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Original article

Hygienic behavior in *Melipona quadrifasciata anthidioides* (Apidae, Meliponini)

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Abstract – Hygienic behavior in stingless bees is a trait of workers that confers colony-level resistance against some brood diseases. Workers of hygienic colonies detect, uncap and remove dead or diseased brood from the nest cells. We examined the hygienic behavior in stingless bees (*Melipona quadrifasciata anthidioides*) from freeze-killed brood assay using liquid nitrogen. Responses were measured at 14 times (3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 h after freeze-killing of the brood). Workers were estimated to remove on average 65% of larvae and 34% of dead pupae within 48 h of freezing. Workers removed dead brood rapidly after uncapping the cells. Strong colonies showed a greater removal of dead pupae, while the size of the population did not influence the removal of dead larvae. These findings report for the first time the hygienic behavior in *M. q. anthidioides* and confirm that workers have more difficulty removing pupae compared with larvae from the combs.

generalized linear models / stingless bees / hygienic colonies / dead brood removal / freeze-killed brood

1. INTRODUCTION

Melipona quadrifasciata Lepeletier ("mandaçaia") is a stingless bee species that occurs in most of Brazil, from the states of Paraíba to Rio Grande do Sul (Moure and Kerr 1950). The higher prevalence of subspecies *M. quadrifasciata anthidioides* is in hot and dry regions, especially in the semi-arid region of the state of Bahia (Nunes et al. 2008). Honey produced by *M. q. anthidioides* is highly appreciated and its colonies present good production when managed rationally, being of great socioeconomic and environmental importance.

In recent decades, bee colonies of *Apis mellifera* (vanEngelsdorp et al. 2007; Neumann and Carreck 2010) and populations of stingless bees have decreased worryingly (Steffan-Dewenter et al. 2005; Slaa et al. 2006; Freitas et al. 2009). There are numerous reasons for these reductions, such as the destruction or changes of habitats, pesticide overuse in agricultural crops, and diseases that affect bees (vanEngelsdorp and Meixner 2010; Reyes-González et al. 2016; Sanchez-Bayo and Goka 2016 and references therein). In the case of diseases, a practical and desirable strategy is that the bee species express high levels of natural resistance, such as the hygienic behavior (HB) (Evans and Spivak 2010; Bigio et al. 2013).

In *A. mellifera*, HB has been described as a process in which bees detect and uncap dead, parasitized or infected brood cells (5th instar, larvae and pupae) and remove them from the nest (Rothenbuhler 1964; Rosenkranz et al. 2010). When quickly performed, this process may prevent the spread of the disease causal agents to healthy brood (Rothenbuhler 1964). HB is a

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significant general resistance mechanism to a number of pathogens, including *Ascosphaera apis* (causal agent of chalk brood) (Gilliam et al. 1988), *Paenibacillus larvae* (causal agent of American foulbrood) (Spivak and Reuter 2001) and the parasitic mite *Varroa destructor* (Rinderer et al. 2010; Rasolofoarivao et al. 2015).

Hygienic colonies have large economic interests in apiculture, because they have been reported to produce more honey and pollen than non-hygienic colonies (Nicodemo et al. 2013). Although HB is a very important feature, it rarely manifests with intensity in populations (Pérez-Sato et al. 2009). Thus, the selection of queen bees related to this characteristic has been intensified in breeding programs (Espinosa-Montaño et al. 2008; Costa-Maia et al. 2011).

Most studies on HB are carried out with *A. mellifera* (Gramacho and Gonçalves 2009; Pérez-Sato et al. 2009; Wilson-Rich et al. 2009; Morais et al. 2010; Stevanovic et al. 2011; Pinto et al. 2012; Bigio et al. 2013; Nicodemo et al. 2013; Pereira et al. 2013; Rasolofoarivao et al. 2015). Few species of stingless bees have been investigated in terms of HB, namely *Melipona beecheii*, *Scaptotrigona pectoralis* (Medina et al. 2009) and *Plebeia remota* (Nunes-Silva et al. 2009).

Therefore, HB investigations in other species of stingless bees is necessary, mainly because of the diversity and specific characteristics of each species, extinction risks for the Meliponini and the growth of meliponiculture which can increase the risks of pathogen transmission (Venturieri et al. 2012), especially to species of greater economic interest. In addition, no study on HB in *M. q. anthidioides* has been conducted.

Our hypothesis is that HB in *M. q. anthidioides* occurs in a similar manner to that of bees of the genus *Apis* and that there is difference in the removal time of dead larvae and pupae by worker bees. The objective was to use *M. q. anthidioides* as a model to investigate HB in short time intervals until the complete removal of all the brood (larvae and pupae) killed by freezing in the colonies.

2. MATERIAL AND METHODS

The study was conducted from May to August, 2015 on 40 colonies of *M. q. anthidioides* housed in standard boxes, INPA model, consisting of first deep and second deep (13 × 13 × 6 cm), honey super (13 × 13 × 3.5 cm), outer cover and bottom board (18 × 18 × 2 cm) and internal space to access the compartments (6 × 6 cm).

HB was quantified at the level of the colony using the freezing method with liquid nitrogen (N_2) (described in Spivak and Reuter 1998), adapted to the nest dimensions of the species under study. For that purpose, an area with seven cells containing black-eyed pupae and seven larvae in the last instar (both capped cells) was delimited and marked. A control area was also delimited and marked to discount the natural removal rate of diseased, dead or parasitized pupae and larvae. A PVC cylinder (2 cm diameter × 3 cm high) was positioned over the areas to be tested and 12 mL of liquid nitrogen were poured to kill the brood. Each colony was monitored until the worker bees performed the uncapping and totally removed the brood.

The experiment was installed in a complete randomized block design with 14 treatments repeated 40 times (colonies) in each block, totaling 1680 records. The blocks were made of three different observation periods for each colony, with intervals of 30 days between each observation. The treatments or measurement times occurred up to 264 h, as follows, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 h after liquid nitrogen application. The variables analyzed were: accumulated average proportion of dead larvae removal (LR) and dead pupae removal (PR), and accumulated average proportion of uncapped cells with not yet removed dead larvae (UL) and pupae (UP). All the colonies assessed for HB had the population size estimated before each repetition according to Ihering (1932) and after measuring the number of brood cells (Aidar 2010).

Generalized linear models (GLM) with binomial error structure and *logit* link function expressed by $g(\mu) = \ln(\mu/1\text{-}\mu)$ were used to test the effect of time and population of the colonies on HB. The model parameters were estimated using the method of maximum likelihood by maximizing the log-likelihood function through generalized estimating equations (GEE). The GEE considered the dependence between the observations measured within the times (hours), which characterized repeated measures in the experimental units (colonies). The responses obtained between experimental units from different periods (blocks) were assumed to be statistically independent. The deviance analysis (ANODE) was used for the GLM adjustment, from a maximal model, represented by systematic portion

$\eta = g(\mu) = \mu + T_i + B_j + P + \varepsilon_{ij}$, where μ is the general average effect, T_i is the time levels effect in hours (i = 1, 2, …, 14), B_j is the blocks effect (j = 1, 2 and 3), P is the effect of the 'colonies population' covariate ,and ε_{ij} is the random error.

The adjustment quality of the models to the observed data and the selection of the best model were based on the higher value of the maximum likelihood function logarithm (*LogLik*). Dispersion parameters were adjusted when overdispersion was found, correcting the standard errors using a *quasibinomial* model, for subsequent comparison between adjusted *LogLik* values of different models. After defining the most appropriate model, the significances of time (T), block (B) and population (P) on the variables were recorded in the type 3 GEE analysis. The effects of time and population on the variables were evaluated by means of the logistic regression models.

After replacing the time values (hours after brood death by freezing) in the models, the identity intercepts and coefficients between the logistic regression models of LR and PR and predictive models of UL and UP was carried out initially by the linear regression model adjustment with 1st degree ($y_i = b_0 + b_1x_i + \varepsilon_i$) of the average values of larvae removal and uncapping (y), estimated at each time ($n = 14$), based on their estimated average removal and uncapping of pupae values (x), using the method of ordinary least squares.

The existence of a linear relationship between the estimated average values of LR and PR, and of UL and UP, was assessed by detecting significance of estimated parameter $\beta 1$ (angular coefficient), verified by the partial *t* test application to test the invalidity hypothesis of $H_0: \beta_i = 0$. The non-acceptance of the invalidity hypothesis to the angular coefficient suggested the influence of the PR and UP estimated average values to explain the variation in the respective LR and UL estimated average values. At the same time, the *F* test was applied (Montgomery et al. 2006) to test the hypothesis of joint nullity for the linear regression parameters ($H_0: \beta_0 = 0$ and $\beta_1 = 1$). The rejection of H_0 indicates an absence of similarity between estimates of average accumulated proportions of LR (y) and PR (x), and UL (y) and UP (x), that is, high magnitude occurs in the residual values ($\varepsilon_i = y - \hat{y}$).

The significance level of 0.05 was adopted in all the analyses. All statistical analyses were performed using the software R v.3.0.2 (R Core Team 2015).

3. RESULTS

We used recommendations of Spivak and Downey (1998) in *A. mellifera* for the parameters to compare HB of the colonies of *M. q. anthidioides* investigated. These authors recommended for hygienic colonies of *A. mellifera* a removal greater than 95% within 48 h in at least two tests using the method of killing the brood by freezing.

For LR, of 120 measurements, 78 (65%) were above 95% and 30 (75%) of 40 colonies showed, in at least two periods, removal greater than 95% within 48 h. For PR, of 120 measurements, 40 (33%) were above 95% and 6 (15%) of 40 colonies showed, in at least two periods, removal greater than 95% within 48 h. The average number of days for the removal of all larvae killed by freezing was 2.90 and for pupae 5.02, with a range of variation of 0–11 days.

The percentage of LR and PR killed by freezing within 48 h ranged from 0 to 100% in the observation periods 1 and 2 and from 14 to 100% in period 3, with a variation coefficient of 42% for LR and 89% for PR. When considering the variation in the percentage of colonies that removed more than 95% of brood killed by freezing within 48 h in each period, LR ranged from 25% of the colonies in period 1 to 85% in period 3 and PR ranged from 4% of colonies in period 1 to 26% in period 3.

There was a positive effect of time and block for LR (time: $\chi^2 = 35.91$, $P < 0.0001$; block: $\chi^2 = 21.03$, $P < 0.0001$). There was a positive effect of time, population and block for PR (time: $\chi^2 = 38.93$, $P < 0.0001$; population: $\chi^2 = 4.92$, $P = 0.0265$; block: $\chi^2 = 27.95$, $P < 0.0001$). There was a positive effect of time for UL ($\chi^2 = 9.61$, $P = 0.0019$). There was a positive effect of time and population for UP (time: $\chi 2 = 7.18$, $P = 0.0074$; population: $\chi^2 = 3.57$, $P = 0.050$).

Figure 1 shows the LR and PR regression models in terms of hours after the brood death by freezing. The LR average estimate ranged from 39.97 to 99.64% within the evaluated times (3–264 h). LR average estimates within 24 and 48 h were 51.97 and 65.32%, respectively. Considering an average population of 806 individuals, the PR average estimate ranged from 14.98 to 98.88% within the measured times (3–264 h). PR average

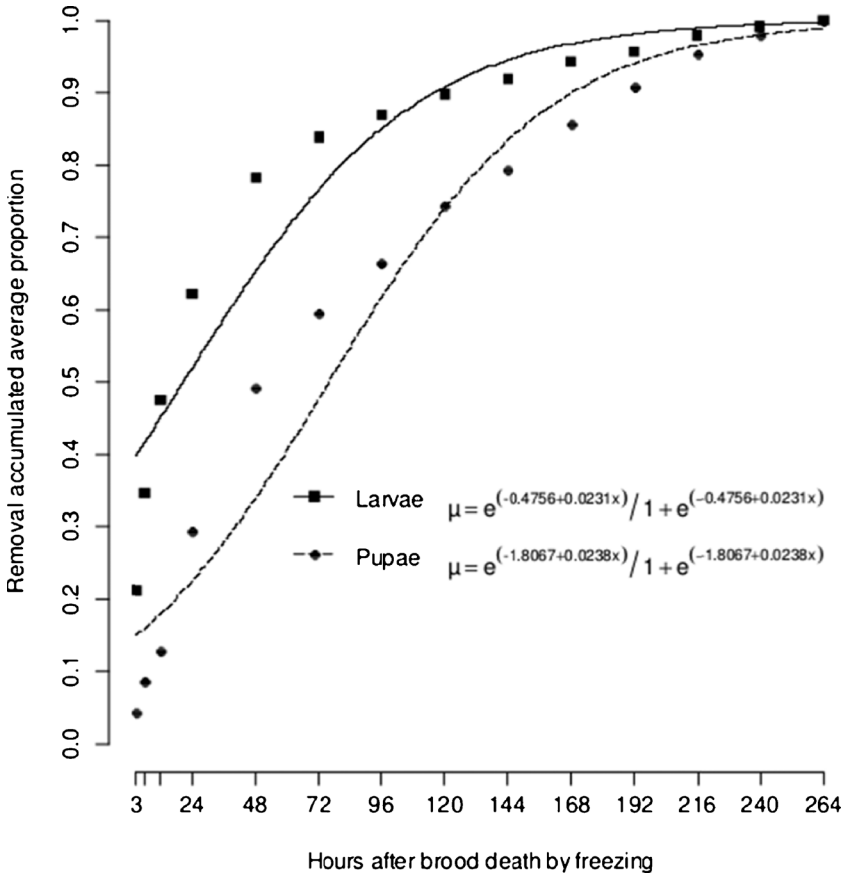

Figure 1. Regression models of the accumulated average proportion of larvae removal (LR) and pupae removal (PR) (average population of PR was 806 individuals) of *M. q. anthidioides* in terms of time values, expressed in hours after brood death by freezing.

estimates within 24 and 48 h were 22.52 and 33.98%, respectively.

Figure 2 shows the regression models for UL and UP in terms of hours after the brood death by freezing. Low percentages of UL and UP with brood killed by freezing not yet removed by worker bees were observed. Only 4.81% UL were estimated within 48 h. The highest UL average value estimated occurred within 264 h (8.82%). When the average estimate of 806 individuals was assessed in the colony, the UP estimated percentage within 48 h was 1.44%. The highest UP estimated average value occurred within 264 h (3.21%).

The differences between the LR and PR average estimates and the UL and UP killed by freezing over time could be statistically detected, because the rejection of the hypothesis of joint nullity was observed for linear regressions parameters (H_0:$\beta_0 = 0$ and $\beta_1 = 1$) when the estimated average values of LR in PR and UL in UP were regressed. In both tests, the significance probability value (P) was lower than 0.0001 ($P < 0.0001$). The estimated 1st degree equations were $LR = 0.360 + 0,\ 682PR$ ($R^2 = 0.9602$) and $UL = 0.015, +2.317UP$ ($R^2 = 0.9987$). It was observed that the inclination angles (inverse tangent of the angular coefficient) of the straight lines for removal and uncapping were 34.29 and 66.66°, different values ($P < 0.0001$) of 45°, ideal angle, a situation that characterizes the lack of similarity between pairs of x and y values.

When a certain time is kept fixed after the brood death by freezing, PR and UP estimates increase as the population in the colonies also increases (Figures 3 and 4, respectively).

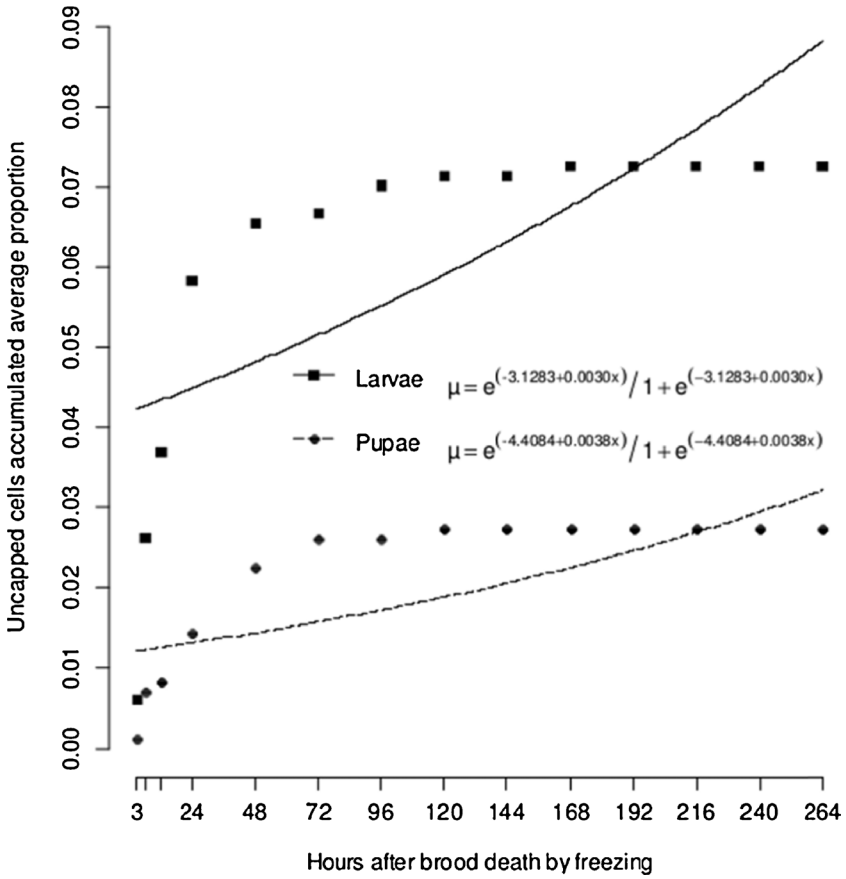

Figure 2. Regression models of the accumulated average proportion of uncapped larvae cells (UL) and uncapped pupae cells (UP) (average population of UP was 806 individuals) of *Melipona quadrifasciata anthidioides* in terms of time values, expressed in hours after brood death by freezing.

4. DISCUSSION

HB sequences observed in unselected colonies of *M. q. anthidioides* confirm the hypothesis that this behavior is similar to that of *A. mellifera*. The main difference is that the *M. q. anthidioides* worker bees destroy brood cells after removing the brood. In addition, although the behavior of partial removal of dead brood in the test areas, which occurs in *A. mellifera*, was not observed, body parts of the brood were observed in the colony, which highlights that such behavior does also occur in *M. q. anthidioides*.

Few uncapped larvae cells and uncapped pupae cells killed by freezing were not immediately removed by worker bees, which is important to avoid possible pathogen dissemination in the colonies if the brood were infected.

On average, there was a gradual increase of LR and PR over time, with 75 and 6%, respectively, of the unselected colonies of *M. q. antidioides* showing removal greater than 95% within 48 h in at least two periods. In *A. mellifera*, Pérez-Sato et al. (2009) and Bigio et al. (2013) found 3 and 0% of colonies with removal percentage greater than 95% within 48 h, respectively. Medina et al. (2009) observed HB in *Scaptotrigona pectoralis* and *M. beecheii* using frozen brood assay and found 100% removal of dead pupae after 2.3 ± 0.6 days and 4.4 ± 2.0 days, respectively. Nunes-Silva et al. (2009) studied HB in *Plebeia remota* using pin-killed brood and found a 96.4% removal after 48 h. Toufailia et al. (2016) found a removal

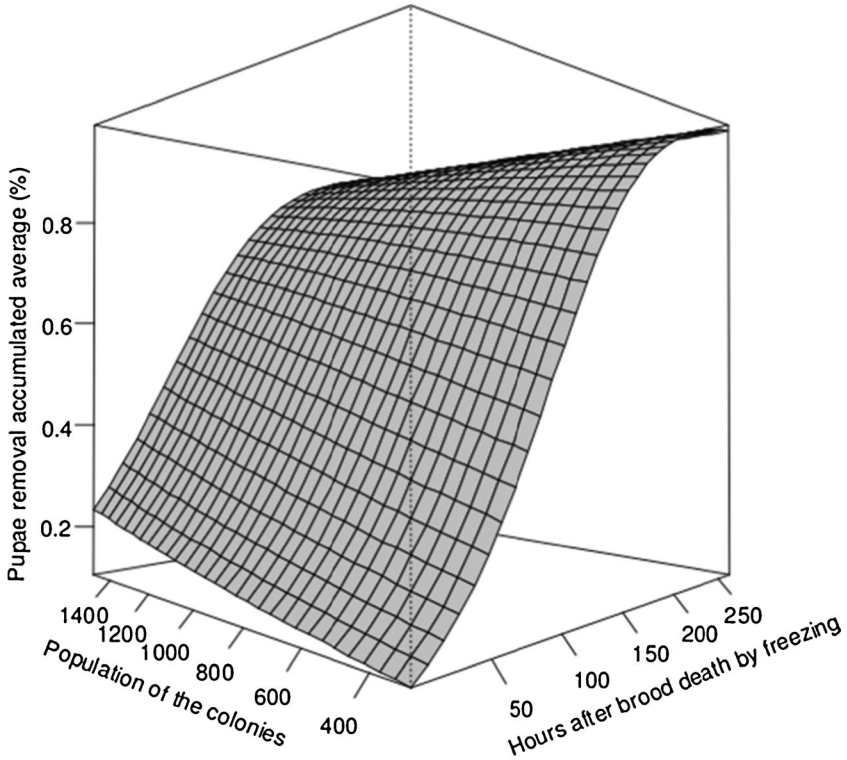

Figure 3. Response surface model of the accumulated average proportion of pupae removal (PR) of *M. q. anthidioides* in terms of time values (expressed in hours after brood death by freezing) and population of the colonies. PR model: $\mu = e^{(-2.4513 + 0.0238T + 0.0008P)} / 1 + e^{(-2.4513 + 0.0238T + 0.0008P)}$.

of freeze-killed brood after 48 h of 99.3% in *M. scutellaris*, 79.5% in *S. depilis* and 62% in *Tetragonisca angustula*.

The workers of *M. q. anthidioides* seem to achieve LR within 48 h from the examination of seven cells of larvae and seven of pupae, although this small sample may arguably have placed a limitation on our conclusions. Compared with other species of stingless bees, it appears that the removal of dead brood is relatively less effective in *M. q. anthidioides*, because, during investigations by Medina et al. (2009), Nunes-Silva et al. (2009), and Toufailia et al. (2016), more dead brood were removed within 48 h from a higher number of cells for their respective species. However, none of these studies made distinctions between the stages of brood development. Differences between the bee species for the brood comb diameter, as well as the different methodologies used to investigate HB are factors that hinder direct comparisons between the different studies and our results. Therefore, any comparison between different species of bees for HB should be made with caution. There was variation in HB between the colonies of *M. q. anthidioides* within each period and between the observation periods. The removal percentage within 6 h, using the method of brood drilling, ranged from 0 to 82% in *A. mellifera unicolor* (Rasolofoarivao et al. 2015), from 1 to 31% in *A. mellifera carnica* (Gramacho et al. 1998) and from 0 to 100% in Africanized honeybee *A. mellifera* using a similar protocol (Fries and Raina 2003). HB is strongly influenced by the time at which the tests are carried out (Uzunov et al. 2014). *A. cerana* colonies showed different HB in various ecological habitats (Athreya and Reddy 2013). However, it is believed that part of the variation in the observed HB for the colonies between observation periods is due to climatic conditions, although they were not investigated.

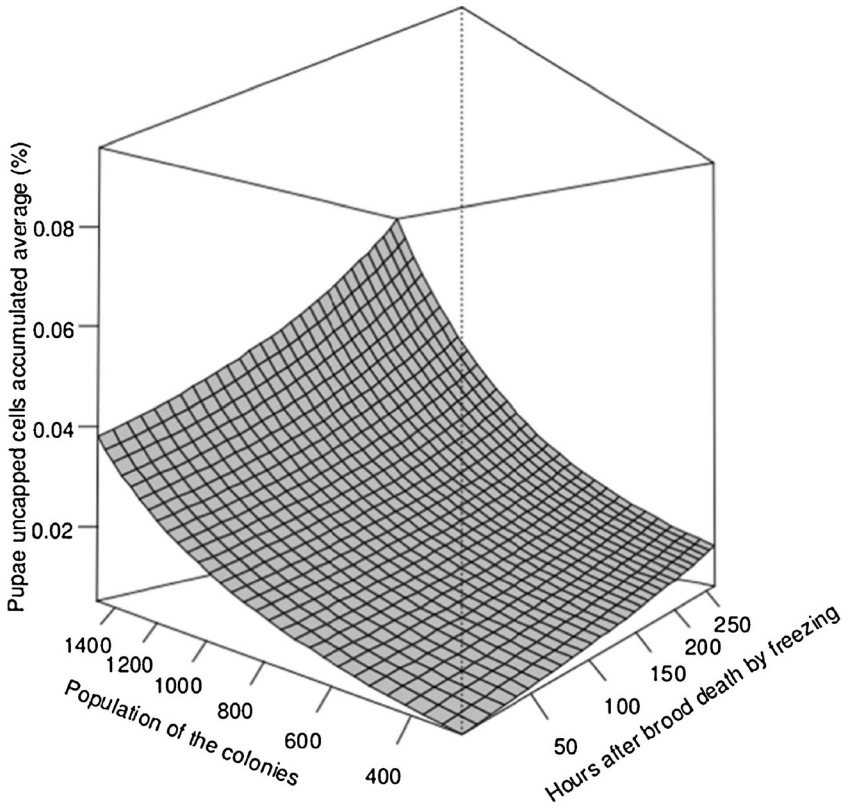

Figure 4. Response surface model of the accumulated average proportion of pupae uncapped (UP) cells of *Melipona quadrifasciata anthidioides* in terms of time values (expressed in hours after brood death by freezing) and population of the colonies. UP model: $\mu = e^{(-5.7781 + 0.0038T + 0.0017P)} / 1 + e^{(-5.7781 + 0.0038T + 0.0017P)}$.

Worker bees removed more killed larvae than pupae from brood cells within 48 h and PR takes a longer time than does LR in *M. q. anthidioides* . In *A. mellifera* , the behavior used to detect and remove pupae killed by freezing is not necessarily the same as that used to detect and remove mite-infested pupae (Spivak 1996). Differences in olfactory sensitivity between hygienic bees can lead to a partition of the uncapping and removal behavior (Gramacho and Spivak 2003). Pores found in opercula have been reported to affect the effectiveness of olfactory detection of bees (Mathur et al. 2010).

At first, we expected that worker bees removed the dead pupae faster than the dead larvae because of the greater amount of wax and resin in the brood disks for larvae, which does not occur in pupal cells due to its removal by worker bees to facilitate adult emergence. The larger amount of wax and resins in cells with larvae could hinder the workers in detecting dead larvae. However, in *M. q anthidioides* , the shorter time for LR than for PR was attributed to the brood integument constitution. Landim (2009) found that pupae integument is more complex and sclerotized than larvae integument. Empirical observations showed that a single worker bee of *M. q. anthidioides* removed dead larvae easily, while for dead pupae, the bees only managed to remove them after tearing the pupa body.

The variation in the estimated population of bees between the colonies did not influence the worker bees in LR and stronger colonies had greater PR. When there is an increase in the colony population, more bees assist in the PR activity of a single cell, reducing removal time.

This is the first study that has investigated HB in *M. q. anthidioides* . The findings confirmed HB in *M. q. anthidioides* and showed that its stages

are identical to those previously reported for *A. mellifera*. However, there is a difference in behavior by the worker bees for LR and PR. Also, strong colonies can shorten the time to remove dead pupae in the colony. This information can be useful for genetic improvement programs of *M. q. anthidioides* colonies on resistance to pests and diseases.

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Contributions GSS and CALC conceived this research and designed experiments; JNJ, EDC and NTEO performed experiments and analysis; and EDC and NTEO wrote the paper. All authors read and approved the final manuscript.

Comportement hygiénique chez *Melipona quadrifasciata anthidioides* (Apidae, Meliponini)

Modèle linéaire généralisé / abeille sans aiguillon / colonie hygiénique / élimination du couvain mort / couvain tué par congélation

Hygieneverhalten bei *Melipona quadrifasciata anthidioides* (Apidae, Meliponini)

Generalisiertes lineares Modell / Stachellose Bienen / hygienische Bienenvölker / Entfernen toter Brut / freeze-killed Brut

REFERENCES

Aidar, D.S. (2010) A mandaçaia: biologia e manejo de abelhas com ênfase à Melipona quadrifasciata Lep. FUNPEC, Ribeirão Preto

Athreya, S.V.R., Reddy, M.S. (2013) Variation of hygienic behaviour (nest cleaning behaviour) in honey bee, *Apis cerana indica* F. in different eco habitats of South India. Curr. Biot. **7**, 101–104

Bigio, G., Schürch, R., Ratnieks, F.L.W. (2013) Higienic behaviour in honey bees (Hymenoptera: Apidae): effects of brood, food, and time of the year. J. Econ. Entomol. **106**, 2280–2285

Costa-Maia, F.M., Toledo, V.A.A., Martins, E.N., Lino-Lourenço, D.A., Sereia, M.J., Oliveira, C.A.L., Faquinello, P., Halak, A.L. (2011) Estimates of covariance components for hygienic behaviour in Africanized honeybees (*Apis mellifera*). Rev. Bras. Zootec. **40**, 1909–1916

Espinosa-Montaño, L.G., Guzmán-Novoa, E., Sánchez-Albarrán, A., Montaldo, H., Correa-Benítez, A. (2008) Estudio comparativo de tres pruebas para evaluar el comportamiento higiénico en colonias de abejas (*Apis mellifera* L.). Vet. Mex. **39**, 39–54

Evans, J.D., Spivak, M. (2010) Socialized medicine: individual and communal disease barriers in honey bees. J. Invertebr. Pathol. **103**, S62–S72

Freitas, B.M., Imperatriz-Fonseca, V.L., Medina, L.M., Kleinert, A.M.P., Galetto, L., Nates-Parra, G., Queza-Eúan, J.J.G. (2009) Diversity, threats and conservation of native bees in the Neotropics. Apidologie **40**, 332–346

Fries, I., Raina, S. (2003) American foulbrood and African honey bees (Hymenoptera: Apidae). J. Econ. Entomol. **96**, 1641–1646

Gilliam, M., Taber III, S., Lorenz, B.J., Prest, D.B. (1988) Factors affecting development of chalkbrood disease in colinies of honey bees, *Apis mellifera*, fed pollen contaminate *Ascosphaera apis*. J. Invertebr. Pathol. **52**, 314–325

Gramacho, K.P., Gonçalves, L.S. (2009) Comparative study of the hygienic behavior of Carniolan and Africanized honey bees directed towards grouped versus isolated dead brood cells. Genet. Mol. Res. **8**, 744–750

Gramacho, K.P., Spivak, M. (2003) Differences in olfactory sensitivity and behavior responses among honey bees for hygienic behavior. Behav. Ecol. Sociobiol. **54**, 472–479

Gramacho, K.P., Gonçalves, L.S., Rosenkranz, P. (1998) Study of temperature of brood killed by the pin-killing method in worker bees of *Apis mellifera carnica*. Apiacta **2**, 35–41

Ihering, H. (1932) A uruçu na apicultura nordestina. Chác Quintais **46**, 292–296

Landim, C.C. (2009) Abelhas: morfologia e função de sistemas. Editora UNESP, São Paulo

Medina, L.M., Hart, A.G., Ratnieks, F.L.W. (2009) Hygienic behavior in the stingless bees *Melipona beecheii* and *Scaptotrigona pectoralis* (Hymenoptera: Meliponini). Genet. Mol. Res. **2**, 571–576

Montgomery, D.C., Peck, E.A., Vining, G.G. (2006) Introduction to linear regression analysis, 4th edn. Wiley, New York

Morais, M.M., Francoy, T.M., Borissevitch, I.E., Goncalves, L.S. (2010) A scientific note about spectroscopic analysis of honey bee brood comb cappings in hygienic and non-hygienic honey bee colonies. Apidologie **41**, 531–533

Moure, J.S., Kerr, W.E. (1950) Sugestões para a modificação da sistemática do gênero *Melipona* (Hymen.- Apoidea). Dusenia **1**, 105–129

Neumann, P., Carreck, N.L. (2010) Honey bee colony losses. J. Apic. Res. **49**, 1–6

Nicodemo, D., de Jong, D., Couto, R.H., Malheiros, E.B. (2013) Honey bee lines selected for high propolis production also have superior hygienic behavior and increased honey and pollen stores. Genet. Mol. Res. **12**, 6931–6938

Nunes, L.A., Araujo, E.D., Carvalho, C.A.L., Waldschmidt, A.M. (2008) Population divergence of *Melipona quadrifasciata anthidioides* (Hymenoptera: Apidae) Endemic to the Semi-arid Region of the State of Bahia, Brazil. Sociobiology **52**, 81–93

Nunes-Silva, P., Imperatriz-Fonseca, V.L.P., Gonçalves, L.S. (2009) Hygienic behavior of the stingless bee *Plebeia remota* (Holmberg, 1903) (Apidae, Meliponini). Genet. Mol. Res. **2**, 649–654

Pereira, R.A., Morais, M.M., Francoy, T.M., Gonçalves, L.S. (2013) Hygienic behavior of Africanized honey bees *Apis mellifera* directed towards brood in old and new combs during diurnal and nocturnal periods. Insects **4**, 521–532

Pérez-Sato, J.A., Châline, N., Martin, S.J., Hughes, W.O.H., Ratnieks, F.L. (2009) Multi-level selection for hygienic behaviour in honeybees. Heredity **102**, 609–615

Pinto, F.A., Puker, A., Barreto, L.M.R.C., Message, D. (2012) The ectoparasite mite *Varroa destructor* Anderson and Trueman in southeastern Brazil apiaries: effects of the hygienic behavior of Africanized honey bees on infestation rates. Arq. Bras. Med. Vet. Zootec. **64**, 1194–1199

R Core Team (2015) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Rasolofoarivao, H., Delatte, H., Ravaomanarivo, L.H.R., Reynaud, B., Clémencet, J. (2015) Assessing hygienic behavior of *Apis mellifera unicolor* (Hymenoptera: Apidae), the endemic honey bee from Madagascar. Genet. Mol. Res. **14**, 5879–5889

Reyes-González, A., Camou-Guerrero, A., Gómez-Arreola, S. (2016) From extraction to meliponiculture: a case study of the management of stingless bees in the West-central region of Mexico. In: Chambó, E.D. (ed.) Beekeeping and bee conservation: advances in research, pp. 201–223. InTech, Rijeka

Rinderer, T.E., Harris, J.W., Hunt, G.J., de Guzman, L.I. (2010) Breeding for resistance to Varroa destructor in North America. Apidologie **41**, 409–424

Rosenkranz, P., Aumeier, P., Ziegelmann, B. (2010) Biology and control of *Varroa destructor*. J. Invertebr. Pathol. **103**, 96–119

Rothenbuhler, W.C. (1964) Behaviour genetics of nest cleaning in honey bees. I. Responses of four inbred lines to diseased-killed brood. Anim. Behav. **5**, 578–583

Sanchez-Bayo, F., Goka, K. (2016) Impacts of pesticides on honey bees. In: Chambó, E.D. (ed.) Beekeeping and bee conservation: advances in research, pp. 77–97. InTech, Rijeka

Slaa, E.J., Chaves, L.A.S., Malagodi-Braga, K.S., Hofstede, F.E. (2006) Stingless bees in applied pollination: practice and perspectives. Apidologie **37**, 293–315

Spivak, M. (1996) Honey bee hygienic behavior and defense against *Varroa jacobsoni*. Apidologie **27**, 245–260

Spivak, M., Downey, D.L. (1998) Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). J. Econ. Entomol. **91**, 64–70

Spivak, M., Reuter, G.S. (1998) Performance of hygienic honey bee colonies in a commercial apiary. Apidologie **29**, 291–302

Spivak, M., Reuter, G.S. (2001) Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. Apidologie **32**, 555–565

Steffan-Dewenter, I., Potts, S.G., Packer, L. (2005) Pollinator diversity and crop pollination services are at risk. Trends Ecol. Evol. **20**, 651–652

Stevanovic, J., Stanimirovic, Z., Lakic, N., Aleksic, N., Simeunovic, P., Kulisic, Z. (2011) Safety assessment of sugar dusting treatments by analysis of hygienic behavior in honey bee colonies. Arch. Biol. Sci. **63**, 1199–1207

Toufailia, H.A., Alves, D.A., Bento, J.M.S., Marchini, L.C., Ratnieks, F.L.W. (2016) Hygienic behaviour in brazilian stingless bees. Open Biol . doi:10.1242/bio.018549

Uzunov, A., Costa, C., Panasiuk, B., Meixner, M., et al. (2014) Swarming, defensive and hygienic behaviour in honey bee colonies of different genetic origin in a pan-European experiment. J. Apic. Res. **53**, 248–260

vanEngelsdorp, D., Meixner, M.D. (2010) A historical review of managed bee populations in Europe and United States and the factors that may affect them. J. Invertebr. Pathol. **103**, 80–95

vanEngelsdorp, D., Underwood, R., Caron, D., Hayes Jr., J. (2007) An estimate of managed colony losses in the winter of 2006–2007: a report commissioned by the apiary inspectors of America. Am. Bee J. **147**, 599–603

Venturieri, G.C., Alves, D.A., Villas-Bôas, J.K., Carvalho, C.A.L., de Menezes, C., Vollet-Neto, A., Contrera, F.A.L., Cortopassi-Laurino, M., Nogueira-Neto, P., Imperatriz-Fonseca, V.L. (2012) Meliponicultura no Brasil: situação atual e perspectivas futuras para o uso na polinização agrícola. In: Imperatriz-Fonseca, V.L., Canhos, D.A.L., Alves, D.A., Saraiva, A.M. (eds.) Polinizadores no Brasil: contribuição e perspectivas para a biodiversidade, uso sustentável, conservação e serviços ambientais, pp. 349–360. EDUSP, São Paulo

Wilson-Rich, N., Spivak, M., Fefferman, N.H., Starks, P.T. (2009) Genetic, individual and group facilitation of disease resistance in insect societies. Annu. Rev. Entomol. **54**, 405–423