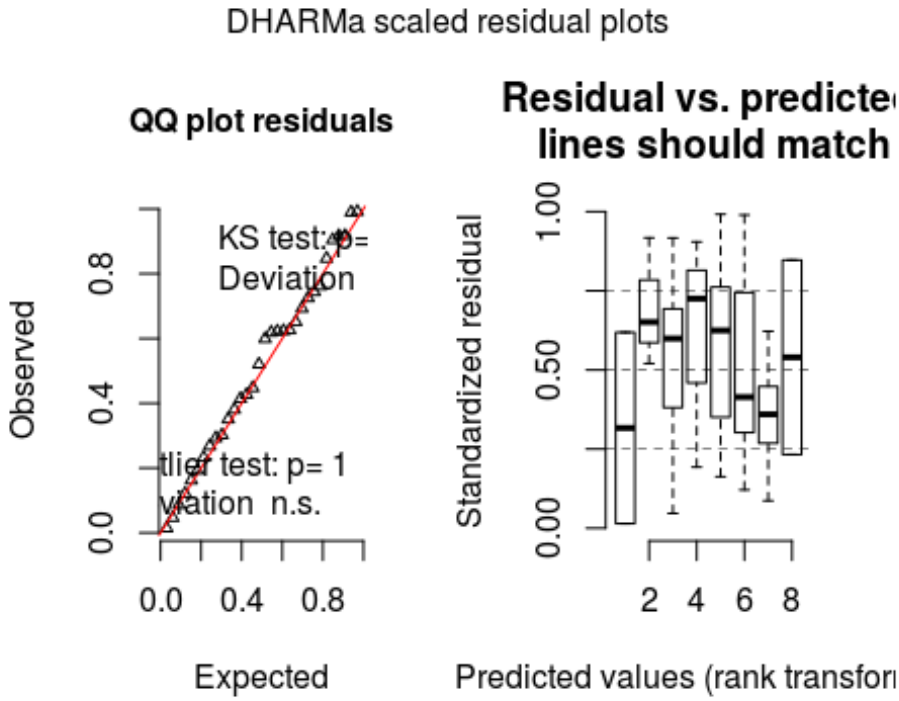
in both trials 96% of ants choose the correct smell. they clearly have learned

now to the model for testing. I drop antID because I kept only one observation for each ant

```
sing<-
subset(EpisodicMemoryPretraining,EpisodicMemoryPretraining$VisitNumber==9)

mExp<-glmer(Firstbin~VisitColor*CorrectSide*CorrectSmell+(1|
Colony),data=sing,family="binomial", glmerControl(optimizer="bobyqa", optCtrl =
list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
```

too many factors to use in the random model. I will try removing random effect just to see if there is any important effect to consider.

```
mExp<-
glm(Firstbin~VisitColor*CorrectSide*CorrectSmell,data=sing,family="binomial")
simres<-simulateResiduals(mExp) #standard seed for random values is 123
plot(simres, asFactor=T)
```

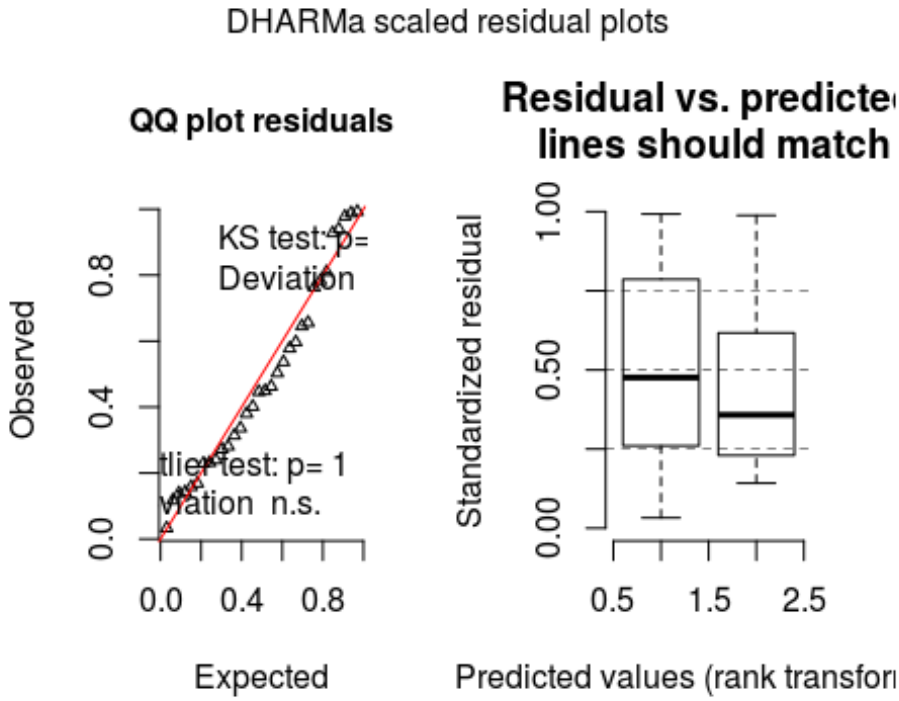


model is good here

```
Anova(mExp)
## Analysis of Deviance Table (Type II tests)
##
## Response: Firstbin
##                                      LR Chisq Df Pr(>Chisq)
## VisitColor                             0.3571  1     0.5501
## CorrectSide                            4.2917  1     0.0383 *
## CorrectSmell                           1.6404  1     0.2003
## VisitColor:CorrectSide                 0.0646  1     0.7993
## VisitColor:CorrectSmell                1.9750  1     0.1599
## CorrectSide:CorrectSmell               2.4969  1     0.1141
## VisitColor:CorrectSide:CorrectSmell    0.0000  1     1.0000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

I see an effect of side. put it back in the model.

```
mExp<-glmer(Firstbin~CorrectSide+(1|Colony),data=sing,family="binomial",
glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
simres<-simulateResiduals(mExp) #standard seed for random values is 123
plot(simres, asFactor=T)
```




```
meanobj <- emmeans(mExp,~CorrectSide, type="response")
(test(meanobj,adjust="bonferroni"))
##  CorrectSide  prob    SE  df z.ratio p.value
##  left        0.814 0.101 Inf 2.220   0.0529
##  right       0.500 0.127 Inf 0.000   1.0000
##
## P value adjustment: bonferroni method for 2 tests
## Tests are performed on the logit scale
```

when the correct is on the left they slightly do more correct choices (n.s.). when is on the right they go completly random. This has to be expected, because ants have a bias towards left side, that they follow when they have no other reliable information (e.g. memory). ants have not learned the task. Will do general probability that I will write in the main paper, as it is the main result.

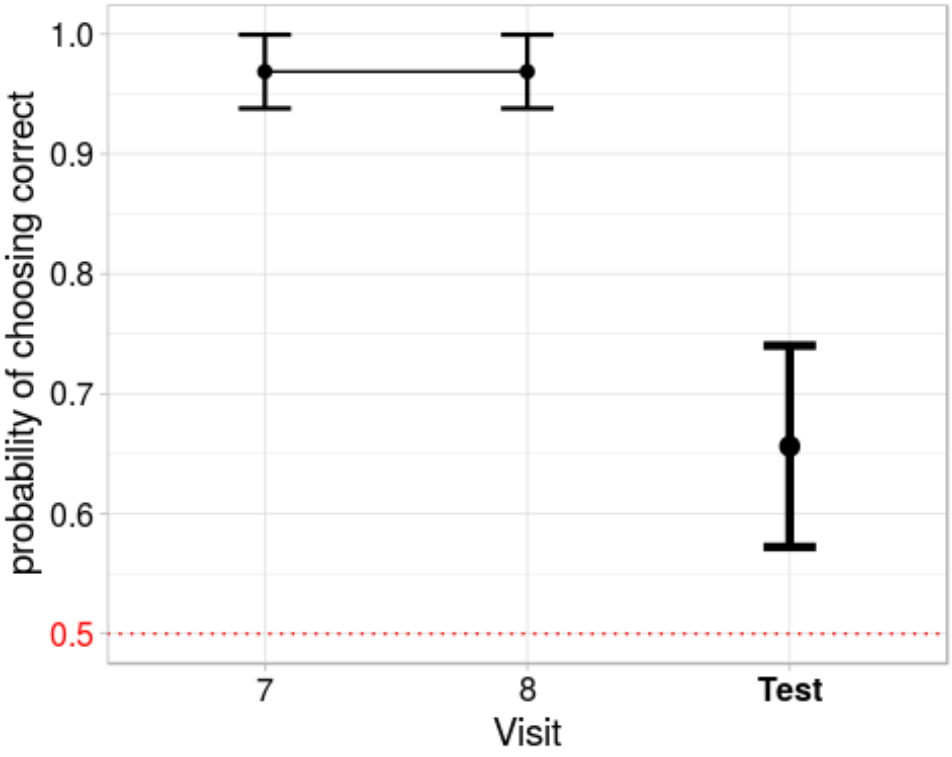
```
mExp<-glmer(Firstbin~1|Colony,data=sing,family="binomial",
glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
meanobj <- emmeans(mExp,~1, type="response")
toplot2<-as.data.frame(meanobj)
(test(meanobj))
##  1        prob    SE  df z.ratio p.value
##  overall 0.656 0.084 Inf 1.737   0.0823
##
## Tests are performed on the logit scale
```

65% of ants choose correclty

Plotting

I want to plot probability of correct choice both for training and for testing

```
toplot <-data.frame(Visit=c(7,8,"Test"),
                    group=c("train","train","test"),
                    prob=c(toplot1$prob,toplot2$prob),
                    SE=c(toplot1$SE,toplot2$SE))
toplot$Visit = factor(toplot$Visit, levels=c("7","8","Test"))
ggplot(toplot,aes(x=Visit,y=prob,group=group))+
  geom_point(size=c(2,2,3))+
  geom_line()+
  geom_errorbar(aes(ymin=prob-SE,ymax=prob+SE),width=0.2,size=c(0.8,0.8,1.5))+
  geom_hline(yintercept=0.5,linetype="dotted",color="red")+
  scale_y_continuous(breaks=seq(0.3,1.1,0.1))+
  ylab("probability of choosing correct")+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black",face=c(1,1,2)),
        axis.text.y =
element_text(size=12,colour=c("red","black","black","black","black","black")),
        axis.title.x = element_text(size=14),
        axis.title.y = element_text(size=14),
        plot.title = element_text(size=18))
```



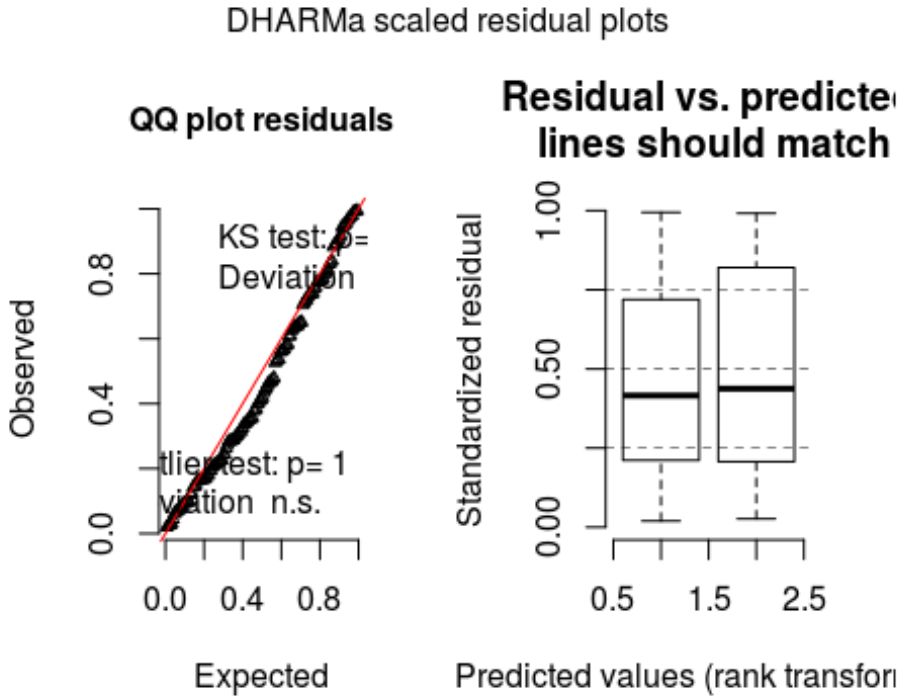
Pheromone deposition

I will look at the pheromone deposited on the way to the drop and back to the nest. I am now adding visit value (sugar or water), given the fact that now visits alternate in quality. will remove visits 7

and 8 from the analysis because are different. Being only 6 visits, I have only 3 and 2 visits for each value: not enough to see change across visits. I will merge the 2/3 visits per value together.

To the drop

```
strain<-
subset(EpisodicMemoryPretraining,EpisodicMemoryPretraining$VisitType=="Strain")

mpExp<-glmer(pherGo~VisitValue+(1|Colony/antID),data=strain,family="poisson",
glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
simres<-simulateResiduals(mpExp) #standard seed for random values is 123
plot(simres, asFactor=T)
```



the model is fine

```
Anova(mpExp)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: pherGo
##             Chisq Df Pr(>Chisq)
## VisitValue 15.618  1   7.75e-05 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
summary(mpExp)
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
```

```
## Formula: pherGo ~ VisitValue + (1 | Colony/antID)
##    Data: strain
## Control:
## glmerControl(optimizer = "bobyqa", optCtrl = list(maxfun = 1e+05))
##
##      AIC      BIC   logLik deviance df.resid
##    196.3    208.4    -94.1    188.3      148
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.9131 -0.3089 -0.1904 -0.1603  2.9279
##
## Random effects:
##  Groups       Name        Variance  Std.Dev.
##  antID:Colony (Intercept) 3.195e+00 1.788e+00
##  Colony       (Intercept) 2.351e-14 1.533e-07
## Number of obs: 152, groups:  antID:Colony, 35; Colony, 4
##
## Fixed effects:
##                 Estimate Std. Error z value Pr(>|z|)
## (Intercept)      -1.9677     0.5417  -3.633 0.000281 ***
## VisitValuewater  -1.0014     0.2534  -3.952 7.75e-05 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr)
## VisitValwtr -0.196
## convergence code: 0
## boundary (singular) fit: see ?isSingular
meanobj <- emmeans(mpExp,~VisitValue, type="response")
(pairs(meanobj))
##  contrast      ratio   SE  df z.ratio p.value
##  sugar / water  2.72 0.69 Inf 3.952   0.0001
##
## Tests are performed on the log scale
```

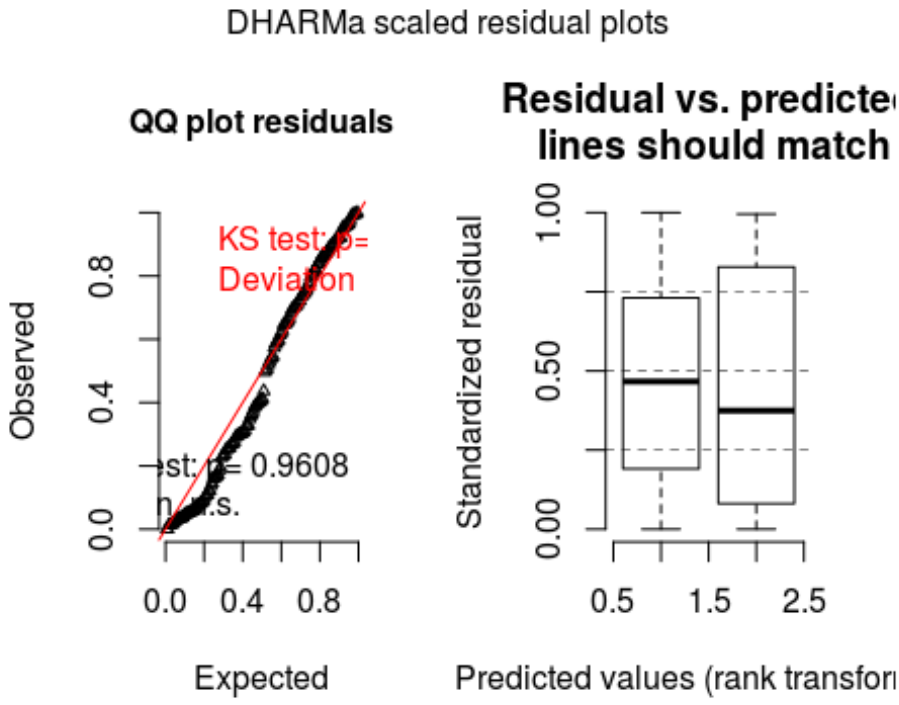
as expected, ants deposit more pheromone for the sugar over the water.

```
phg2<-ggplot(strain,aes(x=VisitValue,y=pherGo))+
  labs(title = "A")+
  ylab("Pheromone deposited to the drop")+
    xlab("Visit value")+
  ylim(0,8)+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black"),
        axis.text.y = element_text(size=12,colour="black"),
        axis.title.x = element_text(size=14),
        axis.title.y = element_text(size=14),
        plot.title = element_text(size=18),
        legend.position="none")+
```

```
  geom_violin()+
  geom_jitter(width = 0.2,height=0)
```

Back to the nest

```
mpExp<-glmer(pherBk~VisitValue+(1|Colony/antID),data=strain,family="poisson",
glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
## boundary (singular) fit: see ?isSingular
mpExp<-glmer(pherBk~VisitValue+(1|antID),data=strain,family="poisson",
glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
## Warning in checkConv(attr(opt, "derivs"), opt$par, ctrl = control
## $checkConv, : unable to evaluate scaled gradient
## Warning in checkConv(attr(opt, "derivs"), opt$par, ctrl = control
## $checkConv, : Model failed to converge: degenerate Hessian with 1 negative
## eigenvalues
mpExp<-glm(pherBk~VisitValue,data=strain,family="poisson")
simres<-simulateResiduals(mpExp) #standard seed for random values is 123
plot(simres, asFactor=T)
```



the model is zero inflated. maybe that is why it did not converge

```
mpExp<- zeroinfl(pherBk ~ VisitValue + 1 | Colony/antID, data = strain)
## Warning: glm.fit: algorithm did not converge
## Warning: glm.fit: fitted probabilities numerically 0 or 1 occurred
## Error in solve.default(as.matrix(fit$hessian)): Lapack routine dgesv: system
is exactly singular: U[34,34] = 0
```

again do not converge. dropping colony as random factor

```
mpExp<- zeroinfl(pherBk ~ VisitValue + 1 | antID, data = strain)
```

```
## Error in solve.default(as.matrix(fit$hessian)): il sistema è numericamente
singolare: valore di condizione di reciprocità = 1.43037e-18
```

again. dropping random effect at all.

```
mpExp<- zeroinfl(pherBk ~ VisitValue , data = strain)
## Error in solve.default(as.matrix(fit$hessian)): il sistema è numericamente
singolare: valore di condizione di reciprocità = 1.03101e-23
```

there seems to be no solution here. We do have a suspect: maybe there are all zeros in one of the groups. let's look at the raw data.

```
tapply(strain$pherBk,strain$VisitValue,summary)
## $sugar
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.    NA's
##   0.000   0.000   2.000   2.353   4.000   8.000      28
##
## $water
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##       0       0       0       0       0       0
```

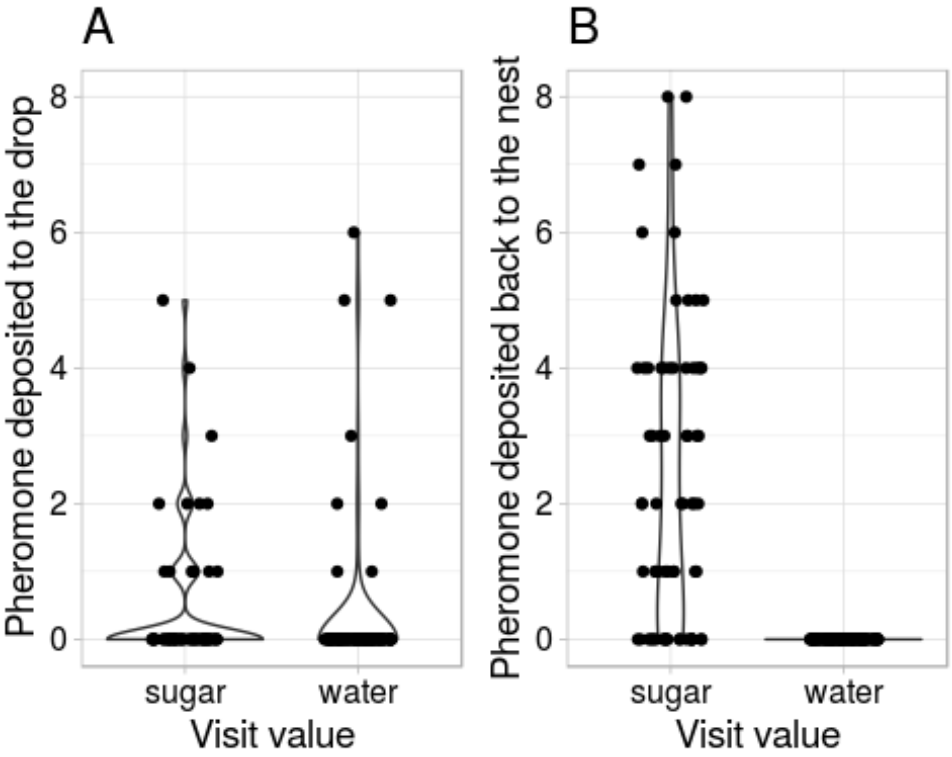
here is the reason. No ant deposited any pheromone on the way back from water in ANY visit. Statistical models cannot grasp complete separation of data. We are confident in saying that ants deposited more pheromone for sugar than for water, even without having a p-value backing it up

```
phb2<-ggplot(strain,aes(x=VisitValue,y=pherBk))+
  labs(title = "B")+
  ylab("Pheromone deposited back to the nest")+
  xlab("Visit value")+
  ylim(0,8)+
  theme_light()+
  theme(axis.text.x = element_text(size=12,colour="black"),
        axis.text.y = element_text(size=12,colour="black"),
        axis.title.x = element_text(size=14),
        axis.title.y = element_text(size=14),
        plot.title = element_text(size=18),
        legend.position="none")+
  geom_violin()+
  geom_jitter(width = 0.2,height=0)
```

I will plot the two together.

Graph together

```
plot_grid(phg2, phb2, nrow=1) #could also label them here with
labels=c("A","B")
## Warning: Removed 41 rows containing non-finite values (stat_ydensity).
## Warning: Removed 41 rows containing missing values (geom_point).
## Warning: Removed 28 rows containing non-finite values (stat_ydensity).
## Warning: Removed 28 rows containing missing values (geom_point).
```


A
Pheromone deposited to the drop
8
6
4
2
0
sugar
water
Visit value
B
Pheromone deposited back to the nest
8
6
4
2
0
sugar
water
Visit value

Appendix 5 – Data Analysis of study: Visual discrimination learning and amodal completion in the jumping spider *Phidippus regius*

This supplement provides the entire R script and output of the statistical analysis we performed and figures produced, in their original form. It is presented in the spirit of open and transparent science, but has not been carefully curated.

Setup

Load packages

```
library(readODS)
library(knitr)
library(lme4)
library(car)
library(emmeans)
library(DHARMa)
library(pscl)
library(ggplot2)
library(ggsignif)
library(MASS)
```

Analysis

Preliminary analysis: number of answered tests

A total of

```
nrow(data)/2
## [1] 802
```

trials have been recorded. They are divided into the three conditions according to the following list:

```
summary(data$cond)[1]/2
## Extinct
##     131
summary(data$cond)[2]/2
## Illusion
##      133
summary(data$cond)[3]/2
## Reward
##    538
```

However, in not all the trials the spiders drank either drop.

```
tapply(data$drinkbin,data$cond, table)
## $Extinct
##
##  0  1
## 11 24
```

```
##
## $Illusion
##
##  0  1
## 14 13
##
## $Reward
##
##   0   1
##   4 102
```

in total, we recorded answer for 35 extinct trial, 27 Illusion trials and 106 reward trials. Out of the total, they represent:

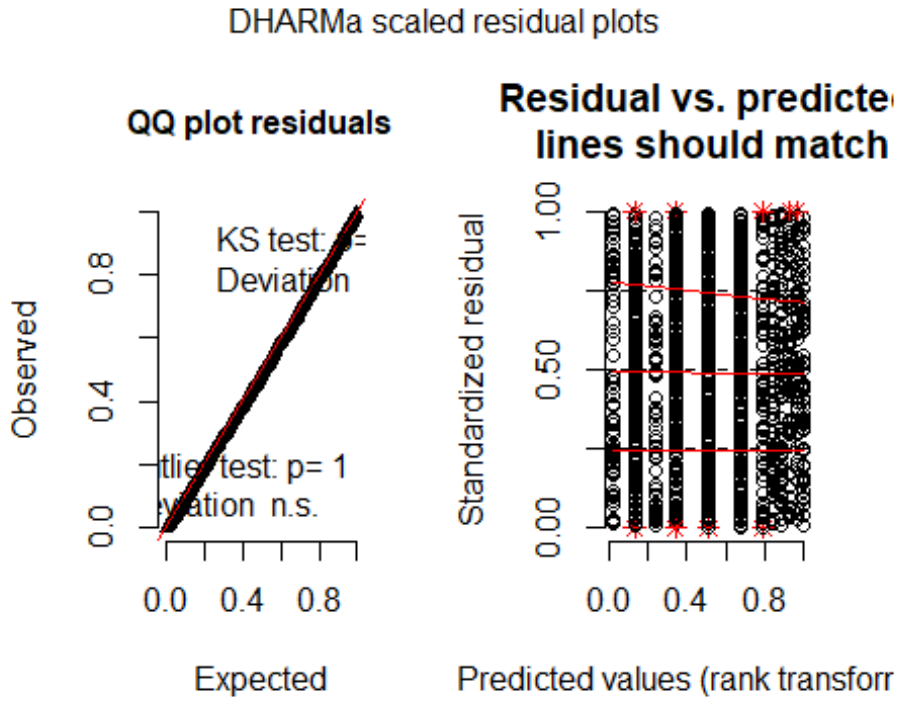
```
35/131
## [1] 0.2671756
27/133
## [1] 0.2030075
106/538
## [1] 0.197026
```

from this count we have already removed 3 trials from the extinct condition, since the spider drank both drops. We interpreted this case as a lack of choice, as much as drinking neither.

Preliminary analysis: difference between sides

At first, I want to see if there is a difference in the number of times spider drank the drops depending on their position in space (left, right, top, bottom). I will test it in interaction with the condition, as I expect the spiders to only drink the correct ones in rewarded trials, as the wrong ones are unpalatable.

```
ms<-glmer(drankit~cond*side+(1|subj),data=data, family= binomial,
          glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
simres<-simulateResiduals(ms) #standard seed for random values is 123
plot(simres)
```




```
Anova(ms)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: drankit
##             Chisq Df Pr(>Chisq)
## cond      5.1650  2    0.07559 .
## side      6.2788  3    0.09881 .
## cond:side 2.0193  6    0.91791
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
e<-emmeans(ms, ~cond*side)
pairs(e, simple="side")
## cond = Extinct:
##  contrast      estimate    SE  df z.ratio p.value
##  down - left   1.70e-01 0.525 Inf  0.324  0.9883
##  down - right  1.70e-01 0.525 Inf  0.324  0.9883
##  down - top   -4.98e-01 0.503 Inf -0.991  0.7543
##  left - right -3.40e-06 0.504 Inf  0.000  1.0000
##  left - top   -6.68e-01 0.486 Inf -1.375  0.5148
##  right - top  -6.68e-01 0.486 Inf -1.375  0.5148
##
## cond = Illusion:
##  contrast      estimate    SE  df z.ratio p.value
##  down - left  -1.76e-01 0.658 Inf -0.268  0.9933
##  down - right -7.07e-01 0.600 Inf -1.179  0.6404
##  down - top   -6.56e-01 0.585 Inf -1.122  0.6759
##  left - right -5.31e-01 0.602 Inf -0.882  0.8141
##  left - top   -4.80e-01 0.588 Inf -0.815  0.8474
```

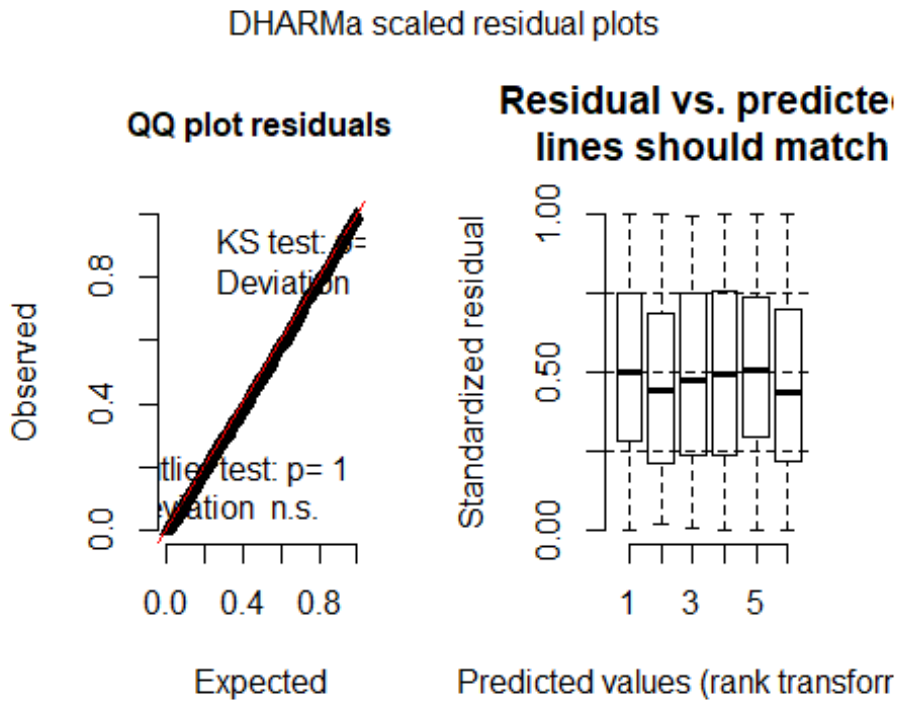
```
##  right - top   5.12e-02 0.523 Inf  0.098  0.9997
##
## cond = Reward:
##  contrast      estimate    SE  df z.ratio p.value
##  down - left  -1.31e-01 0.315 Inf -0.414  0.9760
##  down - right -4.29e-01 0.299 Inf -1.433  0.4784
##  down - top   -4.71e-01 0.295 Inf -1.596  0.3807
##  left - right -2.98e-01 0.292 Inf -1.021  0.7372
##  left - top   -3.40e-01 0.289 Inf -1.180  0.6397
##  right - top  -4.24e-02 0.271 Inf -0.157  0.9986
##
## Results are given on the log odds ratio (not the response) scale.
## P value adjustment: tukey method for comparing a family of 4 estimates
```

There seems to not be any preference for any side.

Preliminary analysis: difference between shapes

At first, I want to see if there is a difference in the number of times spider drank the drops depending on their shape, independently from their value (correct or wrong). Also here I will include condition, both for the same reason as before and because in the "illusion" condition there is no shape

```
msh<-glmer(drankit~cond*shape+(1|subj),data=data, family= binomial,
          glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
simres<-simulateResiduals(msh) #standard seed for random values is 123
plot(simres)
```



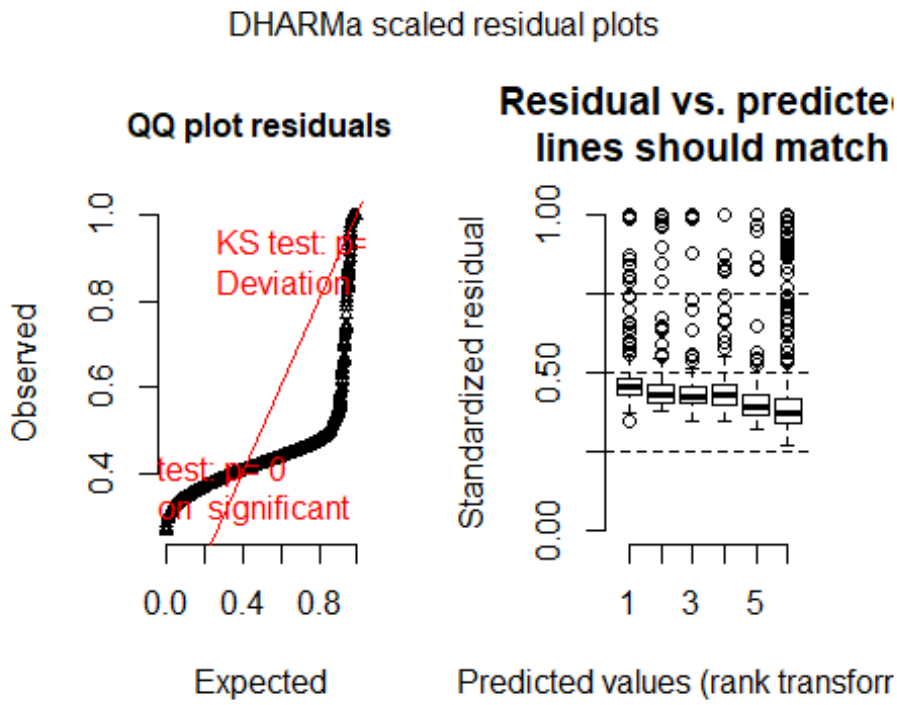
```
Anova(msh)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: drankit
##             Chisq Df Pr(>Chisq)
## cond       5.0621  2    0.07958 .
## shape      0.1718  1    0.67854
## cond:shape 2.0617  2    0.35671
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
e<-emmeans(msh, ~cond*shape)
pairs(e, simple="shape")
## cond = Extinct:
##  contrast estimate    SE  df z.ratio p.value
##  O - X     -0.3775 0.356 Inf -1.061  0.2885
##
## cond = Illusion:
##  contrast estimate    SE  df z.ratio p.value
##  O - X      0.0833 0.406 Inf  0.205  0.8375
##
## cond = Reward:
##  contrast estimate    SE  df z.ratio p.value
##  O - X      0.2121 0.205 Inf  1.032  0.3021
##
## Results are given on the log odds ratio (not the response) scale.
```

There seems to not be any preference for any shape.

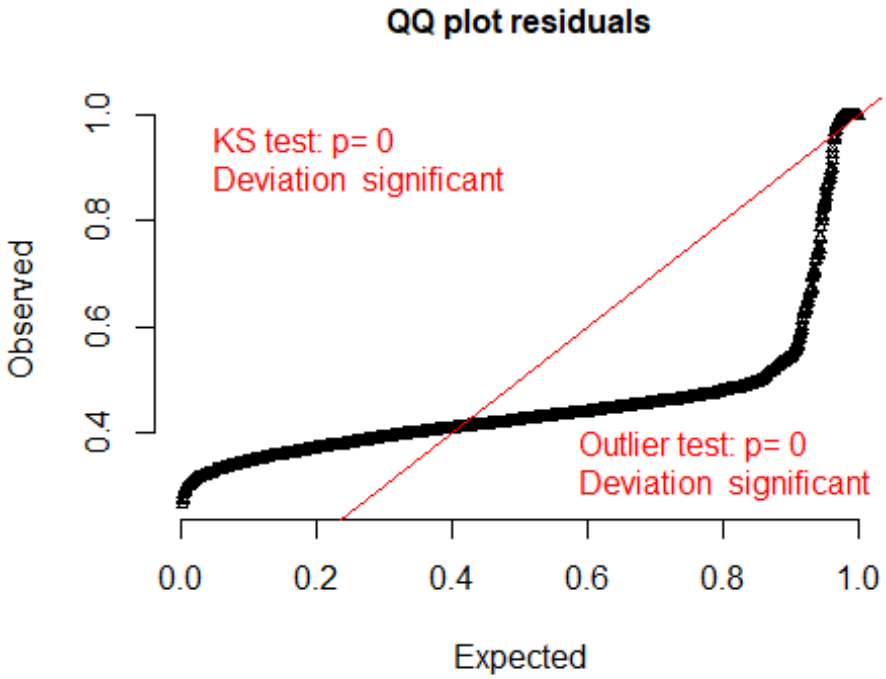
Preliminary analysis: time spent behind either screen

At this point, let's see if the spiders spent more time behind the correct screen, and in turn in the proximity of the correct drop. this is a measure less precise than the binomial choice with drinking, since the spiders wander a lot, but it may still be of interest

```
mt<-lmer(behind~cond*value+(1|subj),data=data)
simres<-simulateResiduals(mt) #standard seed for random values is 123
## Model family was recognized or set as continuous, but duplicate values were
detected in the response. Consider if you are fitting an appropriate model.
plot(simres)
```




```
testUniformity(simres)
```




```
##
##  One-sample Kolmogorov-Smirnov test
```

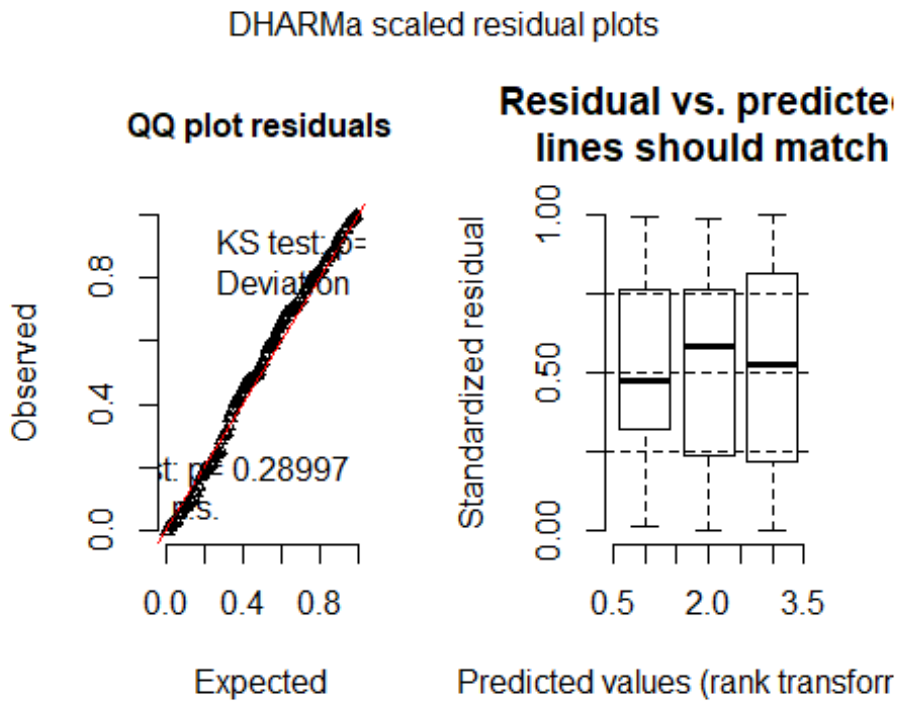
```
##
## data:  simulationOutput$scaledResiduals
## D = 0.35711, p-value < 2.2e-16
## alternative hypothesis: two-sided
mt<-glmmPQL(behind~cond*value, ~1|subj, data=data, family = gaussian)
## iteration 1
## iteration 2
Anova(mt)
## Analysis of Deviance Table (Type II tests)
##
## Response: zz
##             Chisq Df Pr(>Chisq)
## cond       0.3151  2   0.854252
## value      7.8629  1   0.005046 **
## cond:value 8.2563  2   0.016113 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
e<-emmeans(mt,~cond*value,type="response")
pairs(e, simple="value")
## cond = Extinct:
##  contrast        estimate   SE   df t.ratio p.value
##  correct - wrong     0.79 30.4 1581  0.026  0.9793
##
## cond = Illusion:
##  contrast        estimate   SE   df t.ratio p.value
##  correct - wrong   -29.32 30.2 1581 -0.972  0.3313
##
## cond = Reward:
##  contrast        estimate   SE   df t.ratio p.value
##  correct - wrong    58.31 15.0 1581  3.888  0.0001
```

I see a difference in the time spent behind the correct and the wrong screen only for the reward condition. This is probably directly related to the time spent drinking, as I expect them to almost not spend any time near the wrong drop

Main analysis: all trials

now to the main analysis.

```
m0<-glmer(drinkbin~cond+(1|subj),data=data,family="binomial")
## boundary (singular) fit: see ?isSingular
simres<-simulateResiduals(m0) #standard seed for random values is 123
plot(simres)
```




```
Anova(m0)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: drinkbin
##       Chisq Df Pr(>Chisq)
## cond 27.427  2  1.107e-06 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

there is a difference between the conditions. I will not test each one against chance level

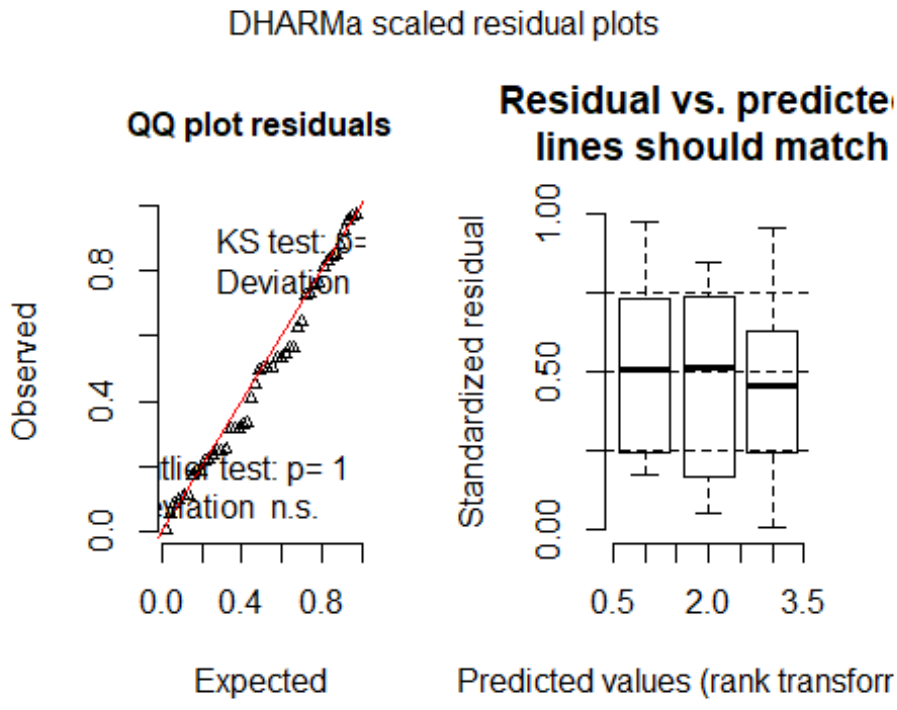
```
e<-emmeans(m0,~cond,type="response")
toplot1<-as.data.frame(e)
test(e, adjust="bonferroni")
##  cond      prob     SE  df z.ratio p.value
##  Extinct  0.686 0.0785 Inf  2.143  0.0964
##  Illusion 0.481 0.0962 Inf -0.192  1.0000
##  Reward   0.962 0.0185 Inf  6.354  <.0001
##
## P value adjustment: bonferroni method for 3 tests
## Tests are performed on the logit scale
```

As expected, the spider drink almost exclusively the correct drop in the “rewarded” condition. The only few contacts with the wrong one are probably accidental ones. In the extinct condition the probability of drinking the correct drop over the wrong one is of 0.686. even thought it does not reach significance, probably due to the low number of subjects

Main analysis: only first trial

there is the chance that the insertion of extinction trials decreases the motivation of subject, that after having experienced for the first time the water drop may stop to perform as the same level. For this reason we test only the first trial in which each subject experienced for the first time the unrewarded trial with the training shapes (extinct) and the illusory ones (illusion). also the rewarded trials are added for completeness, However we do not expect them to be any different from the full experiment

```
rev<-subset(data, data$cond=="Reward")
rev<-subset(rev,rev$drinkbin>=0)
rev<-rev[match(unique(rev$subj),rev$subj),]

ext<-subset(data, data$cond=="Extinct")
ext<-subset(ext,ext$drinkbin>=0)
ext<-ext[match(unique(ext$subj),ext$subj),]

ill<-subset(data, data$cond=="Illusion")
ill<-subset(ill,ill$drinkbin>=0)
ill<-ill[match(unique(ill$subj),ill$subj),]

first<-rbind(rev,ext,ill)
m1<-glmer(drinkbin~cond+(1|subj),data=first,family="binomial")
## boundary (singular) fit: see ?isSingular
simres<-simulateResiduals(m1) #standard seed for random values is 123
plot(simres)
```



```
Anova(m1)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: drinkbin
##       Chisq Df Pr(>Chisq)
## cond 10.681  2   0.004793 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

there is a difference between the conditions.

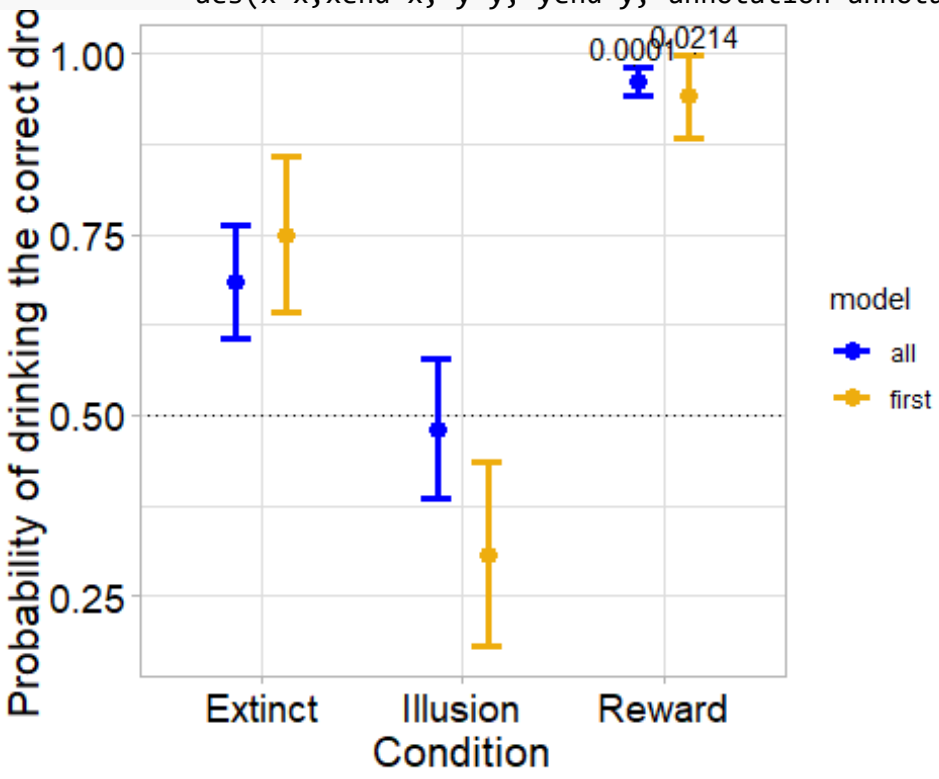
```
e<-emmeans(m1,~cond,type="response")
toplot2<-as.data.frame(e)

test(e, adjust="bonferroni")
##  cond      prob     SE  df z.ratio p.value
##  Extinct  0.750 0.1083 Inf  1.903  0.1712
##  Illusion 0.308 0.1280 Inf -1.349  0.5316
##  Reward   0.941 0.0571 Inf  2.690  0.0214
##
## P value adjustment: bonferroni method for 3 tests
## Tests are performed on the logit scale
```

again, the extinct condition has a probability of 0.75 of choosing the correct drop. However the number of subjects and trial is so low that it does not raise above significance.

Plot the model

```
toplot1$model<-rep("all")
toplot2$model<-rep("first")

toplot<-rbind(toplot1,toplot2)

ggplot(toplot,aes(x=cond,y=prob))+
  scale_color_manual(values = c("blue","darkgoldenrod2"))+
  geom_point(aes(color=model), position=position_dodge(0.5),size=3)+
  geom_hline(aes(yintercept=0.5), linetype="dotted")+
  geom_errorbar(aes(ymin=prob-SE,ymax=prob+SE,
color=model),position=position_dodge(0.5),size=1.3,width = 0.3)+
  xlab("Condition")+
  ylab("Probability of drinking the correct drop")+
  theme_light()+
  theme(axis.text.x = element_text(size=14,colour="black"),
        axis.text.y = element_text(size=14,colour="black"),
        axis.title.x = element_text(size=16),
        axis.title.y = element_text(size=16),
        plot.title = element_text(size=18),
        legend.position="right")+
  geom_signif(stat="identity",tip_length = 0.1,
              data=data.frame(x=c(2.85, 3.15),
```

```
                                y=c(0.985, 1), annotation=c("0.0001", "0.0214")),
          aes(x=x,xend=x, y=y, yend=y, annotation=annotation))
```



Appendix 6 – Data Analysis of the study: design of a low-cost, design and validation of an open source “Skinner-box” system for the study of land arthropods.

This appendix provides the entire R script and output of the statistical analysis we performed and figures produced, in their original form. It is presented in the spirit of open and transparent science, but has not been carefully curated.

Setup

Load packages

```
library(readODS)
library(knitr)
library(lme4)
library(car)
library(emmeans)
library(DHARMa)
library(pscl)
library(ggplot2)
library(ggsignif)
```

Data

This analysis starts from the already summarised data of the experiment. In the data table here loaded each trial has two rows, one for the correct sensor and one for the wrong sensor.

Analysis

Preliminary analysis: raw data vs merged data

While on top of a sensor, the spiders sometimes turned around, changing ever so slightly the portion of the photoresistor that they were covering with their body. Sometimes, when the spider covered the sensor just enough to surpass the activation treshold, the movements on top of the photoresistor caused a fast deactivation and reactivation of it, effectively registering two different presses instead of 1. We belive that it would be improper to consider these multiple actiations as separate, since the spider did not really left and returned to the sensor. To solve this problem, we decided to merge in the raw data every activations of the same sensor that were 0.4sec or below apart, resulting in just one, longer activation, as per the actual behaviour. for transparency, the provided data contains both the raw data and the data after the merging process.

first, let’s see the percentage of trials in which the system registered multiple activations for just one covering.

```
data$havechanged<-data$rawvsclean
data$havechanged[data$havechanged>1]<-1 #0 if they are the same, 1 if they have
changed
mean(data$havechanged)
## [1] 0.1632997
sum(data$havechanged)/2
## [1] 48.5
```

data have been modified in 48.5 trials, 16.33% of the total trials note that the number retrived is not an integer, since we have 2 rows for each trial and it was possible that the modification was done for only one of the two sensors.

now, let's see the percentage divided per sensor (correct or wrong)

```
mp <- glmer(havechanged~sensor+(1|subj),data=data,family=binomial)
Anova(mp)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: havechanged
##         Chisq Df Pr(>Chisq)
## sensor 4.0849  1    0.04327 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
e<-emmeans(mp,~sensor,type="response")
e
##  sensor   prob     SE  df asymp.LCL asymp.UCL
##  correct 0.157 0.0319 Inf    0.1040     0.230
##  wrong   0.104 0.0241 Inf    0.0652     0.162
##
## Confidence level used: 0.95
## Intervals are back-transformed from the logit scale
pairs(e)
##  contrast        odds.ratio    SE  df z.ratio p.value
##  correct / wrong        1.6 0.375 Inf 2.021   0.0433
##
## Tests are performed on the log odds ratio scale
```

there is a higher percentage of multiple activation for the correct sensor. This maybe because spiders tend to spend more time on top of the correct sensor, as it should be preferred. Moreover the drop is dispensed when the spiders cover the correct sensor: this means that they will likely perceive the movement and turn to fixate on the newly dispensed reward, effectivly generating the multiple activation. On the other hand when the wrong sensor is covered nothing happens.

Anyway, since the error was more present for the correct sensor over the wrong one, our merging process will in the worst case scenario make us underestimate the effect, not overestimate. We are confident that our manipulation effectivly decreases the probability of a **type 1 error**

I will now test the times, in order to see if this is the case

Preliminary analysis: time

```
mt<-lmer(time~sensor*blocktrial*block+(1|subj),data=data)
Anova(mt)
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: time
##                            Chisq Df Pr(>Chisq)
## sensor                   18.4995  1  1.699e-05 ***
## blocktrial                0.7935  1     0.3730
## block                     0.2990  1     0.5845
## sensor:blocktrial         0.7259  1     0.3942
## sensor:block              0.5015  1     0.4788
## blocktrial:block          0.3051  1     0.5807
## sensor:blocktrial:block   0.4029  1     0.5256
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

There seems to be a difference in the time spent on each sensor, regardless of trial day or block.

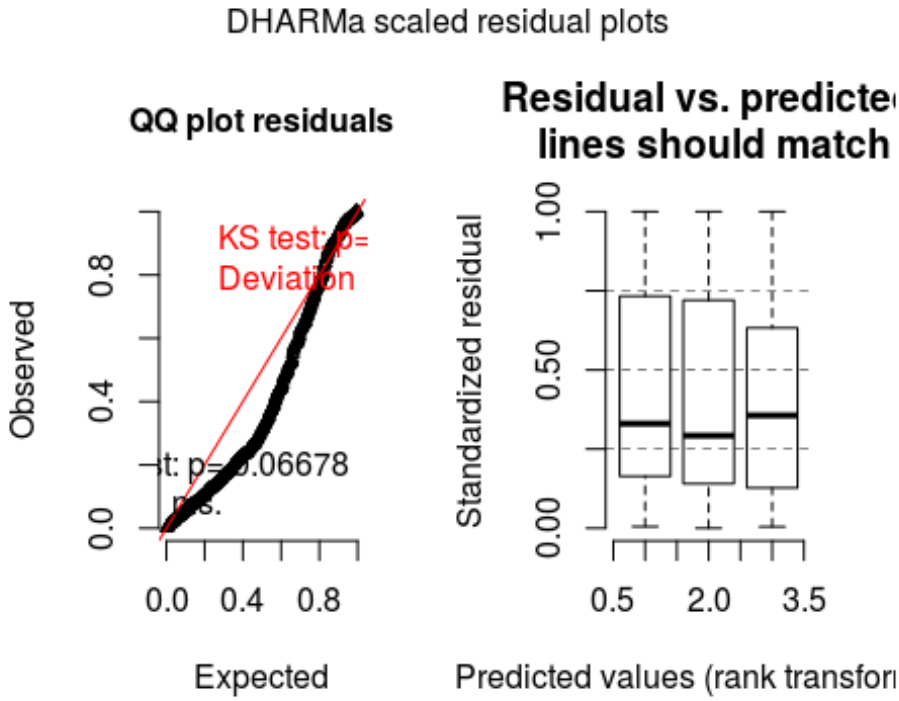
```
e<-emmeans(mt,~sensor,type="response")
## NOTE: Results may be misleading due to involvement in interactions
e
##  sensor  emmean   SE   df lower.CL upper.CL
##  correct 120.73 20.5 83.8     80.1    161.4
##  wrong     6.52 20.5 83.8    -34.1     47.2
##
## Results are averaged over the levels of: block
## Degrees-of-freedom method: kenward-roger
## Confidence level used: 0.95
pairs(e)
##  contrast        estimate   SE  df t.ratio p.value
##  correct - wrong      114 26.5 557 4.309   <.0001
##
## Results are averaged over the levels of: block
```

As expected, the time spent on top of the correct sensor is multiple orders of magnitiude more than the one spent on the wrong sensor

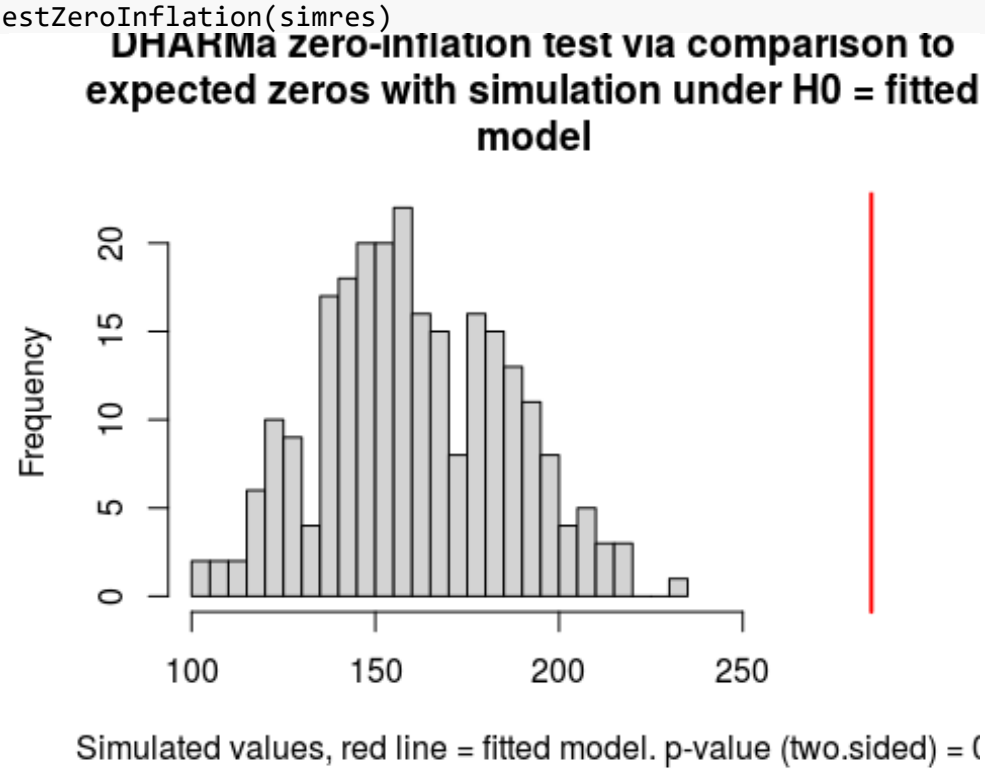
Preliminary analysis: sex differences

before proceeding with the training outcome, we want to see if different sexes (or ages) show a different activity level.

```
msex<-glmer(cover~sex+(1|subj),data=data,family="poisson")
simres<-simulateResiduals(msex) #standard seed for random values is 123
plot(simres)
```




```
testZeroInflation(simres)
```




```
##
##  DHARMa zero-inflation test via comparison to expected zeros with
##  simulation under H0 = fitted model
```

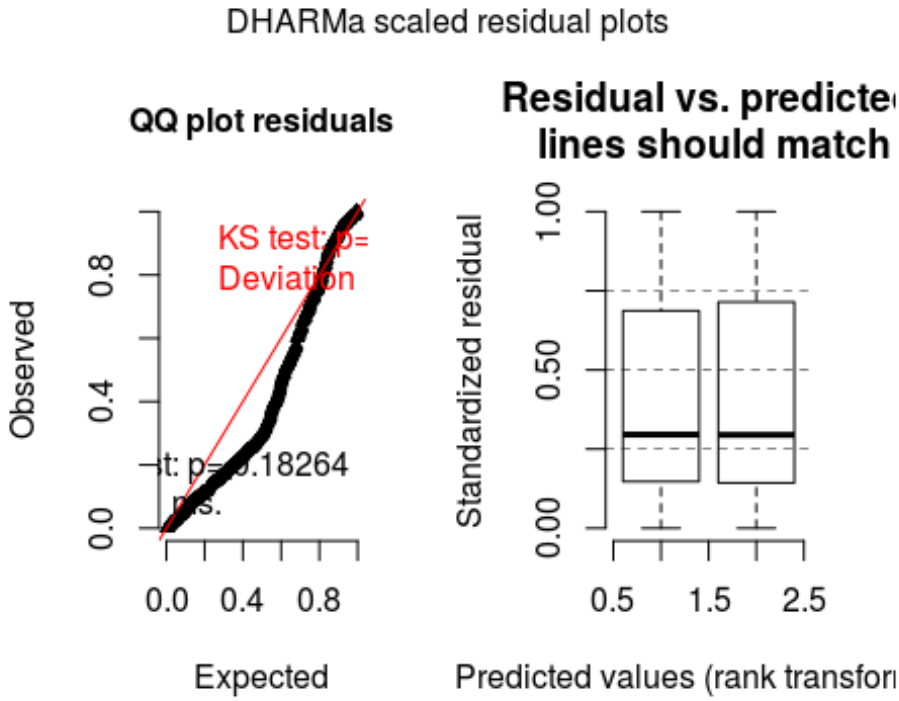
```
##
## data:  simulationOutput
## ratioObsSim = 1.7658, p-value < 2.2e-16
## alternative hypothesis: two.sided
mzsex<-zeroinfl(cover ~ sex + 1|subj, data = data)
Anova(mzsex)
## Analysis of Deviance Table (Type II tests)
##
## Response: cover
##     Df  Chisq Pr(>Chisq)
## sex  2 14.161  0.0008415 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
e<-emmeans(mzsex,~sex,type="response")
## NOTE: A nesting structure was detected in the fitted model:
##     subj %in% sex
pairs(e)
##  contrast          estimate    SE  df z.ratio p.value
##  Female - Juvenile  -1.1605 0.285 Inf -4.078  0.0001
##  Female - Male      -1.1502 0.316 Inf -3.644  0.0008
##  Juvenile - Male     0.0103 0.227 Inf  0.045  0.9989
##
## Results are averaged over the levels of: subj
## P value adjustment: tukey method for comparing a family of 3 estimates
```

there is a clear difference between groups. Males and Juveniles are not different to each other, while females are less active than both males and juveniles. These data has to be taken with caution, since we had only 2 females and 8 males, with the remaining 20 being juveniles. nevetheless the data were worth being presented in the supplemental.

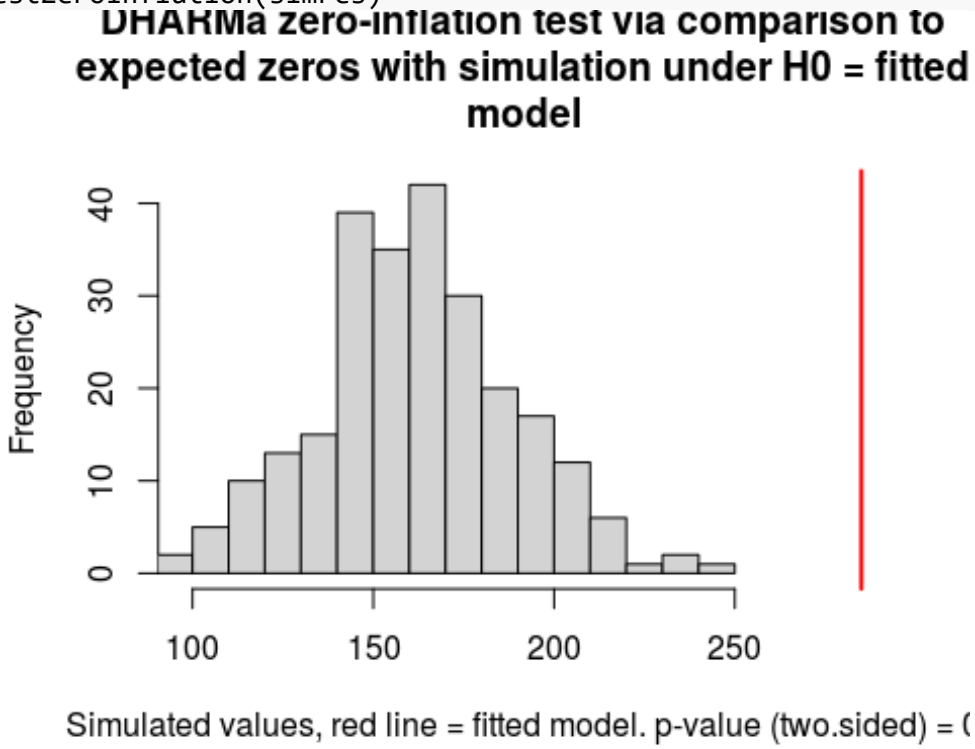
Preliminary analysis: correct side

as before, we want to see if there was any side preference regardless of training outcome.

```
mside<-glmer(cover~side+(1|subj),data=data,family="poisson")
simres<-simulateResiduals(mside) #standard seed for random values is 123
plot(simres)
```




```
testZeroInflation(simres)
```




```
##
##  DHARMa zero-inflation test via comparison to expected zeros with
##  simulation under H0 = fitted model
```

```
##
## data:  simulationOutput
## ratioObsSim = 1.7536, p-value < 2.2e-16
## alternative hypothesis: two.sided
mzside<-zeroinfl(cover ~ side + 1|subj, data = data)
Anova(mzside)
## Analysis of Deviance Table (Type II tests)
##
## Response: cover
##      Df  Chisq Pr(>Chisq)
## side  1 6.5877    0.01027 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
e<-emmeans(mzside,~side,type="response")
e
##  side  emmean    SE  df asymp.LCL asymp.UCL
##  left    2.44 0.123 Inf      2.20      2.68
##  right   2.11 0.114 Inf      1.88      2.33
##
## Results are averaged over the levels of: subj
## Confidence level used: 0.95
pairs(e)
##  contrast     estimate    SE  df z.ratio p.value
##  left - right    0.329 0.128 Inf 2.569   0.0102
##
## Results are averaged over the levels of: subj
```

data shows that left is strongly preferred over right, independently of what sensor is. Is that influenced by the sensor?

```
mzside2<-zeroinfl(cover ~ side*sensor + 1|subj, data = data)
Anova(mzside2)
## Analysis of Deviance Table (Type II tests)
##
## Response: cover
##             Df   Chisq Pr(>Chisq)
## side         1  7.5974   0.005845 **
## sensor       1 44.4151  2.656e-11 ***
## side:sensor  1  2.5102   0.113114
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
e<-emmeans(mzside,~side,type="response")
e
##  side  emmean    SE  df asymp.LCL asymp.UCL
##  left    2.44 0.123 Inf      2.20      2.68
##  right   2.11 0.114 Inf      1.88      2.33
##
## Results are averaged over the levels of: subj
## Confidence level used: 0.95
pairs(e)
```

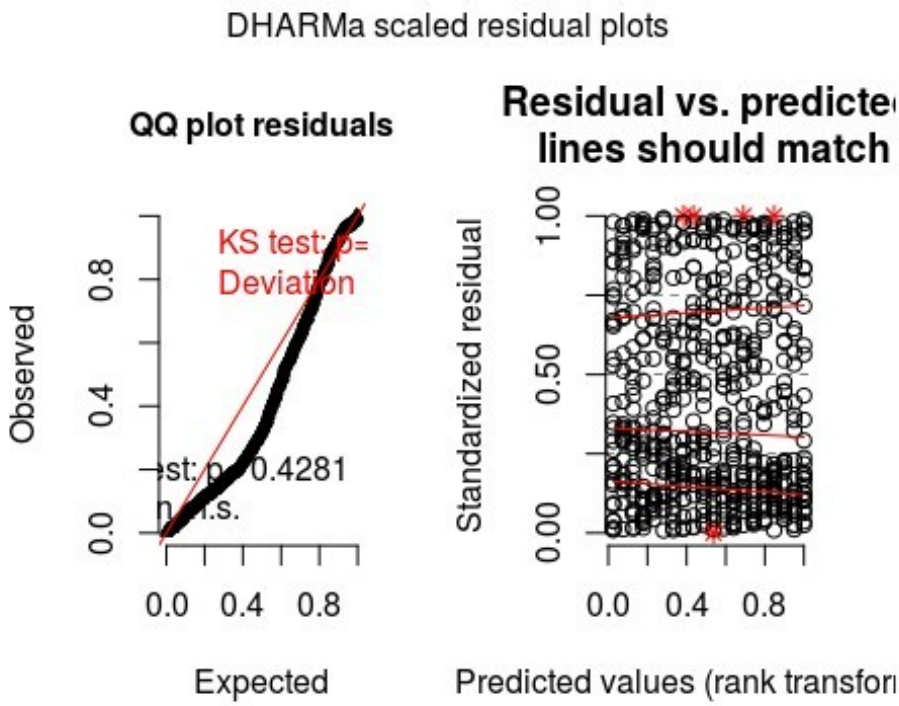
```
##  contrast     estimate    SE  df z.ratio p.value
##  left - right    0.329 0.128 Inf 2.569   0.0102
##
## Results are averaged over the levels of: subj
```

There is a difference between sensors, that we will observe in detail in the main analysis. There is however no interaction between sensor and side, so we can assume that the left bias is independent from the training procedure.

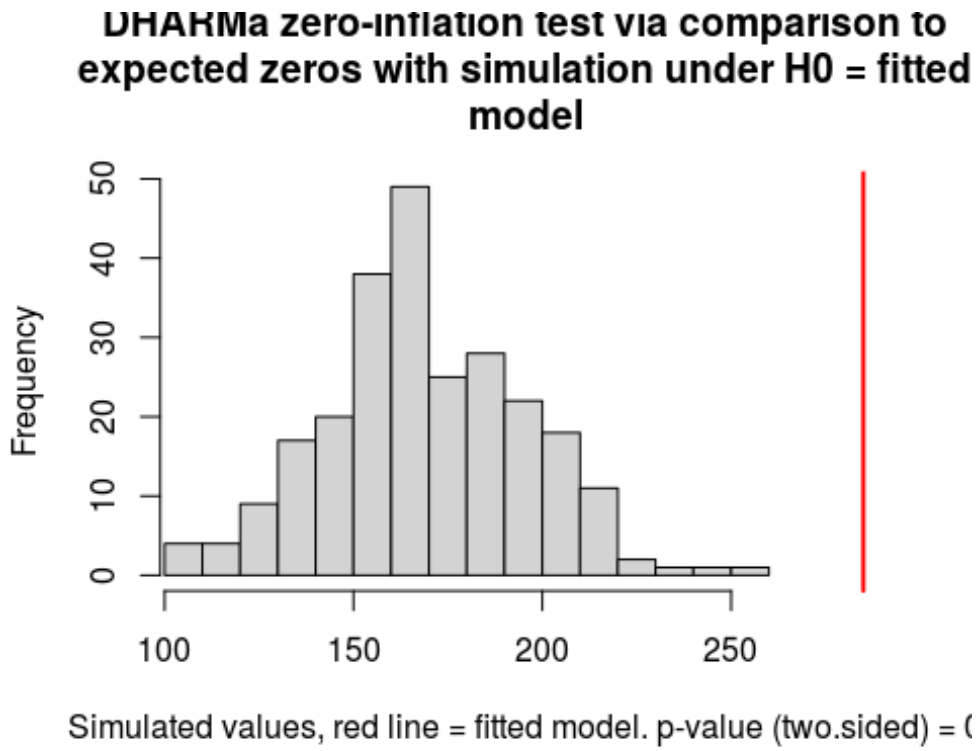
Main analysis

now to the main analysis. I want to see a difference between sensor, that increases with subsequent trials

```
m0<-glmer(cover~sensor*blocktrial*block+(1|subj),data=data,family="poisson",
          glmerControl(optimizer="bobyqa", optCtrl = list(maxfun = 100000)))
simres<-simulateResiduals(m0) #standard seed for random values is 123
plot(simres)
```




```
testZeroInflation(simres)
```




```
##
##  DHARMa zero-inflation test via comparison to expected zeros with
##  simulation under H0 = fitted model
##
## data:  simulationOutput
## ratioObsSim = 1.6832, p-value < 2.2e-16
## alternative hypothesis: two.sided
mz0<-zeroinfl(cover~sensor*blocktrial*block+ 1|subj ,data=data)
Anova(mz0)
## Analysis of Deviance Table (Type II tests)
##
## Response: cover
##                          Df   Chisq Pr(>Chisq)
## sensor                    1 45.2969  1.693e-11 ***
## blocktrial                1  2.4558  0.1170895
## block                     1 12.6204  0.0003816 ***
## sensor:blocktrial         1  1.1841  0.2765197
## sensor:block              1  0.4075  0.5232293
## blocktrial:block          1  0.5963  0.4399892
## sensor:blocktrial:block   1  0.8246  0.3638489
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

there is a difference between the sensors and between the blocks. Follow up with a post hoc

```
e<-emmeans(mz0,~sensor*block,type="response")
## NOTE: Results may be misleading due to involvement in interactions
e
```

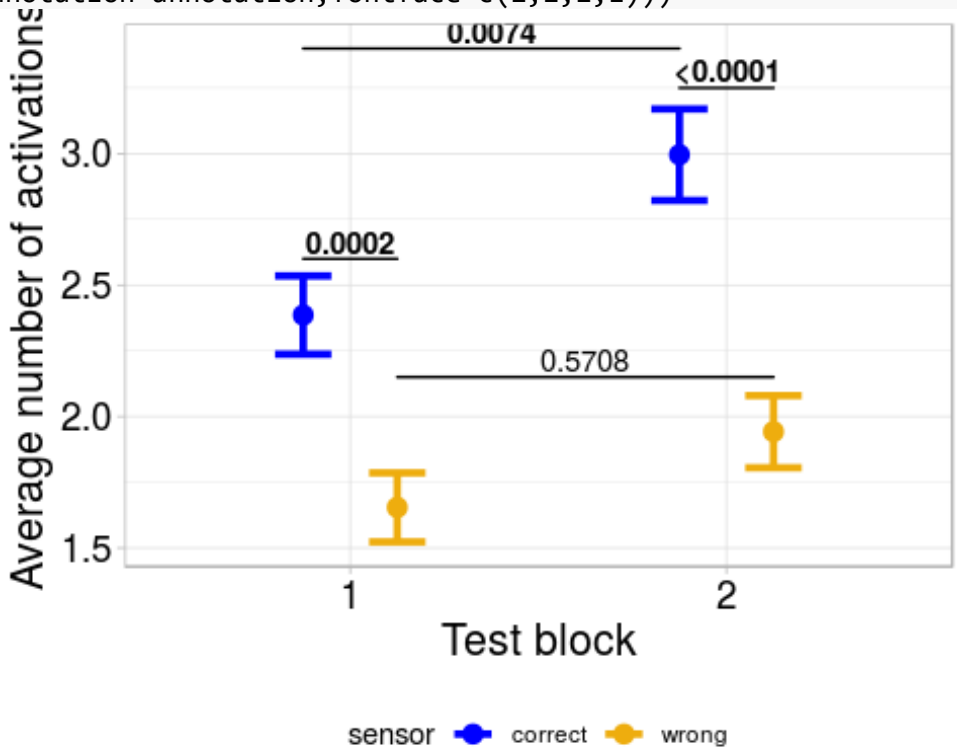
```
##  sensor  block emmean    SE  df asymp.LCL asymp.UCL
##  correct 1       2.39 0.149 Inf      2.09      2.68
##  wrong   1       1.65 0.131 Inf      1.40      1.91
##  correct 2       3.00 0.174 Inf      2.65      3.34
##  wrong   2       1.94 0.137 Inf      1.67      2.21
##
## Results are averaged over the levels of: subj
## Confidence level used: 0.95
contrast(e, adjust="bonferroni", list(C1vsW1=c(1,-1,0,0),
                                      C2vsW2=c(0,0,1,-1),
                                      C1vsC2=c(1,0,-1,0),
                                      W1vsW2=c(0,1,0,-1),
                                      CvsW=c(0.5,-0.5,0.5,-0.5),
                                      week1vsweek2=c(0.5,0.5,-0.5,-0.5)))
##  contrast     estimate    SE  df z.ratio p.value
##  C1vsW1          0.732 0.177 Inf  4.127  0.0002
##  C2vsW2          1.053 0.189 Inf  5.560  <.0001
##  C1vsC2         -0.610 0.189 Inf -3.233  0.0074
##  W1vsW2         -0.288 0.173 Inf -1.669  0.5708
##  CvsW            0.893 0.132 Inf  6.751  <.0001
##  week1vsweek2   -0.449 0.128 Inf -3.494  0.0029
##
## Results are averaged over the levels of: subj
## P value adjustment: bonferroni method for 6 tests
```

the correct sensor is preferred over the wrong sensor both in block 1 and block 2. the preference in block 1 may be due to the habituation phase (blue colour is already rewarded) or to a very fast learning: if the spiders learned already at trial 2 the whole block would result as different. We cannot exclude with our experiment a pre-existing preference for the colour blue over the colour yellow. However the focus of our experiment is not colour discrimination, but the efficacy of the learning paradigm. between block 1 and block 2 the amount of correct activation increases, while the amount of wrong activations remain the same. this is clear evidence of learning. the wrong sensor has no effect, so we expect it to be covered at random for the whole test length. Since the correct sensor instead produce a desired effect, we expected an increase in contacts. Even postulating a pre-existing preference for the blue colour in block 1, the increased responses in block 2 clearly show that the training procedure succeeded.

Plot the model

```
toplot<-as.data.frame(e)

ggplot(toplot,aes(x=block,y=emmean))+
  scale_color_manual(values = c("blue","darkgoldenrod2"))+
  geom_point(aes(color=sensor), position=position_dodge(0.5),size=3)+
  geom_errorbar(aes(ymin=emmean-SE,ymax=emmean+SE,
color=sensor),position=position_dodge(0.5),size=1.3,width = 0.3)+
```

```
  xlab("Test block")+
  ylab("Average number of activations")+
  theme_light()+
  theme(axis.text.x = element_text(size=14,colour="black"),
        axis.text.y = element_text(size=14,colour="black"),
        axis.title.x = element_text(size=16),
        axis.title.y = element_text(size=16),
        plot.title = element_text(size=18),
        legend.position="bottom")+
  geom_signif(stat="identity",tip_length = 0.1,
              data=data.frame(x=c(0.875, 1.875,0.875,1.125), xend=c(1.125,
2.125,1.875,2.125),
                              y=c(2.6, 3.25,3.4,2.15), annotation=c("0.0002",
"<0.0001","0.0074","0.5708")),
              aes(x=x,xend=xend, y=y, yend=y,
annotation=annotation,fontface=c(2,2,2,1)))
```



Anecdotal data – trial by trial comparison

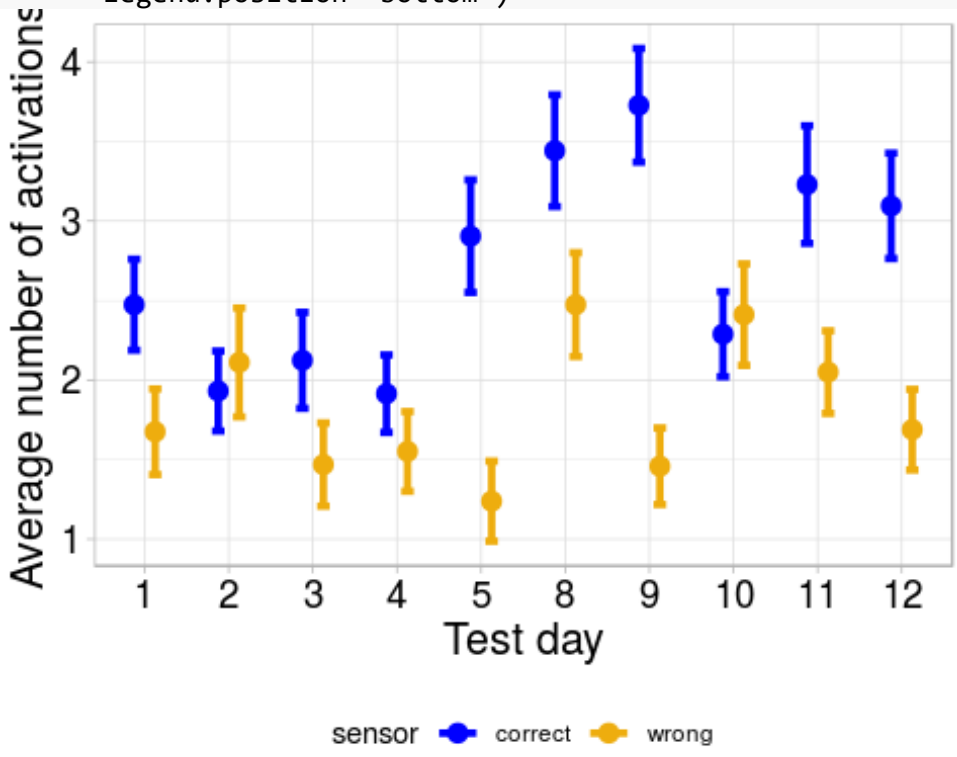
the model showed that there is no difference between trials in each single block. This is probably due to the high amount of zeros in the model, making impossible to produce reliable average number of activation in each single trial. However, just for completeness, I will plot the average number of activation per trial, without any statistical testing

```
data$dayn<-as.factor(data$dayn)
mz1<-zeroinfl(cover~sensor*dayn+ 1|subj ,data=data)
```

```
e<-emmeans(mz1,~sensor*dayn,type="response")
```

Plot the model

```
toplot<-as.data.frame(e)

ggplot(toplot,aes(x=dayn,y=emmean))+
  scale_color_manual(values = c("blue","darkgoldenrod2"))+
  geom_point(aes(color=sensor), position=position_dodge(0.5),size=3)+
  geom_errorbar(aes(ymin=emmean-SE,ymax=emmean+SE,
color=sensor),position=position_dodge(0.5),size=1.3,width = 0.3)+
  xlab("Test day")+
  ylab("Average number of activations")+
  theme_light()+
  theme(axis.text.x = element_text(size=14,colour="black"),
        axis.text.y = element_text(size=14,colour="black"),
        axis.title.x = element_text(size=16),
        axis.title.y = element_text(size=16),
        plot.title = element_text(size=18),
        legend.position="bottom")
```



note: day 6 and 7 are not shown in the graph, as they are the non-testing days between the two blocks