Original Article

POLLEN DIET FOR *IN VITRO* REARING OF AFRICANIZED HONEY BEE LARVAE, *Apis mellifera* (Hymenoptera: Apidae)

DIETA POLÍNICA PARA CRIAÇÃO IN VITRO DE LARVAS DE ABELHAS MELÍFERAS AFRICANIZADAS, Apis mellifera *(Hymenoptera: Apidae)*

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ABSTRACT: Pollen is the major protein source for honey bees, *Apis mellifera*. It is essential for the adults to produce royal jelly to feed the larvae. Young larvae receive the brood food, whereas the older (over 3 days old) larvae receive pollen in addition to brood food. The nutritional value of pollen has been investigated only in adults or at the colony level. Protocols for rearing Africanized honey bee larvae *in vitro* using diets with mixtures of pollen had not been established. We examined different concentrations (2.5, 5 and 10%) of two mixtures of pollen in the larval diet. The effects of pollen diets on larval development were assessed. The survival and development of larvae fed with 10% pollen was impaired; this concentration should be avoided. Concentrations of 2.5 and 5% pollen did not show significant changes in survival, weight, development or the hemolymph protein profile when compared to the controls (without pollen). However, differences in larval survival were observed between the two pollen mixtures (pollen blends 1 and 2), suggesting that a diet with a superior digestibility and greater familial diversity of pollen (blend 2) is more nutritionally adequate.

KEYWORDS: Worker rearing. Survivorship. Honey bee diet. Larval development. Pollen blend.

INTRODUCTION

The adults of honey bees, *Apis mellifera*, like those of other social insects, perform cooperative brood care ("nursing") providing a homeostatic environment (temperature and humidity) and a stable nutritional supply to the larvae (HAYDAK, 1970). Food is provided by nurse bees, which are able to process protein derived from pollen into a high quality larval food (MORITZ; CRAILSHEIM, 1987).

Queen and young worker larvae receive two different food components; clear watery, and milky-white which correspond to different admixtures of the hypopharyngeal gland and mandibular gland secretions produced by the nurse bees. Queen larvae are fed on a 1:1 mixture of the clear and the milky-white secretions throughout most of the larval feeding period, whereas the young worker larvae receive a worker jelly, which is a 3:1 to 4:1 (clear: milky) mixture of the two components (JUNG-HOFFMANN, 1966). Older (over 3 days old) worker larvae receive a mixture of the two glandular secretions with honey and pollen (modified worker jelly) (HAYDAK, 1970). It is estimated that the pollen fed directly to the larvae contributes with about 5% of protein necessary for larval development (BABENDREIER et al., 2004).

Pollen is the main source of proteins for bees (CRAILSHEIM, 1990), and it also provides lipids, vitamins, minerals, starch, and some sugars (WINSTON, 1987). This pollen is ingested by the nurse bees to provide the proteins essential for the production of brood food. It is also important to worker bees to build up their body tissues during the first days after emergence (MAURIZIO, 1954; HAYDAK, 1970). Besides, pollen nutrition is also one of the most important factors influencing the longevity of bees (HAYDAK et al., 1970), reducing the sensitivity to pesticides (WAHL; ULM, 1983), and enhancing immunity (ALAUX et al., 2010).

The protein content of pollen from different plant species and regions varies widely (2.5–61%, ROULSTON et al., 2000), resulting in different nutritive values for bees. Bioassays to determine the nutritional value of pollens for bees are usually conducted with caged adults (e.g., CREMONEZ et al., 1998; PERNAL; CURRIE, 2000; ALAUX et al., 2010; PIRK et al., 2010; HÖCHERL et al., 2012).

The effect of pollen nutrition has also been analyzed in relation to brood production when the adults are fed different pollen diets (e.g., CAMPANA; MOELLER, 1977; DIETZ; STVERSON, 1980). In those cases, nutrition was evaluated at the colony level (BRODSCHNEIDER; CRAILSHEIM, 2010). It is known that in malnourished colonies the bees cannibalize the brood and new brood is not produced (SCHMICKL; CRAILSHEIM, 2001, 2002). Moreover, deficiency in colony nutrition is also considered a factor for recent losses of honey bee colonies (OLDROYD, 2007; NAUG, 2009; BRODSCHNEIDER; CRAILSHEIM, 2010; HUANG, 2012).

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Not only are drastic effects of malnutrition observed, but also their sublethal effects such as short-lived adults or adults with slightly impaired abilities in brood rearing and foraging (MAURIZIO, 1954; MATTILA; OTIS, 2006; BRODSCHNEIDER et al., 2009). At the individual level, experiments on the nutritional values of pollen for brood rearing have been neglected, even though pollen provides an additional source of protein and contributes other nutrients to the diet.

Very few techniques rearing worker larvae in the laboratory using pollen in the diet have been published to date (BABENDREIER et al., 2004; CARVALHO; MESSAGE, 2004; LEHRMAN, 2007; HENDRIKSMA et al., 2011a; CRAILSHEIM et al., 2013). Most of these studies were conducted with the sole purpose of investigating the effects of the consumption of transgenic pollen. One study investigated pollen toxicity on Africanized honey bees (CARVALHO; MESSAGE, 2004). All these studies examined the proportion of pollen in the diet using monofloral pollen types. Therefore, we proposed to evaluate (1) the proportion of mixtures of pollen and (2) the effect of different pollen mixtures in larval diet. To this end, we tested the impact of different on diets the weight, development, protein profile of the hemolymph and survival of honey bee larvae.

MATERIAL AND METHODS

Rearing larvae of bees

Larvae were obtained from an Africanized honey bee (*A. mellifera*) colony maintained at the Apiary of the Department of Genetics, University of São Paulo, at Ribeirão Preto, Brazil. Larvae were categorized by their weight and head capsule diameter according to the criteria of MICHELETTE and SOARES (1993) established for Africanized hybrids. The larvae at the L3 stage were removed carefully from their brood cells with needles, and then transferred to 96-well tissue culture plates. Each well contained 5 µL of food and the larvae were placed on the food at the same side that they were in the brood frame (Figure 1). The food was given two or three times per day depending on its consumption by the larvae. The total amount of food per day was increased to approximately 20 µL, 40 µL and 60 µL, on the second, third, and fourth day, respectively. The plates were placed into a box containing water to ensure high humidity and then maintained in a humid (70-80% RH) incubator at 34°C.

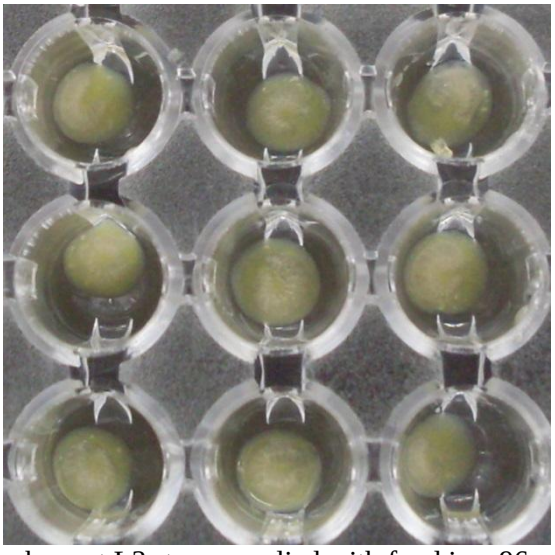

Figure 1. Worker larvae of honey bees at L3 stage supplied with food in a 96-well tissue culture plate.

Pollen diets

The larvae were fed with one of the four diets prepared with different percentages of fresh pollen (0, 2.5, 5 or 10%) added to a basic diet. The control diet had no pollen. The components for a basic diet followed the proportions established for Africanized honey bees by Silva et al. (2009). Fungicide was added to the diet following the suggestion of Herrmann et al. (2008). The variable proportions of the components were: royal jelly (49%, 46.5%, 44% or 39%, depending on the pollen percentage: 0%, 2.5%, 5% or 10%, respectively). Constant proportions were fructose (6.8%), glucose (6.8%), yeast extract (1.1%), fungicide (0.1% of a solution of 50 mg/ml; Nystatin, Sigma) and water. The diet was prepared and stored at -20^{o}C. To feed the larvae, the diet was heated to approximately 30

^{o}C and then gently pipetted into the well under the larva.

Pollen was obtained from the Apiary of the Federal University of Jequitinhonha and Mucuri Valleys, at Diamantina, Brazil (18°12′20″ S, 43°34′13″ W). Pollen loads of the bees were collected using pollen collectors on the hive entrance and frozen at -20^{o}C until use. The pollen loads were collected during two distinct periods: October 2009 and May 2010, comprising two blends with different composition of pollen types. Blend 1 comprised 23 pollen types; the most frequent of which were Asteraceae (77.5%). Pollen blend 2 comprised 20 pollen types; the most frequent of which were *Eucalyptus* (Myrtaceae) (33%), Moraceae (24%) and Cyperaceae (20%).

Assays

Two assays were performed in order to test the effects of the two different blends of pollen (1 and 2) on larval rearing. In each bioassay, twenty four larvae were fed with one of the four diets (described above).

Mortality was checked every day and dead larvae were removed. The experiments ended after five days of treatment, when the larvae were weighed, and developmental stage recorded.

Hemolymph protein profile by SDS-PAGE

The protein profile was investigated in hemolymph samples from the reared larvae at the end of the experiment. Hemolymph of 1-3 workers (ca. 10 μL hemolymph/bee) was used to obtain a pool. Hemolymph samples (1 μL) were subjected to SDS-PAGE (LAEMMLI, 1970) conducted at 15mA, using 7.5% polyacrylamide gels (100x100x1 mm). After electrophoresis, gels were stained with Coomassie Brilliant Blue solution consisting of 50% ethanol, 10% acetic acid and 0.25% Coomassie Brilliant Blue R-250 and de-stained in 45% ethanol and 10% acetic acid.

Statistics

The survival of bees was analyzed with the Kaplan-Meier test for the survival rates of different groups, and with post-hoc comparisons by the Holm-Sidak test. Weight comparisons of the pollen supplied and the control larvae were performed by ANOVA. Analyses were performed with Jandel SigmaStat 3.1 software (Jandel Corporation, USA).

RESULTS

Pollen blend 1 resulted in higher mortality when ingested at a concentration of 2.5, 5 or 10% when compared to its corresponding control (Holm-Sidak; $p<0.05$). In contrast, pollen blend 2 resulted in greater mortality only when administrated at 10% (Holm-Sidak; $p<0.05$; Figure 2A). These results indicate that pollen blend 1 seems to be more detrimental to larval survival.

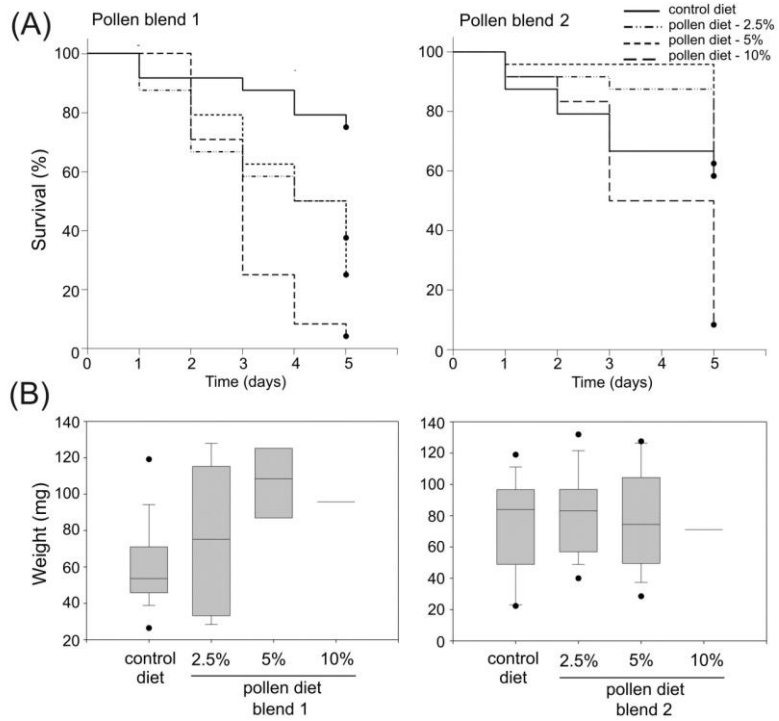

Figure 2. (A) Survivorship and **(B)** weight of larvae fed with one of the three different pollen blend diets (2.5, 5 or 10% pollen) or with no pollen (control diet). The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles.

Pollen in the diet (2.5 and 5%) slightly increased body weight, but no significant differences were observed between bees of the two assays (Figure 2B; Table 1). It is clear however, that 10% of pollen not only resulted in greater mortality but also in a delay in their development reflected in body weight and the absence of the silk threads observed in other pollen-treated groups (Figure 2B; Table 1; Figure 3). After five days of treatment, the larvae were at different developmental stages, observed by the large variation in body weight and in percentage of larvae at the spinning phase (L5S) (Table 1; Figure 3). In both blends, 2.5% pollen in the diet resulted in higher percentage of bees at L5S stage when compared to the controls (Table 1).

Table 1. Number of surviving larvae, mean weight and percentage of larvae at the spinning phase (L5S) after five days of treatment with different diets (twenty four larvae were fed with one of the four diets) prepared with pollen blends 1 and 2.

	Diet	Number of surviving larvae (n)	Mean weight ± SD (mg)	% of larvae at L5S
Pollen blend 1	Control diet – no pollen	18	60.1 ± 22.25	5.6%
	Pollen diet – 2.5%	9	74.8 ± 42.75	22.2%
	Pollen diet – 5%	6	101.98 ± 31.56	33.3%*
	Pollen diet – 10%	1	95.8	100%
Pollen blend 2	Control diet – no pollen	14	73.1 ± 30.83	14.3%
	Pollen diet – 2.5%	15	80.57 ± 25.65	26.7%*
	Pollen diet – 5%	15	109.37 ± 31.51	6.6%
	Pollen diet – 10%	2	71.05 ± 25.81	0

*Presence of visible silk threads

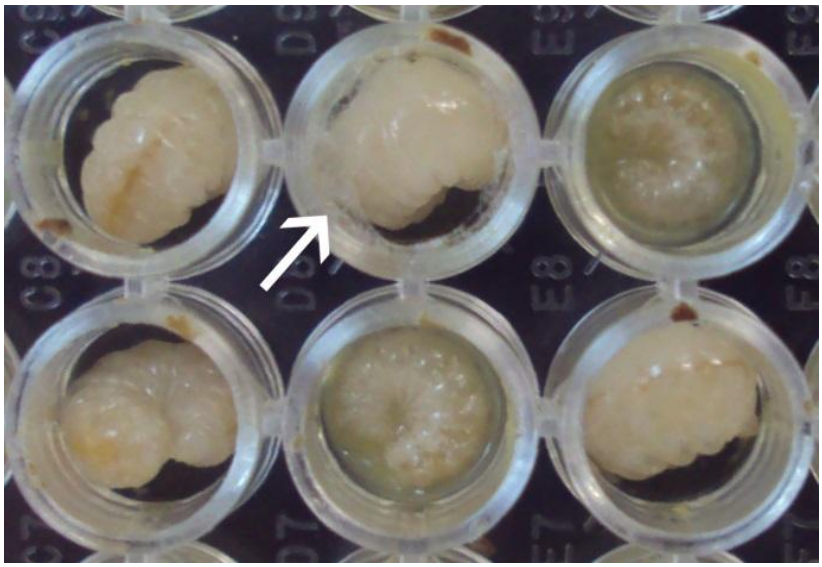

Figure 3. Worker larvae at the L5F (with food in the well) and L5S (without food) stage in a 96-well tissue culture plate after five days of treatment. Arrow indicates the silk threads secreted by the larva at the spinning stage.

Analyzing the protein profile of the hemolymph of treated larvae, we observed that 2.5 and 5% of pollen in the diet did not change the protein profile, showing high amounts of the storage protein Hexamerin 110 and 70 (Figure 4). However, bees supplied with 10% of pollen were moribund and showed degradation of their proteins, as observed by smears in the SDS-PAGE gel (Figure 4).

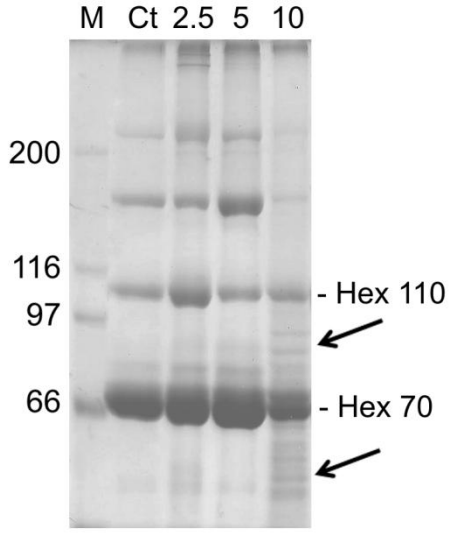

Figure 4. SDS-PAGE of hemolymph proteins from worker larvae fed with 2.5, 5 and 10% of pollen or with no pollen (Ct). M: molecular mass markers (kDa). Hexamerin 110 and 70 are indicated as single bands of 110 and 70 kDa, respectively. Arrows indicate smears which are indication of protein degradation.

DISCUSSION

In the present study we validate different concentrations of pollen in diets for rearing Africanized honey bee larvae. The results show that 2.5 or 5% of pollen in the diet of larvae do not impair survival or development, but 10% of pollen is extremely detrimental to survival and development. Using maize pollen Babendreier et al. (2004) estimated that a worker larva consumes about 1.5 to 2 mg of pollen during their development, whereas Simpson (1955) estimated a wider range of pollen consumption depending on the pollen type: 2.2 to 5.4 mg. Converting the percentage of pollen in the diet and calculating the amount of ingested diet, we provided approximately 3, 6.2 and 12.5 mg of pollen for the 2.5, 5 and 10% diet, respectively. Therefore, 2.5 and 5% of pollen in the diet resembled the pollen consumption of a larvae reared in a normal colony. But, 10% of pollen is more than twice the amount a larva consumes naturally. As shown here, this amount of pollen resulted in fatalities and should be avoided for larval rearing.

Only one study examined different amounts of pollen for rearing honey bee larvae of the *carnica* race (LERHMAN, 2007). In that study, the larvae were fed a diet containing 1% to 4% dried pollen from oilseed rape *Brassica napus*, and larval survival was reduced when larvae were fed with 2% or more (3 and 4%). Then, she used 1.5% dried pollen (corresponding to 4% fresh pollen) in the diet to test the effect of transgenic pollen. The amount of pollen which could be mixed into the food was similar to that used in the present study, which supports the finding that diet containing over 5% fresh pollen is detrimental to larval development (both in Africanized hybrids and in the *carnica* race). However, lower concentrations may have a negative impact on larvae depending on the pollen types. For instance, only 0.5% of toxic pollen in the diet caused larval mortality (CARVALHO; MESSAGE, 2004), as well as 2.5 and 5% pollen from the blend 1 used in the present study (as discussed below).

Our result of the effects of pollen consumption on larval development revealed that the bees responded differently to the various mixtures of pollen. For all the parameters analyzed, only survival was significantly affected, as shown clearly when the bees ingested pollen blend 1. Although this blend included 23 pollen types, pollen of the Asteraceae comprised ¾ of the total. Similarly, pollen blend 2 included 20 pollen types, however, three pollen types (*Eucalyptus*, Moraceae and Cyperaceae) comprised more than ¾ of the total. The nutritional value of pollen depends on its protein content and also on its digestion by the bees. The protein content of pollen varies from 2.5% to 61% (ROULSTON, 2000) and the efficiency of pollen digestion may vary due to its morphology and size (HUMAN et al., 2007). The pollen grains of Asteraceae (in blend 1) may exhibit low digestibility due to its prominent pollenkit layer that has to be digested before the pollen content can be utilized. Low digestion and nutrient assimilation of the pollen of Asteraceae was observed in honey bees (HUMAN et al., 2007) and also in the solitary bee

Osmia lignaria (WILLIAMS, 2003). In these cases, the authors suggested that the pollenkit in pollen of Asteraceae, or chemicals within it, could interfere in its digestion. Conversely, in blend 2, the pollen of *Eucalyptus* was one of the most abundant and, according to BELL et al. (1983), this kind of pollen contains 21-28% protein and digestibility is 52-59%. This type of pollen, together with that of Moraceae and Cyperaceae, seems to be easily digested if we consider that they have a thinner exine, which is contrary to the first blend. Besides the protein content and digestibility of pollen, the diversity of pollen types is also important for a bee´s nutrition. In honey bees, a low diversity in pollen mixtures decreases immunocompetence (ALAUX et al., 2010) and survival against the parasite *Nosema ceranae* (DI PASQUALE et al., 2013); and in bumble-bees, larvae fed polyfloral diets were heavier than larvae fed on monofloral diet (TASEI; AUPINEL, 2008). Therefore, not only the digestibility and protein content, but also the pollen diversity of the pollen blend 2 renders it more nutritionally effective. Furthermore, we cannot exclude the possibility that some pollen type in the mixture of pollen blend 1 may contain a toxic compound. For instance, the toxic pollen from *Stryphnodendron polyphyllum* (Fabaceae, Mimosoideae) is known to kill brood (CARVALHO; MESSAGE, 2004). However, further studies are needed to evaluate this possibility.

Recently, new techniques have been developed to optimize the rearing of honey bee worker larvae (AUPINEL et al., 2005; HERRMANN et al., 2008; HENDKRISMA et al., 2011b; KAFTANOGLU et al., 2011; CRAILSHEIM et al., 2013). These studies aimed to improve larval development and to reduce mortality. Mortality of the larvae ranged from about 3% (HENDKRISMA et al., 2011b) to about 10% (AUPINEL et al., 2005; HERRMANN et al., 2008) to 20% (KAFTANOGLU et al., 2011). It is clear that a nongrafting method improves larvae survivorship (HENDKRISMA et al., 2011b). Here, we collected the larvae with needles, which probably provoked high mortality (25-50%) of the control group (without pollen), as observed by Evans et al. (2010), using needles to graft the larvae.

Honey bee larvae are better adapted to experiments on diet than are the adult bees. As pointed out by Lehrman (2007), the larvae can eat only what is offered, whereas adults can avoid less palatable foods. Moreover, the larvae need a more complex diet than do the adults, which could make them more sensitive to changes in food composition. Besides, larvae are not cannibalized as they are in malnourished colonies, where adults obtain protein which contributes to the feeding of other larvae (for review see BRODSCHNEIDER; CRAILSHEIM, 2010). Using this method, researchers may access not only the nutritional value of food, but also any lethal or sublethal effects of pollen ingestion. Sublethal effects may be investigated in abnormal larval development or physiology, and also in adult features like changes in their morphology and behavior.

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RESUMO: O pólen é a principal fonte de proteína para as abelhas melíferas, *Apis mellifera*. Ele é essencial para que os adultos produzam geleia real para nutrir as larvas. As larvas jovens recebem geleia real, enquanto que as larvas mais velhas (mais que 3 dias de idade) recebem pólen juntamente com a geleia real. O valor nutricional do pólen tem sido investigado apenas em adultos ou ao nível de colônia. Protocolos de criação de larvas de abelhas africanizadas *in vitro* utilizando dietas com misturas de pólen não foram estabelecidas. Nós examinamos diferentes concentrações (2,5, 5 e 10%) de duas misturas de pólen na dieta de larvas. O efeito das dietas de pólen no desenvolvimento larval foi avaliado. A sobrevivência e o desenvolvimento das larvas alimentadas com 10% de pólen foram prejudicados; esta concentração deve ser evitada. Concentrações de 2,5 e 5% de pólen não mostraram uma mudança significativa na sobrevivência, no peso, no desenvolvimento ou no perfil proteico da hemolinfa, quando comparado com os controles (sem pólen). No entanto, diferenças na sobrevivência das larvas foram observadas entre duas misturas de pólen (mistura 1 e 2), sugerindo que uma dieta com uma digestibilidade superior e maior diversidade de famílias de pólens (mistura 2) é nutricionalmente mais adequada.

PALAVRAS-CHAVE: Criação de operária. Sobrevivência. Dieta para abelha melífera. Desenvolvimento larval. Mistura de pólen.

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