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RESEARCH ARTICLE

Combined secondary compounds naturally found in nectars enhance honeybee cognition and survival

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ABSTRACT

The alkaloid caffeine and the amino acid arginine are present as secondary compounds in nectars of some flower species visited by pollinators. Each of these compounds affects honeybee appetitive behaviours by improving foraging activity and learning. While caffeine potentiates responses of mushroom body neurons involved in honeybee learning processes, arginine acts as precursor of nitric oxide, enhancing the protein synthesis involved in memory formation. Despite existing evidence on how these compounds affect honeybee cognitive ability individually, their combined effect on this is still unknown. We evaluated acquisition and memory retention in a classical olfactory conditioning procedure, in which the reward (sucrose solution) contained traces of caffeine, arginine or a mixture of the two. The results indicate that the presence of the single compounds and their most concentrated mixture increases bees' learning performance. However, memory retention, measured in the short and long term, increases significantly only in those treatments offering combinations of the two compounds in the reward. Additionally, the most concentrated mixture triggers a significant survival rate in the conditioned bees. Thus, some nectar compounds, when combined, show synergistic effects on cognitive ability and survival in an insect.

KEY WORDS: Caffeine, L-Arginine, Learning, *Apis mellifera*, Combined effects

INTRODUCTION

Secondary compounds (SC) present in floral rewards are central in ecology, mediating interactions with pollinators and plant antagonists (Strauss and Whittall, 2006; McArt et al., 2014) that influence both plant reproductive success and pollinator general fitness. Alkaloids, phenolics, terpenoids and peptides represent the main compound groups that have been found in both pollen and nectar (Baker and Baker, 1976; Adler, 2000; Nicolson and Thornburg, 2007; Stevenson et al., 2017). Some SC may play a nutritional role, such as encoded amino acids, especially tryptophan and phenylalanine, which are present across a wide variety of plant families (Palmer-Young et al., 2019). Other SC may act as attractants or deterrents of insect pollinators, depending on both dosage and season (Singaravelan et al., 2005). Despite these findings, few studies focus on how mixtures of SC affect insect pollinator behaviour (Gatica Hernández et al., 2019).

Alkaloids that appear in nectars can induce either aversion or attraction for bees depending on their concentration. Singaravelan and collaborators (2005) showed that honeybees *Apis mellifera* fed more when the sugared reward contained caffeine or nicotine in low concentrations that mimicked natural nectars but were deterred at higher concentrations. Insect pollinators could benefit from the intake of alkaloids as they may play a prophylactic or therapeutic role by reducing the insects' pathogen load (Manson et al., 2010; Baracchi et al., 2015); in fact, honeybees may actively search for alkaloid-enriched nectar to keep pathogens at bay (Gherman et al., 2014). Another plausible explanation for such a preference has been proposed by Wright and collaborators (2013), who have demonstrated that caffeine can trigger stable and long-term memory (LTM) of an olfactory nature, promoting a concentration-dependent effect on honeybee associative learning. Caffeine also improves appetitive behaviour in foraging bees, which is manifested in greater gathering activity and recruiting responses (Couvillon et al., 2015).

Arginine is an essential amino acid that is present in floral nectars and pollens of a wide variety of plants exploited by pollinators (Baker and Baker, 1976; Gardener and Gillman, 2001; Power et al., 2018; Terrab et al., 2007; Taha et al., 2019). In honeybees, it participates in the synthesis of nitric oxide, which is involved in downstream mechanisms that prolong the activity of cAMP-dependent PKA, and therefore promotes protein synthesis during LTM formation and other cellular processes that require such synthesis (Müller, 1996, 1997). Furthermore, arginine seems to have effects on the formation of short-term memory (STM) in bees if it is administered at a concentration of 0.001 mmol l^{-1} (Chalisova et al., 2011; Lopatina et al., 2017). Although arginine is considered an essential amino acid, insects can synthesize it, although with some difficulty, so they must incorporate it through food (House, 1965).

With this in mind, we attempted to study how mixtures of the amino acid arginine, found in many floral nectars of different plants species, such as *Calluna vulgaris* and *Lotus corniculatus* (Gardener and Gillman, 2001; Power et al., 2018), and the alkaloid caffeine, found in the nectars of *Citrus* spp. and *Coffea* spp. (Wright et al., 2013), can affect honeybee cognitive ability, specially focusing on memory formation and survival. The presentation of such different compounds in a mixture may not be unusual in nature, given the complexity of the nectar and pollen composition in many flowers, where it is not uncommon to find different sorts of amino acids along with various alkaloids (Palmer-Young et al., 2019). In addition, the generalist behaviour of *A. mellifera* can assert itself throughout the spring–summer seasons, when the large array of different nectars available for collection can provide a multifloral honey that could affect this pollinator's health and cognitive ability on a daily basis. We hypothesized that if caffeine and arginine were presented in combination, as they have independent mechanisms in

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the memory formation processes, they would have a positive effect in this matter. To achieve this, we conducted olfactory conditioning assays where a given olfactory stimulus (conditioned stimulus) was paired with a reward (unconditioned stimulus), which could contain caffeine, arginine or a mixture of the two in different concentrations within the natural range found in nectar. Olfactory memory was tested 15 min and 24 h after bees were conditioned (STM and LTM, respectively). The survival of the animals, after all the conditioning procedures had been made, was also quantified.

MATERIALS AND METHODS

Study site, animals and chemical compounds

Experiments were carried out at the Experimental Field of the University of Buenos Aires, Argentina (34°32′S, 58°26′W) between January and March 2019. Honeybees workers (*Apis mellifera* Linnaeus 1758) of unknown age were captured as they landed at the entrance of at least 10 different Langstroth hives of 10 frames each and composed of a mated queen, three or four frames of capped brood, food reserves and about 20,000 individuals.

The chemical compounds used to prepare the different treatments (see Table 1) were caffeine (Caff) and L-arginine (Arg) (Sigma-Aldrich, Steinheim, Germany). To prepare the different solutions, we considered the caffeine and L-arginine concentrations previously used in honeybee learning studies (Chalisova et al., 2011; Wright et al., 2013; Lopatina et al., 2017). In the case of caffeine, the concentrations used are within the range found in natural nectars (Kretschmar and Baumann, 1999; Wright et al., 2013). In the case of L-arginine, the concentrations used are below the range found in natural nectars (Gardener and Gillman, 2001).

For classical conditioning assays, we used 1-hexanol and nonanal (Sigma-Aldrich), both pure odours commonly present in floral fragrances (Knudsen et al., 1993) that are known to be similarly learned by honeybees (Guerrieri et al., 2005).

Bee capture and harnessing

Captured bees were anaesthetized in the laboratory at −4°C for 1 min and then individually harnessed in metal tubes. Cold anaesthetization was carried out to reduce stress levels and increase the survival rate (Takeda, 1961; Bitterman et al., 1983; Matsumoto et al., 2012). Afterwards, bees were kept in darkness in an incubator at 28°C and 75% relative humidity, for 1 h prior to the experiments. Harnessing restrained the body movements of the bees but allowed them to freely move their antennae and mouthparts (Bitterman et al., 1983; Guerrieri et al., 2005).

Behavioural assays

To study how caffeine, L-arginine and mixtures of the two could affect associative learning and memory in honeybees, several worker bees were harnessed to undergo a classical conditioning protocol adapted from the proboscis extension response (PER) paradigm (Bitterman et al., 1983; Guerrieri et al., 2005; Felsenberg et al., 2011; Matsumoto et al., 2012). During a PER conditioning procedure, bees learn to associate a given olfactory stimulus (conditioned stimulus, CS) with a reward (unconditioned stimulus, US). Here, the reward contained different concentrations of the above secondary compounds (Table 1). The goal of this study was to follow their effect on three different stages of the associative learning process: acquisition (during the PER conditioning assay) and memory retention, in the short term (15 min after conditioning) and long term (24 h after conditioning).

Table 1. Composition of the sucrose solution and secondary compounds used for the different treatments

Treatment	Concentration
Control	1.8 mol l^{-1} SS
Caffeine	SS+0.05 mmol l^{-1} Caff
	SS+0.15 mmol l^{-1} Caff
L-Arginine	SS+0.01 mmol l^{-1} Arg
	SS+0.03 mmol l^{-1} Arg
Mixture	SS+0.15 mmol l^{-1} Caff+0.01 mmol l^{-1} Arg
	SS+0.15 mmol l^{-1} Caff+0.03 mmol l^{-1} Arg

Sucrose solution (SS; 1.8 mol l^{-1}) was used as the unconditioned stimulus; caffeine (Caff) and L-arginine (Arg) were added to this at the indicated concentrations.

Olfactory classical conditioning of proboscis extension

To perform the PER assays, a device that delivered a continuous airflow (50 ml s^{-1}) was used for the application of the odorant; 4 µl of pure odorant impregnated on 30×3 mm filter paper inside a syringe was delivered through a secondary airstream (6.25 ml s^{-1}) to the head of the bee. A fan extracted the released odours to avoid contamination. Bees underwent 5 training trials of paired CS–US presentations, with an inter-trial interval between CS presentations of 15 min. Each learning trial lasted 39 s. Before odour presentation, bees rested for 16 s in the airflow for familiarization as well as for testing their response to the mechanical stimulus. Only bees that did not respond to the mechanical airflow stimulus were used. For the classical conditioning training procedure, the CS was presented for 6 s. Reward, according to the treatment, was presented for 3 s on the proboscis (mouthparts), 3 s after the onset of the CS. After odour presentation, the learning trial ended with 17 s of clean airflow.

STM evaluation

To evaluate whether the bees had formed a memory immediately after the learning assay, bees stayed harnessed for 15 min and were then subjected to (1) presentation of the CS and (2) presentation of the novel odour, both without reinforcement. The presentation order of the odours during the tests was balanced and a time gap of 15 min was used between each presentation. Although the two odours have been reported to be equally preferred by bees (Guerrieri et al., 2005), they had both been used either as a CS or s novel odour, in a balanced number of events, without showing any differential responses. The PER was considered during the first 3 s of presentation of the test odour. After the last odour presentation, all the bees were also checked for the unconditioned PER to antennal contact with reward (sucrose solution) and were discarded in the case of no response.

LTM evaluation

To evaluate LTM after the learning assay, the trained bees were maintained harnessed and tested with both odours alone 24 h later. To keep them alive for that period, each experimental subject was fed with 1.8 mol l^{-1} unscented sucrose solution 15 min after the behavioural assay. Honeybees were fed with sucrose solution through a micropipette tip, which allowed them to drink until satiation. Then, they were kept in darkness in an incubator at 28°C and 75% relative humidity, and fed again to satiety with the same method 12 h later, maintaining them in the incubator under the same conditions until the 24 h period ended.

Survival under the behavioural assays

In addition, the survival of the experimental subjects exposed to conditioning was recorded 24 h after the end of the training period. The response variable obtained in this case was also of the dichotomous ‘yes/no’ type at the end of the prescribed time.

Statistical analysis

All statistical tests were performed with R v3.3.3 (http://www.R-project.org/). The proposed models have a binomial logistic regression, as their variables respond to a certain number of successful events (individuals that extend the proboscis or manage to survive) over a defined total number of individuals which are part of the experimental group. The PER in the conditioning trials was evaluated using generalized linear mixed effects models (GLMM) and the response in the test and survival stages was evaluated using generalized linear models (GLM), following a binomial error distribution and using the glmer and glm functions of the lme4 package (Bates et al., 2015).

In the conditioning assays, a model with the type of treatment and the trial number as two fixed factors was proposed. The trial factor had 4 levels, which corresponded to those of the 2nd to the 5th trial. The treatment factor had 3 levels depending on the compound used: 0.05 mmol l^{-1} Caff, 0.15 mmol l^{-1} Caff and its control; 0.01 mmol l^{-1} Arg, 0.03 mmol l^{-1} Arg and its control; and 0.15 mmol l^{-1} Caff+0.01 mmol l^{-1} Arg, 0.15 mmol l^{-1} Caff+0.03 mmol l^{-1} Arg and its control. These groups were arranged in this manner given the seasonal shift and the differential ability of bees to learn throughout the seasons (Matsumoto et al., 2012). If experimental groups had been set to compete with one another, then the experiments would have taken a longer time to run, risking a change in the appetitive behaviour due to these environmental shifts. In this way, the group exposed to the caffeine treatments was developed during January–February and the groups exposed to L-arginine and mixtures of caffeine and L-arginine were developed in February–March. Furthermore, each individual bee was considered as a random factor (1|ind, in R coding).

In the STM test, the response to the CS was analysed, considering exclusively those bees that had not extended the proboscis to the novel odour. This was done to distinguish responses that were odour specific to the CS.

For the LTM test, those bees that could survive after 24 h and that had also managed to respond effectively during the first evaluation were used as the total base of individuals to be analysed. Then, only those individuals that had retained their previous behaviour (responding effectively to the CS but not to the novel odour, the same criteria as in the previous test), were registered as having effective responses in this instance.

For the survival analysis, all the bees that ended the training phase and the testing phase performed 15 min after training were followed until they were tested 24 h later. Unlike the previous cases, there was no discrimination in the selection of such individuals regarding their responses.

The GLM and GLMM models were simplified as follows: significance of the different terms was tested starting from the higher-order terms model using anova function to compare between nested models (Bates et al., 2015). Non-significant terms ($P>0.05$) were removed (see Tables S1, S2 and S4). In the case of comparisons between the different levels of treatment during the acquisition phase, *post hoc* comparisons were made using least-squares contrasts (see Tables S3 and S5). For this, the emmeans function of the emmeans package (Bates et al., 2015) was used and the alpha level was set at 0.05.

RESULTS

Caffeine effects

During the acquisition phase, the reward that contained caffeine promoted a significant increase in the bees' PER (minimal adequate model: Response∼Treatment+Trial+1|ind., $P=0.0173$; Table S1; Fig. 1A, top). Bees treated with either concentration of caffeine (0.05 or 0.15 mmol l^{-1}) performed better than those that were administered only 1.8 mol l^{-1} sucrose solution (least-squares contrast, $P<0.05$, $N>62$ for all groups). No significant differences were observed between the different concentrations tested.

In the STM test, the alkaloid generated an increase in the response trend, although with marginally non-significant values (minimal adequate model: Response∼Treatment, $P=0.0521$; Table S2; Fig. 1B, top). The percentage of individuals that could discriminate odours was relatively high: 74%, 91% and 82% for the control, 0.05 mmol l^{-1} Caff and 0.15 mmol l^{-1} Caff group, respectively.

Regarding the LTM test, there was an increase in the percentage of caffeine-treated individuals that managed to retain the associative learning after 24 h, although no significant differences were found between any treatment levels (Table S2; Fig. 1C, top). The response percentages for each group were: 40% for control, 50% for 0.05 mmol l^{-1} Caff and for 60.8% 0.15 mmol l^{-1} Caff groups ($N>23$ for all groups).

L-Arginine effects

The effects of administration of the amino acid L-arginine differed significantly when analysing it as factor in the acquisition phase (minimal adequate model: Response∼Treatment+Trial+1|ind., $P=3,42E{-}08$; Table S1; Fig. 1A, centre). Comparisons between levels yielded significant differences between the two applied L-arginine concentrations and the control (least-squares contrast, $P<0.05$, $N>43$ for all groups), without finding significant differences between these two L-arginine concentrations.

During the STM test, the percentage discrimination reached 63%, 80% and 87% for the control, 0.01 mmol l^{-1} Arg and 0.03 mmol l^{-1} Arg group, respectively (Fig. 1B, centre). Once again, the statistical model that proposed treatment as an explanatory variable showed non-significant differences (minimal adequate model: Response∼Treatment, $P=0.0650$; Table S2; $N>30$ for all groups).

Regarding the effect of L-arginine in the LTM test, an increase in the response could also be observed, with 47% for control, 79% for 0.01 mmol l^{-1} Arg and 80% for 0.03 mmol l^{-1} Arg (Fig. 1C, centre), although the differences were non-significant (minimal adequate model: Response∼Treatment, $P=0.0518$; Table S2, $N>17$ for all groups).

Mixture effects

For the mixture of caffeine and L-arginine, an increase in the response was observed during the acquisition phase when analysing the treatment as a factor (minimal adequate model: Response∼Treatment+Trial+1|ind., $P=0.0177$; Table S1; Fig. 1A, bottom). In this case, the only treatment that generated significant differences was the mixture with the highest concentration of both compounds (least-squares contrast, $P<0.05$, $N>52$ for all groups).

For the STM test, both mixtures caused a significant increase in the response of the treated bees: 65% for control, 86% for 0.15 mmol l^{-1} Caff+0.01 mmol l^{-1} Arg (lower concentration) and 89% for 0.15 mmol l^{-1} Caff+0.03 mmol l^{-1} Arg (higher concentration) group (minimal adequate model: Response∼Treatment, $P=0.0041$, $N>44$ for all groups; Table S2; Fig. 1B, bottom). When comparisons were made, only the higher concentration mixture showed significant differences with respect to the control group (least-squares contrast, $P<0.05$; Table S3). It should be noted that differences between the lower concentration mixture and the control group were really close to the alpha level ($P=0.0502$; Table S3) and that no differences were found between the two different mixtures ($P=0.91$; Table S3).

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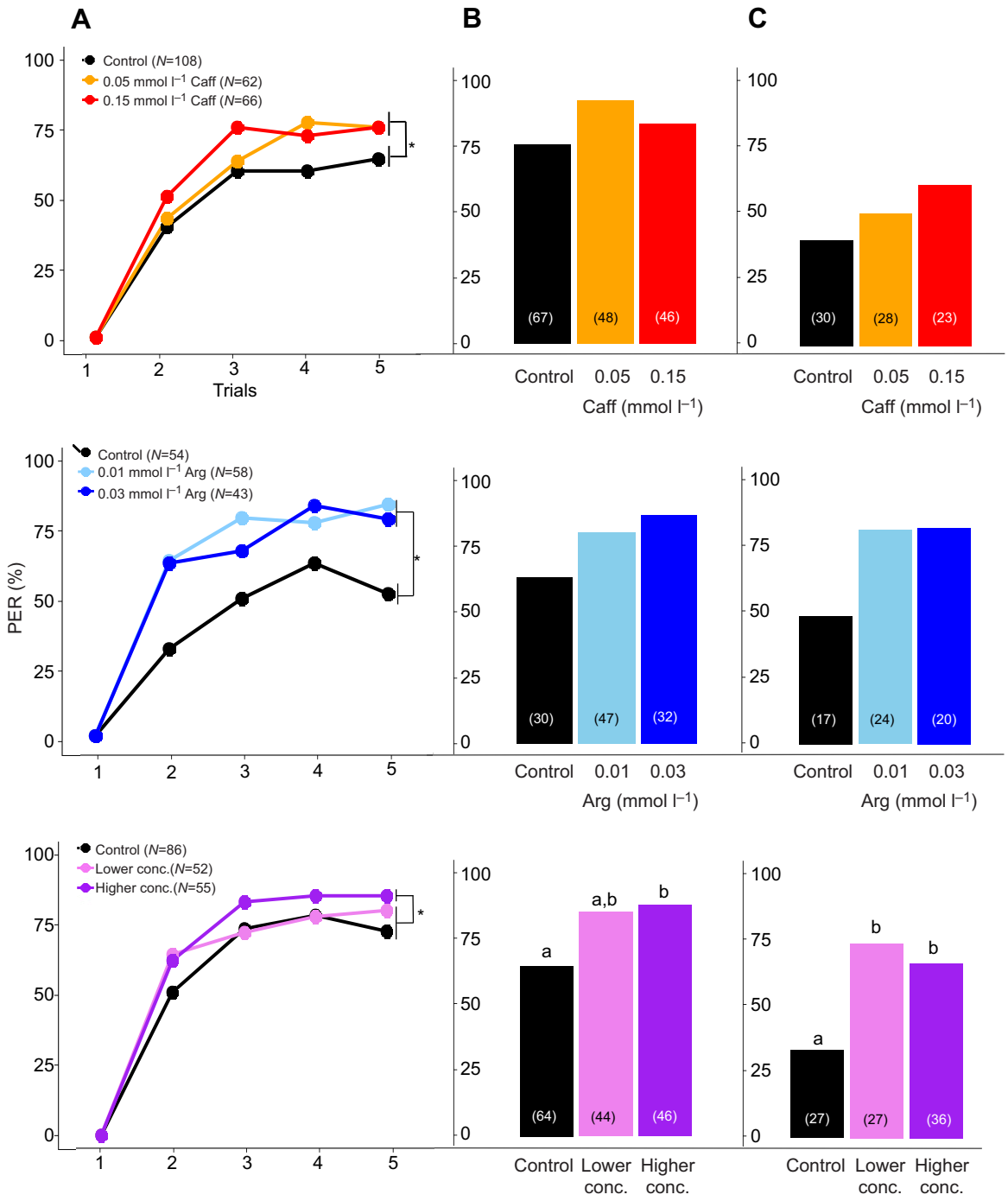

Fig. 1. Effect of secondary compounds on olfactory classical conditioning of proboscis extension in honeybees. The proboscis extension response (PER) towards the trained odour was quantified over the course of 5 acquisition trials (training phase) in which the unconditioned stimulus (US) consisted of 1.8 mol l^{-1} sucrose solution alone (control, 50% w/w) or with the following substances, depending on the treatment: top, 0.05 and 0.15 mmol l^{-1} caffeine (Caff); centre, 0.01 and 0.03 mmol l^{-1} L-arginine (Arg); bottom, 0.15 mmol l^{-1} Caff+0.01 mmol l^{-1} Arg ('Lower conc.') and 0.15 mmol l^{-1} Caff+0.03 mmol l^{-1} Arg ('Higher conc.'). (A) Training phase. (B) Testing performed 15 min after training (short-term memory). (C) Testing performed 24 h after training (long-term memory). The number of bees tested is shown in parentheses. Asterisks in A indicate a significant difference (least-squares contrast, $P<0.05$). Different letters in B and C correspond to significant differences between groups ($P<0.05$).

Application of the mixtures also resulted in significant differences in the LTM test (minimal adequate model: Response~Treatment, $P=0.0045$; Table S2; Fig. 1C, bottom). Regarding the comparisons, significant differences were found between the groups that received either mixture concentration (response: 174% for the lower concentration and 66% for the higher concentration) and the control group (response: 33%) (least-squares contrast, $P<0.05$; Table S3; $N>27$), but there were no significant differences between the two mixture groups.

Survival under the behavioural assays

The survival of the experimental subjects was also analysed, considering the treatment received during the training phase as an exploratory factor. Significant differences were found only in the case

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of the mixture groups (minimal adequate model: Survival~Treatment, P=0.0015; Table S4; Fig. 2C), with the higher concentration group showing the greatest survival rate, reaching up to 98% in comparison to 74% for the control group and 80% for the lower concentration group (least-squares contrast, P<0.05; Table S5).

The caffeine group (including its control) showed ~70% survival (Fig. 2A), whereas for the L-arginine group, the survival rate was around 80% (Fig. 2B). As previously mentioned, in none of these cases were there significant differences between treatments (Table S4).

DISCUSSION

The results presented in this study demonstrate the powerful effect that the oral administration of combined secondary compounds found in nectars can have on cognitive ability and survival in honeybees. Regarding memory retention tested immediately after conditioning (STM), the higher concentration mixture presented significant improvements compared with the lower concentration mixture and the control group. Caffeine and L-arginine offered individually did not show significant effects, although it is worth mentioning that the differences with respect to the null model were marginal, denoting a clear biological pattern. The same was true when memory retention was tested 24 h later (LTM), with only the combined treatment groups differing significantly from the control. When analysing the effect of these substances on the survival of bees, it was found that the mixture of caffeine and L-arginine at their highest concentrations allowed a survival rate of 98% after 24 h. This treatment appeared to be the only one that presented significant differences when compared with their respective control groups.

Effects of single compounds on learning

The results obtained here concur with prior reports showing that the administration of field-realistic doses of caffeine was linked to an improvement in associative learning. It might be surprising that the alkaloid caffeine can cause an increase in the acquisition rate of an appetitive learning procedure, as caffeine is considered an aversive substance for many arthropods (Kretschmar and Baumann, 1999). However, aversion responses were only found in bees that had been exposed to concentrations of this compound higher than 1 mmol l^{-1} (Singaravelan et al., 2005; Wright et al., 2013). In the present study, the addition of caffeine, at either of our chosen concentrations, did not result as an improvement in STM. In previous studies, it was argued that this alkaloid has complex effects on learning and memory in bees, facilitating long-lasting learning processes (Wright et al., 2013; Mustard, 2014). However, under our experimental conditions, memory retention did not change if it was tested either immediately after conditioning or 24 h later.

Regarding the amino acid L-arginine, its use during the acquisition phase caused an increased response for both concentrations offered. Given that it is an essential nutritional component for insects (House, 1965), its potential recognition by bees as a palatable substance would be expected. In line with this, previous studies have shown that the presence of other amino acids offered during or before training affects honeybee learning performance (Kim and Smith, 2000; Simcock et al., 2014; Nicholls et al., 2019). In addition, another study has shown that bees can identify umami taste, and has characterized a chemosensory taste receptor that responds to L-amino acids (AmGr10), among which is L-arginine (Lim et al., 2019). However, none of these studies analysed how these amino acids influence the performance of associative learning of a gustatory nature. Previous reports did show an improvement in STM (Chalisova et al., 2011; Lopatina et al., 2017). In the present study, an improved response with both concentrations of L-arginine was found; however, differences were not significant, even though the P-value was close to the alpha level (P=0.065). In view of these results, it is unclear why the strength of this type of memory should be affected. Its mechanism of action implies an increase in protein synthesis, affecting only long-term processes. However, it should be noted that in *Drosophila* spp., nitric oxide, a product of the arginine pathway, is also involved in the modulation of cholinergic excitatory pathways during the early stages of olfactory processing (Duan et al., 2012).

In honeybees, arginine intervenes not only in nitric oxide synthesis but also in the biochemical processes that trigger protein synthesis and form traceable memory (Müller, 1996, 1997). This is consistent with the results we found in the LTM tests, in which both concentrations of L-arginine resulted in an augmented (but not significant) response. Given that the mechanism of action of arginine promotes the formation of traceable memories, we would expect to find a significant increase in the response when applying this substance. Nevertheless, it is worth mentioning that this is the first report in which a long-lasting response involving appetitive learning has been documented in insects. A previous study in the

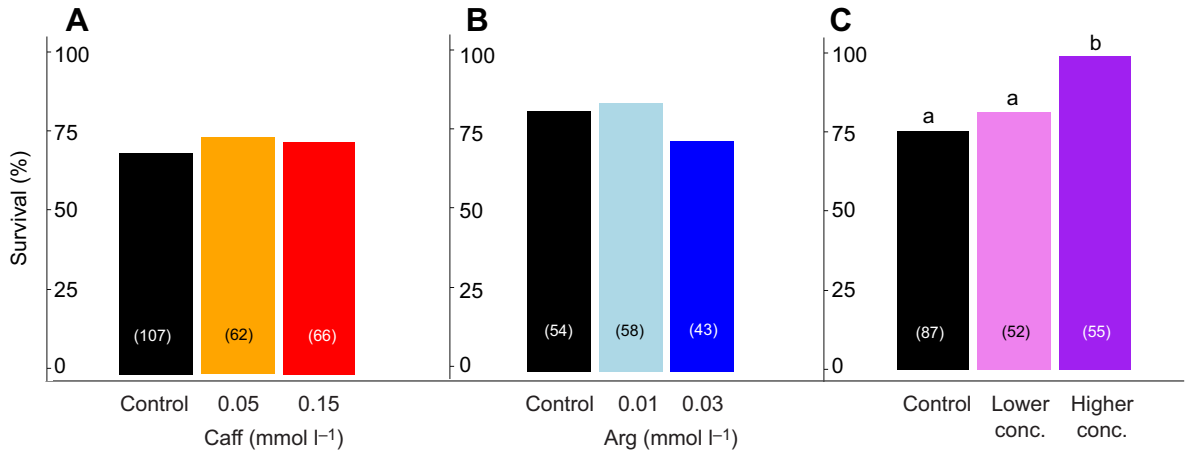

Fig. 2. Effect of caffeine, L-arginine and their combination on the survival of bees in the context of a PER protocol. Survival was assessed 24 h after administration of the secondary compounds. (A) Caffeine group (P=0.82; Table S4). (B) L-Arginine group (P=0.19; Table S4). (C) Caffeine plus L-arginine group (minimal adequate model: Survival~Treatment, P=0.00015; Table S4; least-squares contrasts: P<0.05; Table S5). The number of bees tested is shown in parentheses. Different letters correspond to significant differences between groups (P<0.05).

praying mantis *Stagmatoptera biocellata*, performed in an aversive context (simulation of a predatory attack), found that arginine facilitates LTM formation (D'Alessio et al., 1982). However, because of the relevance of arginine as precursor in the biochemical cascades involving in LTM processes, it is not surprising that this improvement in the responses was detected.

Effects of combined compounds on learning

The mixture with the highest concentration of L-arginine was the only one that showed a significant effect when comparing it with the rest of the treatments during the acquisition phase. The reasons why this mixture generated a differential response cannot be explained solely by the individual effect of L-arginine, given the results found for application of L-arginine alone. Thus, it is possible there is some combined effect on the acquisition phase when this mixture is administered. A similar pattern was observed for STM, although in this case the differences between the lower concentration mixture and the control group were almost significant (P=0.0502) and there were no differences found between the two mixtures (P=0.91).

Regarding the LTM test, both mixtures showed significant differences from the control when memory retention was evaluated. In view of these results and considering the excitatory effect of caffeine on the Kenyon neurons (Wright et al., 2013), which are involved in high-order associative memory, and the participation of arginine pathways enhancing protein synthesis involved in memory formation (Müller, 1996, 1997), it is worth considering future studies that take into account both underlying processes to clarify this issue.

Survival of harnessed bees

The only significant increase in the survival rate under the PER protocol was recorded for the mixture with the highest concentration, where survival of individuals reached 98% (compared with 74% of control bees and 80% of those that received the lower concentration mixture). It is interesting that when these compounds were administered individually, they did not generate any effect, but when they were administered together, such a noticeable change occurred. It is likely that there is a conjunction of the independent effects of each substance. On the one hand, there is the beneficial antioxidant action of caffeine (Lee, 2000; Kriško et al., 2005; León-Carmona and Galano, 2011), which has been shown to increase honeybee survival when administered *ad libitum* at a concentration of 0.25 mmol l^{-1} (Strachecka et al., 2014). On the other hand, there is the effect of arginine that, despite the lack of records of its influence on the survival of honeybees, is known to increase survival rate in vertebrate embryos (Bérard and Bee, 2010), while its product, nitric oxide, improves the immune system in certain insects (Rivero, 2006; Negri et al., 2013).

In summary, in the present work we have demonstrated for the first time that honeybee cognitive ability can be enhanced by combining two different sorts of secondary compounds that occur in natural floral nectars. These two substances are well known to participate directly or indirectly in different mechanisms of action underlying associative learning processes in honeybees (Müller, 1996, 2000; Wright et al., 2013). Because of this unexpected finding, it is worth focusing on neurobiological approaches to obtain a better understanding of this combined effect on the mechanisms involved. The understanding of these processes might even have implications at the social scale, given the highly social nature of the honeybees (Müller, 1996). If we consider learning as the ability to focus on the connection between a series of events and to retain that information (Mackintosh, 1994), it would be expected that the processes that enhance this at the individual level will also facilitate learning mediated by social interactions with nestmates such as mouth-to-mouth food exchange or trophallaxis (Farina et al., 2005). Honeybees combine these social learning events with complex communication systems such as the waggle dance to obtain multicomponent information about resources (Grüter and Farina, 2009). Thus, fast information propagation related to profitable food sources promotes recruitment and increases the foraging force of the whole colony (Grüter and Farina, 2009). Within this framework, secondary compounds acting on individual appetitive responses would promote faster collective output, as demonstrated when caffeine presented in the diet increased behavioural responses at the individual and social level (Couvillon et al., 2015).

Finally, if we consider that social bees are involved in pollination of more than 70% of crops across the globe (Aizen and Harder, 2009), the present findings may have implications for pollination ecosystems. Recently, it has been demonstrated that the use of synthetic mimic odours of the floral crop learned inside the beehive is a suitable procedure to improve yield in commercial crops (Farina et al., 2020). If it were possible to complement this with these secondary compounds that act as memory enhancers, pollination efficiency might be improved further.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: F.P., W.M.F.; Methodology: I.L.M.; Validation: I.L.M., F.P., W.M.F.; Formal analysis: I.L.M., F.P.; Investigation: I.L.M.; Resources: W.M.F.; Data curation: I.L.M., F.P., W.M.F.; Writing - original draft: I.L.M., F.P., W.M.F.; Writing - review & editing: I.L.M., F.P., W.M.F.; Visualization: F.P., W.M.F.; Supervision: F.P., W.M.F.; Project administration: W.M.F.; Funding acquisition: W.M.F.

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Data availability

Data from this study are available in the Dryad digital repository (Marchi et al., 2021): q573n5thg.

Supplementary information

Supplementary information available online at
https://jeb.biologists.org/lookup/doi/10.1242/jeb.239616.supplemental

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Table S1. Olfactory classical PER conditioning. Set of variables considered in the logistic regression models (GLM) effects models explaining bees' responses in the olfactory PER conditioning to different compounds.

Compound	Phase	Variable	Chi sq	P-values
Caffeine	Acquisition	TrialxTreatment	33.13	0.7687
		Treatment	21.93	**0.0173**
		Trial	57.23	**2.30E-09**
Arginine	Acquisition	TrialxTreatment	4.67	0.5865
		Treatment	48.22	**3.42E-08**
		Trial	22.61	**0.0486**
Mixtures	Acquisition	TrialxTreatment	3.31	0.7690
		Treatment	80.72	**0.0177**
		Trial	37.13	**4.32E-05**

Table S2. Short-term memory and long-term memory tests. Statistical evaluation of the logistic regression models (GLM) that include treatment as the only explanatory variable in the tests of short-term memory (STM) and long-term memory (LTM). Values in **bold** represent P-values<0.05, and in *italic* P-values>0.05 by no more than one order of magnitude of difference.

Compound	Phase	Variable	Deviance	P-values
Caffeine	STM	Treatment	59.08	*0.0521*
	LTM	Treatment	22.92	0.3180
Arginine	STM	Treatment	5.47	*0.0650*
	LTM	Treatment	59.22	*0.0518*
Mixtures	STM	Treatment	74.34	**0.0041**
	LTM	Treatment	10.80	**0.0045**

Table S3. Short-term memory and long-term memory tests. Comparisons. Z ratio values (below diagonal) and p-values (above diagonal) obtained from comparisons between treatments given by the mixtures group since it was the only one that showed significant differences in the GLM that included the treatment as an explanatory variable. "Lower conc." stands for the mix between Caf. 0,15mM + Arg. 0.01mM, and "Higher conc." stands for the mix between Caf. 0.15mM + Arg. 0.03mM. Values in **bold** represent P-values<0.05, and in *italic* P-values>0.05 by no more than one order of magnitude of difference.

STM			
Treatment	SS Mixtures	Lower conc.	Higher conc.
SS Mixtures		*0.0502*	**0.0196**
Lower conc.	2.34		0.9157
Higher conc.	-2.69	-0.40	
LTM			
Treatment	SS Mixes	Lower conc.	Higher conc.
SS Mixtures		**0.0102**	**0.0277**
Lower conc.	2.90		0.8021
Higher conc.	-2.56	0.63	

Table S4. Survival. Statistical analysis of the logistic regression models (GLM) that include treatment as the only explanatory variable of the survival of individuals over 24 hours after the olfactory classical conditioning and tests protocols. "Lower conc." stands for the mix between Caf. 0,15mM + Arg. 0.01mM, and "Higher conc." stands for the mix between Caf. 0.15mM + Arg. 0.03mM

Compound	**Phenomenon analyzed**	**Variable**	**Deviance**	**P-values**
Caffeine	Survival	Treatment	0.386	0.82450
Arginine	Survival	Treatment	3.293	0.19270
Mixtures	Survival	Treatment	17.646	**0.00015**

Table S5. Survival. Comparisons. Z ratio values (below diagonal) and p-values (above diagonal) obtained from comparisons between treatments given by the mixtures group since it was the only one that showed significant differences in the GLM that included the treatment as an explanatory variable. "Lower conc." stands for the mix between Caf. 0,15mM + Arg. 0.01mM, and "Higher conc." stands for the mix between Caf. 0.15mM + Arg. 0.03mM

Treatment	SS Mixtures	Lower conc.	Higher conc.
SS Mixtures		0.691	**0.014**
Lower conc.	2.34		**0.044**
Higher conc.	2.69	0.40	