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New method to Proboscis Extension Reflex to the assessment of gustatory responses for stingless bees

Novo Método de Reflexo de Extensão da Probóscide para avaliação de respostas gustativas para abelhas sem ferrão

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ABSTRACT

The proboscis extension reflex (PER) applied to restrained individuals is an important method to assess the sucrose responsiveness under laboratory conditions. Several authors have used the PER bioassay to assess behavioral effects of pesticides. A lot of them reported the difficult to use this method with non-*Apis* bees showing that this basic technique cannot be applied for all bees. The aim of this study was to evaluate the sucrose responsiveness of two brazilian stingless bees, *Melipona scutellaris* and *Scaptotrigona postica* using two different protocols: the traditional one and the new one where bees have free movements. In both cases, the bees were anesthetized (freezing) and inserted into plastic tubes with the tip cut out. After a starvation period were offered an increasing concentration of sucrose-water solution (w/v). Between the solutions, were offered water. With the traditional method, the sucrose responsiveness were observed only in *M. scutellaris* bees (12.5% of tested bees) in just one sucrose concentration (75%). Using the methodology with free movements, both species showed sucrose responsiveness in all concentrations (25%, 50% and 75%) tested. The number of *M. scutellaris* bees that had sucrose response ranged from 53.7% to 76.2% depending on the sucrose concentration. And the number of *S. postica* ranged from 54% to 79%. These results showed that using the methodology with free movements the sucrose responsiveness can be assessment non-Apis bees.

Keywords: non-*Apis* bees, sucrose responsiveness, *Melipona sctutellaris*, *Scaptotrigona postica*.

RESUMO

O reflexo de extensão de probóscide (REP) aplicado a indivíduos restritos é um método importante para avaliar a capacidade de resposta da sacarose em condições laboratoriais. Vários autores usaram o bioensaio REP para avaliar os efeitos comportamentais dos pesticidas. Muitos autores relataram a dificuldade de usar esse método com abelhas sem ferrão, mostrando que esta técnica básica não pode ser aplicada para todas as abelhas. O objetivo deste estudo foi avaliar a capacidade de resposta de sacarose de duas abelhas sem ferrão brasileiras, *Melipona scutellaris* e *Scaptotrigona postica,* usando dois protocolos diferentes: o tradicional e o novo onde as abelhas têm movimentos livres. Em ambos os casos, as abelhas foram anestesiadas (congeladas) e inseridas em tubos plásticos com a ponta cortada. Após um período de inanição foram oferecidas concentrações crescentes de solução de sacarose-água (p/ v). Entre as soluções, foram oferecidas água.

Vol. 7, No. 1, 69-76 (2018)
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Com o método tradicional, a resposta de sacarose foi observada apenas nas abelhas de *M. scutellaris* (12,5% das abelhas testadas) em apenas uma concentração de sacarose (75%). Usando a metodologia com movimentos livres, ambas as espécies apresentaram capacidade de resposta à sacarose em todas as concentrações (25%, 50% e 75%) testadas. O número de abelhas de *M. scutellaris* que apresentaram resposta à sacarose variou de 53,7% a 76,2%, dependendo da concentração de sacarose. E o número para a espécie *S. postica* variou de 54% a 79%. Esses resultados mostraram que, usando a metodologia com movimentos livres, a capacidade de resposta à sacarose pode ser a avaliação de abelhas sem ferrão.
Palavras-chave: abelhas sem ferrão, resposta de sacarose, *Melipona sctutellaris*, *Scaptotrigona postica*.

INTRODUCTION

In general, the Proboscis Extension Reflex (PER) considers that the bees extend their proboscis reflexively (unconditioned response) when the chemoreceptors in their antennae, buccal apparatus or tarsi are stimulated with sucrose solution (unconditioned stimulus) (Takeda, 1961; Bitterman et al., 1983; Menzel, 1999).

This assay is considered a quantifiable and reliable method since it reproduces the bee-plant interaction: when the bee lands on the flower its gustative receptors are stimulated by the nectar and in response, the bee extends its proboscis, collecting the nectar and memorizing the floral odours that, once memorized, are fundamental for the recognition of flowers in the next foraging. PER is also a test of ecological importance, since it is a requirement for the foraging success, with the involvement of the entire colony (Decourtye et al., 2005; Desneux et al., 2007).

It has also been demonstrated that the PER data are well correlated with the olfactory responses of the bees under free flight conditions, suggesting that the effects found in the PER responses in laboratory conditions can reflect the effects that would occur in real field situations (Pham-Dèlegue et al., 2002). The PER procedure has been performed in several studies with honeybees. In these bees, strong similarities were observed between the results with the contained and in free flight individuals (Mauelshagen and Greggers 1993; Pham-Dèlegue et al., 1993).

Several studies with non-*Apis* bees have also used the PER methodology and, mostly, the percentage of bees that respond positively to PER is lower when compared to *Apis mellifera* Linnaeus, 1758 (Abramson et al., 1999; McCabe et al., 2007). Studies of PER with stingless bees (Meliponinae) had responses 35% to 75% lower than *A. mellifera* (McCabe et al., 2007; McCabe and Farina 2009, 2010; Roselino and Hrncir, 2012). Only 5% to 44% of the *Bombus terrestris* Linnaeus, 1758 and *Bombus impatiens* Cresson, 1863 bees presented positive responses when submitted to PER while 70% to 100% of the honeybees have positive responses (Laloi et al., 1999; Toda et al., 2009). *Bombus* ssp., *Megachile* ssp. and *Osmia* ssp. bees did not exhibit responses to the PER test (Vorel and Pitts-Singer, 2010).

Recent results with bees of the *Scaptotrigona postica* Latreille, 1804 species have shown the use of behavioral studies as a means of developing protective measures aiming at the conservation of Brazilian bees (Silva et al., 2016).

Considering that this methodology was developed for the bee *A. mellifera* (Toda et al., 2009) this PER protocol cannot be perfectly adequate for non-*Apis* bees (McCabe et al., 2007). This fact could be a likely reason for the poor performance of non-*Apis* bees when subjected to this test (McCabe and Farina 2010). Thus, more experimental adjustments are necessary to adapt the PER assay for different species of bees (Roselino and Hrncir, 2012).

Still, the ability of bees to feed regularly even when contained is a condition needed to the PER (Abramson et al., 1999). *A. mellifera* bees respond promptly to PER using the traditional harness retention system proposed by Takeda (1961) and Bitterman et al. (1983) and is a basic methodology to test the PER in honeybees (Bitterman et al., 1983), but that cannot be universally used for all species of bees (Vorel and Pitts-Singer, 2010).

A suitable protocol for stingless bees becomes a useful tool for understanding the learning process of these bees, the olfactory mechanisms involved in the orientation of floral sources, the study of their cognitive capacities,

allowing subsequent correlations between the behaviour and the underlying physiological mechanisms of the learning processes (Abramson et al., 1999; Laloi et al., 1999; Menzel, 1999).

In this study we sought to develop a PER methodology that would be best suited for stingless bees by the evaluation of unconditioned gustatory response tests to know if these bees: i) presented a clear proboscis extension and; ii) identify the sensitivity of these bees to sucrose concentrations.

Thus, the aim of this study was to evaluate the gustative responses of foragers of stingless bees using as model a representative of the tribe Meliponini (*Melipona scutellaris* Latreille, 1811) and a representative of the tribe Trigonini (*S. postica*) using two protocols of PER: traditional harness (Takeda 1961; Bitterman et al., 1983) and the methodology proposed of free movement (in plastic tube – like eppendorf).

MATERIAL AND METHODS

The experiments were conducted at the Laboratory of Ecotoxicology and Conservation of Bees (Laboratório de Ecotoxicologia e Conservação de Abelhas - LECA), of the Centre for the Study of Social Insects (Centro de Estudos de Insetos Sociais - CEIS) of the Universidade Estadual Paulista (UNESP), campus of Rio Claro from January to April and from October to December 2012. Three colonies of *M. scutellaris* and three colonies of *S. postica* were used. The colonies were maintained in the meliponary of UNESP and were already established in this place for more than one year. During the experiments the health of the colonies was monitored as well as the queen oviposition activity, the brood combs, foraging activity and food availability. Data were submitted to Analysis of Variance (ANOVA) and followed by Tukey test ($p<0.05$) (SigmaPlot 12©) to compare if there was significative difference between the methodologies tested.

Collection and Preparation of the Bees

The bees were collected at the exit of the colonies, placed in plastic pots in groups of ten, and maintained in B.O.D. incubator at 29ºC ± 1°C and R.H. 70% ± 5% for 4 hours. Before conducting the assay, water was offered to the bees in order to avoid the effect of thirst.

M. scutellaris bees were divided into two groups: traditional REP (n = 100) and REP in plastic tube – eppendorf (n = 100). *S. postica* bees were subjected only to the REP test in plastic tube – eppendorf (n = 100) (due to the difficulty of submitting these bees to the traditional PER test since they are very small).

Traditional PER Harness

The experimental protocol was based in the methodology used by Takeda (1961) and Bitterman et al. (1983). In order to put bees into plastic tips, bees were anesthetized (freezing) for 30 seconds. *M. scutellaris* bees were individually placed in plastic tips adequately cut that restricted the movement of the body but allowed the free circulation of the antennae and buccal apparatus (Figure 1)

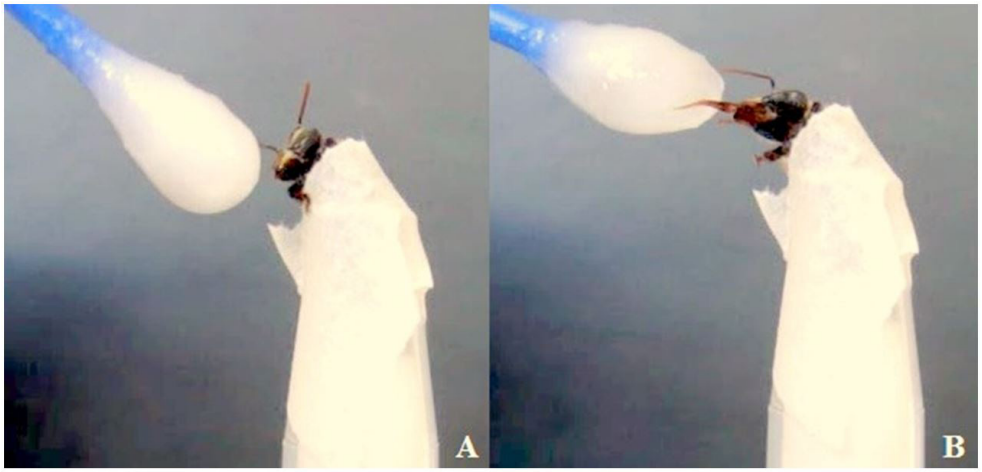

Figure 1. Forager of *M. scutellaris* subjected to the traditional PER harness test, with the body trapped with adesive tape and with free antennae and buccal parts. A – Offering water without proboscis extension; B – Offering sucrose solution 75% with proboscis extension.

Vol. 7, No. 1, 69-76 (2018)
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In order to investigate the sensitivity of all bees to increased sucrose concentrations, after fasting of one, two and four hours, at room temperature, sucrose solution at 25%, 50% and 75% was offered, during ten seconds (Bitterman et al., 1983). The lowest concentration in which a bee responds with the extension of its proboscis is interpreted as its sucrose response threshold (SRT). Each sucrose solution was tested by touching it on the antennae of the bee with a flexible rod with cotton tip soaked in the solution, and it was considered as positive response when the bee extended totally its proboscis (Figure 1B).

Between each sucrose solution, all the bees were tested regarding their response to the water as a control on the potential effects of the repetitive stimulation that could lead to the sensitization or habituation affecting subsequent responses (Page Junior et al., 1998).

PER Free in the Plastic Tube

M. scutellaris and *S. postica* bees were individually placed in plastic tubes like eppendorf of 2 mL, with a hole in the bottom, where the bee had access to the sucrose solution and could extend its proboscis in a visible manner. The bees remained with their bodies free inside the tube (Figure 2A and C). Each sucrose solution was tested allowing the bees to touch the antennae in the flexible rod with cotton tip soaked in the solution, and it was considered as positive response when the bee extended totally its proboscis (Figure 2B and D).

For *M. scutellaris*, sucrose solutions at 25%, 50% and 75% were offered in crescent order during ten seconds, interspersed with water, after fasting between one hour and a half and two hours. For *S. postica* the same sucrose solutions were offered in crescent, decrescent and random order to avoid learning, after 1 hour of fasting, during 10 seconds and interspersed with water offering. As in the traditional PER methodology, the water was used as control on the potential effects of repetitive stimulation that could lead to sensitization or habituation affecting subsequent responses (Page Junior et al., 1998).

RESULTS AND DISCUSSION

The mortality rate of the *M. scutellaris* bees was of 20%, both for the traditional PER and for the PER in tube like eppendorf. Now, for the *S. postica* bees, the mortality rate in the PER test in the plastic tube was of 6%.

In the traditional PER only 12.5% of the total bees of *M. scutellaris* tested (n= 80) presented positive response only to the sucrose solution at 75%, after 4 hours of fasting. For the sucrose solution of 25% and 50% there

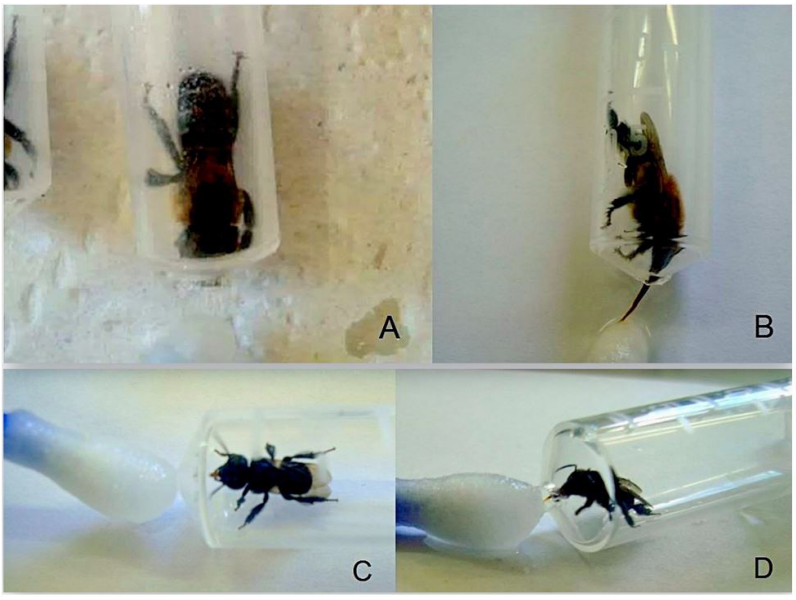

Figure 2. Forager of *M. scutellaris* and *S. postica* subjected to the PER test in plastic tube (eppendorf), with free-moving bodies. A and B: Forager of *M. scutellaris*; in A without offering the sucrose solution and; in B offering sucrose solution 50%, with proboscis extension. C and D: Forager of *S. postica*; in C without offering the sucrose solution and; in D offering sucrose solution 50%, with proboscis extension.

Table 1. Stingless bees tested for the Proboscis Extension Reflex (PER) by the traditional harness method and by the methodology proposed (plastic tube - eppendorf). *M. scutellaris*: n = 80 (for each test); *S. postica*: n = 94.

Bee	Sucrose concentration %	Positive PER (%)	
		Traditional	Eppendorf
M. scutellaris	25	0	53.5
	50	0	71.5
	75	12.5	76.5
S. postica	25	-	79
	50	-	65
	75	-	54

was no positive response (Table 1). During the test, most of the bees tested tried to escape from the tip, as it was also reported by Toda et al. (2009) with *B. impatiens*.

Considering the new methodology of PER in tubes like eppendorf for *M. scutellaris*, the percentage of positive responses was of 53.75% for the sucrose solution at 25%; 71.25% for the sucrose solution at 50%; and 76.25% for the sucrose solution at 75% (Table 1). This protocol enabled a sixfold increase in the number of positive responses exhibited for *M. scutellaris* bees when it was offered sucrose solution at 75% when compared with traditional PER.

Still, there was an increase of over 50% and 70% for the sucrose solutions at 25% and 50%, respectively, concentrations that in the traditional PER test no bee had responded. There was no significant difference for all the sucrose concentrations with the new methodology (ANOVA, $F = 16.6825$; Tukey, $p < 0.01$, Figure 3). Also, the bees fasting period with this new methodology had a reduction in approximately 50%, from 4 hours in the traditional PER tests to one hour and a half and two hours in the free-moving test.

The percentage in the positive responses of the *S. postica* bees (with the new PER methodology in a tube like eppendorf) was of 79% for the sucrose solution at 25%; 65% for the sucrose solution at 50%; and 54% for the sucrose solution at 75% (Table 1).

McCabe et al. (2007), evaluating the classic olfactory conditioning for in bees of the genus *Scaptotrigona* (*S.* aff. *depilis*) obtained 67% of positive responses to the PER for the sucrose solution 50%, similar to our data. In our results, the decrease in the concentration of the sucrose solution for 25% provided an increase of 14% of the positive responses for *S. postica*.

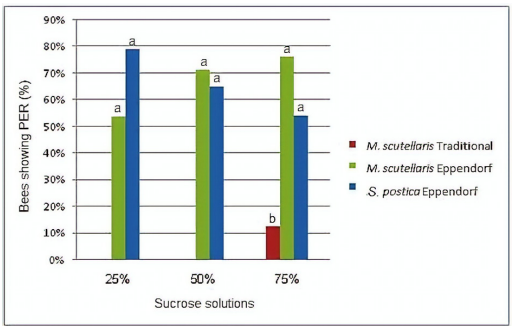

Figure 3. Analysis of sucrose responsiveness in stingless bees foragers (%) exposed to traditional PER and in the plastic tube tests. different letters show statistical differences**.**

Roselino and Hrncir (2012) carried out the traditional PER protocol for a classic olfactory conditioning (where the bees extend the proboscis when the antennae get in touch with an odour associated with the offering of sucrose solution) with foragers of *M. scutellaris*. None of the bees tested responded in a positive form to the PER and less than 20% of the bees responded positively to the short-term memory test, also assessed by the same protocol.

Evaluating the classic olfactory conditioning by the PER in bumblebees (*B. terrestris*), Laloi et al. (1999) observed that the increase in the concentration of the sucrose solution induces the increase of the performance in this test, differing from the results that were herein presented for *M. scutellaris* in the traditional PER. Still, with sucrose solution at 75% these authors obtained a positive response in the PER of, at maximum, 44% of the bumblebees tested, a percentage considered low when compared to the results of *A. mellifera* (70%-100% of positive responses) and with our results with the new methodology for *M. scutellaris* – 76.5%.

Comparing the classic olfactory conditioning by the PER with Africanized *A. mellifera* bees and two species of stingless bees (*Melipona quadrifasciata*, Lepeletier, 1836 and *S.a* aff. *depilis*, Moure, 1942), McCabe et al. (2007) also observed that stingless bees present lower responses (69.9% and 67%, respectively, of the stingless bees responded positively to the sucrose solution at 50%) while 95.5% of the honeybees showed positive responses.

McCabe and Farina (2009) used the PER protocol to assess the classic olfactory conditioning of *M. quadrifasciata* bees and observed that a very low number of bees responded spontaneously to this protocol. In 2010, the authors also observed low positive responses of PER in *Tetragonisca angustula* Latreille, 1811 bees (69%) and a maximum of 20% of positive responses when these bees were subjected to the conditioned PER (McCabe and Farina, 2010).

Evaluating the response to the PER for non-*Apis* bees (*Bombus* spp., *Megachile rotundata* (Fabricius, 1787), *M. pugnata* Say, 1837 and *Osmia lignaria* Say, 1837) in comparison with honeybees, Vorel and Pitts-Singer (2010) observed that, while the honeybee readily respond with the proboscis extension when their antennae are stimulated with sucrose solution (25% and 50%) the bees tested did not exhibit a positive response to the same test.

Experiments of Abramson et al. (1999), corroborate our results obtained with the traditional PER and show that the unconditioned PER cannot be useful for foragers of *M. scutellaris* and that sucrose would not be an effective stimulus for these bees.

The authors, in observation in field test, reported the preference of these bees for honey of their own species, in comparison to sucrose. They suggest that this is a fail in the technique for *M. scutellaris*, proposing two hypotheses: i) develop new methodologies for this species of stingless bee; or ii) these bees simply did not respond to the PER protocol.

One of the possible causes of this low performance of positive responses showed by the stingless bees in the traditional PER methodology could be the high stress level or weakening of the individuals when they have their bodies trapped to perform the PER test (McCabe et al., 2007; Roselino and Hrncir, 2012). Also, a neuro-anatomic study of the antennal lobe of *M. scutellaris* showed that the central olfactory structure of these bees is very different from the honeybees, which could be one of the reasons for the absence of a positive response of these bees to the traditional PER test (Roselino, 2009; Toda et al., 2009).

The lack of positive response to the traditional PER of the stingless bees could be due to the fact that this methodology was developed for the species *A. mellifera* (McCabe et al., 2007; Toda et al., 2009) and the PER traditional protocol is considered a limited process, which does not seem to provide adequate variables for a quantitative evaluation in species of stingless bees (McCabe and Farina, 2009).

Toda et al. (2009) obtained a tenfold increase in the positive responses of PER for *B. impatiens* using a similar methodology than the one proposed herein (in a tube like eppendorf), where the bees remain inside a plastic capsule, but their bodies do not stay trapped, with movement restriction, like in the traditional PER.

Our results for the PER in the tube like eppendorf (Table 1) corroborate Laloi et al. (1999), who observed that an increase in the concentration of sucrose solution induced an increase in the performance in the test for *B. terretris*, and here are the oppose to the results presented by Roselino and Hrncir (2012) where *M. scutellaris* bees respond to the PER in a positive form only to the sucrose solution from 55%.

This significant increase of positive responses with the PER methodology in tube like eppendorf, with offering of the same sucrose concentrations used in the traditional harness PER shows that the preference of these bees for the honey of their own species in comparison with the sucrose solution, according to the suggested by Abramson et al. (1999), is not a determining factor in the efficiency of the PER methodology.

The PER methodology in plastic tube (eppendorf) proposed for the unconditioned gustative stimulus was efficient for stingless bees *M. scutellaris* and *S. postica*. The significant increase in the percentage of positive responses to unconditioned PER for *M. scutellaris* facilitates the selection of bees for the conditioned PER test. The increase in the sucrose concentration increases

the number of *M. scutellaris* bees that respond positively to the proposed PER protocol (plastic tube). Now, the *S. postica* bees respond better to the gustative stimulus with sucrose concentration of 25%, with increased positive responses with decreasing the sucrose concentration.

This new method for stingless bees may bring new insights to the assessments of the impacts of chemical substances on the behavior of stingless bees and give greater subsidies for the conservation of Brazilian species, which currently uses the Africanized hybrid introduced in Brazil as an experimental model.

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