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**HOW DOES A SOLITARY BEE FIND ITS NEST?
A CASE STUDY WITH *CENTRIS (HETEROCENTRIS)*
ANALIS (FABRICIUS) (HYMENOPTERA, APIDAE,
CENTRIDINI)**

PEDRO REIS ANTUNES

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**Dissertação apresentada como requisito para
obtenção do título de Mestre em Ecologia e
Conservação de Recursos Naturais da
Universidade Federal de Uberlândia.**

**Orientadora: Profa. Dra. Solange Cristina
Augusto
Coorientadora: Dra. Francismeire Jane Telles
da Silva**

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“If you assume that there is no hope, you guarantee that there will be no hope. If you assume that there is an instinct for freedom, that there are opportunities to change things, then there is a possibility that you can contribute to making a better world.”

A handwritten signature in black ink, appearing to read "Ronald Reagan". The signature is written in a cursive style with a large, prominent "R" at the beginning.

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ABSTRACT

Antunes, Pedro R. 2019. How does a solitary bee find its nest? A case study with the solitary bee *Centris (Heterocentris) analis* (Fabricius) (Hymenoptera, Apidae, Centridini). MSc thesis. UFU. Uberlândia-MG. 33 p.

The knowledge of the sensory and cognitive capacity of insects is, mostly, restricted to studies with social species. However, more than 85% of bees are solitary. Using the bee *Centris (Heterocentris) analis* (Fabricius) as a model, we aimed to understand which information (spatial or visual), this tropical solitary bee mostly relies on in the context of nest recognition. Using a homogeneous background, we evaluated the effect of continuous and varied nest position in the presence and absence of coloured stimuli, and the possible effect of colour (blue or green) and training on accuracy and search time of returning females. Bees were able to adapt to new cues to find their nests, but performance varied among treatments. Our results have shown that besides spatial information, bees can use the presence of a coloured stimulus to enhance the accuracy during nest location, and that odour is a secondary cue in this process. Overall, as females gained experience with a task, they would require more time before making a choice, a result that seems to be modulated by treatments associated with the presence of visual stimuli.

Keywords: Nest localization, Visual cues, Search time, Chromatic and Achromatic contrasts.

1. INTRODUCTION

The knowledge of the sensory and cognitive ability of insects is, mostly, restricted to studies with social species. Considering Hymenopterans, the major volume of these studies is concentrated in three Apidae bees: *Apis mellifera*, *Bombus terrestris* and *Bombus impatiens*. Indeed, several important discoveries in the field were first made with these model species, such as colour vision (FRISCH, 1914), visual detection of ultraviolet light (DAUMER, 1956), multisensory integration (LEONARD; MASEK, 2014), electromagnetic detection (CLARKE; MORLEY; ROBERT, 2017), and complex and collaborative communication (GIURFA, MARTIN, 2012; LOUKOLA *et al.*, 2017). Besides, if we consider the diversity of bees, and the ecological and evolutionary differences among them (MICHENER, 2007), we will notice that these species represent only a tiny fraction of the world's bee diversity, with many major groups completely omitted. In fact, more than 85% of all bee species are solitary (BATRA, 1984), with about 14000 species worldwide (NEFF, 2008).

Studies of bee sensory ecology typically occur in controlled laboratory conditions (GIURFA, 2007), or semi-natural conditions (TELLES *et al.*, 2017), in order to reduce environmental noise and have a better control of individual performance (SANDOZ, 2011). Usually, the methods are framed in the context of the foraging activity (appetitive conditioning). In this system, experimental commercial colonies are deprived of food (nectar or pollen), and individuals are conditioned to associate a specific stimulus, such as visual or chemical, with the presence of a reward (GIURFA, MARTIN, 2007). However, attempts to replicate these well-established protocols designed for social species to solitary species are difficult. While eusocial bees live in large perennial colonies with a clear division of labour, with one female exclusively responsible for reproduction, and several others responsible for rearing the larvae (nurses) and foraging (workers) to feed the colony (MICHENER, 2007; WINSTON; MICHENER, 1977), for solitary species, a single female constructs her own nest and provides food for herself and her offspring (MICHENER, 2007). Thus, the motivational state of social (or semi-social) and solitary individuals broadly vary. Associated to it, is the limited knowledge of the ecology and natural history of solitary bees, especially in Tropical areas (MICHENER, 2007), which could provide important information about specific requirements of bees to perform the foraging activity. For instance, it can be difficult to attract and keep solitary

females' attention when we do not understand their specific reproductive, nesting and provisioning requirements.

As many other Hymenoptera species, solitary bees are central-place foragers that leave and return to fixed positions in their environment, such as their nests and foraging sites (GUÉDOT *et al.*, 2013). Thus, while for social species studies are focused on their foraging activity, for solitary bees, nest location has been used as focal point (Table 1). This activity involves the animal's capacity of perception, learning, memory and use of different sensory systems. Before leaving the nest, bees perform one to several flights around it to then fly off (DEGEN *et al.*, 2015; INOUYE, 2000). This behaviour, known as exploratory flights (also called as learning, orientation or recognition flights), enables the bee to identify relevant cues that can latter lead to its nest and surroundings. At long and mid-distance, bees follow familiar landmarks to reach the vicinity of their nests (DEGEN *et al.*, 2016; INOUYE, 2000), by using a series of visual images, or "snapshots," of the environment acquired *en route* (COLLETT, T. S.; FRY; WEHNER, 1993; COLLETT, T S, 1996; COLLETT, THOMAS S.; KELBER, 1988; JUDD; COLLETT, 1998; WEHNER; MICHEL; ANTONSEN, 1996). By comparing the currently viewed scene with the appropriate stored image, the bee is able to ascertain whether it is on the correct path and make any necessary correction (ZHANG; SRINIVASAN, 2004).

Among the solitary species, the few knowledge regarding their sensory and cognitive ability is mostly concentrated in the family Megachilidae (Table 1). Given the effectiveness of some species of this family as pollinators of a variety of crops and thus their economic relevance (JAMES; PITTS-SINGER, 2013; KOH *et al.*, 2018), a better understanding of the biology and ecological requirements became necessary for management purpose in agricultural systems.

TABLE 1: Literature survey of studies considering the sensory and cognitive system of solitary bees. Table presents the family, species, region of occurrence, sociality classification (according to PLATEAUX-QUÉNU, 2008), sensory system investigated and the context of the study.

Family/Species	Region	Sociality	Sensory System	Study context	Reference
Apidae					
<i>Epicharis metatarsalis</i>	Central America (Costa Rica)	Solitary and Aggregations	Visual and Olfactory	Nest Position, Chemical Profile and Landmark Cues	(INOUYE, 2000)
<i>Xylocopa flavorufa</i>	Africa (Tanzania)	Subsocial	Visual and Olfactory	Nest Position and Chemical Profile, Spatial and Landmark Cues	(ANZENBEKGER, 1986)
<i>X. imitator</i>					
<i>X. torrida</i>	Asia (Israel)	Subsocial	Visual and Olfactory	Nest Position, Chemical Profile and Spatial Cue	(HEFETZ, 1992)
<i>X. pubescens</i>					
Halictidae					
<i>Lasioglossum figueresi</i>	Central America (Costa Rica)	Solitary and Social Nests	Visual and Olfactory	Nest Position, Chemical Profile, and Circular Cues Associated to the Nest	(WCISLO, 1992)
<i>Lasioglossum malachurum</i>	Europe (Germany)	Primitively Eusocial	Visual	Landmark Cues	(BRÜNNERT; KELBER; ZEIL, 1994)
Megachilidae					
<i>Megachile rotundata</i>	North America (USA)	Solitary	Visual	Stimuli Shape, Colour Contrast, and Spatial information	(FAURIA, KARINE; CAMPAN; GRIMAL, 2004)
<i>M. rotundata</i>	North America (USA)	Solitary	Visual	Vertical and Horizontal Visual Cues	(GUÉDOT; BOSCH; KEMP, 2005)
<i>M. rotundata</i>	North America (USA)	Solitary	Visual	3D Cues and Colour Contrast	(GUÉDOT; BOSCH; <i>et al.</i> , 2006)
<i>M. rotundata</i>	North America (USA)	Solitary	Visual	3D Cues and Colour Contrast	(GUÉDOT; BOSCH; KEMP, 2007)
<i>Osmia cornuta</i>	North America (USA)	Solitary	Visual	Stimuli Shape and Contrast Around Nest	(FAURIA, K; CAMPAN, 1998)
<i>O. lignaria</i>					
<i>O. lignaria</i>	North America (USA)	Solitary	Olfactory	Nest Chemical Profile	(GUÉDOT; PITTS-SINGER; <i>et al.</i> , 2006)
<i>O. lignaria</i>	North America (USA)	Solitary	Visual	Colour of Nesting Boxes	(ARTZ <i>et al.</i> , 2014)
Melittidae					
<i>Dasygaster hirtipes</i>	Europe (Germany)	Solitary	Visual	Landmarks Cues	(BRÜNNERT; KELBER; ZEIL, 1994)

Except by *Epicharis metatarsalis* and *Dasygoda hirtipes*, all solitary bees listed in Table 1 can be attracted to trap-nests. Trap nests utilize the nesting preferences of several bee species that naturally build their nests in pre-existing cavities (KROMBEIN, 1967; MICHENER, 2007). The use and occupancy of trap nests by solitary bees facilitate studies related not only to the natural history of species, but general ecology and more recently, studies related to cognitive capacities (Table1). In South America, the genus *Centris* shows a wide distribution, especially in Brazil (MOURE; URBAN; MELO, 2012), and is considered one of the most abundant bees in tropical regions (230 species, VIVALLO; MELO, 2009), alongside with species from the Tetrapedini tribe (SILVEIRA; MELO; ALMEIDA, 2002). Most of the *Centris* bees nest in the ground, however, females belonging to the subgenera *Heterocentris*, *Hemisiella* and *Xanthemisia* construct their nests in a variety of pre-existing cavities, including trap-nests (AGUIAR; GARÓFALO, 2004; ALVES-DOS-SANTOS; MACHADO; GAGLIANONE, 2007; PINA; AGUIAR, 2011; RABELO, *et al.*, 2015).

From the *Centris* genus, *Centris (Heterocentris) analis* and *Centris (Hemisiella) tarsata* are considered key pollinators of several plant species, either native (GOTTSBERGER, 1986) or of agricultural importance in Brazil (GIANNINI *et al.*, 2015; OLIVEIRA *et al.*, 2013). Currently, due to the capacity of these bees to be attracted to trap-nests (DA ROCHA-FILHO *et al.*, 2017; JESUS; GARÓFALO, 2000), and their potential economic relevance (GIANNINI *et al.*, 2015; SAZAN *et al.*, 2014), general knowledge of the nesting behaviour (ALONSO; SILVA; GARÓFALO, 2012; JESUS; GARÓFALO, 2000), larval food provision (LIMA *et al.*, 2017; RABELO *et al.*, 2012; RABELO *et al.*, 2014), and the relation between resource availability and reproductive rate (DA SILVA *et al.*, 2017) have been accumulated, but nothing is known about their sensory and cognitive capacities.

Different from social species, where nest entrance can be ornamented (CHITTKA, L *et al.*, 1997; ROUBIK, 1983), or perhaps signalled by guards (COUVILLON *et al.*, 2007; GRÜTER; KÄRCHER; RATNIEKS, 2011), solitary bees' nest entrances are usually holes in hollow structures or in the ground without any distinct visual characteristic, at least from a distance (FRANKIE *et al.*, 1988; MICHENER, 2007). Under natural conditions, a nesting returning solitary bee must have to face and deal with a variety of stochastic events around its nesting site. At the same time, she cannot afford

to rely on chance while searching for her nest given all the fitness costs involved on it (SEIDELMANN, 2018). A variety of cues present at both surrounding and nest itself might help the bee to optimize its nest identification at both far and close ranges (ARTZ *et al.*, 2014; FAURIA; CAMPAN, 1998; INOUE, 2000). Thus, any information capable of improving nest localization or identification might be learned and memorized by solitary bees. For instance, nests might possess a particular odour and entrance size, or occupy a specific spatial position in relation to other objects in its near and far vicinity (BRÜNNERT; KELBER; ZEIL, 1994; FAURIA; CAMPAN; GRIMAL, 2004; GUÉDOT; PITTS-SINGER; *et al.*, 2006).

But, what information, visual or chemical, do tropical solitary bees mostly rely on when performing such a task? Are they flexible on the information they use to identify their nest? Can solitary bees learn a particular cue that results in a more reliable guide to its nest at a close range? In the present study we aimed to answer these basic, but yet open questions for a truly solitary tropical species (MICHENER, 1969), using the bee *Centris (Heterocentris) analis* (Fabricius) as model. Specifically, we aimed to answer the following questions: (1) How does this solitary bee choose and memorize its nest position? (2) Does this bee species use any potential olfactory information from the nest as a cue? (3) Can this species associate its nest entrance with a visual stimulus? and (4) Can the visual stimulus improve nest localization?

2. MATERIALS & METHODS

Model species

Centris (Heterocentris) analis (Fabricius, 1804) is a fast-flying bee with 1.27 cm of length in average, and is considered a truly solitary species, which means there is no generations overlap (CARDINAL; DANFORTH, 2011; VIEIRA-DE-JESUS; GARÓFALO, 2000). A truly solitary female has no contact with her brood developing in constantly closed cells, the mother leaves the nest before the offspring emergence (PLATEAUX-QUÉNU, 2008). The species is widely distributed in South America, from south of Brazil to Mexico (MOURE; URBAN; MELO, 2012). It is considered a multivoltine species (GAZOLA; GARÓFALO, 2009; PINA; AGUIAR, 2011), but in some areas occurs at specific times of the year, commonly on the hot/wet season

(MESQUITA; AUGUSTO, 2011), probably due to resource availability and climatic conditions (ALONSO; SILVA; GARÓFALO, 2012; DA SILVA, *et al.*, 2017). Although solitary, it is possible to aggregate individuals in the same nesting site using trap-nests (DA ROCHA-FILHO *et al.*, 2017; RABELO, *et al.*, 2014). Each female produces from three to nine brood cells per nest (JESUS; GARÓFALO, 2000), and has the capacity to fill up to 18 cells during its lifespan (ALONSO; SILVA; GARÓFALO, 2012).

Study area and Trap-nests

Experiments and observations were conducted from January to December 2018 at the Ecological Station of Panga (ESP - 19°09'20"19°11'10" S, 48°23'20"—48° 24'35" W) at the vicinity of Uberlândia, Brazil. ESP is an area of permanent preservation (IBAMA nº 072/97) with 4.038,500 m² of different vegetal physiognomies of Brazilian Savanna (Cerrado), which includes gallery forest, semi-deciduous forest, cerradão, woody savanna (cerrado *sensu stricto*), grassland and palm swamps (MORENO; SCHIAVINI; HARIDASAN, 2008). To attract bees we used as trap-nests hollow bamboo canes with one of the sides naturally closed by a nodal septum (RABELO, L S *et al.*, 2012). In order to standardize the diameter of trap-nests entrance during experiments, we initially offered to bees bamboo canes with diameters ranging from 4.0 to 8.0 mm, and variable lengths. After occupancy by *Centris analis*, we measured the most external diameter of closed nests to determine the inner diameter of canes utilized by the bees in our study area. The average inner dimension of bamboo canes occupied by bees was of 5.72 mm ± 0.56 (mean ± SD; n = 67). Thus, during the experiments we offered trap-nests with diameters ranging from 5 to 6.5 mm.

General Setup, Bee Nest Selection and Visual Stimuli

In order to homogenise the background and nest distribution, trap-nests were presented in a Styrofoam panel (60 x 60 cm), covered with a grey Ethylene Vinyl Acetate foam (EVA, DUB flex®). Panels (n = 3) presented 30 holes with trap-nests separated 5 cm from each other and 10 cm from the borders (Figure 1). We fixed the panels to different areas using the backside of the field station houses (30 m from each other), where they received natural illumination but were protected from rain.

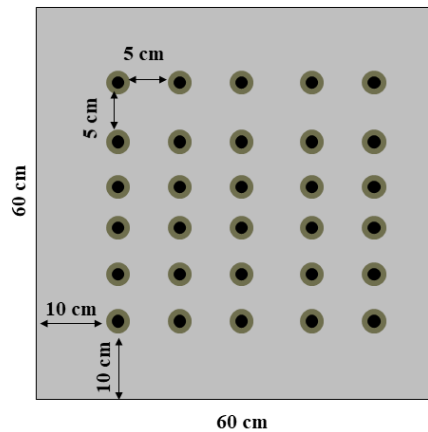


FIGURE 1: Nesting panel. Trap nests were equidistantly separated from each other and from the borders of panels.

We followed bees approaching and inspecting the panel to determine the behaviour of individuals when selecting a nest, and the position (centre or edges, superior or inferior, left or right) of the selected nest. After occupancy and depending on its original position, we changed the selected nest to a more central position on the panel, to avoid (or at least to reduce) the use of proximal information during experiments, such as any contrast produced by the edges of the panel and the wall. Then, we waited at least 12 h, so the female could learn precisely the new position of her nest. After this period, we marked the thorax of selected individuals with a non-toxic paint (Acrilex®) and started the experimental sessions. To avoid interference, once the panel was occupied by a female, other individuals were not allowed to nest on the same panel.

Some treatments consisted on the presentation of a coloured stimuli surrounding the nest entrance. We used two colours: blue and green. Coloured stimuli were selected to vary on the contrast produced with the background, with blue presenting the highest contrast and green the smallest contrast (Table 2). We selected both stimuli to understand whether there was an effect of chromatic and achromatic properties on the accuracy of bees. To calculate the contrast produced between stimuli and the background, we measured the spectral reflectance of both using a USB2000+UV-VIS spectrometer, with a Balanced Deuterium Tungsten Source (DH-2000-BAL, Ocean Optics Inc., Dunedin, FL, USA), positioning probe and light source at an angle of 45°. The spectrometer was calibrated with a standard white (BaSO₄) and blocking light input as black standard. We

limited the readouts to wavelengths from 300 to 700 nm, a range that encompasses the visible spectrum of most Hymenoptera (PEITSCH *et al.*, 1992).

We averaged three spectral measurements to calculate the photoreceptor excitation values (E) using the hexagon colour vision model (CHITTKA, 1992), considering a standard daylight illumination (D65), and our grey background. The E -value reflects the relative excitation (physiological receptor voltage signals) of each photoreceptor in the visual system of the observer when looking at the stimulus, allowing us to calculate the contrast produced by the stimulus, at close (chromatic) and long (achromatic) distances, when against the background (for details of calculations see, TELLES, RODRÍGUEZ-GIRONÉS, 2015). Considering that sensitivity of photoreceptors of *Centris analis* is not available, we used the spectral sensitivity of the closest related species, *Apis mellifera* as surrogate (BOSSERT *et al.*, 2019). To obtain chromatic and achromatic values, we applied the *vismodel* function, from the *pavo* package (MAIA *et al.*, 2013) in the software R (TEAMCORE-R, 2016).

Table 2: Photoreceptor excitation (E) values and the contrasts produced with the background. Achromatic values close to 1 indicates small perceptual differences.

Stimulus	E (UV)	E (Blue)	E (Green)	Colour Contrasts Against the Background	Achromatic
Blue	0.37	0.60	0.41	0.21	0.83
Green	0.17	0.16	0.49	0.33	0.98

Experimental procedure

Focal observations were made from 06:00 h to 19:00 h, or until the bee finished the experimental session. For some treatments, colonised nests were continuously manipulated. We performed manipulations always when the bee was absent to then, record the behaviour exhibited by returning females when attempting to localize their nests (Table 3). For all treatments we videotaped the bee's choice time (s) and decision (incorrect, correct or even choices to the previous nest position). Choice time was considered as the time elapsed since the bee left the previous chosen nest until the next choice, or the time elapsed since the bee entered the panel area and its first choice. The camera (Canon EOS 100D, 18 Mega Pixel) was positioned 45 cm away from the panel, and the observer was at the same distance. We started recordings once approaching bees

were noticed, by means of the typical wingbeat sound pattern of the species. During video analyses (frame-by-frame, 25 frames per second), we considered as a choice, whenever a bee touched the nest entrance, either with its front legs or with its antennae (given the possible use of chemical cues, as reported for different solitary species, FAURIA & CAMPAN, 1998; GUÉDOT *et al.*, 2006). Recordings stopped whenever a bee left the panel area or when it found its nest.

Table 3: Behavioural ethogram for *Centris analis* during experimental sessions.

Behaviour	Description
Hovering flight	The female performed a hovering or zigzagging flight in front of the nest entrance;
Direct flight	The female went straight into the nest.

To avoid visual interference of the camera and observer during experimental sessions (INOUYE, 2000), and to be sure that bees were engaged on cell construction and provisioning after the 12 h period, we assumed a permanent position (as well as the camera) and followed the bees during five consecutive foraging trips. As a trip we considered the trajectory nest-foraging bout-nest. After that, the experimental phase began. Experimental phase consisted of control and nest treatments during which bees had to perform five consecutive foraging trips:

1. *Control (C)*: This experiment was carried out with bees familiarised with their nest position and surroundings to serve as the optimal behaviour for both search time and bees' choice in a situation without nest manipulation. The results of subsequent tests were later compared with this data.
2. *Learning a new position (LNP)*: In this experiment, we tested the bee's capacity to learn a new nest location. For that, following control, we changed the nest position once, while the female was engaged on a foraging trip, and recorded the behaviour of returning females. This experiment was meant to understand the process of spatial memory acquisition, and how females behave under such situation.
3. *Continuous change of the nest position (CCP)*: In this experiment, we tested the bee's capacity to find her nest when its position was continuously changing. We changed the nest position during the five consecutive foraging trips and recorded the behaviour of returning females. This experiment was meant to understand whether bees were able to detect and rely on chemical cues from their nests.

4. *Learning a new nest position in the presence of a visual stimulus (LNP-VS)*: In this experiment, we tested the bee's capacity to associate her nest with a visual stimulus. In the absence of females, we changed the nest position once and set a coloured stimulus around the nest entrance. This experiment was meant to understand whether bees were able to associate a visual stimulus with their nest entrance, and whether the contrast produced by different stimuli had an effect on the behaviour. Also, we aimed to compare the accuracy and choice speed of bees with those of (LNP) experiment.
5. *Continuous change of the nest position in the presence of a visual stimulus (CCP-VS)*: In this experiment, we tested the bee's capacity to associate her nest with a visual stimulus in the absence of spatial information. We changed the nest position during the five consecutive foraging trips and recorded the behaviour of returning females. The aim of this experiment was to understand the behaviour of bees when in the presence of visual stimuli, compared to the absence of it under continuous nest change (CCP). Also, we aimed to compare the results from experiment (LNP-VS), when nest remained in the same position.
6. *Continuous change of the nest position in the presence of a visual stimulus using an empty nest (CCP-VS_{em})*: In this experiment, we tested the bee's capacity to find her nest when in the absence of possible chemical cues from their nests. To that, following the previous experiment (CCP-VS), we exchanged the female's nest by an empty one. All empty nests presented the same diameter as the one colonised by the female. We changed the nest position during the five consecutive foraging trips and recorded the behaviour of returning females. The aim of this experiment was to understand the possible role of chemical cues on nest recognition, compared to experiment (CCP-VS). Once females made a correct choice, we replaced her nest.

Most solitary female tested (13 out of 17) experienced all treatments (repeated measures) in a sequential order (from control to CCP-VS_{em}). For treatments where a visual stimulus was used around the nest entrance, we divided bees in two groups: one group was tested with a blue disc (n = 7) and the other with a green disc (n = 6). For treatments with nest position manipulation, we used a pseudorandom order to decide where the following position of nest would be (considering imaginary columns and lines in the panel). In total, a female would make 30 foraging trips. From the first to the last treatment bees took in average two days.

Video Analyses

We analysed the video recordings to determine the accuracy and choice time of bees during treatments. To extract the behavioural information, we used the software 2bTracked (Kämpgen and Telles, unpub., available under request). During analyses, we ignored the time spent in what we classified as reorientation flights, i.e., when a female left the panel area to later approach it again. Thus, only flights and choices made in the panel area were considered. From the video analyses we obtained information of correct, incorrect and previous nest position choices as well as the time spent by bees to make a choice.

Data Analyses

To understand whether bees increased their performance within foraging trips, data from treatments were restricted to the first, third and fifth trips (henceforth “round”). Data were analysed by fitting different models depending on the distribution of the response variable.

The accuracy of bees was analysed in terms of the proportion of correct choices (response variable) among treatments and round (explanatory variables). During a second bout of analysis, we considered only those treatments with the presence of a visual stimuli (Treatments 4 to 6) and tested whether accuracy (proportion of correct choices = response variable) differed between colours (explanatory variable). In a third bout of analysis, we considered the proportion of choices bees made to the previous nest position among treatments 2 to 6, and whether it also varied between rounds. For all three analyses, we fitted the data to a generalised linear mixed model (GLMM), assuming a binomial distribution and logit link function.

For the time analyses, we divide the total time spent by a bee during a foraging trip by the total number of choices made during that trip, to obtain the “choice time” (an estimate of the time a bee required to choose a nest). To understand how the time spent by bees was affected by treatment and experience (round), and by the colour in treatments with the presence of a visual stimuli (Treatments 4 to 6), we fitted both analyses to a linear mixed model (LMM). Choice time was log-transformed (\log_{10}) to improve homoscedasticity and normality.

Bee identification was used as random term in all mixed models. Differences between levels of significant factors were estimated applying a post-hoc Tukey test. We used the *glmer* and *lmer* functions, both from the *lme4* package (BATES *et al.*, 2015) for running GLMM and LMM models, respectively. Post-hoc analyses were undertaken applying the *glht* function, from the *multcomp* package (HOTHORN; BRETZ; WESTFALL, 2008). We applied type II sum of squares for all analyses, using the *Anova* function from the *car* package (FOX; WEISBERG, 2011). Following Zuur *et al.* (2009) and Harrison *et al.* (2018), we have checked for model adjustment by means of residual plots and applying the *dispersion_glmer* function (GLMM models) from the *blmecco* package (KORNER-NIEVERGELT *et al.*, 2015) and *overdisp_fun* function (LMM model) from the *lme4* package. None of the models fitted showed overdispersion (in all cases the ratio of residual deviance to residual degrees of freedom was <1.2 ; (ZUUR *et al.*, 2009). Statistical analyses were performed using the 3.4.0 version of the R software (TEAMCORE-R, 2016).

3. RESULTS

Nest selection by bees

Most of the females nesting on the experimental panel selected trap-nests on the edges, regardless of the position (Table 4). Before choosing the suitable trap-nest, females inspected several others, even when they presented the same diameter. Since we did not control for the length, females could also have been selecting nests based on it. After selecting one nest, females could exhibit two behaviours: stay inactive inside the nest, or continuously move between the nest entrance and the back, repeating this behaviour few times before starting the foraging activity. Once a nest was selected, females never abandoned it, even during our experimental manipulations.

Table 4: Nest position choice of *Centris analis* on the experimental panel.

Nest Position	Number of nests occupied
Centre	4
Edge	
Superior	
Left	5
Right	5
Inferior	
Left	5
Right	3
Total	22

The effect of treatment on the proportion of correct choices

The performance of bees varied among treatments ($\chi^2 = 181.73$; $df = 5$; $P < 0.001$; Figure 2A). Control was significantly different from most treatments, but when learning a new nest position (LNP-VS) and continuous change of nest position using an empty nest (CCP-VS_{em}), both in the presence of a visual stimuli (Tukey: $Z \leq -2.43$; $P > 0.05$). The proportion of correct choices of females learning the new nest position (LNP) and dealing with continuous nest change in the absence (CCP) and in the presence of a visual stimulus (CCP-VS) was lower when compared with control (Tukey: $Z \leq -10.13$; $P < 0.001$). The accuracy of bees when in the presence of a visual stimuli (LNP-VS, CCP-VS and CCP-VS_{em}) did not differ among treatments (Fig. 2A; Tukey: $Z = 1.90$, $P > 0.05$), neither between the new nest position (LNP) and continuous nest change (CCP) treatments when in the absence of a visual stimuli. Learning acquisition increased with foraging trips (Fig. 2B; $\chi^2 = 107.41$; $DF = 2$; $P < 0.001$), indicating that at the end of experimental sessions, bees were more accurate on their choices.

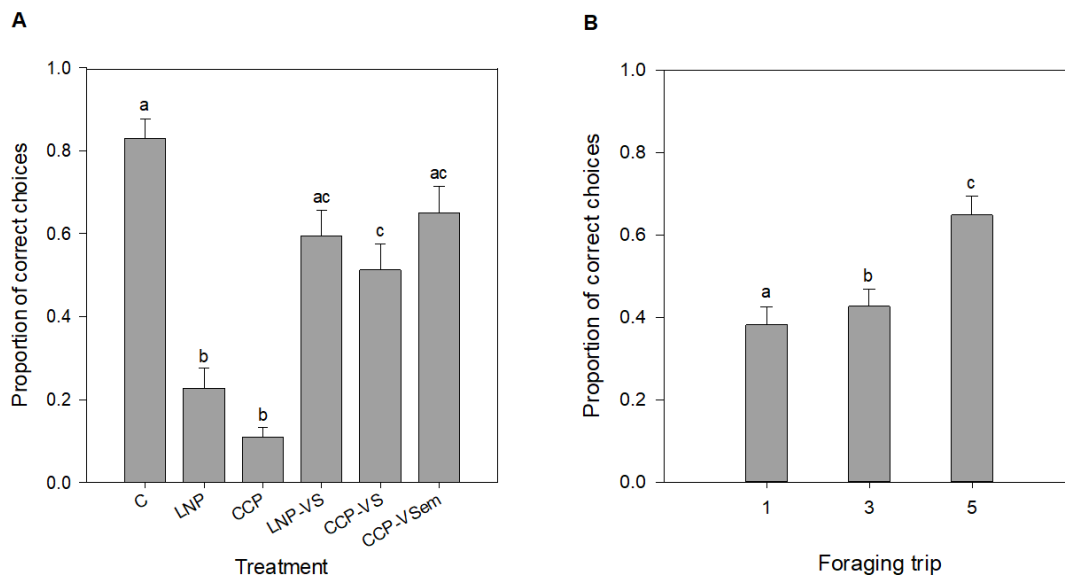


Figure 2: Average proportion of correct choices (A) among treatments and (B) foraging trips. Letters indicate differences based on a post-hoc Tukey test ($p < 0.05$). Bars denotes means \pm s.e.m. C: Control, $N = 16$; LNP: Learning a new position, $N = 15$; CCP: Continuous change of the nest position, $N = 15$; LNP-VS: Learning a new nest position in the presence of a visual stimulus, $N = 15$; CCP-VS: Continuous change of the nest position in the presence of a visual stimulus, $N = 13$; CCP-VSem: Continuous change of the nest position in the presence of a visual stimulus using an empty nest, $N = 13$.

The capacity of bees to associate their nest entrance with a coloured stimulus varied depending on the colour (Fig. 3; $\chi^2 = 9.40$; $DF = 1$; $P < 0.01$). Females trained with the blue colour were more certain about their nest position in comparison with females trained with green (Fig. 3).

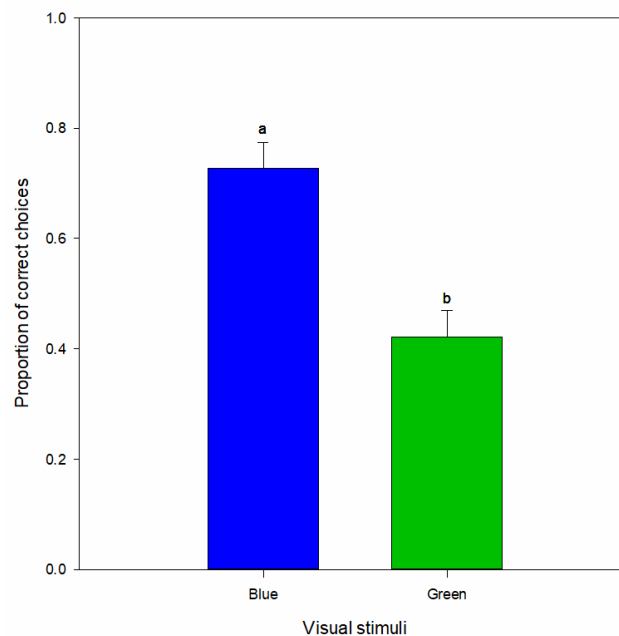


Figure 3: The effect of colour on the proportion of correct choices. Differences based on a post-hoc Tukey test: $P < 0.05$. Bars denotes means \pm s.e.m. Blue, $N = 7$ and Green, $N = 6$.

The proportion of choices bees made to the previous nest position varied according to the treatment (Fig. 4A; $\chi^2 = 99.09$; DF = 4; $P < 0.001$) and round (Fig. 4B; $\chi^2 = 34.06$; DF = 2; $P < 0.001$). Choices to the PNP varied between treatment LNP and CCP, LNP-VS, CCP-VS and CCP-VSem, but not between treatments CCP and CCP-VS. In the presence of a visual stimuli, treatment LNP-VS differed from CCP-VS (Fig. 4A). Choices bees made to the previous nest position were reduced with round (Fig. 4B and Fig. 5).

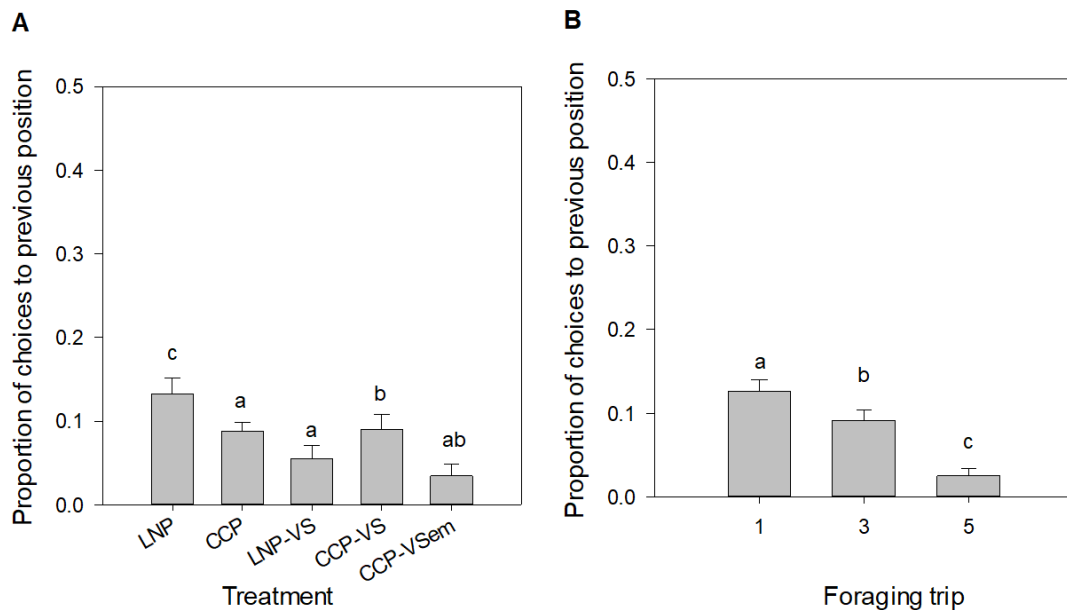


Figure 4: The effect of treatment on the proportion of (A) choices to the previous nest position and (B) foraging trips. Letters indicate differences based on a post-hoc Tukey test ($P < 0.05$). Bars denotes means \pm s.e.m. C: Control, N = 16; LNP: Learning a new position, N = 15; CCP: Continuous change of the nest position, N = 15; LNP-VS: Learning a new nest position in the presence of a visual stimulus, N = 15; CCP-VS: Continuous change of the nest position in the presence of a visual stimulus, N = 13; CCP-VSem: Continuous change of the nest position in the presence of a visual stimulus using an empty nest, N = 13.

The effect of treatment on the choice time

The time required for female bees to make a choice varied among treatments (Fig. 5A; $\chi^2 = 11.23$; DF = 5; $P < 0.05$), but not with foraging trip (Fig. 5B; $\chi^2 = 1.52$; DF = 2; $P > 0.05$). The difference was only between control and treatment CCP-VSem (Tukey: $Z = 3.23$, $P < 0.05$). There were no significant differences in the time spent by females to find their nests among the control (C), most treatments associated to visual stimuli (LNP-VS and CCP-VS, Tukey: $Z_{LNP-VS} = 0.75$ and Z_{CCP-VS} ; $P > 0.05$), and treatments in the absence of visual stimuli (LNP and CCP, Tukey: $Z_{LNP} = 1.40$ and $Z_{CCP} = 1.22$; $P > 0.05$).

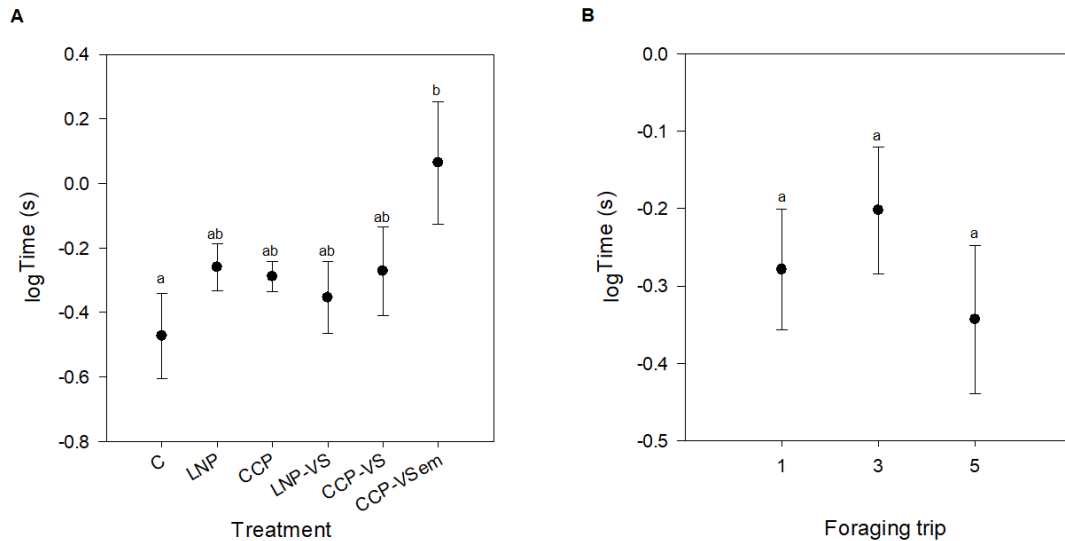


Figure 5: Average time (log) bees took to make a choice depending on (A) treatment and (B) round. Letters indicate differences based on a post-hoc Tukey test ($P < 0.05$). Symbols denotes means \pm s.e.m. C: Control, $N = 16$; LNP: Learning a new position, $N = 15$; CCP: Continuous change of the nest position, $N = 15$; LNP-VS: Learning a new nest position in the presence of a visual stimulus, $N = 15$; CCP-VS: Continuous change of the nest position in the presence of a visual stimulus, $N = 13$; CCP-VSem: Continuous change of the nest position in the presence of a visual stimulus using an empty nest, $N = 13$.

In the analysis of the influence of stimuli colour on the time spent by bees to make a choice, we found a significant difference between blue and green stimuli (Fig. 6; $\chi^2 = 4.64$; $DF = 1$; $P < 0.05$).

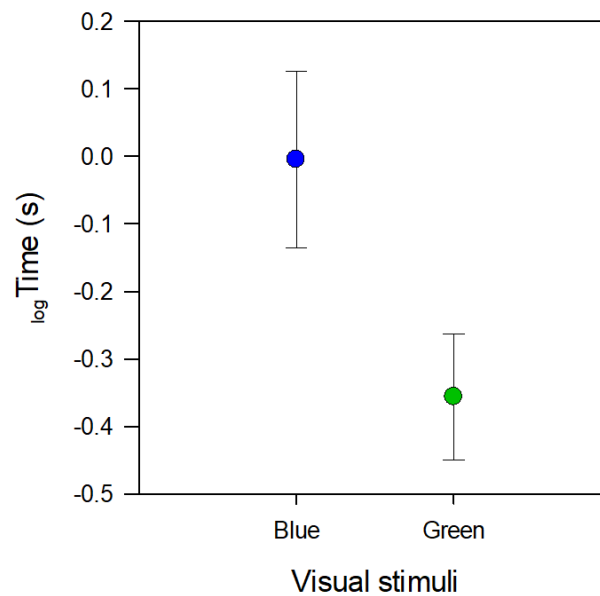


Figure 6: Effect of the stimuli colour (blue and green) on the average time (log) required for females to make a choice. Differences based on a post-hoc Tukey test: $P < 0.05$. Symbols denotes means \pm s.e.m. Blue, $N = 7$ and Green, $N = 6$.

4. DISCUSSION

The presence of a visual stimulus around the nest reflected in a higher proportion of correct choices when compared with treatments where nest location solely relied in learning a new spatial information and/or chemical profile of nests. Control treatment was the exception, given that bees had the chance to familiarize the nest location for a long period before recordings. This pattern is noticed in the round analysis, where accuracy was positively influenced by the amount of trials: at the end of the experimental sessions, bees were more certain of their choices. The accuracy of bees also varied with colour: females trained with blue colour were more certain about their nest position in comparison with females trained with the green stimulus. In this situation, however, accuracy had a cost regarding the time spent by females to make a choice: the more accurate their choices, the more time invested in choosing one coloured stimuli. When comparing choice time among treatments, however, difference was only between control and the last experimental situation.

Studies of the natural behaviour of bees, either foraging or homing, indicate that individuals have the capacity to learn and remember not only the colour and shape of relevant stimuli, but also how to recognize it in a particular spatial relation to other objects, in its near and far environment (COLLETT, *et al.*, 1997; LEHRER, 1994). *Centris analis* females seem to naturally rely on spatial information to localize their nest positions. When given the chance, most of the females selected and colonised trap-nests on the edges of the experimental panel, which positions offered a better contrast to other visual landmarks on its surrounding. Besides, during all sets of experiments, bees were able to adapt to new cues to find their nests, but performance varied among treatments, which indicates that although spatial information, colour and possible chemical cues (from nests) can provide relevant information regarding nest location and identity, the reliability varies.

When presented to a new experimental situation, returning females usually arrived in front of the previous nest position (PNP). After assessing the surrogate nest interior, they engaged in a systematic search around PNP. The frequency of inspections around the PNP was higher when the new experimental situation preceded treatments of permanent nest position (for instance, LNP, which preceded control, and CCP-VS x LNP-VS), but, overall, reduced with experience (round analysis). After a while, bees expanded

the search to the entire panel (or even to the vicinity of the panel) until finding the correct nest. Before that, bees never gave up, even when they had chosen wrong nests for more than 200 times during a single foraging trip. It might seem expected that nest relocation (given natural circumstances) and its further recognition is well developed in solitary bees, but that is not always the case. Females of *Osmia lignaria*, were unable to find their nest on a wooden block when it was moved from one side of a tree trunk to the other (TORCHIO, 1991). In turn, *O. cornuta* was capable to find its nest, after the nesting shelter has been rotated 90° from its original position. Females initially searched in the PNP of the shelter where the cavity entrances originally were, but they all had the capacity to relocate their cavity and continued to nest normally (VICENS; BOSCH, 2000).

Context learning offers the possibility of treating the same stimulus in two or more different ways, thereby enabling the animal to interact more flexibly with its environment (PAHL *et al.*, 2007). For *Centris analis* bees, sensory and cognitive capacities were essential for returning females to find their nests under different contexts. Besides spatial information and coloured stimuli, nest chemical profile seems to act as a cue on this task. During the treatment of continuous nest changing position in the absence of coloured stimuli (CCP), whenever a female inserted its head or directly entered into surrogate empty nests, she quickly retracted to continue the search for her own nest. This behaviour suggests that females were using olfactory cues to recognize nests, as demonstrated for several other solitary bee species (Table 1). The nest profile can be based on abdominal and/or mandibular secretions (ANZENBEKGER, 1986; GUÉDOT; PITTS-SINGER; *et al.*, 2006), and could be individual-specific (GUÉDOT *et al.*, 2013). Although we did not specifically quantify or test for it, during some few foraging trips outside experiments sessions, we placed alien nests belonging to females nesting in a different panel in our experimental panel. Whenever the target female (N = 12) entered the alien nest, she rejected it and kept her search. A previous experiment with *Osmia lignaria* demonstrated that the removal or manipulation of olfactory cues from nest elicited temporary or permanent rejection of the bee's nesting cavity, clearly indicating the presence of individual-specific olfactory cues during nest recognition (GUÉDOT; PITTS-SINGER; *et al.*, 2006).

Given its nesting ecology under natural conditions (VIEIRA-DE-JESUS; GARÓFALO, 2000), *Centris analis* seems to cope with nest location and identity based

on the synergy between visual and olfactory cues. The wide taxonomic distribution of the use of both chemical and visual traits among bees implies that all bees can use both kinds of cues for either nest location or recognition (INOUYE, 2000). However, any particular colour possesses particular properties such as hue, chroma and brightness, affecting the contrast produced with the background and ultimately, affecting bee behaviour. The capacity of bees to associate their nest entrance with a stimulus varied with the colour of the stimulus. Differences in stimuli contrast could explain the low accuracy of bees trained with green colour. Both selected stimuli were above the perceptual colour contrast threshold (0.05 hexagon units; Table 2) established for Hymenoptera species (THÉRY, 2007). While colour contrast was high, achromatic contrast against the background varied from 0.83 for blue to 0.98 (hexagon units) for green. Honeybees can detect visual stimuli using colour contrast if the subtended visual angle of the object is large (ca. 15°); for smaller visual angles, bees deploy the green contrast alone (GIURFA *et al.*, 1996). From the recording point (45 cm away from the panel), stimuli presented a visual angle of $\approx 5^\circ$, which means that achromatic contrast at this distance could have been used. Nevertheless, green stimulus didn't present a good achromatic contrast with the background. Thus, bees had to be close enough to detect it (15 cm, $\approx 15^\circ$). For blue colour, bees could have relied on the achromatic information provided when approaching the panel, later confirming its position and choosing more often the correct nest.

In the foraging context, the ability of social bees to make accurate decisions is often tightly correlated with the amount of time allocated to a task (DYER; CHITTKA, 2004; SKORUPSKI; SPAETHE; CHITTKA, 2006). The time required for *C. analis* to make a choice did not vary among most experimental manipulations, which turned to be an interesting result considering the proportion of correct choices. We can speculate whether the time was masked by the number of choices a bee made during a certain treatment, considering that we divided the total time by the number of visits. If we consider the total time (Fig. 7), when in the absence of a visual stimuli (LNP and CCP), bees spent more time to find their nests, given the difficulty of the task, also increasing the number of choices (average number of choices \pm s.e.m: C= 6.4 ± 2.3 ; LNP= 57 ± 10 ; CCP= 49.2 ± 8 ; LNP-VS and CCP-VS= 11.5 ± 3 , CNP-VS_{em}= 5.8 ± 1.3). When females lost their reference cue or couldn't permanently rely on it (spatial information in treatments in the absence of colour), they started a systematic search in the panel area. The best solution from the perspective of females was to 'guess' their nest, a strategy that resulted

in low decision accuracy and influenced the time they took to make a choice (CHITTKA; SKORUPSKI; 2009). Besides, the potential costs of errors were low (aside of time and energy): bees were not facing predation risk or aversive reinforcement associated to errors. In the absence of visual stimuli, poor accuracy seems to not be a simple error based on attention, but a ‘deliberate’ compromise in the absence of alternatives.

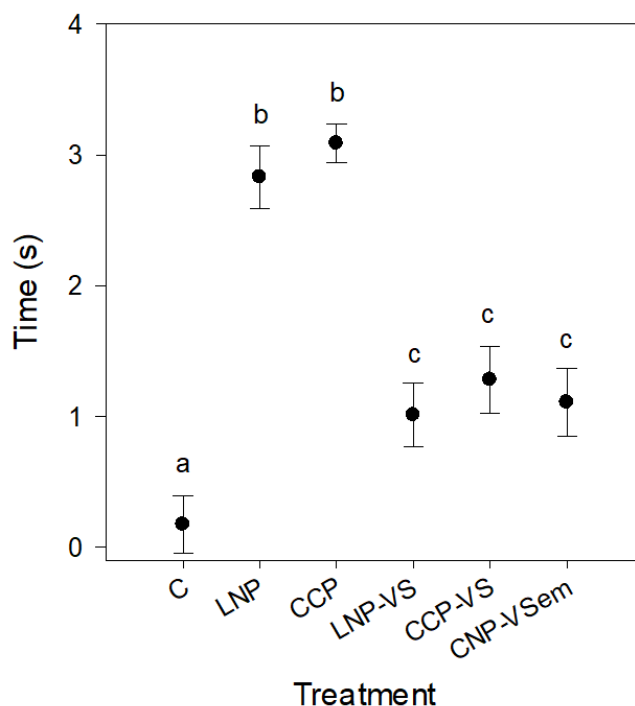


Figure 7: Average time of bees during the five consecutive foraging trips depending on treatment. Letters indicate differences based on a post-hoc Tukey test ($P < 0.05$). Symbols denotes means \pm s.e.m. C: Control, N = 16; LNP: Learning a new position, N = 15; CCP: Continuous change of the nest position, N = 15; LNP-VS: Learning a new nest position in the presence of a visual stimulus, N = 15; CCP-VS: Continuous change of the nest position in the presence of a visual stimulus, N = 13; CCP-VSem: Continuous change of the nest position in the presence of a visual stimulus using an empty nest, N = 13.

Studies on the sensory ecology of native species with economical potential, can be relevant not only for conservation, but also for a better management. Several species of solitary bees have been successfully introduced as managed pollinators (BOSCH; KEMP, 2002; JUNQUEIRA; HOGENDOORN; AUGUSTO, 2012; OLIVEIRA; SCHLINDWEIN, 2009). In Brazil, Centridine females are essential to the pollination of numerous plant species of Cerrado, Caatinga, Amazonian, and Atlantic rain forests (OLIVEIRA; SCHLINDWEIN, 2009). They are morphologically and behaviourally specialized in collecting floral oils, being the sole pollinators of hundreds of floral oil-producing species, including species of economic relevance such as West Indian cherry (MAGALHÃES; FREITAS, 2012; OLIVEIRA; SCHLINDWEIN, 2009; VILHENA *et*

al., 2012). The presence of landmarks (ANZENBEKGER, 1986; BRÜNNERT; KELBER; ZEIL, 1994; INOUYE, 2000), vertical and horizontal cues (GUÉDOT; BOSCH; KEMP, 2005), and the colour contrast produced between stimuli and their backgrounds (FAURIA, K; CAMPAN, 1998; GUÉDOT; BOSCH; *et al.*, 2006; GUÉDOT; BOSCH; KEMP, 2007) can influence the time spent for solitary bees to locate their nests. In homogenous and large landscapes, reducing this time may implicate in more investment by the females in nest construction and provisioning, which in other worlds, can increase flower visits, affecting the pollination rate. ARTZ *et al.* (2014) demonstrated that the colour of the bees' nesting box shelter, did not only influenced nest colonization by females, but the reproductive success of bees in a commercial almond orchard. In their study, *Osmia lignaria* provisioned up to 2.6 times more cells in blue-coloured shelter when compared to yellow and orange. For instance, enhancing pollinators' density in crop fields has been associated with improvements in productive in West Indian cherry (MAGALHÃES; FREITAS, 2012), Passion fruit (JUNQUEIRA, CAMILA N J; AUGUSTO, 2017) and Rabbiteye Blueberry (DEDEJ; DELAPLANE, 2003).

5. CONCLUSION

In summary, *C. analis* have demonstrated the ability and flexibility to learn how to localize and identify their nests according to the context. Our experiments demonstrate that the presence of a coloured visual stimulus contributed to enhance the accuracy of bees during nest localization and that odour is a secondary cue in this process. Furthermore, this knowledge could be applied in commercial crops, once it contributes to management practices for this bee species. Nonetheless, the experimental treatments reported here do not yet fully capture the complexity of the sensory and cognitive ecology of these bees but opens space for further work.

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