HUDSON VANER VENTURA TOMÉ

IMPROVEMENT AND USE OF LABORATORY METHODS FOR TOXICOLOGICAL ASSESSMENTS ON EUSOCIAL BEES

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

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BIOGRAFIA

Hudson Vaner Ventura Tomé, filho de Valdina Vilma Ventura Tomé e Jair Tomé, nascido no dia 11 de maio de 1985, no município de Ponte Nova, Minas Gerais, Brasil. Ingressou na Universidade Federal de Viçosa em março de 2004 onde iniciou o curso de Agronomia. Realizou estágio e foi bolsista de iniciação científica nos anos de 2007 e 2008 sob supervisão do professor Marcelo Coutinho Picanço, graduando-se Engenheiro Agrônomo em Janeiro de 2009.

No período de 2009 a 2011 realizou o curso de mestrado pelo Programa de Pós-Graduação em Entomologia da Universidade Federal de Viçosa, sob a orientação do Professor Raul Narciso Carvalho Guedes. Em março de 2011 iniciou o doutorado em Entomologia pela mesma universidade sob orientação do professor Gustavo Ferreira Martins. No período entre julho de 2013 e agosto de 2014 realizou parte do seu doutorado na University of Florida Gainesville, FL-USA, onde desenvolveu estudos em colaboração do professor James D. Ellis.

INDEX

RESUMO

TOMÉ, Hudson Vaner Ventura, D.Sc., Universidade Federal de Viçosa, fevereiro de 2015. **Plasticidade cerebral e comportamental e danos múltiplos de pesticidas em abelhas eussociais.** Orientador: Gustavo Ferreira Martins. Coorientadores: Maria Augusta L. Siqueira, James D. Ellis e Raul Narciso C. Guedes.

A importância das abelhas para a polinização é inquestionável. Apesar disso, populações desses organismos têm sofrido consideráveis reduções no mundo por diversos fatores que incluem o uso de pesticidas. Abelhas eussociais geralmente vivem em ninhos, onde parte do desenvolvimento pós-embrionário ocorre em favos fechados, constituindo empecilho para o estudo do desenvolvimento. Portanto, para se estudar o efeito de pesticidas nesses organismos sem interferência de fatores ambientais é necessário a criação em condições controladas em laboratório, sendo que em condições naturais parte da exposição a pesticidas ocorre via alimentação larval. Nesse sentido, o aprimoramento de métodos de criação pode contribuir para o entendimento de como pesticidas afetam processos morfofisiológicos e moleculares nas abelhas mediante exposição das larvas a esses compostos. A presente tese objetivou desenvolver e/ou melhorar técnicas de criação de abelhas *in vitro* e realizar testes toxicológicos para avaliar a plasticidade cerebral e comportamental em abelhas sem ferrão. Além disso, desordens ocasionadas por pesticidas em abelhas sem ferrão e abelhas melíferas também foram estudadas. De maneira geral, os resultados obtidos na presente tese permitiram concluir que: 1) a criação de abelha sem ferrão *M. quadrifasciata* em laboratório retarda o desenvolvimento de algumas regiões cerebrais em relação às abelhas mantidas em condições naturais; 2) o inseticida sintético imidaclopride e o bioinseticida spinosade são altamente tóxicos a abelha sem ferrão *M. quadrifasciata* e também promovem efeitos subletais em operárias dessa espécie; 3) é possível reduzir consideravelmente a mortalidade de *Apis mellifera* criadas *in vitro* mediante o uso de procedimentos descritos de forma inédita no presente estudo e 4) todos os pesticidas causaram mortalidade e efeitos subletais (reduções no peso corporal, alterações na atividade de genes associados a destoxificação, distúrbios no desenvolvimento e estruturas das antenas) em abelhas melíferas em virtude da exposição de concentrações realistas de campo durante a fase larval.

ABSTRACT

TOMÉ, Hudson Vaner Ventura, D.Sc., Universidade Federal de Viçosa, February, 2015. **Brain and behavioral plasticity and multiple pesticide disorders in eusocial bees.** Adviser: Gustavo Ferreira Martins. Co-advisers: Maria Augusta L. Siqueira, James D. Ellis and Raul Narciso C. Guedes.

The importance of bees for pollination is unquestionable. Nevertheless, populations of these organisms have suffered considerable reductions in the world by several factors including the use of pesticides. Eusocial bees usually live in nests, where part of the post-embryonic development occurs in closed combs, what makes difficult the study of bee development. Therefore, to study the impact of these pesticides without interference from environmental factors, the rearing of these organisms under controlled conditions in laboratory is necessary whereas part of pesticide exposure occurs via larval feeding in natural conditions. In this sense, the improvement of rearing methods can contribute to the understanding of how pesticides affect morphophysiological and molecular processes in bees by exposure of larvae to these compounds. This work aimed to develop and/or improve *in vitro* bee rearing techniques and perform toxicological tests to assess brain and behavioral plasticity in stingless bees. In addition, disorders caused by pesticides in stingless bees and honeybees were also studied. In general, it can be concluded that: 1) the rearing of stingless *M. quadrifasciata* laboratory decreased development of some bees' brain regions compared with bees kept in natural conditions; 2) the synthetic insecticide imidacloprid and bio-insecticide spinosad are highly toxic to stingless *M. quadrifasciata* and also promote sublethal effects on workers of this species; 3) the mortality of *Apis mellifera* reared *in vitro* can be greatly reduced by using procedures described in the present study and 4) all pesticides tested caused mortality and sublethal effects (reductions in body weight, changes in gene activity associated with detoxification, developmental disorders and structures of the antennas) in honey bees after exposure during the larval stage to realistic field pesticide concentrations.

INTRODUCTION

Bees, including honey bees, bumble bees, stingless bees and solitary bees are ecologically and economically the most important group of pollinators worldwide (Klein et al., 2007; Freitas et al., 2009; Gallai et al., 2009). However, reductions in populations of these pollinators, have been drawing continuous attention and have been linked to diverse stressors, including climate change, habitat fragmentation, exotic species introductions, parasites, pathogens, malnutrition, and pesticide use (Goulson et al., 2008; Freitas et al., 2009; Potts et al., 2010ab; Bryden et al., 2013; Vanbergen et al., 2013). Although no consensus has emerged regarding the main causes of honey bee colony decline, the multifactorial hypothesis has received support where the combination of two or more stressors have been extensively investigated (Alaux et al., 2010; Ratnieks and Carreck, 2010; Mullin et al., 2010; Nazzi et al., 2012; Pettis et al., 2012; DeGrandi-Hoffman et al., 2013; diPrisco et al., 2013). These findings have led to the establishment of methods to assess the influence of these factors, such as pesticides, in laboratory due to more controllable conditions compared to field conditions (Aupinel et al., 2007; Crailsheim et al., 2013).

Laboratorial methods standardization, such as *in vitro* larval rearing, is very useful to isolate common variations from field conditions. In particular, the testing of oral toxicity of pesticides on brood is limited to laboratory, because the peculiar uptake of food with the testing compound is not feasible using in-hive methods (Aupinel et al., 2005, 2007; Crailsheim et al., 2013). Variations in temperature and humidity, presence of parasites and pathogens, heterogeneity of diets and interactions between bees are some interferences found into the hive. The use of diets with the quantities of components adapted to each stage, the standardized volume of food dispensed to individuals, and dialy mortality evaluation for a long period allow the calculation of pesticide doses and the evaluation of its effects under laboratorial conditions (Aupinel et al., 2005, 2007; Gregorc and Ellis, 2011; Zhu et al., 2014). It is not clear if the negative effects of pesticides in laboratory is representative of their effects in the field (Thompson et al., 2007), laboratory assays can be a start point to identify dangerous and safe compounds to the bees in nature.

Substantial progress has been achieved in the development of laboratory standardized tests in honey bees (Peng et al., 1992; Aupinel et al., 2005, 2007; Charpentier et al., 2014; Zhu et al., 2014), but the difficulties involved *in vitro* rearing lead to high levels of mortality (~30⁄) until adult emergence, overestimating the risk assessments. Due to this inconsistency, studies reporting pesticide effects on bees reared *in vitro* have their reliability questionable. Due to the difficulties of rearing larvae in laboratory, works focusing the adult exposure are much

more numerous than works with larval exposure. Most studies reporting pesticides disturbs in honey bees focus mainly on mortality and sublethal effects on learning, memory, longevity and behaviors as flight by exposure in adults (Medrzycki et al., 2003; Aliouane et al., 2009; Decourtye et al., 2003, 2004ab; Faroqui, 2013). For the neotropical stingless bees studies considering both *in vitro* larval rearing and toxicological tests in adult bees are still scarce (Tomé et al., 2012; Del Sarto et al., 2014).

The most famous laboratorial standardized method used among different groups of bees is the classic proboscis extension reflex (PER). Developed over the last three decades ago, PER has been responsible to reveal how learning and memory are important for bees (Bitterman et al., 1983; Riveros and Gronenberg, 2009; Mc Cabe and Farina, 2009). However, its use has been intensified especially in pesticide toxicity studies, where sublethal concentrations of various compounds have shown impairments (Decourtye et al., 2003, 2004ab; Frost et al., 2012; Williamson and Wright, 2013). Besides PER, other laboratorial methods adopting morphology have been useful to study age and experience mediated brain plasticity linked to behavior and labor division in bees with different social levels (Mares, et al., 2005; Fahrbach 2006, Krofczik et al., 2008; Smith et al., 2010). Dependent-experience brain enlargements in honey bee workers, for example, have been well understood by use of environment richness and its manipulation in the field and laboratory (Farris et al. 2001; Krofczik et al., 2008; Maleska et al., 2009). Older honey bees change nest tasks (e.g., cleaning, nursing and honeycomb construction) to more complex tasks outside the colony (e.g., foraging), resulting in age polyethism (Robinson 1992). Therefore, honey bees change from a relatively signal-poor environment (inside the nest) to an external signal-rich environment leading to the increase in brain volume (Farris et al. 2001; Krofczik et al. 2008). Although many studies have been published addressing brain plasticity (e.g. enlargements in brain associated to labor division in honey bees), neuroanatomical changes of brain dependent on age and complexity of environment stimuli are still missing in stingless bees.

The first chapter of the present work assess the effect of aging and rearing conditions (field vs. laboratory) in the development of the mushroom bodies and the antennal lobes of adult workers of the stingless bee *Melipona quadrifasciata* (L.). The second chapter assess the lethal and sub-lethal effects of imidacloprid and spinosad in adult individuals of this species. The third chapter reports a novel, improved and detailed protocol for *in vitro* rearing of *Apis mellifera* (L.). Finally, the last chapter evaluate the lethal and sublethal pesticide disorders in honey bees. Adopting our improved method of rearing honey bees *in vitro*, it was possible to assess the pesticide disorders in a broad range, including reductions on survival, decreasing of

body weight, midgut cell damage, malformations on the antennal structure and changes on expression of several detoxification genes in workers.

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AIMS

- Assess the influence of aging and rearing conditions (field vs laboratory) in the development of mushroom bodies and antennal lobes in the brain of adult workers of the native stingless bee *Melipona quadrifasciata*.

- Assess the effect of acute toxicity of spinosad (and imidacloprid as a positive control) and disorders in the respiration, overall group activity, and flight behavior in adult workers of *M. quadrifasciata*.

- Improve the *in vitro* rearing of larvae of workers of *Apis mellifera* to optimize individual's survival until adult emergence.

- Assess the survival, development, body weight, antennal structure, midgut cell damage and gene expressions of honey bee workers exposed to field-level pesticide concentrations *in vitro*.

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Original article

Age-mediated and environmentally mediated brain and behavior plasticity in the stingless bee *Melipona quadrifasciata anthidioides*

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Abstract – Structural changes in the insect brain related to age and individual experience may underlie the behavioral plasticity that is particularly important in such social insects as bees. This study assessed the influence of age and rearing conditions (field vs laboratory) in mediating changes in the volume of mushroom bodies and antennal lobes in the brains of workers of the native stingless bee *Melipona quadrifasciata anthidioides* Lepeletier, a pollinator species with small colonies exhibiting high level of sociability and behavioral versatility. Although only age-dependent enlargement was observed in the antennal lobes, significant increase (21 %) in the neuropils of the mushroom bodies occurred before the foraging age, in contrast to honeybees, and environmental complexity led to a significant increase in both the mushroom body volume and the walking activity. Such differences in the stingless bee *M. quadrifasciata anthidioides* as compared with the honeybee may assist in relating brain evolution and plasticity with the behavior in these social insects.

native stingless bee / walking behavior / mushroom body / native pollinator

1. INTRODUCTION

The brain of both vertebrates and invertebrates exhibits a remarkable ability of structural and functional reorganization in response to external stimuli, and the reasons for these neuroanatomical changes are a main focus of modern neurobiology (Kolb and Wishaw 1998; van Praag et al. 2000; Sokolowski 2010). Nervous system remodeling may occur in response to age and environmental signals as a consequence of the individual's experience (Farris et al. 2001). In addition, an abundance of environmental stimuli, as in social environments, may also induce neuroanatomical changes to affect neurogenesis, dendritic arborization, and learning (Scotto Lomassese et al. 2000; van Praag et al. 2000; Tavosanis 2011). Nonetheless, individual experience and environmental signals are considered preponderant factors of neuroplasticity, altering the dendrite length and size and number of synapses, which has been consistently reported in honeybees (Durst et al. 1994; Kolb and Wishaw 1998; Farris et al. 2001; Tavosanis 2011).

The structural and volume enlargements in the insect brain regions indicate that these changes may also be related to chronological age and behavioral plasticity (Meinertzhagen

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2001; O'Donnell et al. 2004; Fahrbach 2006; Jones et al. 2009). Eusocial bees, such as the honeybee (*Apis mellifera* L.), can exhibit brain growth in consequence of both experience-expectant and experience-dependent components (Farhbach et al. 1998). The former refers to the chronological increase of brain regions with age, and the latter refers to such an increase with behavioral changes (or individual experience) (Farhbach et al. 1998). Older honeybees shift nest tasks from simpler ones (e.g., cleaning, nursing, and honeycomb construction) to more complex tasks outside the colony (e.g., foraging), resulting in age polyethism (Winston 1987; Robinson 1992). Therefore, honeybees change from a relatively signal-poor environment (inside the nest) to an external signal-rich environment leading to the increase in brain volume (Withers et al. 1993; Durst et al. 1994; Farris et al. 2001; Krofczik et al. 2008).

Memory retention of multiple signals is crucial for foraging, which also requires the learning of resource location and celestial and terrestrial orientation during long flights (Menzel 2001; Menzel and Giurfa 2006; Srinivasan 2010). Foraging activity coincides with volume increases in certain brain regions when young, inexperienced bees (still living within the colony) are compared with foraging bees (Withers et al. 1993; Farris et al. 2001; Ismail et al. 2006). Nevertheless, older bees prevented from leaving the nest also exhibit neuroanatomical changes, suggesting that the observed brain structural modifications are influenced by both individual experience and by aging (Withers et al. 1995; Fahrbach et al. 1995, 1998; Farris et al. 2001; Fahrbach 2006).

The mushroom bodies of the insect brain are regions of multisensorial integration and are important in memory and learning (Menzel 2001; Menzel and Giurfa 2001, 2006; Heisenberg 2003; Fahrbach 2006; Wessnitzer and Webb 2006). Neuroplasticity occurs in the mushroom bodies as a consequence of aging, experience, and social interactions (Farris et al. 2001; Fahrbach 2006). Interestingly, the mushroom body calyces of the honeybee are larger in foraging workers than in young bees (Withers et al. 1993; Farris et al. 2001), a consequence of increases in dendritic arborization (Fahrbach et al. 1995; Farris et al. 2001). In addition to the mushroom bodies, specific glomeruli of the antennal lobes in some species can also change in volume with age, particularly when the worker bee begins to forage (Withers et al. 1993; Sigg et al. 1997; Morgan et al. 1998; Brown et al. 2002, 2004).

The present study aimed to assess the changes in the brain and behavior of workers of the stingless bee *Melipona quadrifasciata anthidioides* Lepeletier (Apidae: Meliponinae), a major pollinator in tropical ecosystems, as a function of their age and the environment in which they develop. Colonies of this species are small (300–600 bees), and the workers perform activity within the colonies longer (several days) than the honeybees starting to fly only at advanced age (between 28 and 30 days after emergence). Based on reports for honeybees, an increase in mushroom body volume and increased walking activity was expected with aging, particularly during the foraging age, and increased environmental complexity (Withers et al. 1993; Fahrbach et al. 1998; Farris et al. 2001; Fahrbach 2006; Ismail et al. 2006). However, in contrast to honeybees, the results obtained indicate that increases in neuropils of mushroom bodies of *M. quadrifascista anthidioides* occurred in nurse bees before the stingless bees reached the foraging age.

2. MATERIAL AND METHODS

2.1. Stingless bee colonies

Five colonies of *M. quadrifasciata anthidioides* were collected in Viçosa County (state of Minas Gerais, Brazil; 20°45′S and 42°52′W) and maintained in the Experimental Apiary of the Federal University of Viçosa for subsequent use in the experiments. Although the colonies are currently maintained in the same area, they were originally collected from different fields to allow for representative genetic variability among colonies.

2.2. Field-reared bees

Pupa-containing brood chambers were removed from field nests and maintained under controlled incubators at 28±2 °C, 75±5 % relative humidity, and 24 h of darkness until the emergence of adult workers (similar to their natural conditions). Upon emergence, the workers were marked with nontoxic paint of different colors (Brasilux®, São Paulo, São Paulo, Brazil) for age monitoring and subsequently returned to the colony of origin. The newly emerged adult worker bees (<1 day old) were periodically collected at the ages of 4, 8, and 28 days after emergence, in addition to foraging bees (>32 days old), for histological and behavioral analyses. The bees were collected from the same five nests, each nest corresponding to an independent replicate in each experiment. Although the newly emerged bees (4 and 8 days old) are not able to fly and leave the colony, they can perceive (sun) light at the colony entrance.

2.3. Laboratory-reared bees

Laboratory-reared bees were obtained as described by Tomé et al. (2012) by removing egg-containing brood chambers from field hives and transferring them to artificial cells filled with 130-μL diet (added to 10 μL of water), providing sufficient food for full larval development. The artificial cells were constructed with honeybee wax and placed in the wells of polyethylene microplates (24-well plates), which were individually covered with a (honeybee) wax cap. The larval diet was obtained from the same hives as the larvae, and the brood chambers were maintained at 28±2 °C, 90±5 % relative humidity, and 24 h of complete darkness until the end of the feeding period. The artificial brood chambers were subsequently transferred to the same conditions as described for the field-reared colonies; the adult bees were marked with nontoxic paint upon emergence and transferred to wood microhives (10×10×3 cm) in groups of 10±2 bees. The adult bees received honey syrup and pollen ad libitum, and newly emerged adult workers (<1 day old) and workers at 4 and 8 days of age were collected for histological and behavioral analyses. The adult workers reared in the laboratory died before reaching an age of 28 days after emergence (~15 days after emergence).

2.4. Morphometry of mushroom bodies and antennal lobes

The volumes of the mushroom bodies and antennal lobes of five adult workers, each randomly obtained from a different colony, were measured at newly emerged adult workers, and at 4 and 8 days after emergence for both the field- and lab-reared bees and at 28 days after emergence for the field-reared bees. The volume of these brain regions were measured in these bees reared either in the laboratory or in the field to represent the distinct social environment in which they were reared. The insects were chilled at 2 °C, and their brains were dissected in insect physiological solution; the tissues were then fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h. The fixed samples were rinsed in 0.1 M phosphate-buffered saline (PBS), dehydrated in an increasing series of ethanol concentrations (70–100 %), and embedded in JB4 historesin (EMS) according to the manufacturer's instructions. The brains were subjected to serial sectioning (7-μm-thick slices) using a glass knife on an automatic microtome (Leica RM 2255, Leica Microsystems, Wetzlar, Germany). The brain sections were mounted on glass slides and stained with hematoxylin and eosin before photographing using a digital camera (Canon Power Shot A640, Lake Success, NY, USA) coupled to a light microscope (Axioskop 40, Zeiss, Göttingen, Germany). The measurements were performed at each of six-section intervals with the software Image-Pro Plus™ (MediaCybernetics, Bethesda, MD, USA) following Cavalieri's method (Gundersen and Jemsen 1987; Møller et al. 1990; Withers et al. 1993; Fahrbach et al. 1995; Tomé et al. 2012). For the determination of the volume of the mushroom bodies, the median and lateral calyces and the median and vertical peduncles and lobes were considered; for the volume of the antennal lobes, the total area encompassing all of the glomeruli was considered (Figure 1).

2.5. Walking behavior

Behavioral walking bioassays were performed to assess the walking activity of workers at different ages. Each insect was individually transferred to

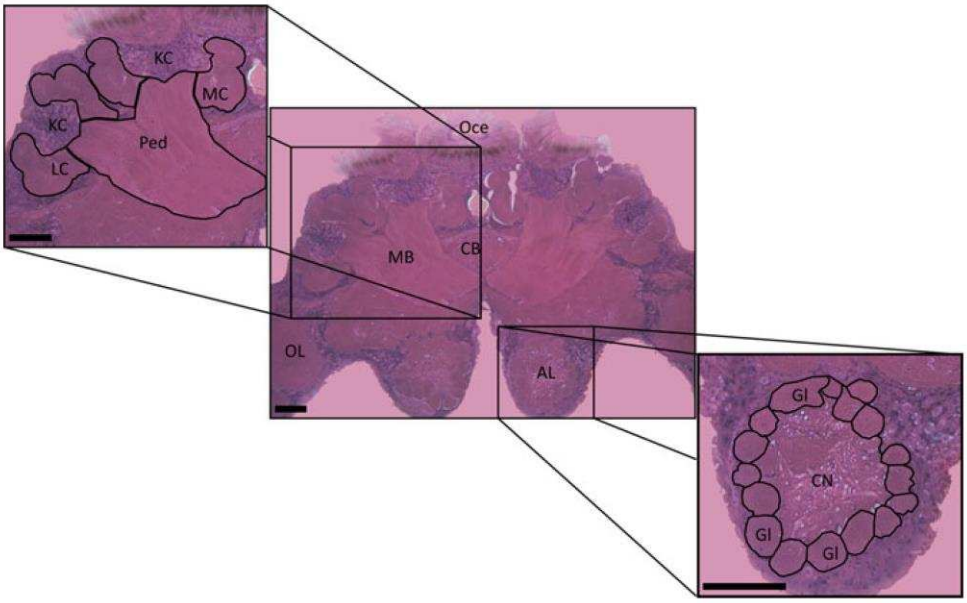


Figure 1 Median transversal section of the brain of a stingless bee (*Melipona quadrifasciata anthidioides*) worker showing the regions of the mushroom bodies and antennal lobes. Both regions are delimited by *black lines* at the higher magnification. *MC* median calyx, *LC* lateral calyx, *KC* Kenyon cells, *Ped* pedunculum, *MB* mushroom body, *Oce* oceli, *AL* antennal lobe, *CN* central neuropil, *Gl* glomerulus, *OL* optical lobe, *CB* central body. *Bars* 100 μm

an open petri dish arena (9-cm diameter and 2-cm high) lined with a filter paper (Whatman no. 1); the inner walls were covered with Teflon® polytetrafluoroethylene (PTFE) (Dupont, Wilmington, DE, USA) to prevent insect escape (the adult workers do not fly at the tested ages). Insect movements were recorded for 10 min and digitally transferred to a computer using an automated video tracking system equipped with a charge-coupled device (CCD) camera (ViewPoint Life Sciences, Montreal, Canada). The walking parameters recorded were distance walked (centimeters), walking velocity (average) (centimeters/second), and resting time (seconds). The walking behavior was recorded for adult workers immediately after emergence and at 4 and 8 days thereafter (before the workers are able to fly; Waldschmidt and Campos (1997)). The walking bioassays were performed at room temperature with an artificial light, a temperature of 25±3 °C, and between 2:00 and 6:00 p.m. Twenty-five adult workers of each age and rearing strategy (field or laboratory) were used in the walking bioassays in which each replicate corresponded to the average of five individual bees from each of the five colonies used (i.e., the colonies were the replicates).

2.6. Statistical analysis

The results for the volume of the mushroom bodies and antennal lobes of field-reared workers up to 28 days old were subjected to a (linear) regression analysis (PROC REG; SAS Institute 2008), with age as the independent variable and volume (of either mushroom bodies or antennal lobes) as the dependent variable. Student's *t* tests were used to compare the volumes of the mushroom bodies and antennal lobes of the worker bees reared in the laboratory or field at 1, 4, and 8 days after emergence ($P<0.05$). The walking behavior data were subjected to repeated measures analyses of variance to test the effect of the rearing environment (field vs laboratory) considering age as pseudoreplicates (in time), and eventual differences in the age intervals were tested by

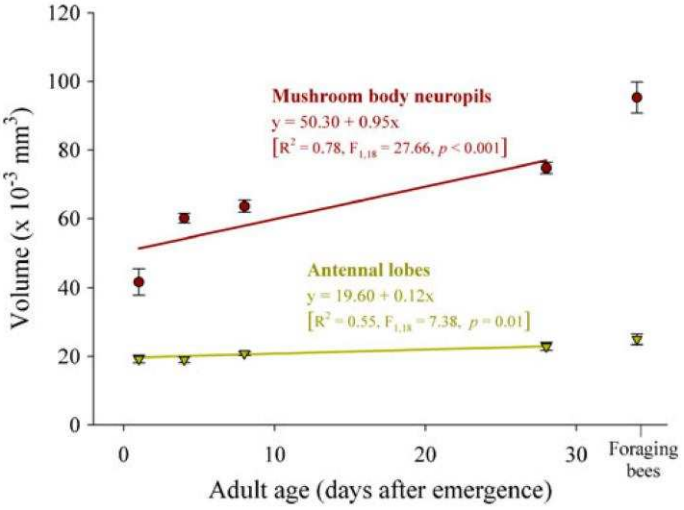


Figure 2 Volume of the mushroom body neuropils and antennal lobes in the brains of stingless bee (*Melipona quadrifasciata anthidioides*) workers at different ages and reared in the field under natural conditions. The foraging bees were not included in the regression analysis because their exact age was unknown. The *symbols* represent the means of five replicates, and the *vertical bars* represent the standard error

Fisher's *F* test (PROC ANOVA with the PROFILE statement; SAS Institute 2008).

3. RESULTS

3.1. Morphometry of mushroom bodies and antennal lobes

The volume of both the mushroom bodies and antennal lobes increased with age in the adult workers of the stingless bee *M. quadrifasciata anthidioides* obtained from the field-reared colonies (Figure 2). Such volume increases with age were particularly larger for the mushroom bodies compared to the antennal lobes, with the foraging bees (i.e., over 32 days old) exhibiting 21.2 % higher mushroom body volume than the 28-day-old worker bees (Figure 2).

The laboratory- and field-reared adult worker bees of *M. quadrifasciata anthidioides* also exhibited significant differences with regard to increases in the mushroom body volume with age (Figure 3). The mushroom body volume was similar in the newly emerged adult workers reared in field and laboratory colonies ($38.10 \pm 10.34 \times 10^{-3}$ mm^3; $t_{1,8}=1.12$, $P=0.29$) but increased to significant levels with aging ($t_{1,8} > 2.44$, $P<0.04$) (Figure 3). In contrast, the antennal lobe volume was similar between the laboratory- and field-reared bees ($17.67 \pm 2.67 \times 10^{-3}$ mm^3 for newly emerged bees, $19.22 \pm 2.10 \times 10^{-3}$ mm^3 for 4-day-old bees, and $20.78 \pm 2.86 \times 10^{-3}$ mm^3 for 8-day-old bees; $t_{1,8}<0.52$, $P>0.61$).

3.2. Walking behavior

Tracks representative of the typical walking behavior of the young adult workers of *M. quadrifasciata anthidioides* are shown in Figure 4. The repeated measures analyses of variance performed for each walking parameter indicated significant differences between the rearing environments (field vs laboratory: $F_{1,38}>11.76$, $P<0.001$), ages (newly emerged, 4 and 8 days old: Wilk's $\gamma>0.33$; $F>35.42$; $df_{num/den}=2/36$; $P<0.0001$), and the interaction between these sources of variation (Wilk's $\gamma > 0.82$; $F>3.80$; $df_{num/den}=2/36$; $P<0.03$). The differences in the walking activity between the field- and laboratory-reared bees increased with

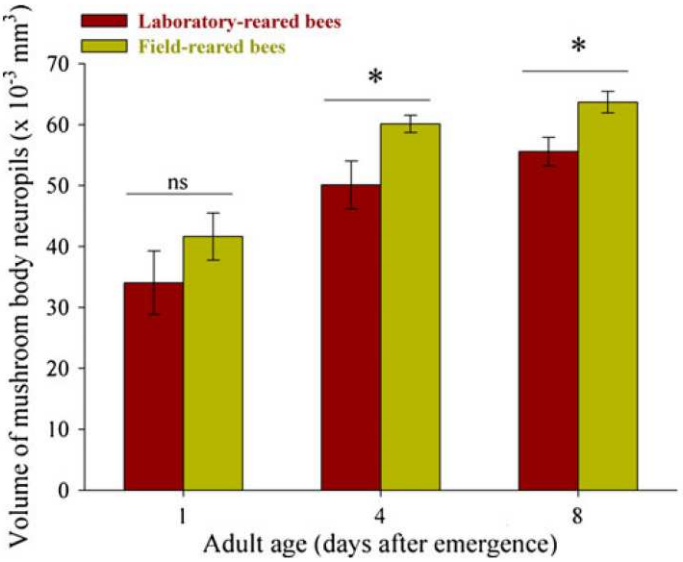


Figure 3 Volume of the mushroom body neuropils in the brains of stingless bee (*Melipona quadrifasciata anthidioides*) workers at different ages and reared in the field under natural conditions or in the laboratory. The *histogram bars* represent the means of five replicates, and the *vertical bars* represent the standard error. The *asterisk* at the top of the bar indicates the significant differences between the field- and laboratory-reared bees by Student's *t* test ($P<0.05$)

age, particularly between 4 and 8 days after emergence ($F_{1,38}>4.43$; $P<0.03$) (Figure 5). The field-reared adult stingless bees exhibited increased walking activity in comparison with the laboratory-reared bees after reaching 4 days after emergence (Figure 5a, b), and the field-reared bees began their walking activity soon after emergence, exhibiting a lower resting time (Figure 5c).

4. DISCUSSION

Volume changes in brain regions may be related to age and experience and may modulate behavioral changes in both vertebrates and invertebrates (Kolb and Wishaw 1998; Farris et al. 2001; O'Donnell et al. 2004; Fahrbach 2006). Here, we demonstrated for the first time how this process occurs in the stingless bee *M. quadrifasciata anthidioides* native to Brazil. The volumes of the mushroom bodies and antennal lobes in the brain of adult *M. quadrifasciata anthidioides* workers increase with age. In addition, higher mushroom body volume and walking activity (with larger volume and increased activity) were observed for field-reared versus laboratory-reared bees. However, in contrast to the mushroom body of the stingless bees, the volume of the antennal lobes was not affected by the rearing environment.

The increase in the mushroom body volume of newly emerged *M. quadrifasciata anthidioides* workers was significant already in the first 4 days after emergence, with an 80 % increase in volume at 28 days after emergence. These results indicate a high increase in mushroom body volume when the worker bees are still constrained to within-colony tasks, prior to the foraging age. In contrast, the increase in mushroom body volume of honeybee workers occurs mainly at the transition from nursing to foraging activities (Withers et al. 1993; Farris et al. 2001; Fahrbach 2006; Ismail et al. 2006). However, honeybees remain within the hive for only between 1 and 3 weeks, performing various tasks before beginning to forage (Winston 1987); *M. quadrifasciata anthidioides* workers represent a distinct contrast, as they remain longer within the hive during their adult

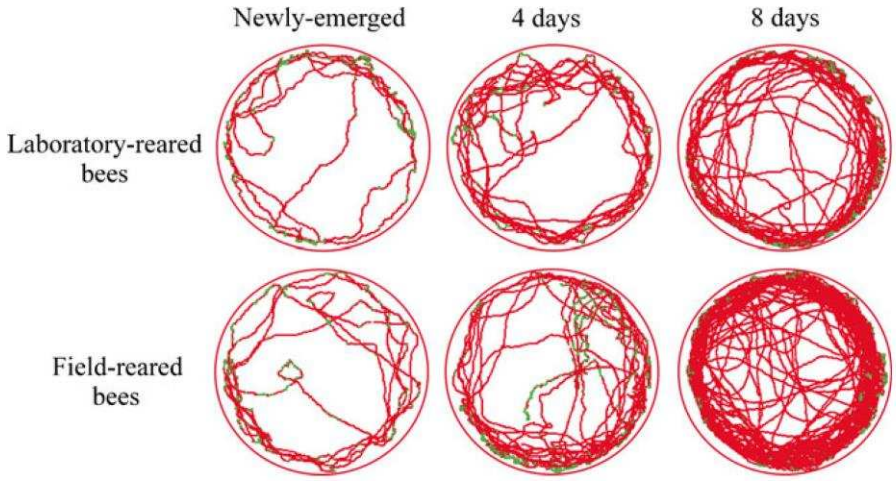


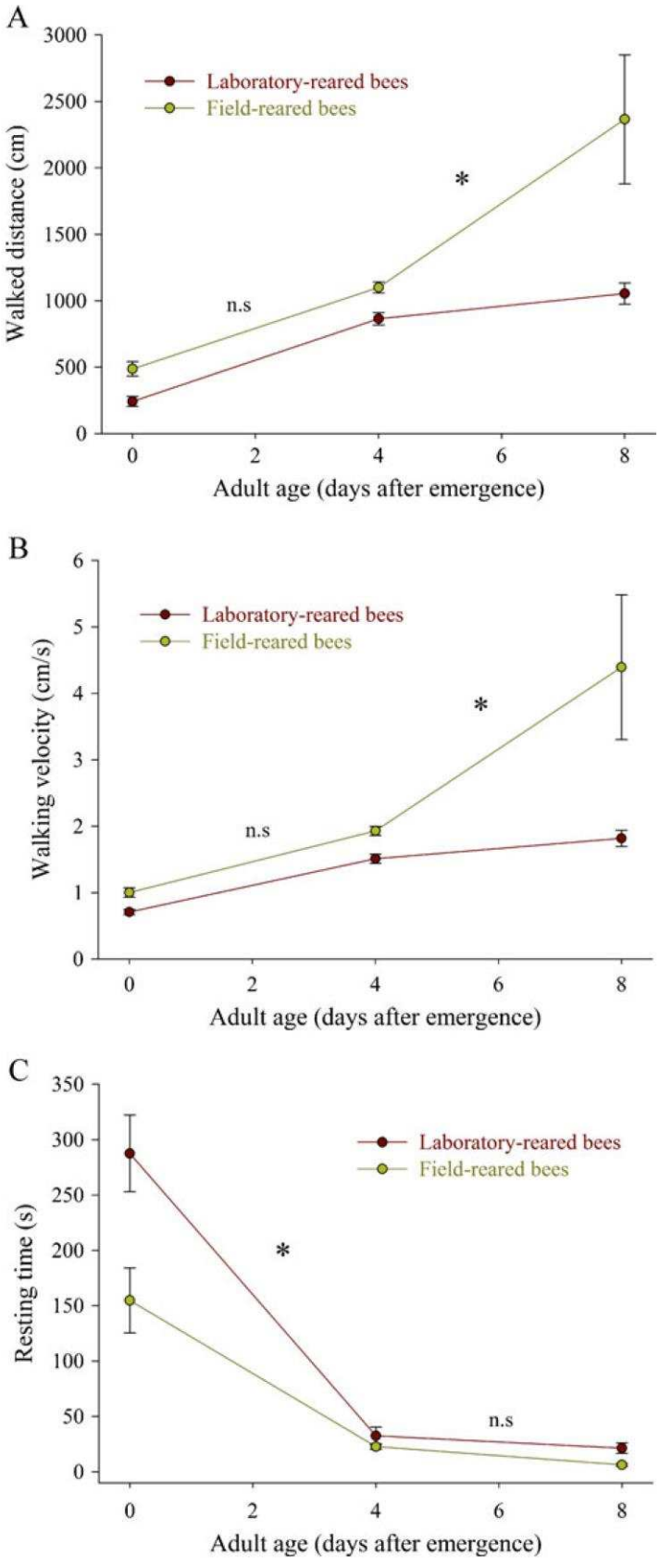
Figure 4 Representative walking tracks exhibiting the movement (for 10 min) of individual adult workers (at different ages after emergence) of the stingless bee *Melipona quadrifasciata anthidioides* reared in the field under natural conditions or in the laboratory. The *red tracks* indicate high walking velocity, whereas the *green tracks* indicate low walking velocity (Color figure online)

life (~30 days) before gradually shifting their activities outside the hive (Waldschmidt and Campos 1997; Teixeira et al. 2011).

Workers of *M. quadrifasciata anthidioides* exhibit higher plasticity than honeybee workers with regard to the development of their age-associated tasks, reflecting a higher level of idiosyncrasy that appears to be a consequence of the reduced number of individuals in the small colonies of native stingless bees (Waldschmidt and Campos 1997; Hartfelder et al. 2006). In other words, unlike *A. mellifera*, which exhibits small colonies and specialized tasks for workers of different ages, workers of *M. quadrifasciata anthidioides* exhibit high worker versatility as a result of the small colony sizes of this species. As the majority of these tasks require learning, the longer permanence of stingless bee workers within the colony (before reaching the foraging age) and the shifts in their within-colony activities may be the main reasons for the higher increase of their mushroom bodies before they begin foraging.

The neural development of worker honeybees is correlated with the age-related tasks performed (Withers et al. 1993; Durst et al. 1994). However, the most relevant changes in the mushroom body volume observed in honeybee workers are mainly associated with the exploration of the external environment and individual experience regarding the multiple environmental signals associated with navigation, orientation, and memory for resource localization (Menzel 2001; Menzel and Giurfa 2006; Fahrbach 2006). Species with less defined age polyethism represent an important contrast, whereby the plasticity of mushroom bodies is likely associated with other factors (Mares et al. 2005). Although the mushroom body plasticity in *M. quadrifasciata anthidioides* workers is evident in 4- and 8-day-old nurse bees as a likely consequence of their versatility in activity within the hive before reaching the foraging age, environmental complexity (signal-rich) also appears to play a role in favoring these earlier neuromorphological changes in workers. This is so because the bees maintained in complete dark laboratory conditions exhibited mushroom bodies with smaller neuropils than the field-reared bees.

The social environment is considered to be crucial for the increase of mushroom bodies in honeybees, affecting the behavior and circadian rhythms of locomotory activity in young bees


A
Walked distance (cm)
3000
2500
2000
1500
1000
500
0
Laboratory-reared bees
Field-reared bees
*
n.s
0
2
4
6
8
Adult age (days after emergence)
B
Walking velocity (cm/s)
6
5
4
3
2
1
0
Laboratory-reared bees
Field-reared bees
*
n.s
0
2
4
6
8
Adult age (days after emergence)
C
Resting time (s)
350
300
250
200
150
100
50
0
Laboratory-reared bees
Field-reared bees
*
n.s
0
2
4
6
8
Adult age (days after emergence)

◀ **Figure 5** Effect of the rearing environment and age after adult emergence on the distance walked (**a**), walking velocity (**b**) and resting time (**c**) of individual adult workers of the stingless bee *Melipona quadrifasciata anthidioides* reared in the field under natural conditions or in the laboratory. The *symbols* represent the means of five replicates, and the *vertical bars* represent the standard error. The *asterisk* indicates the significant difference in the age interval between the field- and laboratory-reared bees by Fisher's *F* test ($P<0.05$)

(Maleszka et al. 2009; Smith et al. 2010; Teixeira et al. 2011; Eban-Rothschild et al. 2012). Therefore, the greater walking activity and the increase in mushroom bodies observed in field-reared *M. quadrifasciata anthidioides* are likely consequences of their more social complex environment under natural conditions than in the laboratory (Maleszka et al. 2009). This situation occurs because, in the natural environment, direct contact with the queen, larger number of workers and brood, pheromone exposure, and contact by trophallaxis are more important for communication and colony functioning (Meshi and Bloch 2007). This scenario contributes to enhance individual experiences, consequently leading to neural plasticity and favoring the reported changes in walking activity.

The field-reared worker bees of *M. quadrifasciata anthidioides* exhibited higher walking activity than the laboratory-reared bees and began such activity earlier. Again, these results are the likely consequence of the higher exposure with visual and olfactory signals and social interaction observed in the field-reared bees, which is the result of more complex and diversified environment. Such environmental effects seem so important that apparently compromised the length of survival of the laboratory-reared bees, which did not suffer from nutritional deficiency or disease infection in our study. Honeybees, for instance, exhibit improved memory and learning performance when reared in a colony compared with isolated individuals (Ichikawa and Sasaki 2003; Maleszka et al. 2009). Conspecific interactions also contribute to modulating circadian rhythms in the nurse honeybees (Shemesh et al. 2010; Eban-Rothschild et al. 2012). In *M. quadrifasciata anthidioides*, the contact of nurse bees with foraging bees and the presence of the queen are important for circadian rhythm synchronization and also influence gene expression in young nurse bees to initiate colony activities (Hartfelder et al. 2006; Teixeira et al. 2011). Although we did not explore a broad behavior repertoire in field-reared bees compared with laboratory-reared bees, our results regarding the walking behavior of *M. quadrifasciata anthidioides* suggest that the environmental (social) complexity of the colony may affect the behavioral plasticity of the colony individuals.

In summary, our findings indicate that the neuroanatomical changes of mushroom body volume in *M. quadrifasciata anthidioides* are dependent on age and complexity of the environment stimuli. Signal richness associated with the division of labor within the colony and with the exploration of the external environment also appears important for the plasticity of mushroom bodies of nurse and foraging bees, respectively. This first study on the development of the brain regions of stingless bees and the results obtained provide support for the current model established for social bees in which age and individual experience promote neuroanatomical changes in these insects. Future comparative studies among distinct groups of social bees will be important to elucidate how brain plasticity might contribute to the behavior in these organisms.

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Modifications du cerveau en lien avec l'âge et l'environnement et plasticité comportementale chez l'abeille sans aiguillon *Melipona quadrifasciata anthidioides*

Comportement de marche / corps pédonculés / pollinisateur indigène / Brésil / Meliponinae

Alters- und erfahrungsabhängige Plastizität des Gehirns und des Verhaltens der stachellosen Biene *Melipona quadrifasciata anthidioides*

einheimische stachellose Biene / Laufverhalten / Pilzkörper / einheimische Bestäuber

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Spinosad in the native stingless bee *Melipona quadrifasciata*: Regrettable non-target toxicity of a bioinsecticide

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HIGHLIGHTS

- Imidacloprid and spinosad are highly toxic to adult stingless bee workers.
- The bioinsecticide spinosad was more toxic to the bee workers than imidacloprid.
- Imidacloprid impaired worker respiration, overall group activity and flight.
- Spinosad impaired flight, but not respiration and overall group activity.
- Both insecticides were highly hazardous to the stingless bee *Melipona quadrifasciata*.

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ABSTRACT

The risks imposed by novel insecticides, mainly bioinsecticides, are largely unknown despite their increased use and their perceived environmental safety, which is based on their natural origin. Furthermore, unlike honeybees, native pollinator species have received little attention. In the present study, the lethal and sublethal effects of the neonicotinoid imidacloprid and the bioinsecticide spinosad were assessed in the stingless bee species *Melipona quadrifasciata*, an important native pollinator in the Neotropical region. The adult stingless bee workers exhibited high oral insecticide susceptibility, with LD_{50}s of 23.54 and 12.07 ng a.i./bee for imidacloprid and spinosad, respectively. Imidacloprid also impaired worker respiration and overall group activity and flight, while spinosad significantly impaired only worker flight despite exhibiting higher oral toxicity to adult workers than imidacloprid. These findings indicate the hazardous nature not only of imidacloprid but also the bioinsecticide spinosad to adult workers of the native pollinator *M. quadrifasciata*. Therefore, bioinsecticides should not be exempted from risk assessment analysis due to their lethal and sublethal components.

1. Introduction

Reductions in pollinator populations, such as honeybees and bumblebees, have been drawing continuous attention and have been linked to diverse stressors, including climate change, habitat fragmentation, exotic species introductions, parasites, pathogens, malnutrition, and pesticide use (Goulson et al., 2008; Freitas et al., 2009; Potts et al., 2010a,b; Vanbergen, 2013). The prevailing general consensus is of a multifactorial effect, leading to the decay and loss of bee colonies (Potts et al., 2010a,b; Vanbergen, 2013). However, exposure to pesticides, mainly insecticides, seems to potentiate the decline of bee colonies (vanEngelsdorp and Meixner, 2010; Becher et al., 2013).

Insecticide use is routine and even indispensable in several agricultural systems (Oerke and Dehne, 2004; Cooper and Dobson, 2007). Pollinators can physically contact and ingest insecticides, and acute lethal toxicity testing of insecticides to honeybee workers is a longstanding requirement for insecticide registration in several countries (Lewis et al., 1998; Whitford et al., 2002). However, there has been an apparent shift in attention to the sublethal effects of insecticides, which may compromise individual fitness and contribute to colony decline (Valdovinos-Núñez et al., 2009; Brittain et al., 2010; Johnson et al., 2010; Bryden et al., 2013). Such effects include developmental alterations, longevity and queen production declines, neural disturbances, memory and learning impairments, and walking and foraging disabilities (Decourtye et al., 2004a,b; Yang et al., 2008; Belzunces et al., 2012; Henry et al., 2012; Tomé et al., 2012). Pyrethroids, the phenylpyrazole insecticide fipronil, and neonicotinoids in particular have been the main focus of these studies, instigating intense debates and

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calls for banning their use in Europe (Blacquière et al., 2012; European Food Safety Authority, 2013; Gross, 2013) and restricting their use in other countries, such as in Brazil (Instituto Nacional do Meio Ambiente e Recursos Naturais Renováveis, 2012).

The use restriction and even banning of synthetic insecticides, in addition to the growing demand for organically produced crops, has increased the pressure and necessity for bioinsecticides (i.e., insecticidal molecules of biological origin) (Isman, 2006; Villaverde et al., 2014). Natural products are valuable for crop protection as stand-alone insecticides, or as templates for development of more efficacious synthetic insecticides. Nonetheless, the common perception that bioinsecticides are safer for humans and the environment due to their (natural) origin and consequently should benefit from "fast-track" registration is disputable (Coats, 1994; Kidd, 2000; Bahlai et al., 2010).

Spinosad is a bioinsecticide made from spinosyns, which is generated as a fermentation product from the actynomycete species *Saccharopolyspora spinosa* (Mertz & Yao) (Sparks et al., 2001). This compound is an agonist of nicotinic acethylcholine receptors and also interferes with receptors of γ-aminobutiric acid (GABA) in the nervous system (Salgado, 1998; Sparks et al., 2001). Spinosad was initially considered safe for several non-target arthropods, and its use was allowed and expanded for crop protection (Miles, 2003; Sarfraz et al., 2005), especially in organic production, but such perceived selectivity has been challenged (Morandin et al., 2005; Biondi et al., 2012).

The assessment of the ecological risk of insecticides to pollinators has largely centered on the honeybee *Apis mellifera* L., which is the bioindicator of choice for other arthropod pollinators (Lewis et al., 1998; Whitford et al., 2002). There have been efforts to encompass other species, and at least some of this research challenges the common use and extrapolation of honeybee results to other pollinators (Thompson and Hunt, 1999; Besard et al., 2011; Tomé et al., 2012; Del Sarto et al., 2014). The native Neotropical stingless bee *Melipona quadrifasciata* (Lepeletier) produces highly valued honey and is an important pollinator for both wild and cultivated plant species (Slaa et al., 2006; Bispo dos Santos et al., 2009). Furthermore, *M. quadrifasciata* is a more suitable species for an insecticide impact assessment on pollinators in tropical America due to its greater ecological relevance than the exotic Africanized *A. mellifera* (Kremen et al., 2002; Tomé et al., 2012). The stingless bee *M. quadrifasciata* is also closely related to *Melipona capixaba* (Moure & Camargo), another Neotropical stingless bee species formally recognized as an endangered species by the Brazilian Ministry of Environment (MAPA Normative Instruction No. 3, May 27th 2003) and the International Union for the Conservation of Nature and Natural Resources (IUCN, 2013). These factors emphasize the importance of *M. quadrifasciata* as a surrogate species in insecticide risk assessments.

Here, we assessed the acute toxicity of spinosad (and imidacloprid as a positive control) to *M. quadrifasciata* adult workers that were orally exposed to the insecticide. The sublethal impact of the bioinsecticide spinosad (and the neonicotinoid imidacloprid) was also assessed with regard to respiration rate, overall group activity and the flight behavior of adult workers. Highly deleterious effects of imidacloprid were expected on orally exposed workers of *M. quadrifasciata* based on recent results with this species (Tomé et al., 2012). In contrast, only a mild effect was expected for spinosad exposure because of its presumed non-target safety (Miles, 2003; Sarfraz et al., 2005).

2. Material and methods

2.1. Insects and insecticides

Three colonies of *M. quadrifasciata* were initially collected at the edge of an area of secondary subtropical forest in Viçosa county (state of Minas Gerais, Brazil; 20°45′S and 42°52′W). They were subsequently established in the experimental apiary of the Federal University of Viçosa, where they are permanently maintained. The colonies of this species are small (300–600 individuals), and the workers performed nest activities for longer than the honeybee workers starting to fly when older, between 28 and 30 d after emergence. These colonies were later used for the bioassays. The adult workers were collected in the hive entrance using glass jars, which were subsequently transferred to the laboratory and maintained for 1 h in wooden cages covered with organza (35 × 35 × 35 cm) in the dark without food and under 25 ± 2 °C and 70 ± 10% relative humidity until the bioassays were established.

The two insecticides were used in their respective commercial formulations as follows: imidacloprid (700 g a.i. L^{-1}, water dispersible granules; Bayer CropScience, São Paulo, SP, Brazil), and spinosad (480 g a.i. L^{-1}, suspension concentrate; Dow AgroScience, Santo Amaro, SP, Brazil). The insecticides were diluted in a 50% sucrose solution using deionized and distilled water to obtain the final insecticide concentrations.

2.2. Dose-mortality bioassays

The dose-mortality bioassays were performed by exposing adult worker bees to different concentrations of each insecticide. A control without insecticide exposure was used to assess natural mortality and subsequently correct the mortality data. The insects that were initially maintained for 1 h in cages were subsequently individualized in cylindrical glass tubes (15 cm length and 1.5 cm diameter) sealed with parafilm (Parafilm M, PPP Co., Chicago, IL, USA) and provided with 10 μL of insecticide-contaminated 50% sucrose solution (bees in the control received uncontaminated sucrose solution). The bees that completely depleted the sucrose solution were transferred to 200 mL plastic containers with an *ad libitum* provision of uncontaminated 50% sucrose solution for 24 h, after which mortality was recorded. The adult workers were considered dead if they were unable to respond when prodded with a fine hair brush. Preliminary experiments allowed the determination of dose ranges for the subsequent dose-mortality bioassays. Therefore, six doses of imidacloprid were used (5.0, 10.0, 30.0, 50.0, 70.0, and 90.0 ng a.i./bee), and five doses of spinosad were used (5.0, 10.0, 17.5, 25.0, and 42.5 ng a.i./bee) for the respective dose-mortality bioassays, in addition to the control without insecticide exposure. Dose-mortality bioassays were blocked by colony using 10 workers per dose of each insecticide per block (i.e., colony); three colonies were used for each bioassay encompassing 30 workers for each dose of each insecticide tested.

2.3. Overall group activity

Bioassays of the overall group activity of *M. quadrifasciata* workers were performed 3 and 24 h after exposure to the respective LD_5s for imidacloprid and spinosad and the non-exposed controls, as previously described. The exposed and unexposed insects were transferred to Petri dishes (9.0 cm diameter and 2 cm high) in groups of 10 individuals from the same colony, and three replicates (i.e., colonies) were used for each determination. The bottom of each Petri dish was covered with filter paper (Whatman No. 1), and the dish was covered with transparent plastic film to prevent insect escape. The overall insect activity, including walking behavior, insect interactions, and the movement of body parts (e.g., trophallaxy, grooming), was recorded for 10 min and digitally transferred to a computer using a video tracking system equipped with a digital CCD camera (ViewPoint LifeSciences, Montreal, QC, Canada). The overall insect activity was recorded as spatial movement of bees over time and registered as pixels $^{-1} \times 10^{-2}$. After the test, the bees were returned to their 200 mL plastic containers with

an uncontaminated sucrose solution *ad libitum* until the 24 h bioassay. An acclimation period of 2 h in the Petri dish was used before the activity recording. The bioassays were performed under 25 ± 2 °C and artificial fluorescent light between 14:00 and 18:00 h in the afternoon.

2.4. Flight bioassays

The worker bees were subjected to two flight bioassays comparing each insecticide effect on worker flight with the control (untreated workers). The same number of colonies (three with 10 workers from each) used in the overall activity bioassays were used in both flight bioassays after 24 h of the insecticide exposure. A 105 cm tower was formed with three stacked wooden cages covered with organza fabric at their sides (35 × 35 × 35 cm each) and opened in their top and bottom parts to allow free insect flight through them. A fluorescent lamp (60 W, 800 lumens) was placed 10 cm above the tower in a dark room. The 1st bioassay explored the vertical bee flight towards the light source after the insect was released in the center bottom of the tower, and the flight take-off (or lack thereof) was recorded within 1 min of worker release. The flight activity was stratified as follows: (I) no flight (i.e., bee remained on the base of the tower), (II) flight up to 35 cm high, (III) flight between 35 and 70 cm high, (IV) flight between 70 and 105 cm high, and (V) flight reaching the light source at 120 cm high.

The 2nd flight bioassay explored the free-fall flight of the workers by individually releasing the bees 5 cm below the light source within the wooden tower and recording their site of landing. The free-fall flight was stratified as follows: (I) free-fall without flight, landing directly on the tower base; (II) initial fall followed by flight, landing up to 35 cm high; (III) initial free-fall followed flight, landing between 35 and 70 cm high; (IV) initial free-fall followed by flight, landing between 70 and 105 cm high; and (V) no-fall and flight towards the light source.

2.5. Respirometry bioassays

Because respiration rate is a measure of the individual level of stress (Kestler, 1991), respirometry bioassays were conducted 3 and 24 h after the workers were exposed or not exposed to an insecticide-contaminated sucrose solution, as previously detailed. The insecticide doses used corresponded to the recorded LD_5 of each insecticide. The respiration rate was determined in three batches (i.e., replicates) of six insects from the same colony. CO_2 production was recorded using a TR3C respirometer equipped with a CO_2 analyzer (Sable Systems International, Las Vegas, NV, USA). Each adult bee was individualized in 25 mL glass chambers connected to a completely closed system. CO_2 production (μmol CO_2/h/bee) was determined after a 3-h period by injecting CO_2-free air into the chamber for 2 min at a flow rate of 600 mL min^{-1}. The air current directed the bee-produced CO_2 to an infrared reader connected to the system. CO_2 production was also determined in a control chamber without any insect.

2.6. Statistical analyses

The data from the dose-mortality bioassays were subjected to probit analyses to estimate the toxicological parameters LD_5 and LD_{50} (PROC PROBIT; SAS Institute, 2008). The overall group activity and the respiration rate were subjected to two-way analyses of variance (time × insecticide treatment) and Tukey's HSD test ($p < 0.05$) when appropriate (PROC GLM; SAS Institute, 2008). As the time interval was assessed in different insect samples, they are not pseudoreplicates in time and therefore subject to regular two-way analyses of variance instead of repeated measures analyses of variance. Normality and homoscedasticity assumptions of analysis of variance were ascertained before such analyses (PROC UNIVARIATE; SAS Institute, 2008), and the overall group activity and respiration rate were transformed to $\log(x + 1)$ to satisfy these assumptions. Neither of the flight bioassay results satisfied the assumptions for the analysis of variance, so they were subjected to the (non-parametric) Kruskal–Wallis test at $p < 0.05$ (PROC NPAR1WAY; SAS Institute, 2008).

3. Results

3.1. Acute insecticide toxicity

The probit model was suitable to the results of the dose-mortality bioassays for both insecticides, imidacloprid and spinosad, based on the low χ^2 and high p-values obtained in the goodness-of-fit tests (Fig. 1). The LD_{50} estimates obtained with the probit model were 23.54 and 12.07 ng a.i./bee for imidacloprid and spinosad, respectively (Fig. 1). Therefore, spinosad was nearly two times more toxic to the *M. quadrifasciata* workers than imidacloprid. The estimated LD_5s for imidacloprid and spinosad (5.38 and 5.29 ng a.i./bee, respectively) were similar, and each was used in the subsequent bioassays.

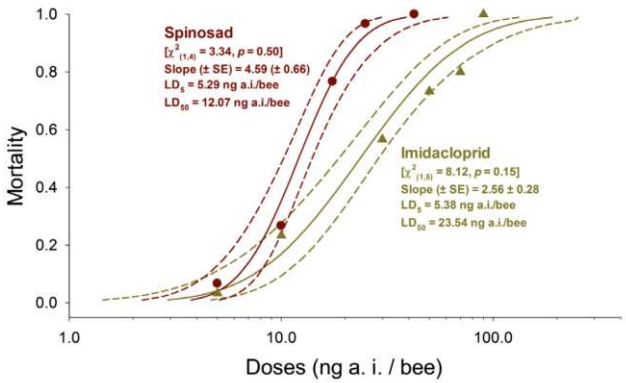

Fig. 1. Dose–mortality curves of adult workers of the stingless bee *Melipona quadrifasciata* orally exposed to imidacloprid and spinosad. The slopes, LD_{50}s and LD_5s are indicated. The dotted lines represent the 95% fiducial limits of each curve.

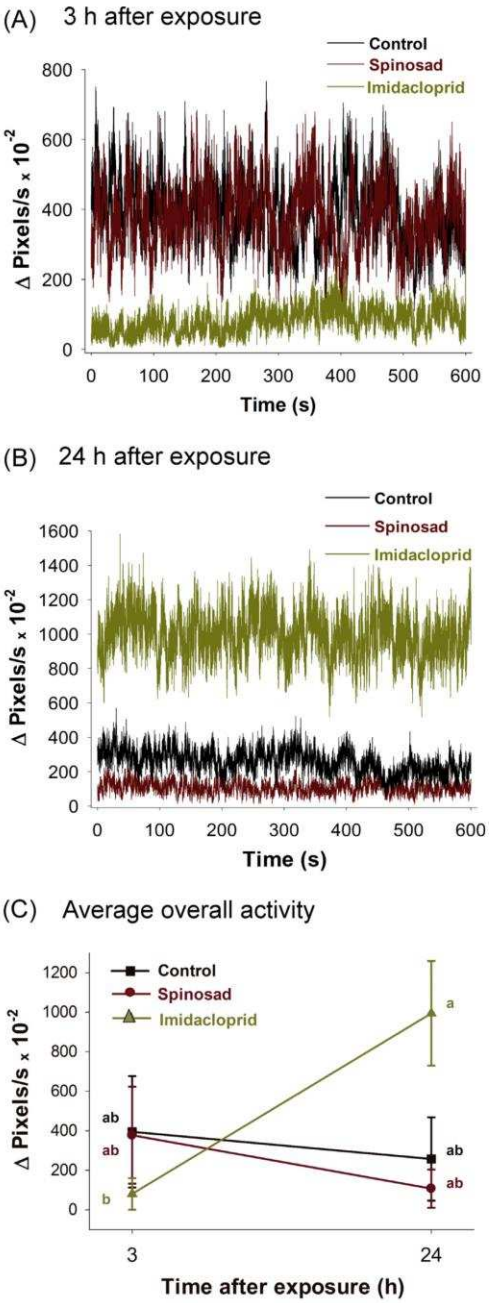

Fig. 2. Overall group activity of groups of ten adult workers of the stingless bee *Melipona quadrifasciata* three and 24 h after being orally exposed to imidacloprid and spinosad. The two upper plots represent the overall activity during 10 min (A and B), while the bottom plot represent the average (±SE) overall activity expressed as changes in registered pixels per second ($\times 10^{-2}$). The The different letters indicate significant differences among insecticide treatments based on Tukey's HSD test ($p < 0.05$).

3.2. Overall insect group activity

The overall worker bee activity was significantly different for insecticide treatments ($F_{2,12} = 6.56$, $p = 0.01$), time ($F_{1,12} = 7.95$, $p = 0.01$) and interaction between treatment and time ($F_{2,12} = 23.88$, $p < 0.001$). The imidacloprid-exposed workers exhibited an average group activity about four times lower than the spinosad-exposed and unexposed workers 3 h after exposure (Fig. 2AC). In contrast, a reversion in overall group activity subsequently took place, and the imidacloprid-exposed workers exhibited nearly five times higher activity than the spinosad-exposed and unexposed workers 24 h after exposure (Fig. 2BC). The group activity of spinosad-exposed and unexposed workers did not change significantly between 3 and 24 h after exposure (Fig. 2).

3.3. Flight activity

Both imidacloprid and spinosad significantly affected the flight performance of workers (Fig. 3). In contrast to the unexposed bees, which were largely able to reach the light source, the flight of the imidacloprid-exposed bees was greatly compromised, with the bees not reaching heights above 35 cm (Fig. 3A). Imidacloprid also significantly impaired the free-fall flight of the workers, which were unable to recover from the initial free-fall after being released, unlike the unexposed workers (Fig. 3C). Spinosad also impaired both vertical and free-fall flights compared to unexposed workers, but the observed impairment was not as drastic as with imidacloprid exposure (Fig. 3BD).

3.4. Respiration rate

The respiration rate of worker bees was significantly different for insecticide treatments ($F_{2,102} = 13.28$, $p < 0.001$), and interaction between treatments and time ($F_{2,102} = 3.50$, $p = 0.03$), but was not different between 3 and 24 h ($F_{1,102} = 0.25$, $p = 0.62$). Imidacloprid significantly reduced the respiration rate of workers both at 3 and 24 h after exposure compared with spinosad-exposed and unexposed workers (Fig. 4). Furthermore, the respiration rate of workers exposed to imidacloprid increased between 3 and 24 h after exposure. Spinosad did not significantly alter the worker respiration rate 3 h after exposure, but the bees exhibited reduction on respiration rates 24 h after exposure (Fig. 4). The respiration rate in the unexposed workers remained at approximately the same levels at the 3 h and 24 h recordings (Fig. 4).

4. Discussion

Both imidacloprid and spinosad were highly toxic to the adult workers of *M. quadrifasciata*, with LD_{50}s in the range of 12.07 and 23.54 ng ingested per bee for spinosad and imidacloprid, respectively. Although imidacloprid is broadly recognized as very toxic to bees, usually with LD_{50}s in the range of 3.8 to over 81.0 ng/bee (Decourtye et al., 2004a,b; Cresswell, 2011; Blacquière et al., 2012), the results with spinosad provide some evidence of deleterious effects on bees (Miles, 2003; Morandin et al., 2005; Besard et al., 2011; Biondi et al., 2012; Gradish et al., 2012a,b). Surprisingly, spinosad exhibited higher acute toxicity than imidacloprid, suggesting its potential impact on *M. quadrifasciata*. The apparently higher susceptibility of stingless bees to spinosad, compared with the honeybee and bumblebee (Mayes et al., 2003; Bailey et al., 2005; Morandin et al., 2005), should also be a matter of concern in future insecticide impact assessments in warmer climates. Lethality, however, is a simplistic indicator of environmental

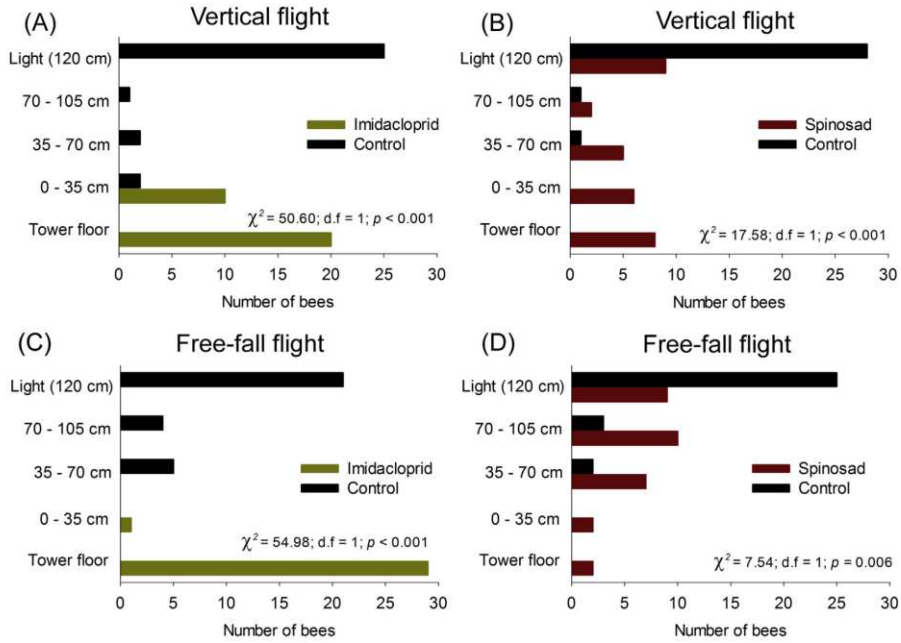

Fig. 3. Flight activity (vertical and free-fall flight) of adult workers of the stingless bee *Melipona quadrifasciata* 24 h after being orally exposed to imidacloprid (A and C) and spinosad (B and D).

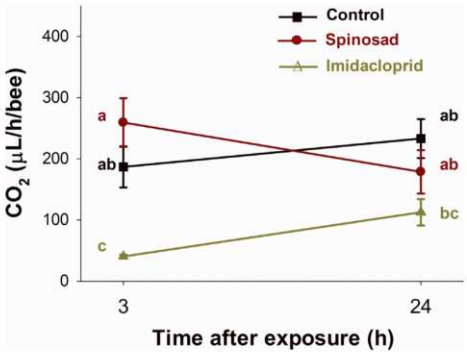

Fig. 4. Respiration rate of individual (±SE) adult workers of the stingless bee *Melipona quadrifasciata* three and 24 h after being orally exposed to imidacloprid and spinosad. The different letters indicate significant differences among insecticide treatments based on Tukey's HSD test ($p < 0.05$).

impact, and sublethal effects of imidacloprid and spinosad in *M. quadrifasciata* were also assessed.

The toxicity of imidacloprid and even more so of spinosad to *M. quadrifasciata*, illustrates the high (acute) mortality caused by insecticide ingestion. However, the sublethal effects of insecticides are even more important than their lethal effect. Imidacloprid, for instance, depressed the overall group activity soon after exposure, with a subsequent increase in activity above normal levels. The reason for this result is unclear because a short-term increase in activity is expected under exposure to this neurotoxic compound. Imidacloprid agonistically interacts with nicotinic acetylcholine receptors, with a subsequent reduction in activity due to the eventual collapse of the excitatory stimulus transmission (Salgado, 1998; Sparks et al., 2001). The initial depression of activity with subsequent hyperactivity may result from secondary neural interactions of imidacloprid within the individual, or lower agonist affinity that may promote excitatory symptoms in insects, what deserves future attention (Salgado, 1998; Tan et al., 2007).

Spinosad does not seem to interfere with the overall group activity of *M. quadrifasciata* workers. However, this insecticide does impair flight activity, which is likely to compromise foraging activity, eventually compromising colony maintenance and ultimate survival (Yang et al., 2008; Blacquière et al., 2012; Henry et al., 2012). This finding is consistent with previous laboratory studies with spinosad reporting its negative impact in honeybees and bumblebees (Mayes et al., 2003; Bailey et al., 2005; Morandin et al., 2005), but such an effect was not evident in field studies (Scott-Dupree et al., 2009; Biondi et al., 2012). Regardless that, the perceived notion of environmental safety of bioinsecticides, and particularly of spinosad, deserves caution.

Respiration rate is an indicator of physiological stress, and insecticides can compromise insect respiration by impairing muscle activity, leading to paralysis (Kestler, 1991; Zafeiridou and Theophilidis, 2006). Indeed, imidacloprid compromised the respiration rate of *M. quadrifasciata* up to 24 h after exposure, likely reflecting in-flight and group activity interference. The insects however seem to undergo some recovery around 24 h after exposure, a likely consequence of the rapid breakdown and excretion of imidacloprid, as recently reported in honeybees and bumblebees (Cresswell et al., 2014). Spinosad represented a distinct contrast at a sublethal exposure in the same range of imidacloprid (5 ng a.i./bee), eliciting only a marginal reduction in respiration rate 24 h after exposure and not affecting the overall group activity. However, spinosad also impaired worker flight, albeit at a lower degree than imidacloprid. The reason for this result may be the high

demands of energy and muscle synchronization and activity required for flight, leading to its more drastic impairment even under low exposure (Candy et al., 1997). However, the impairment of overall group activity in eusocial bees may also have important consequences for the individuals and the colony (Tomé et al., 2014).

The acute toxicity of spinosad in adult workers of the stingless bee *M. quadrifasciata* was even higher than that of imidacloprid, and both compounds elicited deleterious sublethal effects on group and/or flight activities in this species. These findings further challenge the common extrapolation of toxicity assessments with *A. mellifera* to all (native) bee pollinators, which have been recognized as about 10-fold more tolerant to pesticides than stingless bees based on a recent meta-analysis study (Arena and Sgolastra, 2014).

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CHAPTER 3

IMPROVED PROTOCOL FOR *IN VITRO* REARING OF *Apis mellifera* WORKERS

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ABSTRACT

Recently, the *in vitro* methods of rearing honey bee larvae has become increasingly important in honey bee risk assessment, particularly for testing certain compounds on the development. To study the effect of pesticides from the beginning of the post-embryonic development of bees is essential to improve the previews *in vitro* larval rearing methods that still demonstrate high mortality in the controls until emergence of adult workers. Here, *in vitro* rearing *Apis mellifera* method has been improved, and for the first time, the survival of bees until the emergence of adults reached levels of 100⁄ , which means an increasing of 25-30⁄ of survival in comparison to the current literature. Changes in the proportion of the components of diets, high quality of royal jelly, transfer and maintenance of larvae and especially changes in procedures used during larva-pupa transition are the main reasons for the high success achieved here. Due to the high reliability of our method, we recommend the use of it from now in all toxicological bioassays with pesticide exposure in larvae of honey bees in laboratory conditions.

1. INTRODUCTION

The importance of honey bees for pollination and biodiversity is unquestionable (Klein et al., 2007; Gallai et al., 2009; vanEngelsdorp et al., 2010). Nevertheless, populations of these organisms have suffered considerable reductions in the world by several factors including the use of pesticides (Neumann and Carreck, 2010; Vanbergen & The Insect Pollinators Initiative, 2013; Staveley et al., 2014, Fairbrother et al., 2014). Although *Varroa* mites and diseases are the most probable cause of reductions in honey bee populations, pesticides can affect immunity of bees, making them more susceptible to other stressors (Pettis et al. 2012; diPrisco et al. 2013; Staveley et al. 2014). Therefore, an important tool to understand how pesticides can affect honey bees is the rearing of their larvae *in vitro* , where interferences, such as oscillation in temperature and humidity, presence of parasites, pathogens and nurse bees, and heterogeneity of diets are eliminated (Aupinel et al., 2005, Aupinel et al. 2007; Crailsheim et al., 2013).

Substantial progress has been achieved in the development of *in vitro* rearing standardized methods of honey bee (Peng et al., 1992; Aupinel et al., 2005, Aupinel et al. 2007; Hendriksma et al., 2011a; Crailsheim et al., 2013; Charpentier et al., 2014; Zhu et al., 2014). In spite of this, the difficulties involved in *in vitro* rearing lead to high levels of mortality (~30⁄) until adult emergence, overestimating the risk assessments. Due to this limitation, works focusing on the adult exposure are much more numerous than works focusing on larval exposure.

In this study, we improved prior published *in vitro* methods, reaching 100⁄ regarding survival until the emergence of adults, which means about 20-30⁄ more of survival when compared to current literature (Peng et al., 1992; Aupinel et al., 2005, Aupinel et al., 2007; Gregorc and Ellis, 2011; Hendriksma et al., 2011a; Hendriksma et al., 2011b; Crailsheim et al., 2013; Charpentier et al., 2014; Zhu et al., 2014). We provide a clear and updated protocol step by step as a recipe to show readers how to rear *in vitro* honey bee workers. In addition, we present the crucial points of rearing process to achieve high success on method, as changes on transfer and maintenance of insects, especially during larvae-pupae transition, and finally in the usage of high quality of royal jelly.

2. PREPARING SUPPLIES/TOOLS

a. Chinese grafting tool

   i. Chinese grafting tools should be modified when used for transferring honey bee larvae from combs to larval sterile tissue culture plates (STCPs) (Fig. 1A). In these instances, the flexible tip of the tools should be trimmed to

approximately 2.5 mm in width to allow for the larvae to be picked up from the base of the cell unhindered.

ii. The Chinese grafting tools should be modified differently when used to transfer prepupae from larval STCPs to pupal STCPs (Fig. 1B). The tools should be modified by removing their springs and plungers. The plunger and spring are easily removable by pulling firmly on the plunger, and sliding off the plunger and spring from the tool.

b. 48-well sterile tissue culture plates (STCP)

i. Larval STCP

1. Cut the #2 braided cotton dental roll into 3mm long pieces. Place a single 3 mm piece on the bottom of each well in the larval STCP, thus elevating the cell cups from the bottom of the well. This is done to allow for easy removal of the cell cups from the STCP when larvae die during the larval development stage.
2. A single cell cup should be placed in each well, on top of the cotton. The cell cup should be recessed in the STCP well about 1 mm to allow adequate ventilation for the larvae.

ii. Pupal STCP

1. Cut Kimwipes into 2.0 cm × 1.0 cm pieces.
2. Place a piece of Kimwipe at the bottom of each well in a new STCP. A properly placed Kimwipe will cover the bottom of a well and fold up the sides of the well.

c. Tools, materials, and supplies. All tools, materials, and supplies must be sterilized, whenever possible, prior to their use to prevent contamination or the introduction of pathogens to the developing bees.

i. Always wear clean nitrile disposable gloves and a face mask during material preparation.

ii. Place all grafting tools, laboratory tools, STCPs, glass beakers and aluminum foil under a UV light for 15 minutes. This adequately sterilizes all material for use.

iii. Once sterilization is complete, use gloved hands to place plate covers onto the STCPs, cover the glass beakers with the UV-exposed surface of the aluminum foil facing down on the beakers, and wrap tools in aluminum foil (sterile surface contacting the tools) until use.

d. Prepare the desiccators and incubator

i. Clean all interior surfaces of the desiccators and incubator (Fig. 2) with a 10⁄ v/v bleach solution before each new round of larval grafting. Be sure to let the desiccators and incubator dry completely before placing any bees into the rearing environment

ii. Place the desiccators in an incubator with the set-point at 34.5° C. The temperature within the incubators should not vary more than ± 0.5° C from the set-point. Data loggers should be used to confirm temperature within the incubators and temperature/humidity within the desiccators.

iii. Prepare supersaturated salt solutions of K_2SO_4 (for the larval stage) and NaCl (for the pupal stage) by mixing the salts in warm tap water until the salt no longer dissolves into solution.

iv. The referenced desiccators contain a leak-proof tray that can hold the salt solutions. The trays should be filled with about 800 ml of salt solution while the trays are still partially inserted into the desiccators. Once the salt solution has been poured into the tray, slide the tray completely into the base of the desiccator while taking care not to spill the solution. The K_2SO_4 solution is used in desiccators where larval STCPs are held and the solution produces a R.H. of ~94⁄ at the incubator temperature of 34.5° C. The NaCl solution is used in desiccators where pupal STCPs are held, producing a R.H. of ~75⁄ at the incubator temperature of 34.5° C. We recommend that at least two desiccators be used, one for housing larval STCPs and a second for housing pupal STCPs. Closely monitor the amount of salt solution in the tray and be sure to refill when the solution is getting low due to evaporation.

v. All manipulation with the immature bees, including larval grafting and feeding, should be done in a positive flow clean hood (Fig. 3). The hood should be sterilized by with a 10⁄ bleach solution v/v before and after the larvae are fed or inspected. Alternatively, the hood can be equipped with a UV light that can be turned on for 15 minutes prior to any manipulation with immature bees. The hood should be located in close proximity to the incubator in which the larvae/pupae are kept. This will minimize any disturbance to the immature bees during grafting and feeding. The hood should be equipped with a light source for proper illumination of the larvae and a ceramic space heater to keep the hood space warm during larval transfers, feedings, and mortality inspections. All supplies and tools are listed on Table 1.

3. PREPARING THE LARVAL DIET

a. Honey bee larvae are fed a diet of water, sugars, yeast, and royal jelly over a period of six days. The diets should be stored for a maximum of two days at 4° C.

b. The diet should be mixed together, using sterile laboratory tools and beakers. Filtering or heating is not necessary while preparing the diet. Prepare the diet at least one hour prior to larval grafting to allow the diet to settle. Mix the diet at room temperature in the following order:

   i. Measure the amount of ddH_2O (room temperature) needed
   ii. Add the two sugars, D-fructose and D-glucose, and mix until dissolved completely into solution
   iii. Add the yeast and mix all ingredients until dissolved into solution
   iv. Add the royal jelly and mix until reaching a uniform consistency

c. There are three different diets (Diet A, B, and C) fed to the honey bees during larval development. The three diets differ in the amount and proportion of ingredients they contain. The diet recipes are listed in Table 2. The sample amounts of diet given in Table 2 (10 g of diet A, 10 g of diet B, and 50 g of diet C) will feed about 400 larvae.

4. COLLECTION OF 1ST INSTAR LARVAE

a. A honey bee queen in a queen-right colony is confined on a frame of wax comb using a zinc queen excluder push-in cage. It is better to confine queens to wax combs that are less than one year old to limit larval exposure to pesticides in the wax. Worker bees can travel through the excluder walls and face of the push-in cage, thus they are able to tend the developing brood and queen. Such a cage can be used to obtain approximately 400 1st instar larvae, but cage design can vary.

   i. Place the queen inside the queen excluder cage by putting the queen on the comb and securing the cage around her. Press the edges of the cage into the wax comb to prevent the queen from escaping. Place the frame with the excluder cage into the middle of the brood nest to ensure the proper incubation of the eggs prior to hatching.
   ii. Release the queen from the cage after a period of 24 hours. Confirm that the queen has laid eggs in the cells. The age of the eggs when the queen is released is estimated to be t = 12 ± 12 hours (t = 0 ± 12 hr) when the queen has been caged for 24 hours).

iii. Replace the push-in cage on the comb in the same position it was in when it contained the queen. This will prohibit the queen from laying more eggs in the target brood section.

b. First instar larvae (t = 87 ± 12 hr) are transported to a sterile lab environment for grafting

i. Pre-warm two clay-based heat packs (Thermipaq 15 cm × 30 cm) in a 1000w microwave for 90 seconds.

ii. Place the heating packs on the inside base of a five-frame hive box with a telescoping outer cover on both the top and bottom of the hive box. The heat pads will provide the larvae with adequate warmth for about 30 minutes, which should be enough time while moving the larvae from the field to the laboratory.

iii. At t = 87 ± 12 hr, remove the frame from the colony, brush off the bees, and transport the frame to the laboratory in the heated nucleus hive box. Be sure to never shake the frame of 1st instar larvae and to confirm the presence of 1st instar larvae (Fig. 4).

iv. At the laboratory, place the collected frame in an incubator maintained at 34.5° C and constant dark until the time of grafting (no longer than 3 hours).

5. LARVAL GRAFTING AND MAINTENANCE

a. All three diets (A, B, and C) are fed to the larvae during their development. The amount of the diet fed to the larvae varies throughout larval development according to the schedule in Table 4. There are a total of five feedings over a six day period, with no feeding occurring on the second day of rearing.

b. First instar larvae are transferred from the comb to the cell cups in the prepared and sterilized larval STCP.

i. Pre-warm diet A in the incubator until it reaches approximately 34.5°C.

ii. Place the sterilized, prepared larval STCP and grafting tool, nitrile gloves, and face mask in the clean hood.

iii. Turn on the space heater to the low setting and locate the heater about 15 cm from the edge of where the frame will be in the hood. A light source can be used to facilitate seeing the larvae in the cells.

iv. Place the comb with the 1st instar larvae in the hood. The comb should be slightly tilted up from the flat position to allow the grafter to see into the back of the cells.

v. Visually inspect and omit any larvae that have noticeable defects or stunted growth.

vi. If testing pesticides, we recommend mix a specific volume of pesticide stock solution previously prepared. Using a glass vial (10 ml) and a glass stick (10 cm), we recommend mix 1 µL of pesticide solution per each 20 µL of diet A. For feeding one plate with 30 larvae, place 600 µL of diet A and 30 µL of pesticide stock solution to reach concentration needed in the solution, immediately prior feeding process. In controls, use the same volume of acetone, chloroform or ddH_2O, depending of pesticide used on study.

vii. Place 20 µL of diet A (or diet A with pesticide) into one row (8 cell cups) of the larval STCP using a calibrated variable volume pipette.

viii. Dip the flexible tip of the Chinese grafting tool into ddH_2O (to provide lubrication while grafting the larvae).

ix. Insert the flexible tip of Chinese grafting tool (the one modified for grafting larvae) along the side of a cell containing a larva and gently slide the tip underneath the larvae.

x. Gently lift the grafting tool straight out of the cell with the larvae firmly on the end of the tip. Only insert the tool inside the cell once to graft the larvae. Avoid grafting any larvae that were not secured on the first attempt (i.e. a single larva should be manipulated only once and then ignored if she was not secured on the first attempt).

xi. Place the tip of the grafting tool containing the larvae into a sterile cell cup until the tip of the tool is bent slightly against the base of the cup.

xii. Depress the plunger slowly to force the larvae off of the tip of the grafting tool and onto the top of diet A.

xiii. Slowly and gently remove the grafting tool from the cell cup, being careful not to disturb the larvae. The larvae should be laying with the same side facing up as they were in the comb.

xiv. Wash the grafting tool after every time each larva grafted. The tool should be washed in 75⁄ ethanol and then rinsed with ddH_20. Allow the tool to dry by placing it on a Kimwipe before grafting additional larvae. It is better to use

multiple tools in the grafting process so that some are available for use while others are clean and drying.

xv. Confirm that each grafted larvae is sitting on top of the diet (Fig. 5). If any larvae are improperly grafted due to grafting error, remove the larvae/cell cup from the experiment and replace with a new cell cup into which diet A and a larva are placed.

xvi. After grafting eight larvae, fill another eight cell cups with diet A before continuing grafting to prevent the diet becoming cool in the cell prior to grafting.

xvii. It should take less than 30 minutes to graft larvae into one STCP.

xviii. Once larval STCP is filled, place the STCP in the larval desiccator maintained at 94% R.H. using the K_2SO_4 salt solution. Do not move the desiccator suddenly as this can disturb the developing larvae and spill the salt solution.

xix. We do not suggest use exclusion of grafting effect because we obtained constantly 100% of survival on rearing in accordance with prior recommendations.

c. The larval diet is fed to the larvae as outlined in Table 3. The feedings should always be performed in the hood with the space heater on the low setting.

i. Any leftover diet visible at the next schedule feeding is left for the developing larvae. The new scheduled diet gets added to any leftover diet. Be sure not to remove the leftover diet because of the risk of damage to the larvae.

ii. Place the new diet, with pesticides or not, at the anterior end of the larvae near the mouthparts and be careful not to submerge the larvae during application.

iii. Do not touch the larvae with the pipette tips at any time during larval development.

d. Larval survival is monitored daily by removing the larval STCP, placing them in the heated hood (heater setting on low), and visually inspecting the larvae. It is a good strategy to monitor mortality when new diet is being added. This will limit the time that the larval STCP are outside the incubator.

i. Remove and dispose of cell cups containing dead larvae (do not reuse).

ii. Do not touch the larvae while assessing mortality.

iii. Once finished assessing mortality, return the larval STCPs to their appropriate desiccator.

6. PUPAE TRANSFER AND MAINTENANCE

a. Larvae will begin to be transferred from the larval STCP to the prepared pupal STCP 144 hours (six days) after grafting to allow the larvae to pupate into adults. The larvae are ready to be transferred to the pupal STCP once they have consumed all of the available diet. Larval transfer time for each STCP can occur over a period of a few days depending on specific experimental treatments (i.e. pesticides causing delayed development). Only larvae that have consumed their diet are moved (Fig. 6).

   i. Monitor the larvae daily and move them prior to defecation. Otherwise, larvae will defecate in their food, resulting in increased mortality in the developing bees. Significant differences were found between larvae moved prior and after defection (Fig. 7).

   ii. Transferring the mature larvae to the pupal STCP:

      1. Remove the cell cup from the larval STCP.
      2. Gently invert the cell cup at a 45° angle over a well in the pupal STCP (Fig. 8).
      3. Guide the larvae into the well of the pupal STCP using a Chinese grafting tool modified as described in 3.1.2.

b. Once the pupal STCP is filled (i.e. all larvae have been transferred), place the STCP in a desiccator maintained at 75% R.H. using the NaCl salt solution.

   i. The pupal stage of development requires no feedings.

   ii. Do not move the desiccator suddenly as this can disturb the developing pupae and spill the salt solution.

c. Pupal survival is monitored daily by removing the pupal STCP (Fig. 9) and visually inspecting the pupae while in the clean hood.

   i. Remove and dispose of dead pupae with a sterile pair of forceps.

   ii. Do not touch the pupae while assessing mortality.

7. ADULT EMERGENCE

a. After a period of approximately 519 hours (18 days) after grafting, adult bees will begin to emerge (Fig. 10). Check for emerged adults at least once every 24 hours as some adult eclosion may be delayed. Adults can be maintained by feeding the bees with pollen and 50% sugar solution: H_2O (w/v) in an artificial bioassay cage.

8. DISCUSSION AND CONCLUSIONS

Here, we show the critical points to obtain high success on *in vitro* honey bee rearing during whole life cycle. Our results suggest common reductions on survival found in previews protocols probably resulted from infections that start at the beginning of larvae defecation on high humidity environment, which induce posteriorly mortality in prepupae and pupae stage. In addition, the quality of diet components, mainly the royal jelly, careful larvae collection and practical experimenter skills are the most important points to obtain high success on method. The main causes of the observed honey bee survival variance between laboratories may lie in practical experimenter skills, the season, genetic variation and larval age heterogeneity at grafting (Aupinel et al., 2009). However, important points in the protocols, such as the larvae-pupae transition, may be responsible for mortality of bees reared in the laboratory.

Just prior the pupal stage, on the seventh day after the first instar larval grafting, the larvae start defecation. It is important to mention that on that moment, the larvae are maintained in high humidity environment (96% R.H.), and the contact with feces may induce infections (Crailsheim et al., 2013). However, little attention has been given to this crucial point in the larval rearing technique, and information about the best moment to move larvae for pupal plates are omitted or are not clear in previews studies (Aupinel et al., 2005, 2007; Crailsheim et al., 2013). We observed similar mortality levels at prepupal and pupal stages of Aupinel and collaborators (2007), when larvae were moved to new plates after defecation. Contrary, we obtained excellent survival (100%) until emergence of adult bees, when larvae were transferred just prior to the beginning of defecation. Inside the wells in the pupal plates, piece of Kimwipe absorb feces, avoiding the feces spreading and contact with larvae body. In addition, the drier environment (75% R.H.) may contribute with reductions of bacterial and fungal infections. Therefore, moving larvae to pupae plates immediately after the end of diet consumption improve survival, because the defecation together with the high humidity environment may induce high mortality.

The use of nongrafting methods have resulted in larval death between 2 and 8% upon the prepupae (Hendriksma et al., 2011a; Hendriksma et al., 2011b). Supposedly, larva collection and transference with artificial combs (i.e., without grafting), generates lower death when compared to traditional methods. However, in this case the mortalities until adult emergence are still high (~20%) (Hendriksma et al., 2011a). The efficient manipulation or practical experimenter skills during grafting are required to obtain high *in vitro* survival, but it is still not the main point for the larval rearing. We could optimize the larval survival even by using traditional grafting technique to collect larvae from combs. Again, high mortality rates

observed elsewhere probably resulted from infections by contact with feces (Aupinel et al., 2005; Aupinel et al., 2007; Silva et al., 2009; Hendriksma et al., 2011a; Lima et al., 2011; Crailsheim et al. 2013). If cleaning of materials is guaranteed, collecting and feeding larvae by the experimenter is appropriate, mortality during larval stage would be more related to low quality of diet components (e.g., the royal jelly).

Another important issue to obtain high survival in laboratory conditions is the origin of diet components. Differently of the yeast extract and sugars (D-fructose and D-glucose), which can be kept at room temperature, the royal jelly must be kept under low temperatures to avoid degradation. For instance, all of royal jelly sources should be shipped via overnight delivery and stored at -20°C upon arrival. In addition, it should be vetted appropriately prior and after to use. After opening, it is recommended split royal jelly in small vials with specific volume to maintain on fridge at -4°C, avoiding wastes and degradation by thaw. In addition, the composition of royal jelly depends on season and the day of harvesting (Sabatini et al., 2009; Zheng et al., 2011). Finally, the differences in the quality and integrity of royal jelly proteins may influence rearing success or pathogens susceptibility (Crailsheim et al., 2013). Here, we used Stakich brand royal jelly, the best brand tested in our pilot experiments (data not shown). However, we are still conducting experiments to evaluate quality of different royal jelly brands to confirm the high heterogeneity between sources.

In conclusion, the present study showed details of *in vitro* honey bee larval rearing method, which allowed improvements on bee survival along its whole life cycle. Due to the high reliability of the updated method, we recommend the use of it in all toxicological bioassays with honey bees in different life stages, including newly emerged adult workers. Our method suggested that the traditional larvae collection with grafting is not the main reason for mortality of bees. The physical contact between larvae and feces might increase the mortality probability by fungal and bacterial infections. Furthermore, practical experimenter skills and high quality of diet components, mainly royal jelly, are essential for the successful rearing of honey bees in the laboratory.

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Appendix of pictures of *in vitro* rearing protocol

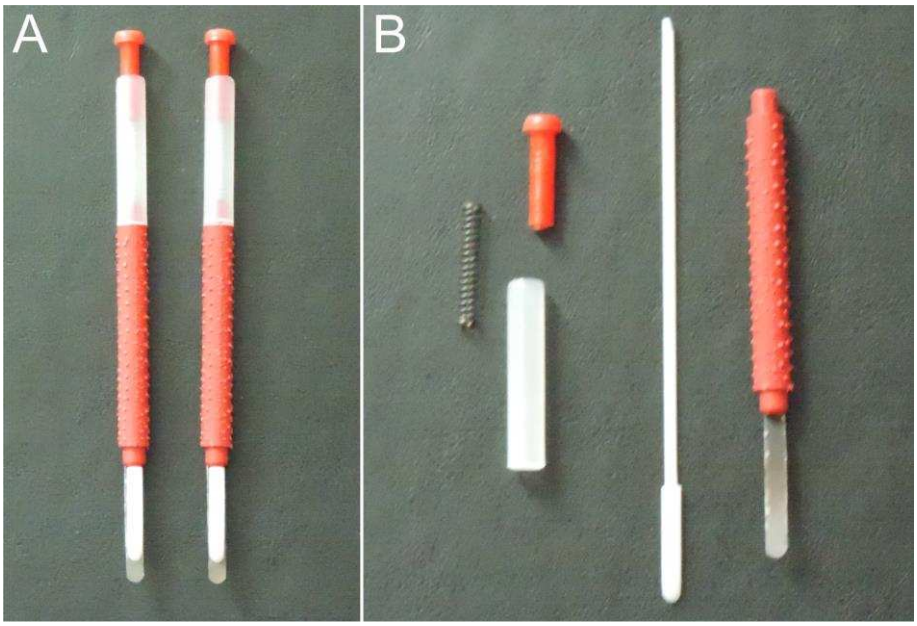

Figure 1. Chinese grafting tool (A) and modified chinese grafting tool (B).

Figure 2. Incubator containing desiccators used to house larval and pupal STCPs.

Figure 3. Hood used for *in vitro* larval grafting, feeding, and survival monitoring.

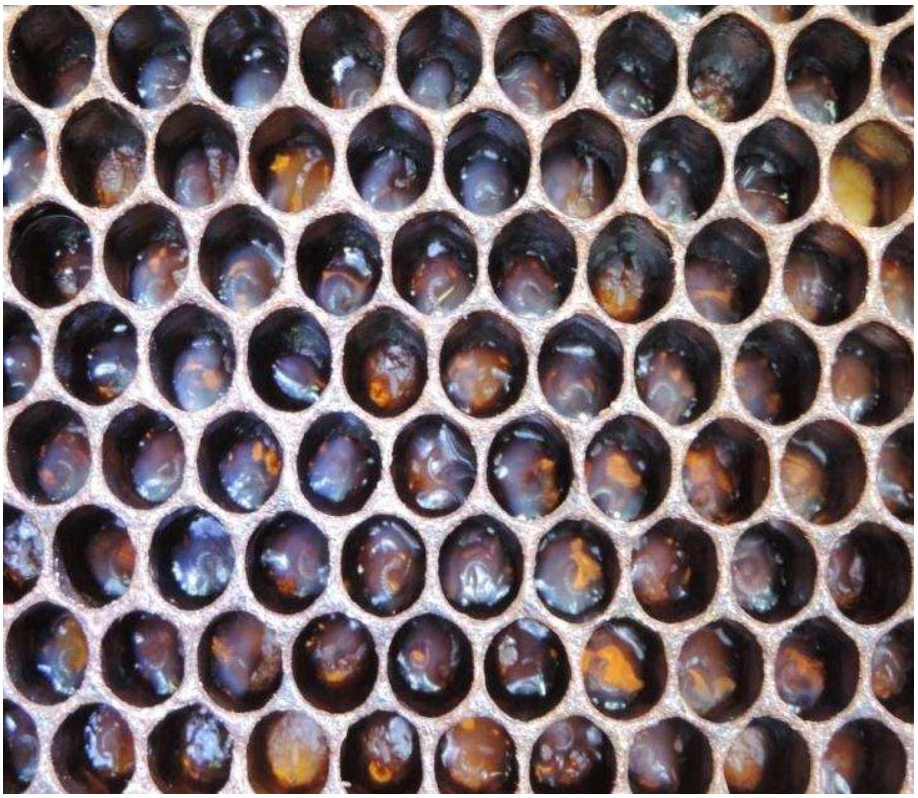

Figure 4. First instar larvae floating on brood food inside the comb (t = 87 ± 12 hr after egg laying).

Figure 5. First instar larvae (t = 87 ± 12 hr after egg laying) after grafting onto artificial larval diet A.

Figure 6. Larvae just prior the grafting from the larval STCP to the pupal STCP (t = 231 ± 12 hr after egg laying).

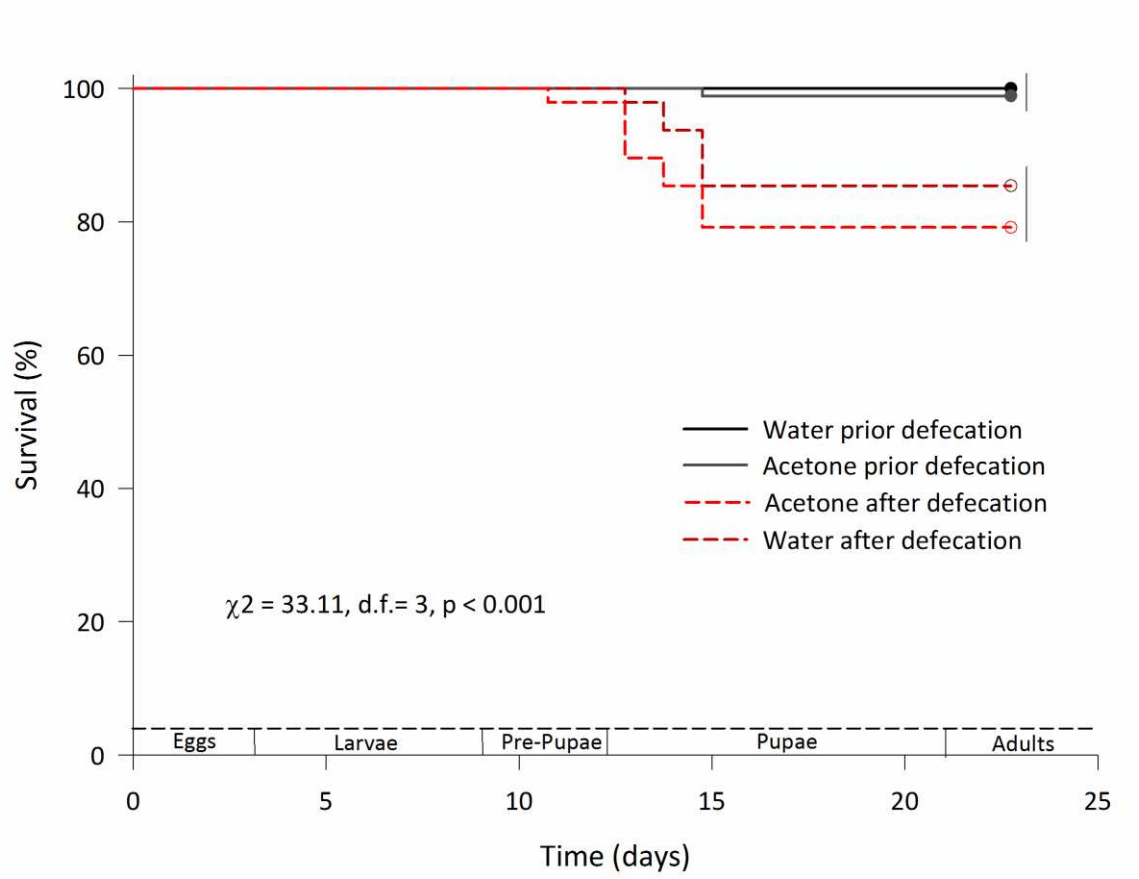

Figure 7. Survival plots of honey bee workers reared *in vitro* and fed diet and solvents (water and acetone) used as vehicle to pesticides. Survival of larvae moved after prior and after defecation was evaluated. Differences in the survival between bees moved prior and after defecation were detected by LOG RANK test. Survival curves grouped by the same line are not significantly different by Holm-Sidak's test ($p > 0.05$).

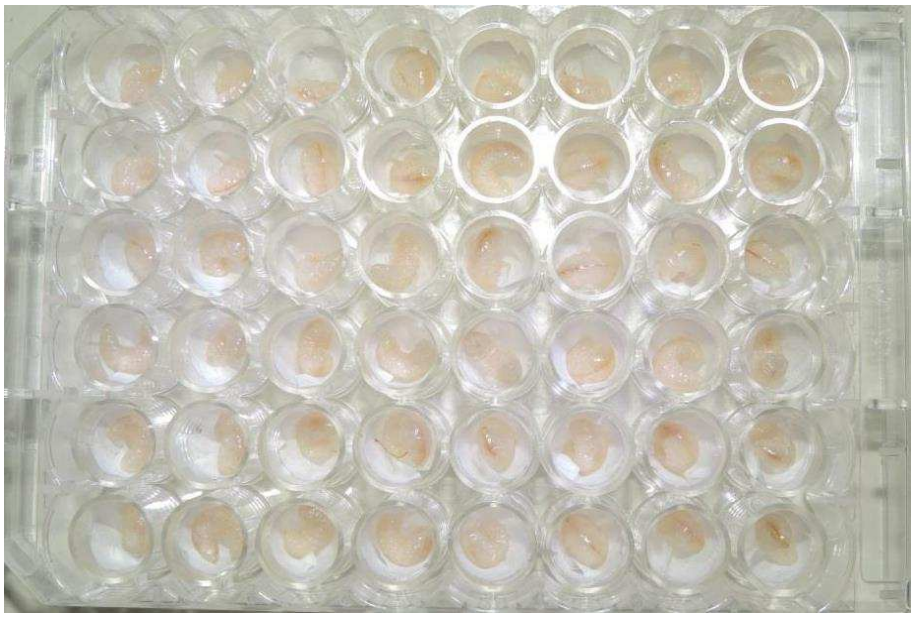

Figure 8. Pre-pupae in the pupal STCP (t = 255 ± 12 hr after egg laying).

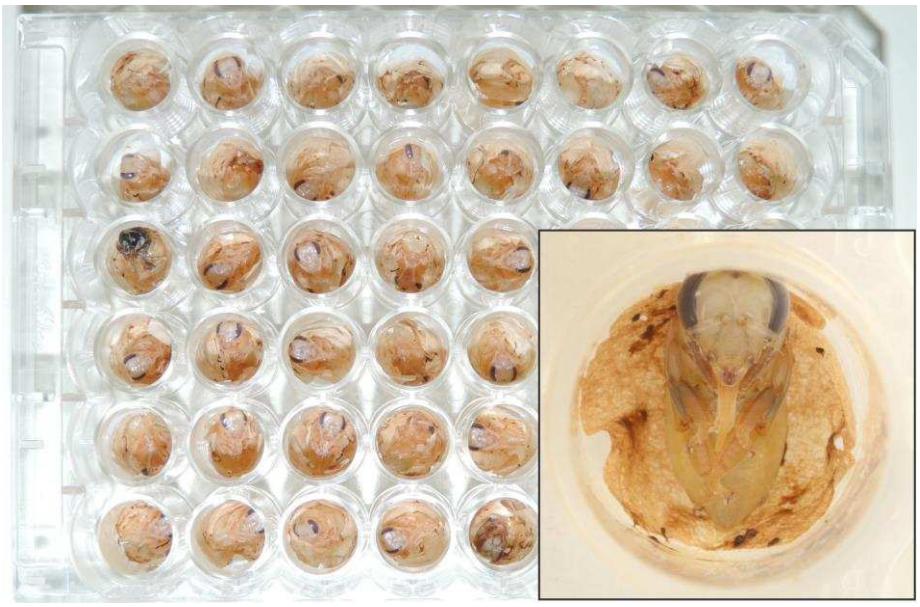

Figure 9. Pupae (t = 423 ± 12 hr after egg laying). Note the pigmented eyes and the dark cuticle (inset).

Figure 10. Newly emerged adult workers (t = 519 ± 12 hr after egg laying).

Table 1. Tools and Supplies Needed

	Item and Description	**Quantity (minimum)**
Equipment	Heratherm incubator (Thermo Scientific, #IMH750-S), or equivalent. The incubator must maintain temperature of ± 0.5°C.	1 unit
	Freezer (-20°C)	1 unit
	Refrigerator (+4°C)	1 unit
	Clean hood, positive pressure (air flow set at 0.5 inches of water)	1 unit
	Microwave, 1000 watt	1 unit
	Space heater, 1500 watt ceramic (Comfort Zone, #C2442WN)	1 unit
	30.5 cm^3 desiccators (Thermo Scientific, #08-642-21)	2 units
	Data loggers (Onset, HOBO #UX100-011)	2 units
Queen caging and transportation	Zinc queen excluder push-in cage (10 cm × 10 cm × 3 cm)	4 cages
	ThermiPaq heat packs, clay based, 15 cm × 30 cm (Medical Supply 123, #201)	2 packs
	Five-frame honey bee hive box with two telescoping outer covers	1 box with two lids
Larval diet preparation	D-fructose (Fisher, #L95-500), store at room temperature	1 container
	D-glucose (Fisher, #D16-500), store at room temperature	1 container
	Bacto yeast extract (Bacto, #288620), store at room temperature	1 container
	Royal jelly (Stakich). The source of royal jelly is of very important and it should be vetted appropriately prior to use. Stakich brand royal jelly has been used successfully and reliably for rearing larvae. All royal jelly should be shipped via overnight delivery. It should be stored at -20°C upon arrival.	1 container
	ddH_2O or autoclaved water.	1 gallon

	Stainless steel laboratory spatula (Fisherbrand, # 14-373)	3 tools
	Stainless steel laboratory scoopula (Fisherbrand, #14357Q)	3 tools
	100 mL glass beakers (Corning Life Sciences, #07-250-054), or equivalent	3 beakers
Honey bee grafting and maintenance	Chinese grafting tools (GloryBee, #14513) or similar grafting tool. The grafting tools should be made of plastic or metal so that they can be sterilized easily. Wooden grafting tools are not acceptable.	10 tools
	48-well tissue culture plates, sterile (Falcon, #353230) or equivalent. Plates wil be prepared in two different ways for larval rearing. The larvae will be reared in larval sterile tissue culture plates (STCP), while the pupae will be transferred to and maintained in pupal STCP.	1 case
	Brown plastic cell cups (GloryBee, #14332). The cell cups need to fit within the STCP wells, therefore other cell cup designs may not work for rearing protocol.	10 bags
	#2 braided cotton dental rolls (Crosstex International, #DNCB)	1 box
	Kimwipes (Kimberly Clark, #06-666A)	1 box
	Sodium chloride (NaCl, Sigma Aldrich, #S7653-1Kg)	1 container
	Potassium Sulfate (K_2SO_4, Sigma Aldrich, #223492-2.5Kg)	1 container
	Fiber optic light source, or equivalent	1 unit
Sterilization	Face mask (Global Industries, #T9F954219)	2 masks
	UV light	1 light
	Nitrile gloves (Fisher Scientific, #19-167-051)	1 box
	Aluminum foil	1 box
	Bleach (10⁄ v/v)	1 container

Table 2. Amount and percentage of diet components

	Amount of Diet Components				**Percentage of Diet Components**		
	Diet A	Diet B	Diet C		Diet A	Diet B	Diet C
Royal Jelly (g)	4.43	4.30	30.00		44.25⁄	42.95⁄	50.00⁄
Glucose (g)	0.53	0.64	5.40		5.30⁄	6.40⁄	9.00⁄
Fructose (g)	0.53	0.64	5.40		5.30⁄	6.40⁄	9.00⁄
Yeast Extract (g)	0.09	0.13	1.20		0.90⁄	1.30⁄	2.00⁄
Water (mL)	4.43	4.30	18.00		44.25⁄	42.95⁄	30.00⁄

Table 3. Sample time frame for acquiring first instar larvae from honey bee colony

Day and Time	**Time after Queen Caging**	**Task**
Monday, 9 am	t= 0	Cage queen
Tuesday, 9 am	t = 24 hr (1 day)	Remove queen from cage
Friday, 12 pm	t= 99 hr (4.125 days)	Collect frame for grafting

Table 4. Schedule of larval feeding for the *in vitro* rearing

Time after Grafting	**Diet**	**Amount (µL)**
t= 0	A	20
t = 24 hr (1 day)	n/a	0
t = 48 hr (2 days)	B	20
t = 72 hr (3 days)	C	30
t = 96 hr (4 days)	C	40
t = 120 hr (5 days)	C	50

Supplemental Table 1. *In vitro* rearing time frame

Day	Age (hr ± 12 hr)	Age (days ± 0.5 days)	Time after grafting (days)	Task
0				Cage - 9 am
1	12	0.5		Release - 9 am
2	36	1.5		N/A
3	60	2.5		N/A
4	87	3.625	0	Graft - 12 pm
5	111	4.625	1	Inspection - 12 pm
6	135	5.625	2	Feeding/Inspection - 12 pm
7	159	6.625	3	Feeding/Inspection - 12 pm
8	183	7.625	4	Feeding/Inspection - 12 pm
9	207	8.625	5	Feeding/Inspection - 12 pm
10	231	9.625	6	Pupal Transfer/Inspection - 12 pm
11	255	10.625	7	Pupal Transfer/Inspection - 12 pm
12	279	11.625	8	Pupal Transfer/Inspection - 12 pm
13	303	12.625	9	Inspection - 12 pm
14	327	13.625	10	Inspection - 12 pm
15	351	14.625	11	Inspection - 12 pm
16	375	15.625	12	Inspection - 12 pm
17	399	16.625	13	Inspection - 12 pm
18	423	17.625	14	Inspection - 12 pm
19	447	18.625	15	Inspection - 12 pm
20	471	19.625	16	Inspection - 12 pm
21	495	20.625	17	Inspection - 12 pm
22	519	21.625	18	Inspection/Adult Emergence - 12 pm
23	543	22.625	19	Inspection/Adult Emergence - 12 pm
24	567	23.625	20	Inspection/Adult Emergence - 12 pm

CHAPTER 4

RELEVANT FIELD PESTICIDE CONCENTRATIONS AND MULTIPLE DISORDERS IN HONEY BEES

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ABSTRACT

The decline of pollinators worldwide is a major concern in recent years and the causes attributed to this phenomenon has been given to a combination of factors including pesticides. Recently, different pesticides were identified in nectar, pollen, and wax from managed honey bee colonies. Here we have examined the effects of pesticides on honey bee larval survival, gene expression, development, midgut cell damage, body weight and antennal structure in the adult workers. Using *in vitro* rearing, we examined the effects of 3 acaricides (amitraz, coumaphos and fluvalinate), 2 insecticides (chlorpyrifos and imidacloprid), 1 fungicide (chlorothalonil) and 1 herbicide (glyphosate) on bees at the field-relevant concentrations found in wax and pollen. We observed significant changes in honey bee survival exposed to all pesticides. Only imidacloprid and glyphosate did not affect the development of bees. The body weight of newly emerged adults treated with amitraz, chlorothalonil and chorpyrifos was significantly lower than controls. In addition, we found impact of these pesticides in the antennal structure and in the activity of different genes related to detoxification.

1. INTRODUCTION

The European honey bee (*Apis mellifera*) is a very important cosmopolitan pollinator able to generates alone more than $200 billion in honey production and pollination of many economically crops (vanEngelsdorp et al. 2010). Nevertheless, honey bee populations have been drastically declining for the last 60 years mainly in United States and Europe where different reasons as natural habitat degradation, malnutrition, diseases, parasites, genetically modified crops and pesticides are indicated as possible reasons of colony declines (Vanbergen & The Insect Pollinators Initiative, 2013; Staveley et al. 2014, Fairbrother et al. 2014). In addition, colony collapse disorder (CCD), a still not understood syndrome, which is characterized by a sudden disappearance of worker bees from the hive, also has been attributed to colony losses since 2006 (vanEngelsdorp et al. 2010). Although Varroa mites, malnutrition and diseases are classified as the most probable concerns for reductions on honey bee populations, beekeepers and other scientists suspect pesticides may contribute for weakening of colonies, disturbing immune system of individuals, making them more susceptible to parasites and diseases (Pettis et al. 2012; diPrisco et al. 2013; Staveley et al. 2014).

Honey bees come in high frequent exposure with a wide range of pesticides in agricultural landscapes (Krupke et al., 2012), and over 120 different pesticides and its metabolites have been identified in hive products (Mullin et al. 2010). Systemic compounds as neonicotinoid insecticides are able to travel throughout the plant and contaminate the pollen and nectar, which are subsequently collected by honey bees while foraging (Oldroyd, 2007; Blacquière et al., 2012; Cresswell et al., 2012). In the same way, compounds routinely applied on crops during blooming time are collected by bees (Mullin et al., 2010). Additionally, beekeepers frequently apply miticides within the colony to control pests, notably the *Varroa* mite (Bogdanov, 2006; Mullin et al., 2010; Rosenkranz et al., 2010; Johnson et al., 2013). These possible routes, separately or together, are responsible to contaminate colonies not only with insecticides and acaricides, but also with herbicides and fungicides (Johnson et al., 2010; Mullin et al., 2010). Although many studies in laboratory or in field conditions have reported pesticides causing mortality and sublethal effects in honey bees, they do not use realistic field-level pesticide concentrations. Disorders on survivability or behavioral impairments promoted by pesticides are reported only in concentrations higher than those residues found within honey bee colonies. Also, the focus of the studies almost always is on exposure in adults what contributes with a lack of important risk assessment on immature (Blacquiere et al., 2012; Henry et al., 2012; Fairbrother et al., 2014).

It is known that contaminations within colonies can affect all bees, including adult and immature. Pesticide residues come in contact with the developing larvae through the feeding of brood food (processed pollen), and through the housing within contaminated wax comb, reducing individual longevity (Mullin et al., 2010; Gregorc and Ellis, 2011; Wu et al., 2011; Charpentier et al., 2012; Gregorc et al., 2012; Zhu et al., 2014). Due to high difficulties involved in *in vitro* rearing techniques, high levels of mortality (~30⁄) have been reported. In addition, there are few data reporting the capabilities of honey bee larvae protecting themselves from pesticide exposure and if this exposure may trigger sublethal effects on immature and newly emerged adults. Here we examined the p450 detoxification and stress gene activities in honey bee workers exposed on larval stage to relevant field-level concentrations of pesticides *in vitro*. Additionally, we assessed the larval survivability to adult to determine whether the energetic cost of protecting individual from pesticide exposure as larvae leads to a reduction in adult emergence and sublethal effects on development, cell deaths on midgut, antennal structure and body weight of newly emerged workers.

2. MATERIALS AND METHODS

In vitro rearing

The experiments were conducted at the Honey Bee Research and Extension Laboratory located within the University of Florida's Entomology and Nematology Department (Gainesville, FL, USA). Honey bee queens were confined on the frame within the hive using a steel queen excluder cage (~10 x 10 x 3 cm) at time = 0 h in order to accurately acquire known-aged eggs. The frame containing the queen was placed at the center of the brood nest where the worker bees can access the queen (Peng et al. 1992; Aupinel et al. 2005; Crailsheim et al. 2013; Zhu et al. 2014). The queen was released after 24 hours and the frame with eggs remained in the hive for additional 72 hours. After 96 hours (from t = 0) the 1st instar larvae had appropriate size to be transported to the laboratory. A therm package clay previously heated in the microwave was used to maintain the temperature about 35 °C in a heated 5-frame nuc box for transportation.

Larvae were grafted and reared *in vitro* according to the standardized methods as described in Tomé et al., 2015 (in prep.). First instar larvae were grafted in queen cups within a 48-well plate (Fisher Scientific®) using Chinese grafting tools while being maintained at ~35 °C by a small portable heater close to the frame. The larvae were grafted with a single gentle movement of the grafting tool into the comb cells to avoid damage on larvae body. Sterilized rearing equipment (cups, plates and Chinese tools for grafting) and proper rearing conditions were used throughout the experiment. Larvae were fed on days 1, 3, 4, 5, and 6 with different

diets, consisting of D-glucose, D-fructose, yeast extract (Fisher Scientific®), royal jelly and double distilled water. The diets were prepared immediately prior to feeding the larvae by mixing sugars in pre-warmed distilled water at 35 °C and adding yeast extract and royal jelly posteriorly.

Larvae were reared in queen cups within a 48-well plate and incubated in the dark at 35 °C ~94⁄ RH (RH maintained using a saturated solution of K_2SO_4). Diet remaining at the beginning of each scheduled feeding was not removed for placing the new diet into the cups. Once the larvae finish feeding all volume of diet on seventh day, they were transferred to a new 48-well plate containing tissue paper (Kimwipes) inside the plates for pupation into adults. In this period, the plates were incubated in the dark at 35 °C ~75⁄ RH (RH maintained using a saturated solution of NaCl) until adult emergence.

Larval exposure to pesticides

Stock solutions were prepared for each 7 different pesticides used in the study. The selected pesticides for the study included 3 acaricides (amitraz, coumaphos, fluvalinate), 2 insecticides (chlorpyrifos, imidacloprid), 1 fungicide (chlorothalonil) and 1 herbicide (glyphosate). Concentrations were chosen based upon their occurrence in the pollen and wax within the hive (Mullin et al. 2010; Wu et al. 2011; Krupke et al 2012), alongside two control diets consisting of a no-treatment and acetone control. The concentrations for pollen and wax of each pesticide were, respectively: amitraz (181/4700 ppb), chlorothalonil (10380/1545 ppb), chlorpyrifos (127/33 ppb), coumaphos (730/11555 ppb), fluvalinate (294/28703 ppb), glyphosate (0.8/54 ppb), and imidacloprid (3.1/377 ppb). The pesticides were integrated individually into the larval diet and fed to the larvae at each of the scheduled feedings beginning at first day. Glyphosate and imidacloprid were dissolved into water while the other compounds into acetone. Each pesticide was added at a volume of 1 µL per bee for each diet. The consumption of diet and amount of active ingredients during larval stage are showed on Table 1.

Survival, developmental time and adult body weight

The survival and development of individual honey bee larvae was monitored daily in each rearing plate, from the 1st instar larval stage until adult emergence. The bees were daily inspected for this purpose; dead individuals identified by the absence of spiracle movements and presence of dark colors were immediately removed from the plates. Three replicates of 30 insects from three colonies were established for each concentration of pollen and wax of each pesticide beyond two controls. The developmental time (days) from 1st instar larval stage until

adult emergence was recorded for each insect. All of the insects that survived pesticide exposure during the larval period were weighted on an analytical scale (Mettler Toledo AL 204) to determine body mass when they reached the 1st day of emergence.

Detoxification genes expression

Newly emerged adult honey bee workers were collected for detoxification gene expression analysis. Eighteen individuals (6 per colony) for each treatment were flash-frozen in liquid nitrogen and kept in the -80°C freezer. Relative expression levels of insects were mearsured for eight genes (five cytochrome p450, one gluthatione s-transferase and two cAMP-dependent protein kinases) associated with detoxification activity in honey bee (Mao et al., 2011; Boncriastini et al., 2012; Schmehl et al., 2014). Bee abdomens were prepared on dry ice, and homogenized in lysis buffer with zirconium beads using a cool-prep FastPrep instrument. RNA was extracted using the Qiagen Miniprep kit, and Qiagen QIAshredder columns. Samples were then quantified using a Nanodrop 2000 and Dnase treated using the Turbo DNA-free reagents. RNA was again quantified and diluted to 2 micrograms to prepare cDNA using High Capacity cDNA Reverse Transcription Kit (Invitrogen 4374966) according to manufacturer's protocol. cDNA was then pooled into four separate groups per treatment to run Real Time quantitative PCR (*q*RT-PCR) using SYBR Green detection method (SsoAdvanced SYBR® Green Supermix polymerase, Biorad). RNA was pooled by grouping 4 individual bee samples into 1 pool (2 µg of each sample was pooled). Sixteen bees splited in 4 replications were quantified per treatment (bees 1,2,3,4 are replication 1, bees 5, 6, 7, 8, are replication 2 and so on with 4 replications). Analysis was based on statistical comparison of cycle threshold (Ct) values of treatment groups. The expression of each eight genes was normalized to the geometric mean (Vandesompele et al. 2002) of the two housekeeping genes *eIF-S8* and *actin* (Grozinger et al., 2003; Huising and Flik, 2005). The ΔΔCT method was used for relative quantification of gene expression. A H_2O and no-enzyme controls were included for each primer to ensure no contamination from DNA or primer dimers.

Cell damage in the midgut

Honey bees were collected at two developmental stages for cell deaths on midgut. Larvae (7-days old) and newly emerged adults for each treatment had their midgut dissected in insect physiological solution (0.1 M phosphate buffer at pH 7.4) and fixed in formalin 10⁄ (pH 7.4) overnight at 4 °C. The fixed samples were dehydrated in a crescent ethanol series (50⁄ – 100⁄) for 1 h in each solution, and embedded overnight in JB-4 historesin without hardener. After this period, the samples were placed into JB-4 historesin with hardener following the

manufacturer's recommendations (Leica RM 2255). Each sample was sectioned into 5 µm-thick slices with a glass knife on an automatic microtome. Two longitudinal slices (top and base) showing whole side view midgut were mounted on glass slides and incubated with proteinase K (10 µg/ml in 10 mM Tris/HCl, pH 7.4) for 1 h at 37 °C. Subsequently, the slices were rinsed 2x with phosphate buffer (pH 7.4) and submitted to the TUNNEL reaction with In Situ Cell Death Detection Kit, Fluorescein (Roche) during 1 h on darkness at 37 °C. Slices without TUNNEL cell detection was used as negative control. Slices treated with DNAse served as positive control. The slides were washed, and covered with anti-fading media (Mowiol, Sigma-Aldrich). The slides exhibiting the cells under study were analyzed under an epifluorescence microscope (Zeiss Axiostar Plus) using a WU filter and photographed by a CCD camera (Zeiss Axiocam MRM). Posteriorly, the percentage of cell with fragmented DNA (in two hundred cells) was counted in the sections of the middle of each midgut using the software Image-Pro Plus™ (MediaCybernetics, Bethesda, MD, USA). The DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich) was used to locate the cell nuclei and contrast with the TUNNEL marking. The average of two slices per midgut of nine bees (three per colony) was used in each treatment.

Antennal topography

The left antenna from fifteen newly emerged adult workers (5 per colony) were randomly collected of each treatment for scanning electron microscopy (SEM). Only bees showing complete extended wings and walking behavior were used in the experiment. The antennae bees were removed and fixed in 10／ formalin buffer (0.1 M, pH 7.2). The samples were washed in phosphate-buffered saline (PBS, 0.1 M), dehydrated in an ascending series of acetone solutions (50 to 100 ／), dried with HMDS (Hexamethyldisilazane), mounted on aluminum stubs, and metallized with gold using a sputter coating device. The samples were visualized using a LEO VP1430 SEM at Núcleo de Microscopia e Microanálise/UFV, Brazil. Images of antennae were acquired with fixed magnification (×240) and resolution (72 pixels/inch). We measured the length of parts of antennae (scape and flagellum) using the Image-Pro Plus program (Media Cybernetics) (Ravaiano et al. 2014).

Statistical Analysis

The results of the mortality bioassays were subjected to survival analysis using the non-parametric procedure LIFETEST (SAS Institute, 2008), in which survival curves are obtained using Kaplan-Meyer estimators. The bees that survived through emergence were treated as censored data. Developmental time, insect body mass, cell deaths on intestine, length of scape and flagellum for antenna were subjected to analysis of variance (ANOVA GLM procedure in

SAS) with Tukey's test HSD when necessary. Gene expression data were subjected to t-test performed for all multiple comparison statistics (SAS Institute, 2008). The assumptions of normality and homoscedasticity were checked before all data analysis (UNIVARIATE procedure in SAS).

3. RESULTS

Survival and development

The survival of honey bee exposed to all pesticides to concentrations found in wax were significantly impaired (Log-rank test: $\chi^2 = 848.01$; df = 8; $p < 0.001$). The stronger survival reductions were found in miticides amitraz, coumaphos and fluvalinate where the last one killed 100⁄ of bees on larval stage (Fig. 1A). Pesticide concentrations found in pollen, except to imidacloprid and glyphosate, also impaired survival of honey bees (Log-rank test: $\chi^2 = 400.36$; df = 8; $p < 0.001$). However, the strongest survival reduction was found on bees exposed to chlorpyrifos where only about 15⁄ of individuals reached adult emergence (Fig. 1B). The developmental time of bees exposed to concentrations found in wax was significantly affected by chlorothalonil and chlorpyrifos (Fig. Spl 1). Similarly, coumaphos, amitraz, chlorothalonil, chlorpyrifos and fluvalinate disturbed development of bees by concentrations found in pollen (Fig. Spl 2). The percentages of honey bees starting pupation and emergence on expected time (i.e, 10th and 21th days onwards, respectively) were lower in these compounds comparing with controls (Fig. Spl 2). Probably these pesticides changed feeding behavior or promoted any antifeedant effect on larvae whereas diets remained on cups on last day on larval stage (Fig. Spl 3), inducing higher lengthening of developmental time up to emergence of adults.

Detoxification genes expression

The overall gene expression of newly emerged adults exposed to pesticides on larval stage was not significantly different in comparison with adult workers exposed to water and acetone. However, the detoxification gene activities were significantly different in some treatments (Figs. 2 and 3). In the bees exposed to concentration found in wax, the gene PKA-C1 was down- and up-regulated by imidacloprid and chlorothalonil, respectively (Figs. 2A, 2D). The expression levels of CYP9S1 were up-regulated in response to chlorpyrifos and down-regulated in response to glyphosate (Figs. 2B, 2C). In addition, the genes PKA-R1 and CYP9Q2 were down- and up-regulated by imidacloprid and chlorpyrifos, respectively (Figs. 2A, 2C). In the bees exposed to concentration found in pollen, the genes CYP9S1 and CYP9Q2 were up-regulated in response to amitraz and chlorothalonil (Figs. 3A, 3C). Also, the gene PKA-R1 was up-regulated by amitraz, chlorpyrifos and chlorothalonil (Figs. 3A, 3B, 3C). PKA-C1 was up

regulated by chlorpyrifos. In addition, the GST-D1 activity was down-regulated in response to glyphosate (Fig. 3D). Excepting for chlorothalonil and chlorpyrifos that changed transcriptional levels for both wax and pollen concentrations, amitraz, glyphosate and imidacloprid impacted gene activities at higher concentrations. Fluvalinate affected the gene expression at lower concentration, while results with its highest concentration were not analyzed.

Body weight and antennal structure

The body weight of newly emerged adult bees exposed to imidacloprid, glyphosate, coumaphos and fluvalinate was not significantly different comparing with controls. However, the body weight of bees exposed to amitraz, chlorothalonil and chlorpyrifos was significantly lower than controls for both concentrations found in wax and pollen (Fig. 4). In addition, deformations on antennal structure were observed in adult bees exposed to all pesticides, except amitraz (Table 3). Twisted and malformed antennae were observed among newly emerged workers after pesticide treatment (Fig. 5). Uncompleted ecdysis and shortening of flagellum and scape were the more frequently aberrantions observed in the antenna (Fig. 5). There were no significant differences in the size of flagellum and scape of antenna between bees treated with pesticides and controls. In addition, it was not observed significant difference in the total number of sensillae between treated bees and control.

Cell damage in the midgut

Even though the pesticides disturbed survival, development, body weight and gene expressions of the bees, the pesticides did not induce cell damage in the midgut of larvae and adult bees (Fig. Spl 3). The number of cells (including regenerative and digestive cells) with nuclear DNA fragmentation was not significantly different between treatments and controls ($p > 0.05$).

4. DISCUSSION

Honey bees larvae can be exposed to a wide range of pesticides within colonies, where nectar, pollen and wax are frequently contaminated with insecticides, fungicides and miticides. Previous studies have been addressing pesticide impacts on honey bee larval survivorship (Aupinel et al., 2007; Charpentier et al., 2012; Zhu et al., 2014). Our findings have demonstrated that the *in vitro* chronic exposure to amitraz, coumaphos, chlorothalonil, chlorpyrifos, fluvalinate, imidacloprid and glyphosate via diet did reduce significantly the survival of honey bee workers. Additionaly, we have showed that pesticides caused multiple disorders at realistic concentrations found within colony pollen and wax in the field.

The symptom differences found in our study might be result from variations between mechanisms of action and concentration of each pesticide. It is not clear yet how the fungicide chlorothalonil and the herbicide glyphosate affect physiology of honey bees, while the modes of action of other pesticides tested here are well known (Haarmann et al., 2002; Burley et al. 2008; Boncristiani et al. 2012; Zhu et al., 2014). For example, amitraz is a formamidine, which acts as octopaminergic agonist in neural system of mite and insects (Yu, 2008). Coumaphos and chlorpyrifos are organophosphates, which inactivate acetylcholinesterase in neural synaptic clefts, and fluvalinate (a pyrethroid) blocks the voltage-gated sodium and calcium channels in neuron axions. Imidacloprid is a neonicotinoid that acts agonistically in the nicotinic acetylcholine receptors (Yu, 2008).

Although honey bees have substantially fewer xenobiotic detoxification enzymes than other insect species (Claudianos et al., 2006), the contact of bees with different compounds in nature not always represents a risk to colony healthy. However, the increase of stress associated with overloading these detoxifying mechanisms elicited by chronic pesticide exposure, such as miticide applications to control *Varroa* mites, can affect the colony fitness. Studies have reported that pesticide impact in several physiological pathways in adult honey bee workers, including changes in the activity of genes associated to detoxification, immunity and development (Mao et al., 2011; Mao et al., 2013; Boncristiani et al., 2012; Derecka et al., 2013; Schmehl et al., 2014). However, little is known about pesticide exposure on larval stage (Gregorc et al., 2012; Derecka et al., 2013).

According to our results, the expression of genes of three families of metabolic enzymes (cytochrome P450, gluthatione s-transferase and protein kinases) was significantly changed in newly emerged bees previously exposed to pesticides during larval stage. In the family CYP, the expression levels of CYP9S1 were up-regulated in response to amitraz, chlorothalonil and chlorpyrifos and down-regulated by glyphosate, whereas, the gene CYP9Q2 was up-regulated by amitraz, chlorothalonil and chlorpyrifos. These results were also found by Schmehl et al. (2014) but were not observed in other studies (Mao et al., 2011). Here, GST-D1 activity was down-regulated in response to fluvalinate, but was not significantly impacted in a prior study (Schmehl et al., 2014). On the other hand, pesticides did interfere with the expression levels of the other tested P450s (CYP305D1, CYP9Q1, and CYP9Q3), despite having been reported their expression increase in honey bees in response to pesticides (Mao et al., 2011, Schmehl et al., 2014). In the family PKA, the gene PKA-C1 was down-regulated by imidacloprid and up-regulated by chlorothalonil and chlorpyrifos. The gene PKA-R1 was down-regulated by imidacloprid, and up-regulated by amitraz, chlorothalonil and chlorpyrifos which corroborate with expression in adult bees exposed to miticides (Boncristiani et al., 2012).

Accordingly, there is evidence that pesticides can modify gene activities, but there is also a significant complexity in the responses to different pesticides, possibly modified by bee genetics, environment, nutritional conditions, and other factors (Schmehl et al., 2014).

Pesticides effects in honey bees may arise in different fashions, including mortality as well as a wide range of sublethal effects (Bryden et al., 2013; Fairbrother et al., 2014; Pisa et al., 2015). However, our results and other studies have showed that honey bee workers can activate detoxification genes responsible to metabolize these stressors (Mao et al., 2011, Boncristiani et al., 2012; Schmehl et al., 2014). Although the majority of detoxification mechanisms are induced after exposure, it is assumed that this process are energetically and metabolically expensive. Evidences of metabolic costs due to pesticide breakdown are sparse. However, changes gluconeogenesis and glycolysis pathways in honeybee larvae treated with imidacloprid and disturbs in trehalose metabolism of honey bees exposed to fipronil have been detected (Derecka et al., 2013; Aufauvre et al., 2014). Corroborating with this, we observed significant body weight reductions on newly emerged bees exposed on larval stage to amitraz, chlorothalonil and chlorpyrifos, which induced changes in expression levels of detoxification genes at the beginning of the adulthood. Although impacts on gene expression levels occur mainly during exposure in the larval stage (data not shown), the long period (larvae up to adult) expressing different enzymes of detoxification may require substantial amount of energy, which would affect the gain of body weight and the post-embryonic development of bees.

Despite pesticides can affect cell proliferation, cell death in the honey bee midgut and DNA damage in other organisms (Gregorc and Ellis, 2012; Forkpah et al., 2014; Bakry et al., 2015), the realistic field pesticide concentrations used here did not elicited DNA fragmentation in the midgut cells. However, the developmental time of bees exposed to amitraz, coumaphos, chlorothalonil, chlorpyrifos and fluvalinate was slightly longer than controls. Delay on development may be related to antifeedant effect (Boina et al., 2009; Barbosa et al., 2015), because part of the larvae did not consume contaminated diet on expected time. However, only honey bees exposed to amitraz, chlorothalonil and chlopyrifos exhibited lower body weight, showing no relation between weight and antiffedant effects. There are positive correlations between body weight, flight capacity and cognitive skills of forager bees, where heavy bees demonstrate better yield in such as activities (Scheiner, 2012; Ding et al., 2013). Thus, weight reductions caused by amitraz, chlorothalonil and chlopyrifos may affect flight, learning and memory of honey bees workers. It was observed deformations on antennal structure in newly emerged adults exposed to all pesticides, except amitraz. Although there were not significant differences in the size and number of sensillae, torsions in the anntenae

may induce sensorial systems impairments and consequently at learning, memory, communication and orientation of bees. Sublethal effects in the developmental time, body weight and ecdysis process (affecting antennae) may be related with protein kinase pathways, which are involved with ecdysteroid synthesis (Rybczynski et al., 2001; Gilbert et al., 2002; Rybczynski and Gilber, 2003). Thus, the changes in the levels of hormonal production may affect development and molting of bees.

In conclusion, the present study demonstrated that realistic field concentration of different pesticides (commonly found in pollen and wax) can disrupt survival of honey bee workers when exposed during the larval stage. Besides mortality, the pesticides promoted multiple disorders, such as changes in gene expression pattern, alterations in the duration of the life cycle, reductions in gain of weight and deformations in the antenna. Therefore, honey bees seem to be more susceptible to pesticides than previously reported, once low pesticide concentrations, including herbicide and fungicide, affected them. We believe that all symptoms observed here are result mainly of pesticide mode of action (most are neurotoxic), but the energetic cost associated with detoxification process and unbalanced transcriptional levels of genes involved with hormonal production could be involved with disturbs in development and morphological anomalies.

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Figure 1.

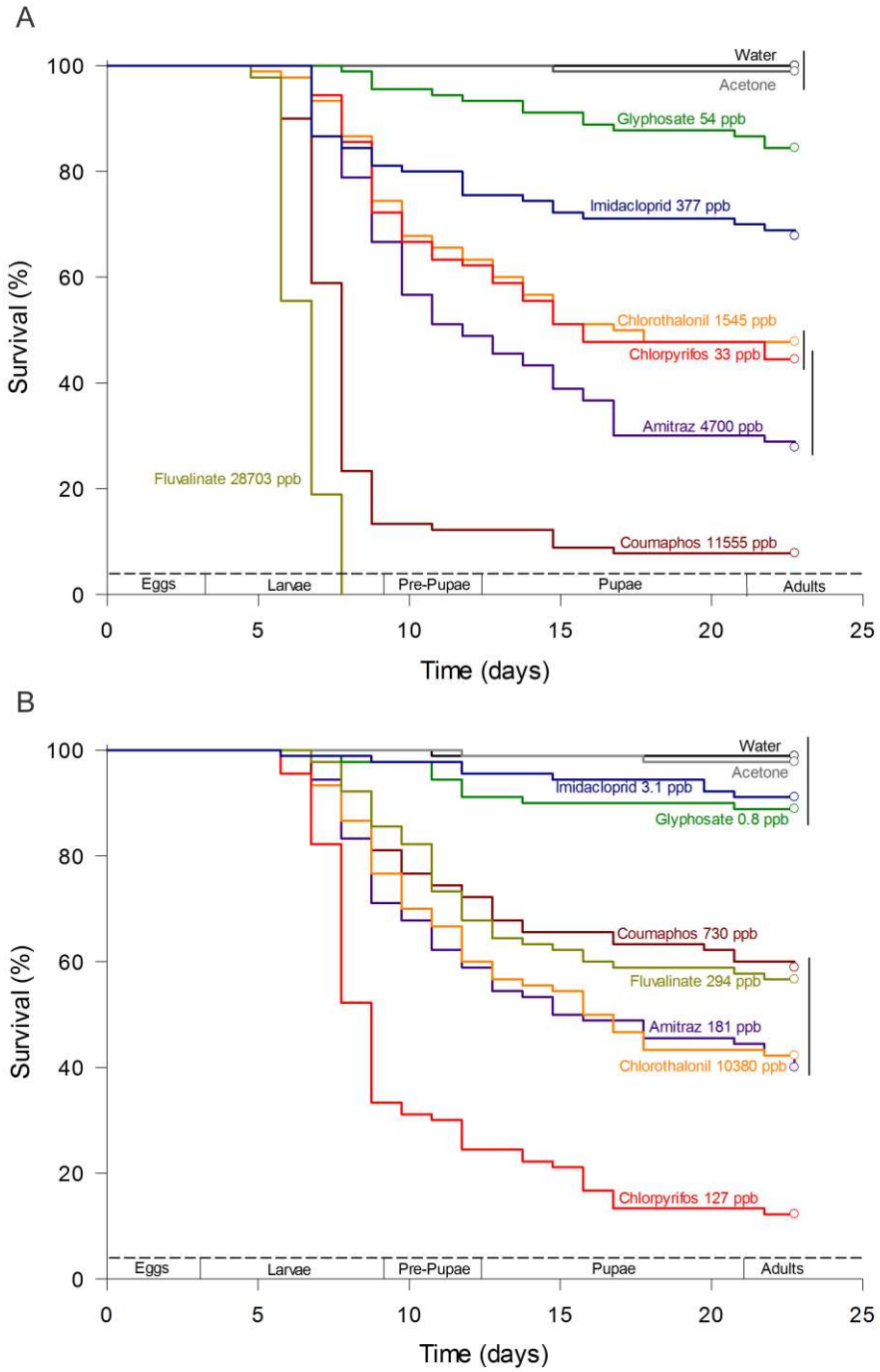

Figure 1. Survival plots of honey bee workers (*Apis mellifera*) fed contaminated diets containing realistic concentrations of pesticides found in wax (A) and pollen (B) during larval stage. Individual larvae were chronically exposed to pesticides for a period of six days *in vitro*. There is no significant difference between water and acetone controls. All pesticides affected survival of bees up to adult emergence, except for imidacloprid and glyphosate with concentrations found in pollen. Survival curves grouped by the same line are not significantly different by Holm-Sidak's test ($p > 0.05$).

Figure 2.

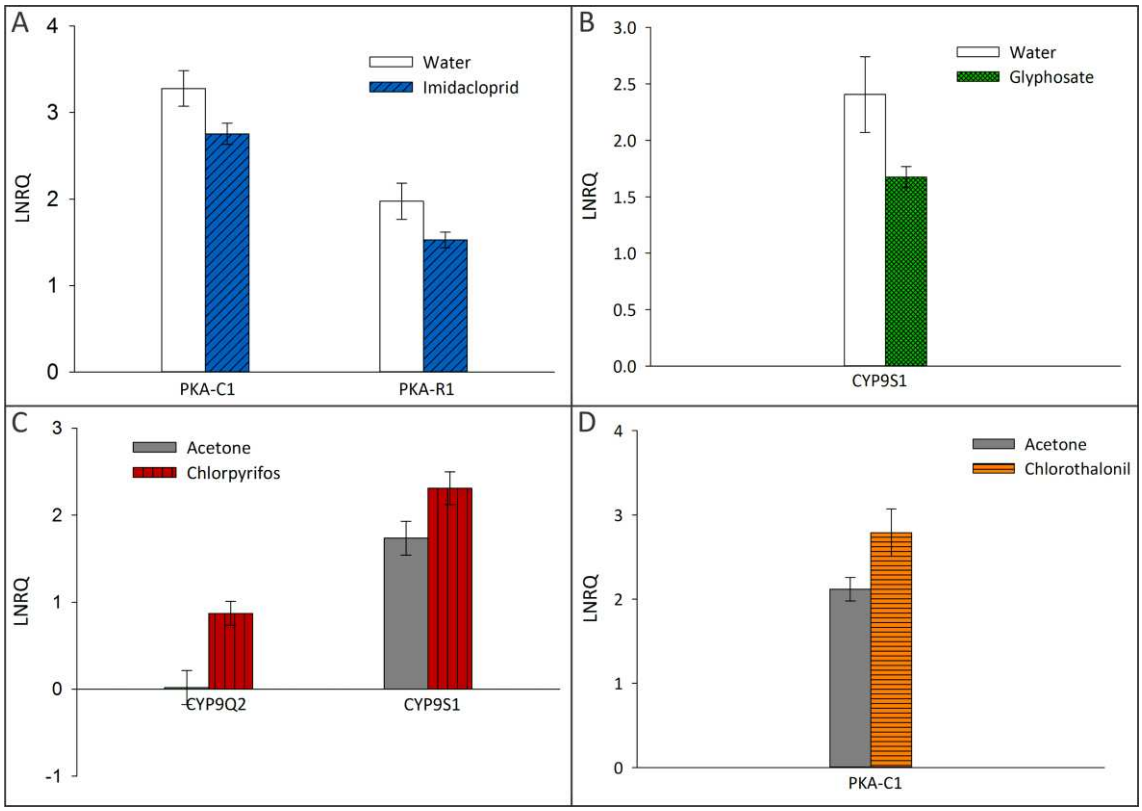

Figure 2. Relative expression levels of genes associated to detoxification process in newly emerged adult honey bee in response to field-relevant pesticide found in wax. Individual larvae were chronically exposed to pesticides for a period of six days *in vitro*. After the emergence, individual adults were homogenized and RNA extracted and expression levels calculated. The gene PKA-C1 was down-regulated and up-regulated by imidacloprid and chlorothalonil, respectively (A, D). The expression levels of CYP9S1 were up-regulated and down-regulated by chlorpyrifos and glyphosate, respectively (B, C). The genes PKA-R1 was down-regulated by imidacloprid and CYP9Q2 was up-regulated by chlorpyrifos (A, C). The means ± standard errors are represented. All graphs show significant differences between treatments and respective controls by t-test ($p < 0.05$).

Figure 3.

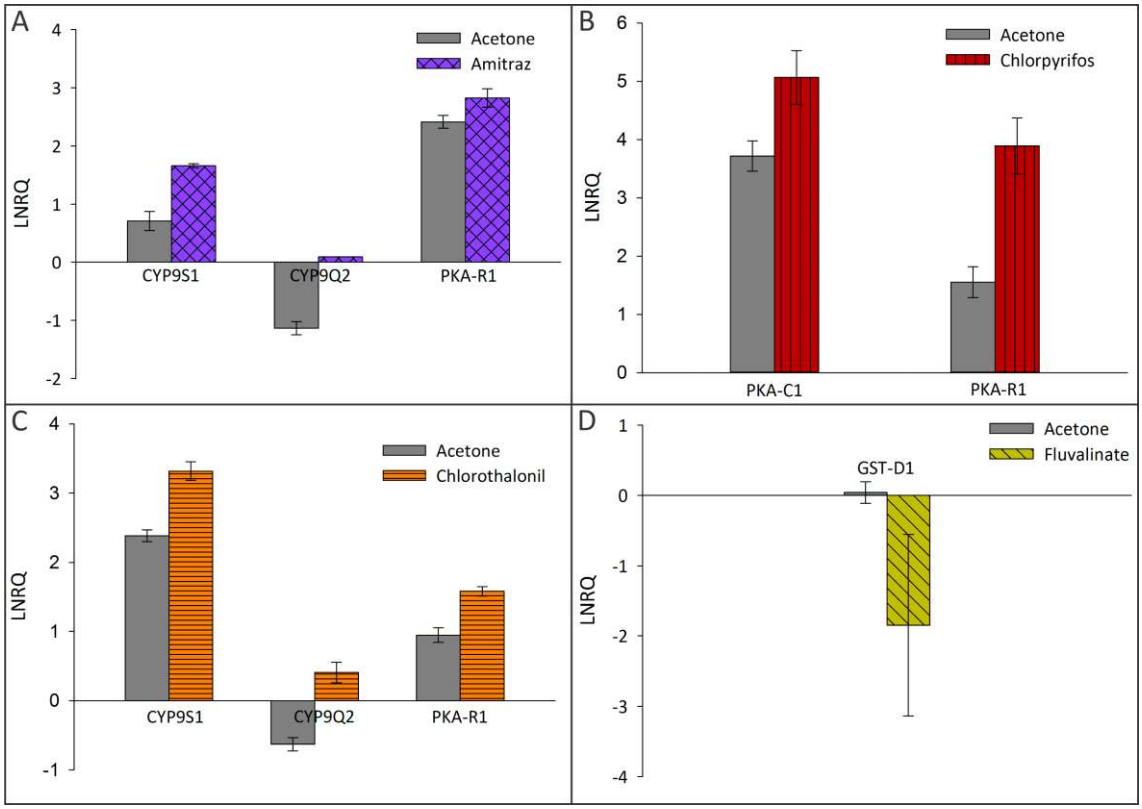

Figure 3. Relative expression levels of genes associated to detoxification process in newly emerged adult honey bee in response to field-relevant pesticide found in pollen. Individual larvae were chronically exposed to pesticides for a period of six days *in vitro*. After emergence, individual adults were homogenized and RNA extracted and expression levels were calculated. The genes CYP9S1 and CYP9Q2 were up-regulated by amitraz and chlorothalonil comparing with controls (A, C). PKA-R1 was up-regulated by amitraz, chlorpyrifos and chlorothalonil (A, B, C). PKA-C1 was up-regulated in response to chlorpyrifos (B). The gene GST-D1 was down-regulated by fluvalinate (D). The means ± standard errors are represented. All graphs show significant differences between treatments and respective controls by t-test ($p < 0.05$).

Figure 4.

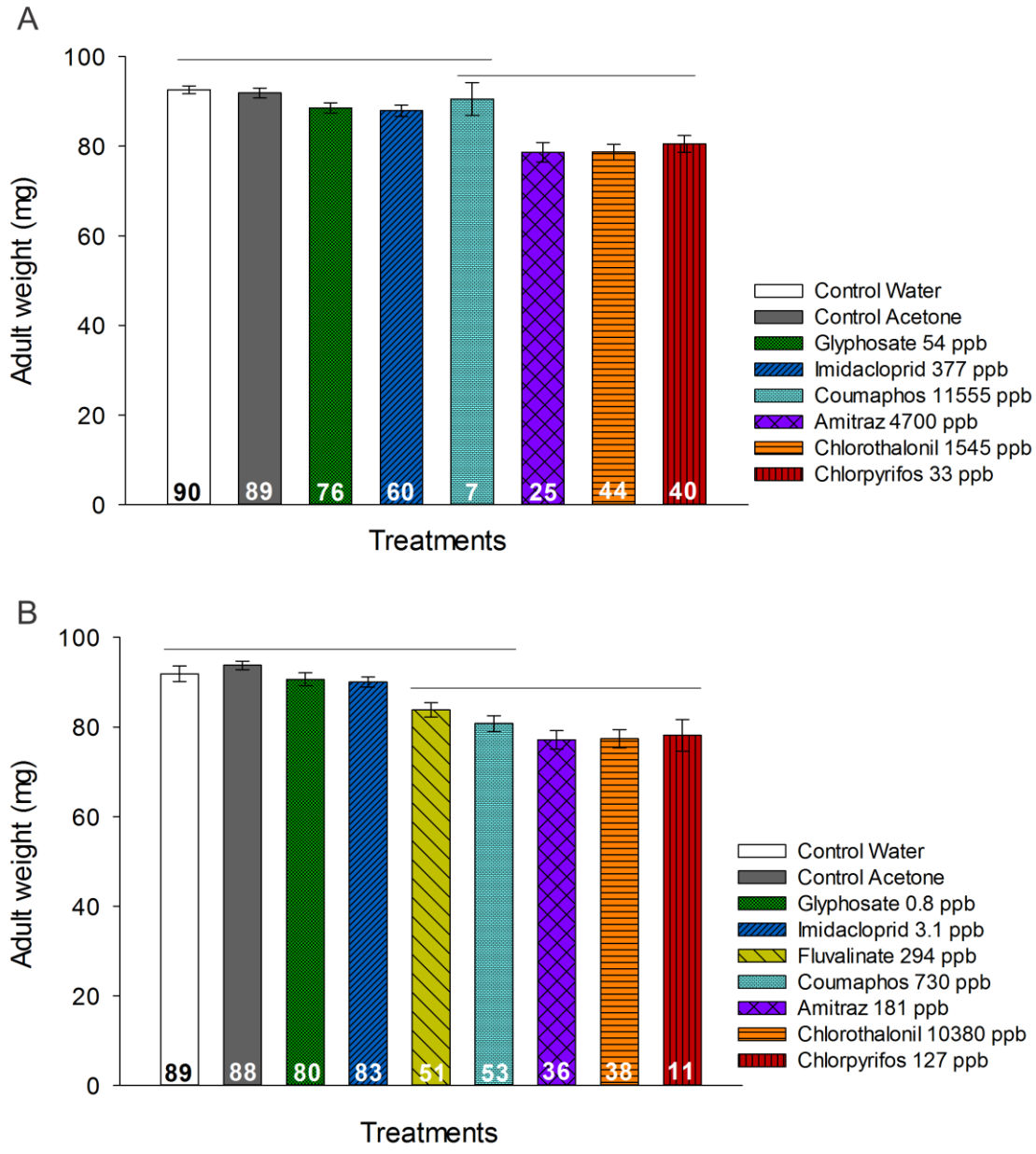

Figure 4. Body weight of newly emerged worker bees (*Apis mellifera*) reared on larval stage with pesticide-contaminated diets containing realistic pesticide concentrations found on wax (A) and pollen (B). The numbers of bees weighted are indicated inside in each treatment bars. Amitraz, chlorothalonil and chorpyrifos reduced significantly the body weight of adult bees comparing with controls. The means ± standard errors are represented. Bars grouped by the same line are not significantly different by HSD Tukey's test ($p > 0.05$).

Figure 5.

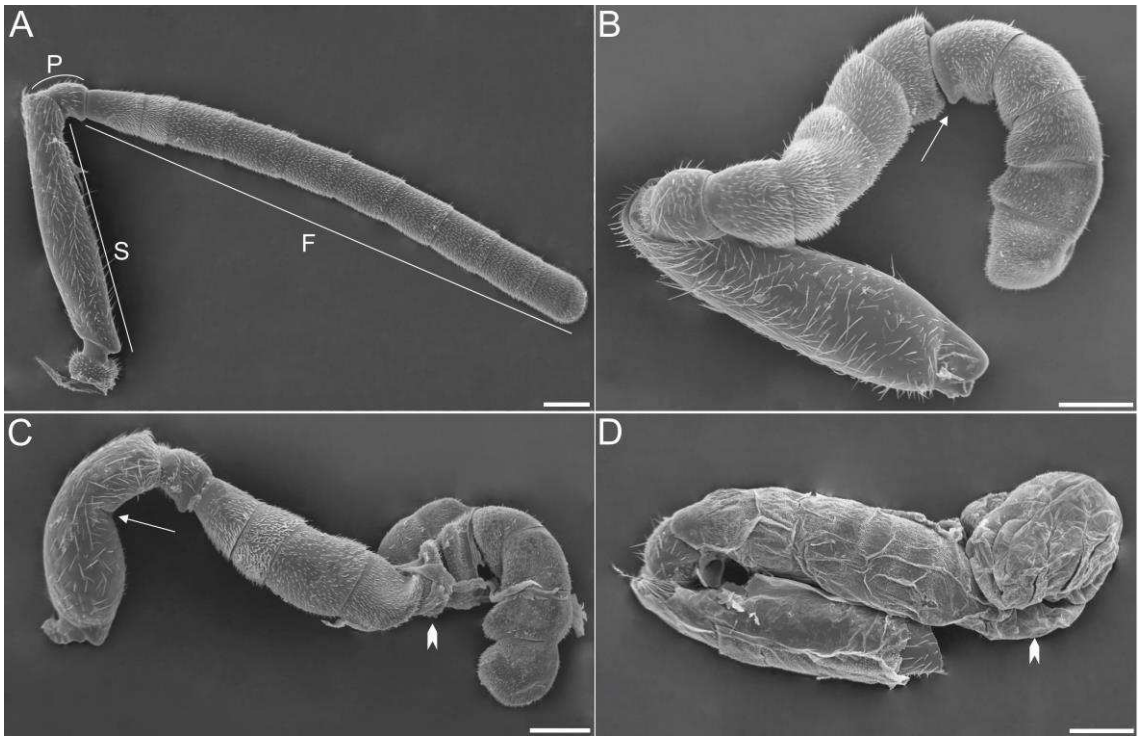

Figure 5. Scanning electron microscopy of antenna of newly emerged adult worker bees (*Apis mellifera*) reared on pesticide-contaminated diets during larval stages. The micrographs correspond to the left antenna of individuals exposed to acetone (A), imidacloprid (B), chorpyrifos (C) and chlorothalonil (D) in pollen concentrations. Solid arrows show twists on flagellum (F) and scape (S). Arrowheads demonstrate reminiscent pupae integument covering adult antennas, which indicate malformation during the ecdysis. P- pedicel. Bar = 200 µm.

Table 1.

			Active ingredients consumed per day (µg)					Total (µg)
Pesticide	**Source**	**Concentration (ppb)**	**D1 + D2 (in 20 µg of diet)**	**D3 (in 20 µg of diet)**	**D4 (in 30 µg of diet)**	**D5 (in 40 µg of diet)**	**D6 (in 50 µg of diet)**	**Active ingredient/larva (in 160 µg of diet)**
Amitraz	Pollen	181	0.00362	0.00362	0.00543	0.00724	0.00905	0.02896
Chloropyrifos	Pollen	127	0.00254	0.00254	0.00381	0.00508	0.00635	0.02032
Chlorothalonil	Pollen	10380	0.20762	0.20762	0.31140	0.41521	0.51911	1.66080
Coumaphos	Pollen	730	0.01461	0.01461	0.02190	0.02921	0.03651	0.11680
Fluvalinate	Pollen	294	0.000036	0.000036	0.000055	0.000074	0.000092	0.00029
Glyphosate	Pollen	0,8	0.000016	0.000016	0.000024	0.000032	0.00004	0.00013
Imidacloprid	Pollen	3,1	0.000023	0.000032	0.000035	0.000047	0.000058	0.00019
Amitraz	Wax	4700	0.09401	0.09402	0.14111	0.18821	0.23502	0.75200
Chloropyrifos	Wax	33	0.00066	0.00066	0.00099	0.00132	0.00165	0.00528
Chlorothalonil	Wax	1545	0.03092	0.03092	0.04635	0.06182	0.07725	0.24720
Coumaphos	Wax	11555	0.23113	0.23111	0.34665	0.46223	0.57775	1.84880
Fluvalinate	Wax	28703	0.57406	0.57406	0.86109	1.14812	1.43515	4.59248
Glyphosate	Wax	54	0.00108	0.00108	0.00162	0.00216	0.00271	0.00864
Imidacloprid	Wax	377	0.00754	0.00754	0.01131	0.01508	0.01885	0.06032

Table 1. Amount of active ingredient consumed by larvae daily. The diet ingested on day 1 and 2 (20 µg) was fully dispensed at the first day. On the second day, we only monitored the survival and did not feed the larvae. From third day onwards, the larvae were fed dialy.

Table 2.

Target gene	Description	Category	Forward primer	Reverse primer	References
CYP9S1	Cytochrome P4509S1	Detoxification	CTAATTTTCGCGTTCCCAAA	CTCCCGTTACGTTTGTCGAT	Schmehl et al., 2014
CYP305D1	Cytochrome P450305D1	Detoxification	TCGATCTTTTTCTCGCTGGT	TTGCTTTGTCCTCCATGTTG	Schmehl et al., 2014
CYP9Q1	Cytochrome P4509Q1	Detoxification	TCGAGAAGTTTTTCCACCG	CTCTTTCCTCCTCGATTG	Mao et al., 2011; Schmehl et al., 2014
CYP9Q2	Cytochrome P4509Q2	Detoxification	GATTATCGCCTATTATTACTG	GTTCTCCTTCCCTCTGAT	Mao et al., 2011; Schmehl et al., 2014
CYP9Q3	Cytochrome P4509Q3	Detoxification	GTTCCGGGAAAATGACTAC	GGTCAAAATGGTGGTGAC	Mao et al., 2011; Schmehl et al., 2014
GST-D1	Gluthatione s–transferaseD1	Detoxification	GCCGCTTCAAAAGAAGTACG	GTGGCGAAAACAAGGATGAT	Schmehl et al., 2014
PKA-C1	cAMP – dependent kinase type 1	Detoxification	TCCATTTTTGGTCTCCTTGC	GTAAAAGCGCGAATGTGGTT	Boncristiani et al., 2012
PKA-R1	cAMP – dependent kinase type 1 regulatory subunit	Detoxification	GAAGCAATTATTCGGCAAGG	TCACCGAAACTTCCACCTTC	Boncristiani et al., 2012
eIF3-S8	Eukaryotic translation initiation factor 3 subunit 8	Housekeeping	TGAGTGTCTGCTATGGATTGCAA	TCGCGGCTCGTGGTAAA	
Actin	Actin protein	Housekeeping	CCTAGCACCATCCACCATGAA	GAAGCAAGAATTGACCCACCAA	

Table 2. Gene descriptions and primer sequences used for *q*RT-PCR to check the relative expression levels of eight selected genes associated to detoxification (5 cytochrome p450 detoxification genes, 1 gluthatione s-transferase and 2 cAMP-dependent kinase genes) in response to field-relevant pesticide exposure.

Table 3.

Treatment	Source	Concentrations (ppb)	Sampled bees (N)	Deformed bee (N)	Deformed bee (/)
Control water	-	-	15	0	0
Control acetone	-	-	15	0	0
Amitraz	Wax	4700	15	0	0
Chlorpyrifos	Wax	33	15	2	13.33
Chlorothalonil	Wax	1545	15	1	6.66
Glyphosate	Wax	54	15	1	6.66
Imidacloprid	Wax	377	15	2	13.33
Control water	-	-	15	0	0
Control acetone	-	-	15	0	0
Amitraz	Pollen	181	15	0	0
Chlorpyrifos	Pollen	127	15	2	13.33
Chlorothalonil	Pollen	10380	15	3	20.00
Coumaphos	Pollen	730	15	1	6.66
Fluvalinate	Pollen	294	15	1	6.66
Glyphosate	Pollen	0.8	15	0	0
Imidacloprid	Pollen	3.1	15	1	6.66

Table 3. Number and percentage of bees with deformed antennal structure in each treatment. Coumaphos and fluvalinate in wax concentrations were not analyzed because few individuals treated with coumaphos reached the emergence and fluvalinate killed 100/ of bees on larval stage.

Figure Spl 1.

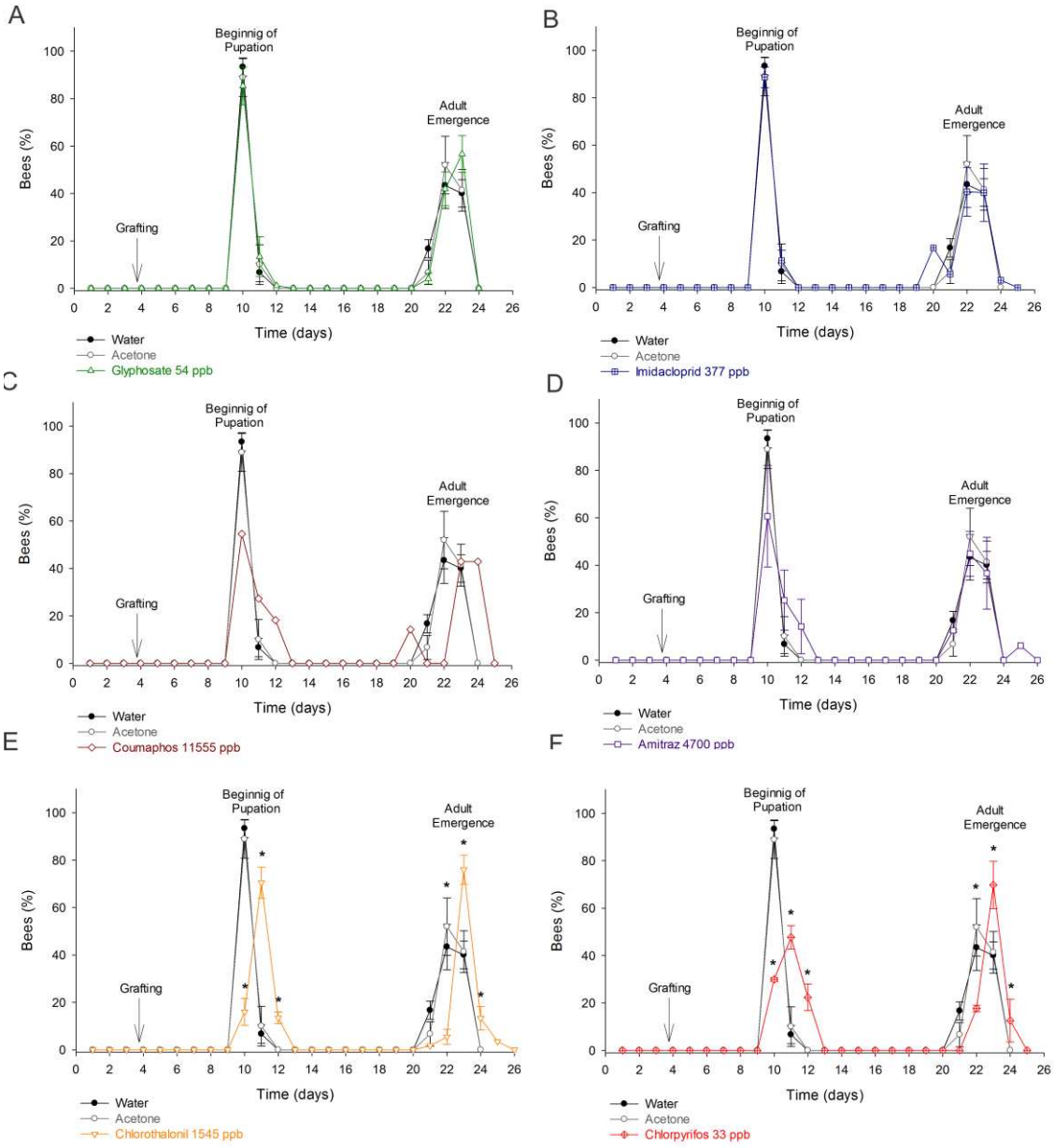

Figure Spl 1. Development of worker bees (*Apis mellifera*) reared on pesticide-contaminated diets containing realistic concentrations of pesticides found in wax. The beginning of pupation and adult emergence times are showed for each treatment. Glyphosate (A) and imidacloprid (B) did not affect development of bees. In the case of coumaphos (C) and amitraz (D), there were not enough alive bees for statistical analyses. Chlorothalonil (E) and chlorpyrifos (F) disturbed the development of bees when compared with the controls. Symbols (*) represent significantly differences on the proportion of bees in each phase between pesticide and controls by analysis of variance - ANOVA ($p < 0.05$).

Figure Spl 2.

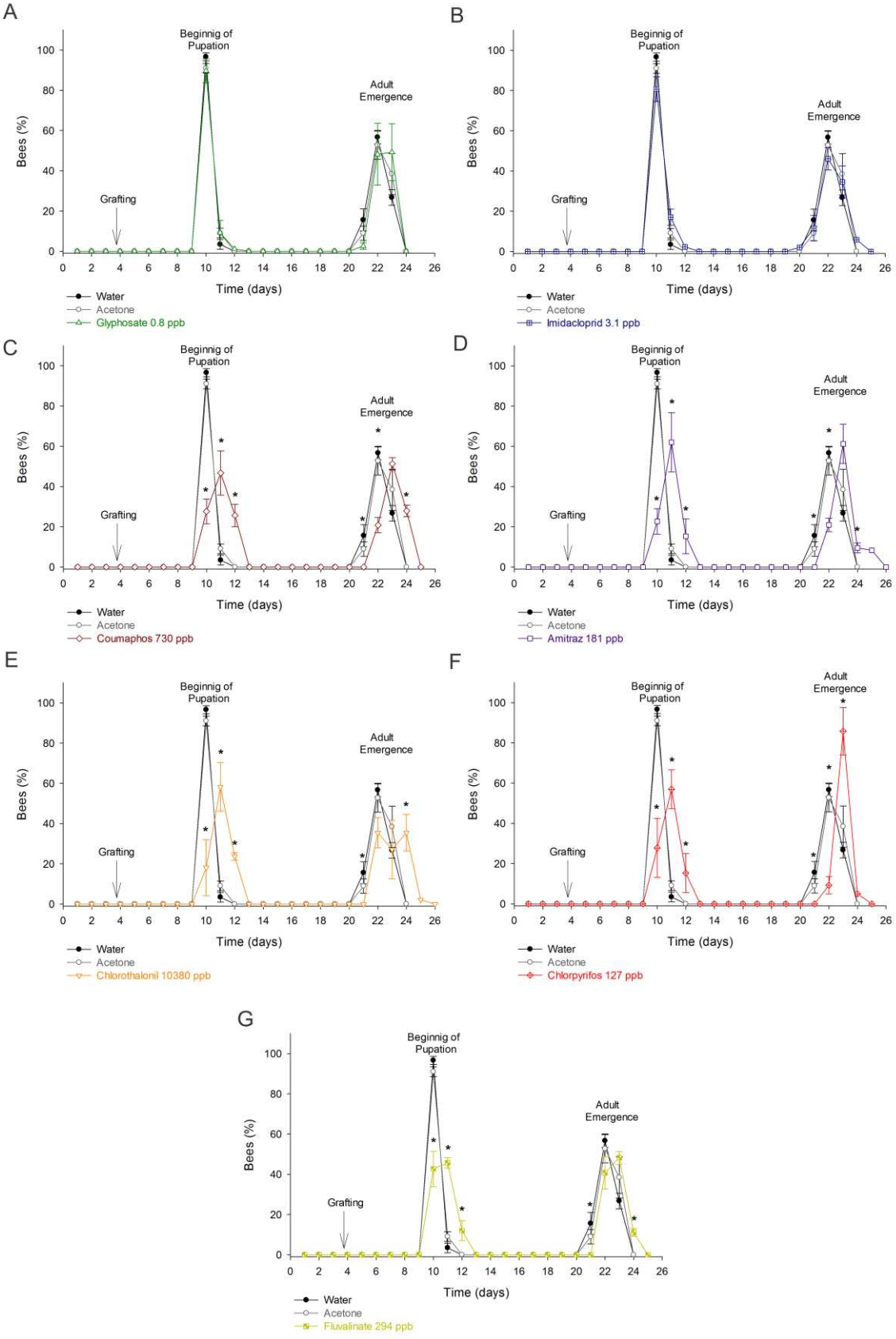
A
Beginnig of Pupation
Adult Emergence
Grafting
Bees (%)
Time (days)
Water
Acetone
Glyphosate 0.8 ppb
B
Beginnig of Pupation
Adult Emergence
Grafting
Bees (%)
Time (days)
Water
Acetone
Imidacloprid 3.1 ppb
C
Beginnig of Pupation
Adult Emergence
Grafting
Bees (%)
Time (days)
Water
Acetone
Coumaphos 730 ppb
D
Beginnig of Pupation
Adult Emergence
Grafting
Bees (%)
Time (days)
Water
Acetone
Amitraz 181 ppb
E
Beginnig of Pupation
Adult Emergence
Grafting
Bees (%)
Time (days)
Water
Acetone
Chlorothalonil 10380 ppb
F
Beginnig of Pupation
Adult Emergence
Grafting
Bees (%)
Time (days)
Water
Acetone
Chlorpyrifos 127 ppb
G
Beginnig of Pupation
Adult Emergence
Grafting
Bees (%)
Time (days)
Water
Acetone
Fluvalinate 294 ppb

Figure Spl 2. Development of worker bees (*Apis mellifera*) reared on pesticide-contaminated diets containing realistic concentration of pesticidess found in pollen. The beginning of pupation and adult emergence are showed for each for treatment. Glyphosate (A) and imidacloprid (B) did not affect the development of bees. However, coumaphos (C), amitraz (D), chlorothalonil (E), chlorpyrifos (F) and fluvalinate (G) disturb the development of bees, when compared to controls. Symbols (*) represent significantly differences on the proportion of bees in each phase between pesticide and controls by analysis of variance - ANOVA ($p < 0.05$)**.**

Figure Spl 3.

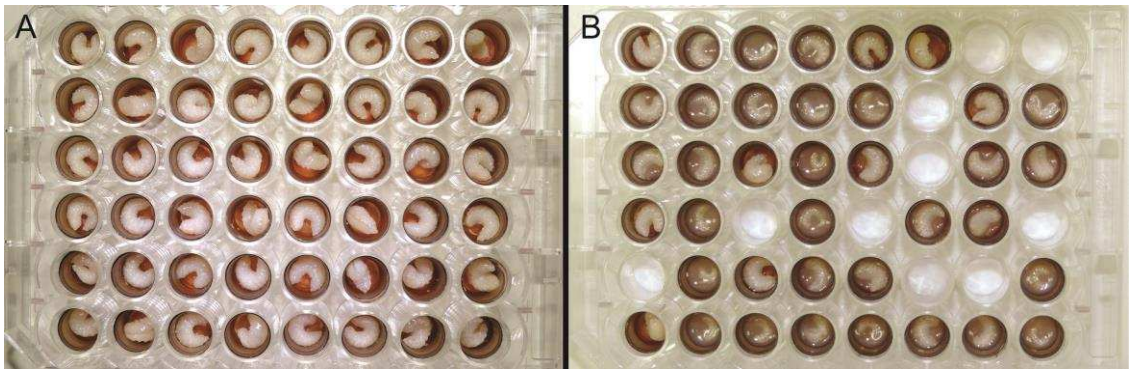

Figure Spl 3. In vitro 48-well plates with honey bee larvae on 7th day of development for the control (A) and chlorothalonil (B). The larvae of the control consumed all diet on expected time, and showed uniform size. At this time, all the larvae in the control are ready to pupate. The plate with diet containing chlorothalonil has some larvae with smaller size than control and diet remain inside the cups. These “late bees” pupated and emerged later than the control. Empty wells represent larval mortality.

Figure Spl 4.

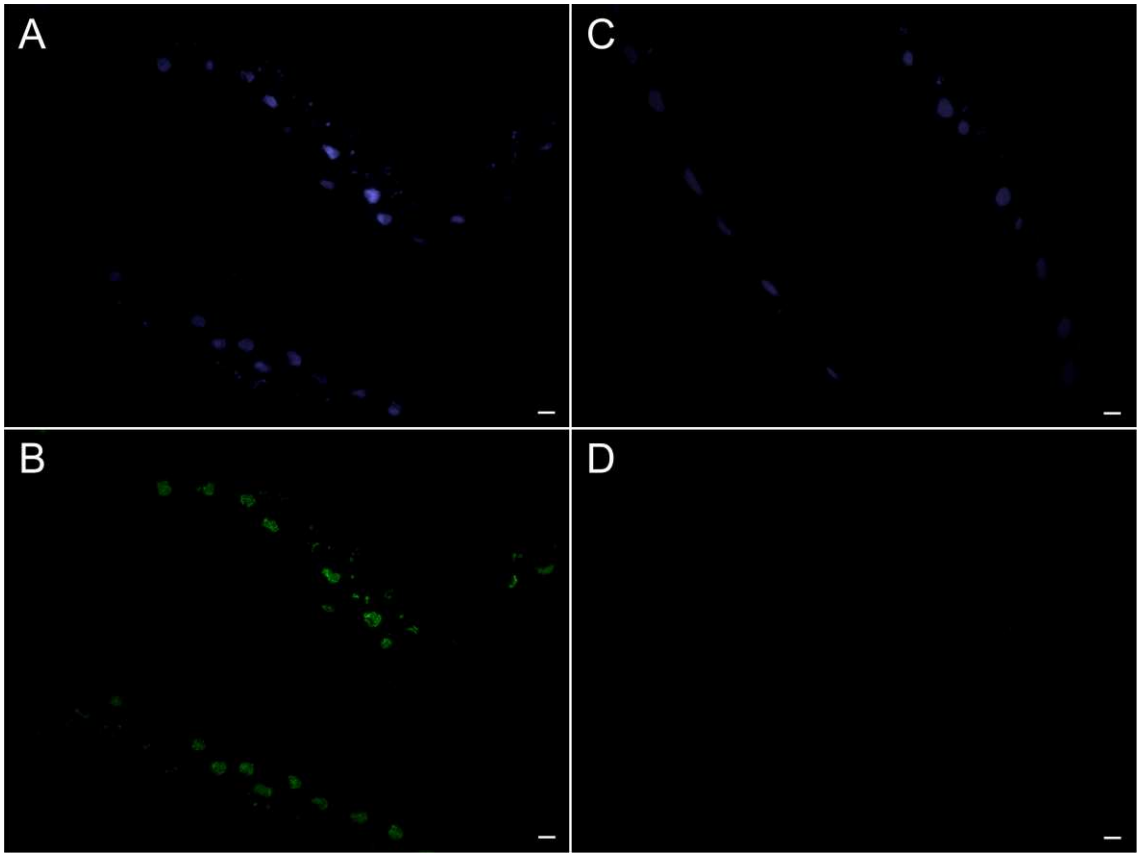

Figure Spl 4. Stained midgut cells of honey bee adult workers exposed to pesticides. A and C show stained cell nuclei with DAPI (blue). B (positive control treated with DNAse) and E (coumaphos) show the cell nuclei (green) with DNA fragmentation (after TUNEL reaction). Bars: 10 µm.

CONCLUSIONS

- Structural changes in the volume of mushroom bodies and antennal lobes in the brains of workers of the native stingless bee *Melipona quadrifasciata* are related to aging and rearing conditions (field vs laboratory). Although only age-dependent enlargement was observed in the antennal lobes, significant volume increase (21 ⁄) in the neuropils of the mushroom bodies occurred before the foraging age, in contrast to honeybees. In addition, the environmental complexity led to a significant increase in both the mushroom body volume and in the walking activity. Such differences in the stingless bees as compared with the honeybee may assist in relating brain evolution and plasticity with the behavior in these highly eusocial insects.

- The adult stingless bee workers exhibited high oral insecticide susceptibility, with LD50s for imidacloprid and spinosad. Imidacloprid also impaired worker respiration and overall group activity and flight, while spinosad significantly impaired worker flight, despite exhibiting higher oral toxicity to adult workers than imidacloprid. Accordingly, bioinsecticides should not be exempted from risk assessment analysis due to their lethal and sublethal components.

- The improved rearing method of honey bee larvae reduced the individual mortality to zero. By avoiding the physical contact between larvae and their feces is crucial to reduce the mortality, maybe reducing the fungal and bacterial infections. In addition, high quality of diet components, mainly royal jelly, is essential for the successful *in vitro* rearing of honey bees.

- Realistic field concentration of different pesticides can disrupt survival of honey bee workers when exposed on larval stage. Besides mortality, the pesticides can promote multiple disorders, such as changes in gene expression pattern, alterations in the life cycle, reductions in the body weight and deformations in antennal structure. These symptoms resulted mainly of pesticide mode of actions. However, the energetic cost associated with detoxification process may disturb the development and morphological abnormalities.