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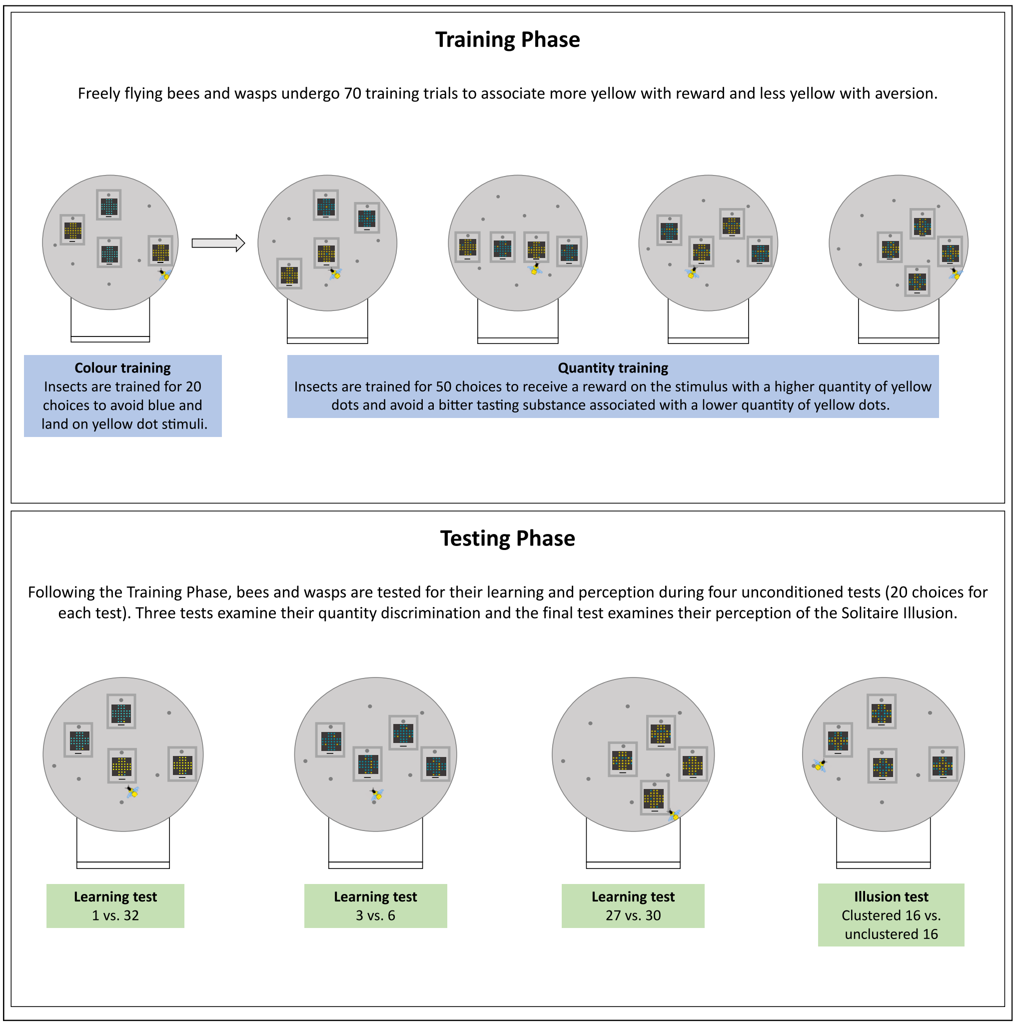

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Highlights

Bees and wasps were trained to choose the larger quantities of yellow vs. blue dots

Insects demonstrated a susceptibility to perceive the Solitaire Illusion

The Solitaire Illusion may be a misperception of quantity and/or other spatial cues

More studies on invertebrates are needed to determine if the perception is conserved

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Quantity misperception by hymenopteran insects observing the solitaire illusion

Scarlett R. Howard[1,4,*] and Adrian G. Dyer[2,3]

SUMMARY

Visual illusions are errors in signal perception and inform us about the visual and cognitive processes of different animals. Invertebrates are relatively less studied for their illusionary perception, despite the insight that comparative data provides on the evolution of common perceptual mechanisms. The Solitaire Illusion is a numerosity illusion where a viewer typically misperceives the relative quantities of two items of different colors consisting of identical quantity, with more centrally clustered items appearing more numerous. We trained European honeybees (*Apis mellifera)* and European wasps (*Vespula vulgaris*) to select stimuli containing a higher quantity of yellow dots in arrays of blue and yellow dots and then presented them with the Solitaire Illusion. Insects learnt to discriminate between dot quantities and showed evidence of perceiving the Solitaire Illusion. Further work should determine whether the illusion is caused by numerical cues only or by both quantity and non-numerical spatial cues.

INTRODUCTION

Visual illusions are errors in signal perception and can cause misperceptions of stimulus size, brightness, color, shape, orientation, motion, or numerosity.[1] Studying the perception of visual illusions in different animals can inform our understanding of the variation in visual processing and the evolution of visual systems.[1] Often the perception of visual illusions can be attributed to whether a species or individual prefers to process visual information separately (local or elemental processing) or as a whole (global or holistic processing).[2] Global processing is suggested to encourage the perception of illusions, while local processing restricts illusionary perception.[2] A well-known size illusion, the Ebbinghaus Illusion, is a misperception of size where a central circle (target) looks larger or smaller depending on the sizes of circles surrounding it (inducers).[3,4] For some species and populations, the Ebbinghaus Illusion is perceived as the target circles of identical size appearing smaller when surrounded by relatively larger circles or larger when surrounded by relatively smaller circles.[5–10] However, some studies have shown certain species perceive the opposite illusion,[11–13] or no illusion at all.[7] For example, baboons (*Papio papio*) have a local preference for processing visual information,[14] and do not perceive the Ebbinghaus Illusion.[7] As discussed further below, studies on animals which do not show perception of illusions may be explained as an artifact of the methods where animals had their viewing distance restricted. This explanation is suggested to describe discrepancies between findings.[6,15,16]

Numerosity illusions are well-studied visual illusions in vertebrates which cause misperceptions of quantity through overestimation or underestimation due to the spatial arrangement of the elements.[17,18] These illusions include nested set illusions, the Regular Random Numerosity Illusion (RRNI), and the Solitaire Illusion. Nested set illusions occur when participants are slower and more inaccurate at judging the quantities of objects, such as circles, when they are nested (contained within) other circles, compared to when the circles do not overlap. The RRNI occurs when participants tend to overestimate the numerosity of items when they are arranged more regularly compared with random arrangements. The Solitaire Illusion occurs when elements of two different colors appear more numerous when clustered together centrally, but less numerous when unclustered. This illusion is suggested to occur as the centrally clustered elements form a single unit, a Gestalt, while the elements forming the perimeter are separated into four clusters[19] (Figure 1). Adult humans perceive the elements in the perimeter, which are of identical quantity to those in the center as being 76% as numerous as the central elements. Thus, the outer, less clustered elements appear about three-quarters of the quantity as more centrally clustered elements.[20] It is currently known that humans, capuchin monkeys,[17] guppies,[21] and bumblebees[22] can perceive this illusion, although there is evidence of individual variation in non-human animal participants. Chimpanzees,[17] rhesus monkeys,[17] and domestic dogs[23] do not show evidence of perceiving the Solitaire Illusion. There also appears to be an age effect in humans, where younger children are less susceptible to the Solitaire Illusion than older children,[24] potentially suggesting an acquired or developmental influence from experience or other cultural factors. While there have been important comparative studies in some vertebrate species, there is currently only one recent study regarding how invertebrates perceive numerosity illusions.[22] Such studies can potentially provide important insight into the evolution of visual systems and visual processing similarities across different species.

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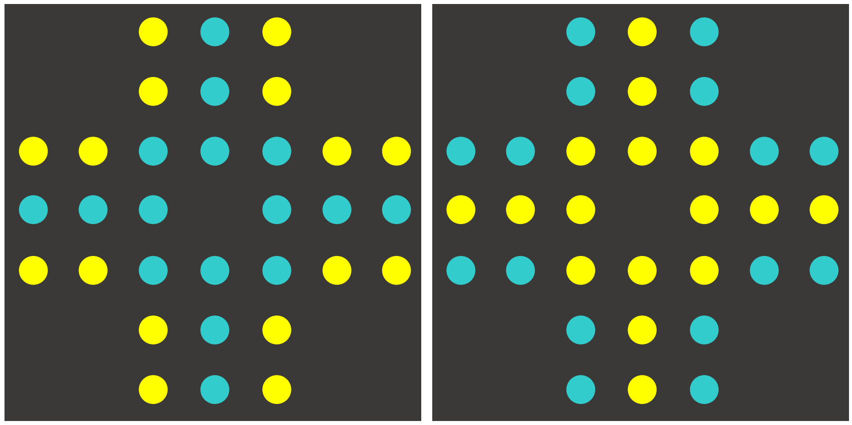

Figure 1. An example of the Solitaire Illusion

The yellow elements generally appear more numerous on the right than the left, despite both images having an identical quantity of yellow and blue elements. Alternatively, this illusion can also be interpreted as the yellow elements appearing more numerous than the blue elements in the right image and vice versa in the left image.

Honeybees (*Apis mellifera*) are model species for testing learning and perception.[25–27] Honeybees are known to perceive a variety of spatial, movement, and color illusions including illusory contours,[28,29] the Delboeuf Illusion,[16] the Benham Illusion,[30] and the Craik–O'Brien–Cornsweet Illusion.[31] As honeybees can process visual information both locally and globally, but demonstrate a global preference for visual information processing,[32,33] this makes them a good candidate for studying their susceptibility to visual illusions. When studying the perception of the Delboeuf Illusion in honeybees, it was found that the restriction of viewing distance significantly impacted whether bees perceived the illusion or not.[16] When bees were allowed to fly freely while inspecting stimuli at an unconstrained distance, they perceived an illusion. However, when the viewing distance of the stimuli was constrained to 6 cm at the closest, bees did not perceive an illusion. This demonstrates that viewing distance and restriction of visual angle impacted the perception of the illusion, which has previously been suggested as a mediating factor of illusion perception.[6] In the current study, we aimed to determine if honeybees and/or closely related wasps (Order: Hymenoptera) could perceive the Solitaire Illusion, and thus allowed the individually trained and tested insects from respective species to fly freely and inspect stimuli at their preferred distance before making a decision on where to land.

Honeybees have shown a range of numerical and quantitative abilities[34–36] from simple to complex tasks. Honeybees are able to discriminate between and order numerosities,[37–40] match identical quantities regardless of shape and color,[41] transfer quantity to size,[42] use rudimentary counting of landmarks to navigate,[43,44] perform simple arithmetic operations,[45,46] match quantities to abstract characters,[47] and there is also evidence that honeybees can categorize odd and even numbers.[48] Recently, honeybees have also been shown to have a left to right mental number line, meaning they prefer to order smaller quantities on the left and larger quantities on the right,[49] similar to newborn chicks,[50,51] and humans.[52,53] Interestingly, the demonstration of numerical abilities in insects has led to much debate on the mechanisms (numerical or non-numerical), evolutionary pathways, cultural factors, neurobiology, and the capacity of bees[49,54–57] to perform numerical and quantitative tasks. Similar debates have taken place for the capacity of non-human vertebrate species to perform numerical tasks.[51,58–64] Informing these debates is recent evidence that quantity processing in the fruit fly (*Drosophila melanogaster*) is enabled in the columnar neurons in the lobula, showing that the neurobiological mechanisms to enable number processing exist in insects,[65] strengthening the notion that invertebrates are capable of numerical tasks. Additionally, a recent study has shown that bumblebees (*Bombus terrestris audax*) are able to perceive the Solitaire Illusion,[22] making a comparison with other insects, particularly other Hymenoptera, now possible.

Some species of wasp have shown learning and visual discrimination abilities, although currently wasp perception is less studied for these tasks than honeybees.[57,66] Nevertheless, as honeybees and many wasps are visual foragers and relatively closely related Hymenoptera, recent research shows comparative studies on these insect species can provide important information on visual processing mechanisms.[67–73] For example, paper wasps (*Polistes fuscatus*) are able to learn the abstract concept of same vs. different.[66] Paper wasps are also capable of learning and recalling unique facial markings of individual conspecifics and transitive inference, which is the ability to infer unknown relationships using information from known relationships (e.g., if A > B and B > C, then A > C).[74] Recently, the yellow-legged hornet (*Vespa velutina nigrithorax*) and the European hornet (*Vespa crabro*) demonstrated the ability to perform differential and reversal learning.[75] The European wasp (*Vespula vulgaris*) has shown the capacity to perform a perceptually challenging color discrimination task following training, which is dependent on the type of conditioning they receive.[76] *V. vulgaris* performs significantly better at a perceptually difficult color discrimination task when trained with appetitive-aversive differential conditioning compared to appetitive differential conditioning or absolute conditioning.[76] Appetitive-aversive differential conditioning provides a reward for a correct stimulus choice and an aversive outcome for an incorrect choice while appetitive differential conditioning provides a reward for a choice of a correct stimulus and no outcome for the choice of the incorrect choice. Finally, absolute conditioning is where the individual receives a reward on the correct stimulus in the absence of the incorrect options and then is tested for the ability to discriminate between the correct and distractor stimulus options.[76] *V. vulgaris* is also known to

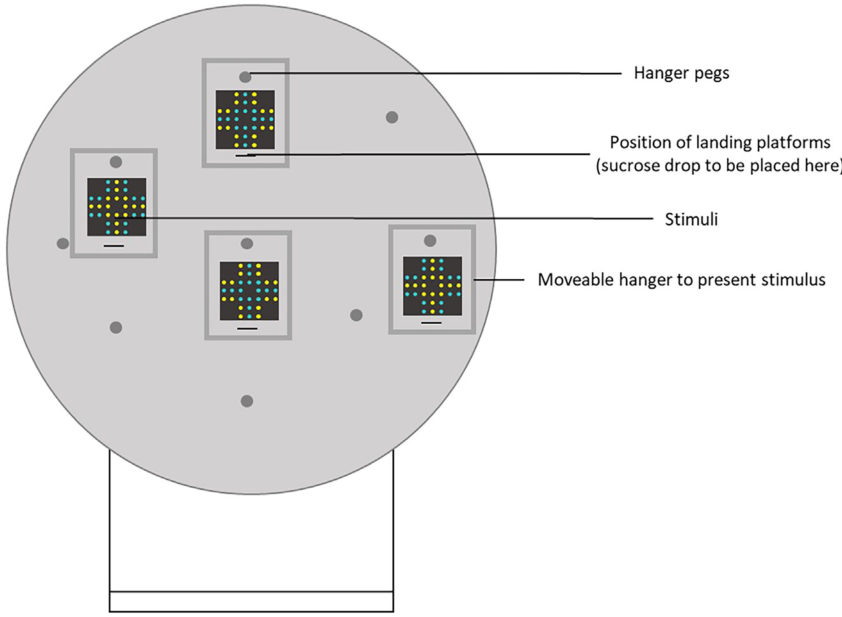

Figure 2. The rotating screen apparatus used to train and test insects during the experiments
Stimuli shown in the figure present elements in configurations known as the Solitaire Illusion.

process certain visual images holistically[69,70,77] in a way analogous to observed honeybee preferences[32,33], thus making them a good candidate to potentially perceive illusions and study visual perception in a comparative way.

In the current study, we tested whether European honeybees (*A. mellifera*) and wasps (*V. vulgaris*) show evidence of perceiving the Solitaire Illusion in a way consistent with human perception. If honeybees and/or wasps perceived the Solitaire Illusion, when trained to go to the 'greater' quantity of elements, we would expect them to choose more clustered elements of a target color compared with unclustered elements of the same target color even when both quantities were the same. If the insects did not perceive the illusion, we would expect them to choose at chance level regardless of element spatial configuration as both quantities were identical. We initially trained individual insects from each species to discriminate between quantities of yellow and blue dots, and then the animals were presented with stimuli depicting the Solitaire Illusion. While honeybees have been tested on their numerical and quantitative abilities,[34–36] wasps are yet to demonstrate numerical or quantitative ability and thus this study additionally serves as an important test of whether they are able to perform quantity discrimination, with the possibility of using non-numerical cues in parallel due to the nature of the stimuli.

RESULTS

We trained and tested honeybees and wasps with the same general procedure. Insects were recruited from gravity feeders dispensing 5–8% sucrose solution. Each individual was color marked for identification then trained and tested separately. Insects were trained to visit a neutral gray circular rotating screen (Figure 2) that had gray hangers presenting stimuli options (two identical incorrect options vs. two identical correct options). The hangers had small landing platforms under the stimuli where insects could receive a reward of sucrose for a correct choice or an aversive outcome of quinine (a bitter tasting substance) for an incorrect choice. Individual insects were first trained to land on the hanger platforms to obtain sucrose solution in the absence of any stimuli. Once insects were landing without assistance from researchers, stimuli were placed on the fresh hangers and the training phase of the experiment commenced. Throughout the experiments, stimuli, hangers, and the apparatus were cleaned with ethanol, water, and dried after each choice.

Training

An initial preference test for color showed that all insects preferred blue and thus yellow (or a higher relative quantity of yellow elements) was used as the rewarding color in subsequent training (Figure 3). Wasps and bees have been shown to require appetitive-aversive differential conditioning to successfully learn cognitively and perceptually challenging tasks, therefore this conditioning type was used throughout all training.[40,76,78] Insects were first trained to an array of 33 yellow circles vs. 33 blue circles presented on laminated cards (stimuli). Insects were trained to avoid their initial color preference (blue) and land on the non-preferred color (yellow; Figure 3). Insects were rewarded with sucrose solution for a correct choice of yellow and received an aversive outcome of quinine for an incorrect choice of blue for 20 color training trials[76,78] (Figure 3). Following these 20 trials, insects were trained for an additional 50 trials to visit stimuli consisting of both yellow and blue circles. Insects were trained that the higher quantity of yellow circles amongst blue circles would result in a reward of sucrose, whereas the alternative stimulus, consisting of a lower yellow to blue ratio would result in an aversive outcome (Figure 3). The sequence of

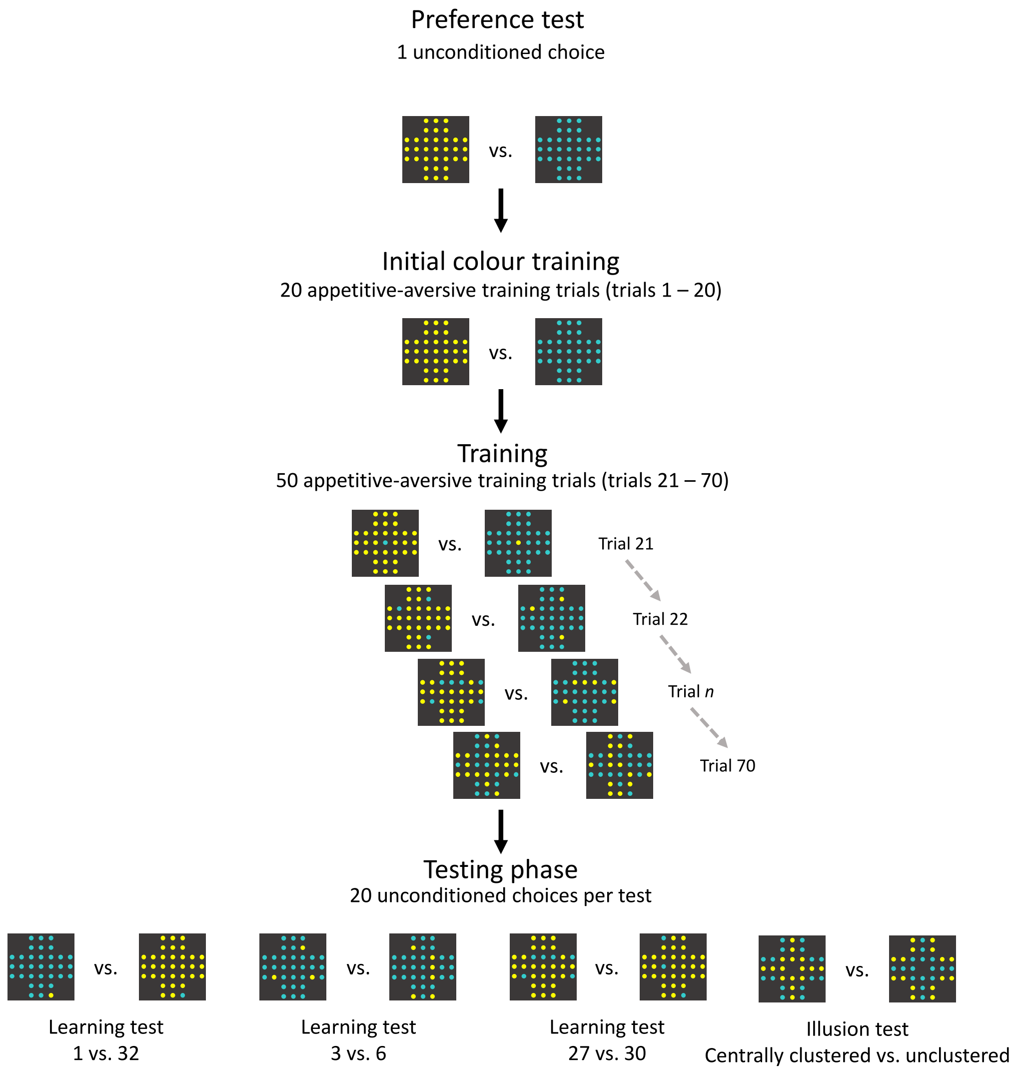

Figure 3. Training and testing process for honeybees and wasps

Honeybees and wasps first underwent a Preference Test to determine the target color (opposite to preference). Insects then underwent 70 appetitive-aversive differential conditioning training trials – the first 20 trials presented the target vs. distractor colors and the following 50 trials (21–70) trained insects to choose the greater quantity of yellow vs. blue dots. We then conducted four tests in sequence from the least to most challenging discriminations, with the final test presenting the Solitaire Illusion, which presented bees with identical quantities of blue or yellow stimuli that were either centrally clustered or unclustered.

presentation was the same for each individual: 1 vs. 32, 3 vs. 30, 6 vs. 27, 14 vs. 19, 1 vs. 6, 3 vs. 6, 27 vs. 30, 19 vs. 30, 3 vs. 19, 6 vs. 30, where all numbers refer to the quantity of yellow dots in the array 33 overall dots (Figure 3). The number of blue dots can be calculated by subtracting the number of yellow dots from 33. Not all insects were exposed to all comparisons as this depended on the length of the individual's bout and number of correct and incorrect choices made during each visit. All insects were trained for 50 appetitive-aversive trials to select the greater quantity of yellow. Altogether, insects each received 70 appetitive-aversive training trials (20 trials of yellow vs. blue; 50 trials of greater vs. lesser quantities of yellow elements; Figure 3).

In the training phase, both honeybees (z = 3.197, p = 0.001; n = 12) and wasps (z = 1.718, p = 0.0857; n = 12) significantly increased their proportion of choices for the correct stimulus option (Figure 4A). The analyses of training was treated as a one-tailed test as the insects underwent extended training to select the correct stimulus and thus a result below 50% correct choices was not expected following the appetitive-aversive conditioning.[78]

The initial model comparing the performances of honeybees and wasps showed a significant effect of trial (z = 3.162, p < 0.002), but no significant effect of species (z = 0.782, p = 0.434) or an interaction between species and trial (z = −1.027, p = 0.304), therefore we removed the interaction term from the model. The new model without the interaction term showed a significant effect of trial (z = 3.459, p = 0.001) but no effect of species (z = −0.191, p = 0.848), demonstrating that bees and wasps had similar performances following the appetitive-aversive conditioning to training stimuli.

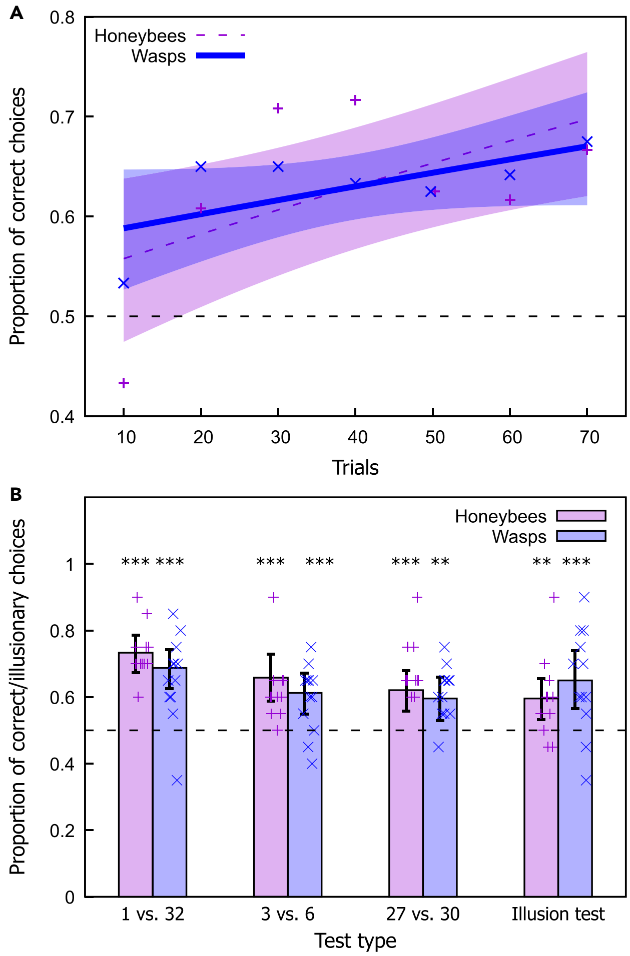

Figure 4. Performance of honeybees and wasps during training and testing phases

Panel (A) shows the performance of honeybees (violet broken line; violet plus signs) and wasps (blue solid line; blue crosses) over the 70 training trials. Shaded areas are 95% confidence intervals. Plus signs and crosses show the mean data of honeybees and wasps per 10 trials. Broken black line at 0.5 shows chance level performance.

Panel (B) shows the performance of honeybees (violet plus signs) and wasps (blue crosses) during tests. Data shown in columns is the mean $\pm$95% confidence intervals, represented by the black error bars. Broken black line at 0.5 shows chance level performance. Plus signs and crosses show the individual performance of each insect during the tests. Bees and wasps performed significantly above chance level in all tests demonstrating learning of 'greater vs. lesser' and perception of the Solitaire Illusion. Significance from chance level performance is indicated by *NS* $\geq$ 0.05, * $\leq$0.05, ** $\leq$0.01, *** $\leq$0.001 (generalized linear mixed model with a binomial distribution).

Testing

Following the 70 training trials, four unconditioned tests were conducted, where insects were not provided with sucrose or quinine for choices. Instead a drop of water was placed on the platform as a neutral outcome, which encouraged insects to land but provided no conditioning. This method allowed us to completely rule out effects of learning differences between solutions and/or scent marking by insects. Insects underwent three learning tests where they were presented with three quantity comparisons experienced during the training phase: 1 vs. 32, 3 vs. 6, and 27 vs. 30 (Figure 3), where the comparisons refer to the quantity of yellow elements in the stimuli. Bees and wasps then underwent the comparison of 16 vs. 16 (Illusion Test), where one stimulus had 16 clustered yellow dots, and one stimulus had 16 unclustered yellow dots in a field of 32 overall dots (Figures 1 and 3). Each unconditioned test consisted of 20 choices (touches of the platforms or stimuli).

Honeybees

In learning tests, honeybees demonstrated that they had learnt the rule of "choose more yellow dots" by choosing the correct stimulus option in all three learning tests. In the test of 1 vs. 32, bees chose the correct option in 73.33% of choices (MPCC: 0.733; Confidence Intervals [CIs]: 0.674, 0.786; $z = 6.930$; $p < 0.001$; $n = 12$). In the test of 3 vs. 6, bees chose the correct option in 65.83% of choices (MPCC: 0.661; CIs: 0.587, 0.728; $z = 4.139$; $p < 0.001$; $n = 12$). In the test of 27 vs. 30, bees chose the correct option in 62.08% of choices (MPCC: 0.621; CIs: 0.558, 0.680;

$z = 3.706$; $p < 0.001$). In the illusion test, bees chose the stimulus that would indicate illusion perception in 59.58% of choices (MPCC: 0.596, CIs: 0.533, 0.656; $z = 2.951$; $p = 0.003$; $n = 12$; Figure 4B). Thus, honeybees successfully chose the greater quantity of yellow dots in all three learning tests and also demonstrated perception of the Solitaire Illusion consistent with past studies of some humans and non-human animals.[17,21,22]

Wasps

In learning tests, wasps demonstrated evidence of learning the rule of "choose more yellow dots" by choosing the correct stimulus significantly more than the incorrect stimulus in all three learning tests. In the test of 1 vs. 32, wasps chose the correct option in 68.75% of choices (MPCC: 0.688; CIs: 0.626, 0.743; $z = 5.662$; $p < 0.001$; $n = 12$). In the test of 3 vs. 6, wasps chose the correct option in 61.67% of choices (MPCC: 0.612; CIs: 0.549, 0.672; $z = 0.133$; $p < 0.001$; $n = 12$). In the test of 27 vs. 30, wasps chose the correct option in 60% of choices (MPCC: 0.596, CIs: 0.529, 0.661; $z = 2.783$; $p = 0.005$; $n = 12$). In the illusion test, wasps chose the stimulus that indicated illusionary perception in 65.42% of choices (MPCC: 0.658; CIs: 0.565, 0.740; $z = 3.275$; $p = 0.001$; $n = 12$; Figure 4B). These results showed that wasps significantly chose the greater quantity of yellow dots in all three learning tests and also appeared to perceive the Solitaire Illusion.

Comparison

To determine whether bees and wasps performed differently during each of the tests, we compared their performance. In the learning tests and illusion test, bees and wasps did not perform significantly different from each other in terms of correct choices for the higher quantity of yellow dots or choice of a stimulus that would indicate the perception of the Solitaire Illusion (all tests: $p > 0.05$).

DISCUSSION

Studying the perception of illusions across vertebrate and invertebrate species can inform us about the evolution of the visual system and illusion perception. In the current study, we show that two invertebrates, both from the order Hymenoptera, perceive a numerosity illusion in a similar way to humans, capuchin monkeys,[17] guppies,[21] and bumblebees.[22] Conversely, research shows that chimpanzees, rhesus monkeys,[17] and domestic dogs[23] do not perceive the illusion. This suggests one of two evolutionary pathways: (1) conserved evolution, with the loss of the perception in some vertebrate species, or (2) convergent evolution in some species of vertebrates and invertebrates. It would be valuable to test the perception of the Solitaire Illusion in other invertebrates outside of Hymenoptera, and a wider variety of vertebrates, to further inform us of the evolutionary mechanism of perception.

A potential contributing mechanism enabling illusion perception is global visual processing. Honeybees are known to prefer to process globally rather than locally.[32,33] Some wasps process certain visual images holistically[69,70,77]; however, there is variation in the strength of global visual processing in wasps. For example, a study comparing holistic processing in European honeybees (*A. mellifera*) and European wasps (*V. vulgaris*) determined the capacity of both species to holistically view stimuli presenting human faces or Navon-like parameterized stimuli.[69] Both honeybees and wasps showed a preference for processing human face stimuli holistically. Interestingly, honeybees demonstrated a greater preference to process the Navon-like parameterized stimuli holistically compared to wasps; however, wasps did still show a global preference for processing the visual information. Thus, it appears from past studies that both bees and wasps process visual images in a holistic way and prefer to use global perception.[69] These previous findings suggest that one contributing mechanism enabling the perception of the Solitaire Illusion by honeybees and wasps is their capacity and preference to process visual information globally.[2]

The nature of the stimuli for the Solitaire Illusion means that the discrimination between two stimuli of differing elements can be achieved by using numerical information (quantity of elements), although other non-numerical cues such as surface area (amount of yellow), line length, perimeter, or other cues could play a role in the process. It is possible that insects are using a combination of numerical and non-numerical cues to discriminate between quantities during training and learning tests, and revert to only using numerical cues for differentiating between the illusionary stimuli. It is also possible that the Solitaire Illusion could induce the perception of size, line length, or perimeter illusions as well as being a numerosity illusion, thus we are unable to currently disentangle the use of numerical or non-numerical cues in the learning phase or the perception of the illusion. Further work should aim to unravel the use of different spatial cues during the perception of the Solitaire Illusion to determine if it is truly an illusion of numerosity or if it may also be an illusion of other spatial cues. The use of non-numerical cues is a potential reason that we suggest for the ability of honeybees and wasps to differentiate between 27 vs. 30 yellow elements (ratio of 0.900) during the learning test, as this is otherwise a challenging comparison for animals to successfully perform. Few animals have shown a capacity to discriminate between two numbers with a ratio of 0.900. Some animals show an ability to discriminate ratios of over 0.8 including elephants (*Elephas maximus*) (ratio: 0.833),[79] great apes (*Pan paniscus*, *Pan troglodytes*, *Gorilla gorilla*, *Pongo pygmaeus*) (ratio: 0.900),[80] Western scrub jays (*Aphelocoma californica*) (ratio: 0.875),[81] North Island robins (*Petroica longipes*) (ratio: 0.875),[82] and guppies (*Poecilia reticulata*) (ratio: 0.833).[83] Honeybees have also shown an ability to discriminate between 11 vs. 12 in a parity task (0.917),[48] and such a task excludes the use of non-numerical cues such as surface area, spatial frequency, perimeter, line/edge length, and others. However, we do suggest that honeybees may not be performing a purely numerical task during parity categorization.[48] A recent study on bumblebee perception of the Solitaire Illusion found that bumblebees could discriminate up to a ratio of 0.780 (14 vs. 18 elements).[22] Based on these past studies, it is possible that insects may use a combination of cues for difficult quantity comparisons, such as 27 vs. 30. Furthermore, recent work shows that free-flying honeybees appear to weight numerosity cues above non-numerical cues during some numerical tasks.[49] However, the use of numerical and non-numerical cues is an issue unlikely to impact our ability to compare the current findings with other species tested on their perception of the Solitaire Illusion experiments, as the typical Solitaire Illusion image used in those studies[17,19–21,23,24] was also used in this study.

The current study presents evidence that wasps can potentially use numerical and/or quantitative information to perform discrimination between two quantities. While there could be non-numerical cues playing a role in quantity discrimination (discussed above), our results suggest that wasps may possess numerical/quantity discrimination abilities. This means that wasps would join many animals which are known to perform quantity discrimination ranging across invertebrates and vertebrates. Our findings strengthen arguments by other authors that numerical abilities in many animal species are a result of evolutionary convergence[35] or evolutionary conservation.[36] However, the study of numerical competency in a greater range of invertebrates and an analysis of where in the insect brain numerical tasks may be processed[65,84] would be necessary to better understand the evolution of numerical ability across diverse animal species.

Limitations of the study

This study presents experiments testing whether European honeybees and European wasps can perceive the Solitaire Illusion, which is suggested to cause a misperception of numerosity. This study, and studies testing different species for their perception of the Solitaire Illusion, cannot determine if animals were using numerical or other spatial cues (e.g., surface area, line length, perimeter, etc.) to learn to choose the correct option during the training phase. Therefore, in addition to being a misperception of quantity, the Solitaire Illusion could also potentially be an illusion of non-numerical cues such as surface area. Further examination of what spatial cues drive learning and illusion perception in animals will be necessary to disentangle these cues and show what visual cues cause the illusion.

STAR★METHODS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Raw data	This study	https://github.com/DrScarlettRHoward/Publications-Quantity-misperception-by-hymenopteran-insects-observing-the-Solitaire-Illusion/tree/main
Data analysis code	This study	https://github.com/DrScarlettRHoward/Publications-Quantity-misperception-by-hymenopteran-insects-observing-the-Solitaire-Illusion/tree/main
Experimental models: Organisms/strains		
European honeybee foragers (*Apis mellifera*)	Colonies managed by Johannes Gutenberg-Universität Mainz, Germany	N/A
European wasps (*Vespula vulgaris*)	Wild insects at Johannes Gutenberg-Universität Mainz, Germany	N/A
Software and algorithms		
R Studio (R version 4.2.0)	https://www.r-project.org/	N/A

RESOURCE AVAILABILITY

Lead contact

Additional information and requests for resources should be directed to and will be fulfilled by the lead contact: Scarlett Howard, scarlett.howard@monash.edu.

Materials availability

This study did not generate new materials.

Data and code availability

- Raw data is available on GitHub at this URL: https://github.com/DrScarlettRHoward/Publications-Quantity-misperception-by-hymenopteran-insects-observing-the-Solitaire-Illusion/tree/main.
- All original code, R scripts, are available on GitHub at this URL: https://github.com/DrScarlettRHoward/Publications-Quantity-misperception-by-hymenopteran-insects-observing-the-Solitaire-Illusion/tree/main.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

A total of 12 free-flying European honeybee foragers (*Apis mellifera*) and 12 free-flying European wasp foragers were involved in this study. All insects were adult non-reproductive female foragers. Bees were approximately 22–42 days old, wasps were likely less than 3 weeks old. All insects were living on campus in nests or hives at the Johannes Guttenberg University of Mainz in Germany. Honeybee hives were managed by Johannes Guttenberg University of Mainz. Wasps were wild and not maintained by the institution. One subject was excluded as it was identified as being the wrong wasp species following an experiment. Individuals had not previously been involved in other experiments.

METHOD DETAILS

Study species

Honeybee foragers (*A. mellifera*) from three hives maintained at the Johannes Guttenberg University of Mainz in Germany were used in the experiment. Bees were recruited to a von Frisch-style gravity feeder containing 5–8% sucrose solution by volume. Bees were then collected from the feeder onto transparent plexi-glass spoons containing 20% sucrose solution by volume and transported to a rotating screen apparatus 50 cm in diameter enabling vertical presentation of stimuli at pseudo-random positions (Figure 2).[69,85] Each test bee was color marked following standard procedures to enable us to identify the individual and record individual choices.

Wild wasps (*V. vulgaris*) were also recruited from the gravity feeders at the Johannes Guttenberg University of Mainz in Germany. The wasps were also collected onto spoons containing 20% sucrose solution and placed onto the landing platforms of the rotating screen apparatus (Figure 2). *V. vulgaris* has previously been shown to act as a central place forager, which enables the training and testing of individually marked insects.[69]

One individual was trained and tested at a time. The experiment took between 2 and 3 h to complete for individuals of respective species. Individual insects were marked with colored dots to differentiate between them. Experiments were conducted from August to September 2022. We trained and tested 12 honeybees and 12 wasps (n = 24 insects). No individuals were excluded from analyses.

Apparatus

A circular gray plexi-glass rotating screen, 50 cm in diameter, was used for the experiments (Figure 2).[85] The screen was positioned vertically and could be rotated to randomise stimuli positions on it. The screen consisted of pegs which were used to hold gray plexi-glass hangers. The hangers were 6 × 8 cm and had a small landing platform for bees and wasps to land and taste solutions (e.g., sucrose or quinine solution or water). The stimuli could be presented on these hangers and thus associated with a reward of 20% sucrose solution for a correct choice or an aversive outcome of 6 mM quinine solution for an incorrect choice. Four hangers were presented to bees at a time. The hangers contained no stimuli when training individual bees or wasps to land and return to the apparatus location. After insects had learnt to land on the platforms, training and testing began. During training and testing, two hangers showing identically correct stimuli were presented against two hangers showing identically incorrect stimuli. Training length was determined in pilot testing.

Stimuli

Stimuli consisted of salient blue or yellow dots on a gray background presented on laminated cards (Figure 3). The respective blue and yellow colors are shown to be easily discriminable from each other by honeybees and the colors have been measured when used in a past experiment.[46] The gray color acted as a neutral background for bees.[86] We assumed wasps would also be able to discriminate these color stimuli as they have a similar trichromatic visual system to honeybees,[87] and this was confirmed during training and testing.

Stimuli were 6.5 × 6.5 cm square cards. Stimuli used during preference testing and training trials 1–20 consisted of cards containing either 33 yellow dots, or 33 blue dots. Training stimuli for trials 21–70 and testing stimuli consisted of 33 dots (Figure 3) of different quantities of yellow and blue. Training stimuli contained yellow:blue dot ratios of 1:32, 32:1, 3:30, 30:3, 6:27, 27:6, 14:19, and 19:14. Individuals were presented with comparisons of a target color (e.g., yellow) and shown a higher or lower quantity of yellow and were then required to choose the higher quantity. Each time an insect touched a stimulus or hanger, all stimuli and hangers were removed, cleaned with 30% ethanol solution, water, and dried to exclude the use of scent marking.

Preference test

Individuals were first given a preference test to determine their preferred color. This preference tested lasted for one single spontaneous choice (landing onto a hanger platform). Insects were not rewarded for a choice in this test. The stimuli presented contained either 33 yellow dots vs. 33 blue dots. If an insect chose blue in this preference test, they would be trained to associate more yellow elements with sucrose and less yellow elements with quinine. If an insect chose yellow, they would be trained to associate more blue elements with a reward and less blue elements with quinine. However, as all 12 bees and 12 wasps tested had a spontaneous blue preference, consistent with previous research,[88] all individuals were trained to associate more yellow dots with a reward of sucrose solution.

Training

Training trials consisted of appetitive-aversive differential conditioning, where a correct choice is rewarded with 20% sucrose solution and an incorrect choice results in bees tasting 6 mM quinine solution, a bitter-tasting substance that promotes visual learning in free-flying Hymenopterans[76,78,89] and improves quantity discrimination in European honeybees.[40]

After the preference test, we conducted 70 training trials. The first 20 trials trained insects to select yellow and avoid blue stimuli using the 33 yellow stimuli vs. the 33 blue stimuli (Figure 3). Insects were rewarded for selecting yellow with sucrose solution and received an aversive outcome of quinine for selecting blue. Throughout training, stimuli were randomly moved to control for positional cues on the rotating screen. A choice was defined as a landing and touch of the solution on the hanger platform with the insects' antennae, leg, or proboscis. Insects generally made about 2–6 correct choices per bout until satiated. A bout was defined as a visit from the hive to the apparatus to make choices.

After the first 20 trials, each insect underwent 50 training trials to select the larger quantity of yellow on stimuli. The insects were shown sequences of comparisons that changed each bout. The sequence of presentation was the same for each individual: 1 vs. 32, 3 vs. 30, 6 vs. 27, 14 vs. 19, 1 vs. 6, 3 vs. 6, 27 vs. 30, 19 vs. 30, 3 vs. 19, 6 vs. 30, where all numbers refer to the quantity of yellow dots in the array 33 dots (Figure 3). The number of blue dots can be calculated by subtracting the number of yellow dots from 33. Not all insects saw all comparisons as it depended on the length of the individual's bout and number of correct and incorrect choices made during each visit. All insects were trained for 50 appetitive-aversive trials to select the larger quantity of yellow. Altogether, insects each received 70 training trials (20 trials of yellow vs. blue; 50 trials of greater vs. lesser quantity of yellow elements; Figure 3).

After a correct choice was made, each insect was collected onto the plexi-glass spoon from the landing platform, and placed behind an opaque gray screen located 1 m in front of the apparatus. Insects received sucrose behind the screen, on the spoon, while stimuli and hangers were replaced (cleaned and dried) and moved, and solutions were replenished while the screen was rotated. The insect was then allowed to make another choice, or return to its hive if satiated. If an insect made an incorrect choice and tasted quinine, it was not interfered with and allowed to continue choosing until it made a correct choice. Once it had made a correct choice, the normal protocol for a correct choice was followed. All incorrect and correct choices were recorded for analysis.

Testing

Insects underwent four unconditioned tests (no sucrose or quinine solution was present) consisting of three learning tests using stimuli quantities they had viewed during training, and one illusion test to determine if they perceived the Solitaire Illusion. A drop of water was placed on each platform to act as additional motivation encouraging insects to land in the absence of sucrose or quinine solution. The water acted as a neutral solution during testing. In sequence, insects received increasingly difficult tasks during testing (so that more difficult tasks did not impact performance on less challenging ones). Each test consisted of 20 unreinforced trials for each test per insect. They were shown comparisons of 1 vs. 32 (Learning Test 1), 3 vs. 6 (Learning Test 2), 27 vs. 30 (Learning Test 3), and 16 vs. 16 (Illusion Test), where one stimulus had 16 clustered yellow dots, and one stimulus had 16 unclustered yellow dots in a field of 32 overall dots. Between each test of 20 unreinforced choices, insects were provided with one bout of refresher trials (same stimuli and procedure as in training) to motivate them to return for the next unconditioned test.

QUANTIFICATION AND STATISTICAL ANALYSIS

The 70 choices made during the training phase were analyzed to determine whether honeybees and wasps demonstrated significant learning during the appetitive-aversive differential conditioning phase of the experiment. Data from the 70 appetitive-aversive differential training trials were analyzed with a generalized linear mixed-effects model (GLMM) with a binomial distribution using the 'glmer' package within the R environment for statistical analysis.[90] The full model was first fitted with choice as the categorial response variable with two levels (correct; incorrect) and trial number as a continuous predictor (1–70). Subject (insect ID) was included as a random factor to account for repeated choices of individual insects. This analysis was treated as a one-tailed test as the insects underwent extended training to select the correct stimulus and therefore a result below 50% correct choices was not expected following the appetitive-aversive conditioning, and any such finding would only be a random effect considering how bees are known to learn.[78] A P-vale of $\leq$0.100 was thus considered as significant for the analysis of whether training resulted in choices significantly different from chance expectation.[91]

To compare whether learning differed significantly between honeybees and wasps, we analyzed the data using a GLMM with a binomial distribution using insect choice as the categorical response variable with two levels (correct; incorrect). Individual trial number, species, and an interaction term between these two predictors were included in the model. Subject (insect ID) was included as a random factor to account for repeated choices of individual insects.

To determine whether the insects learnt to choose the higher quantity of yellow dots in learning tests, we employed a GLMM with a binomial distribution including categorial response variable with two levels (correct; incorrect) for all three learning tests. For the Illusion Test, the categorical response variable had two levels (choice for clustered elements; choice for unclustered elements). Subject ID was included as a random factor to account for repeated choices of individual insects. The proportion of choices for the correct color (MPCC) recorded from the tests was used as the response variable in the model. The Wald statistic (z) tested if the mean proportion of correct choices recorded from the test, represented by the coefficient of the intercept term, was significantly different from chance expectation, i.e., H_0: Mean Proportion of the Correct Choice (MPCC) = 0.5.

The models were estimated using the routine "glmer" available as part of the "lme4" package written for the R statistical language, run in R version 4.0.3.[90,92]

For Figure 4, significance from chance level performance is indicated by $NS \geq 0.05$, * $\leq$0.05, ** $\leq$0.01, *** $\leq$0.001 (generalised linear mixed model with a binomial distribution).