WAGNER FARIA BARBOSA

TOXICITY ASSESSMENT OF REDUCED-RISK (BIO)PESTICIDES ON NON-*Apis* BEES

Thesis presented to the Universidade Federal de Viçosa, as part of the requirements for the degree of *Doctor Scientiae* in Entomology, in co-tutelage with the Faculty of Bioscience Engineering of Ghent University.

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“When you resemble Christ in what you do,
there is your vocation”

René Kivitz

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Quoting a worldwide-known Brazilian singer so called King Roberto Carlos: "*I have so much to say but I can't put it in words*". Indeed, I have so many grateful feelings that I don't how properly say for all relatives, friends, colleagues and promoters of mine that took part of my life during these almost 4.5 years as PhD student. By God's provision, I believe I was greatly blessed for all these people and with this opportunity I would like to acknowledge and thank them all.

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BARBOSA, Wagner Faria, D.Sc., Universidade Federal de Viçosa, janeiro de 2015. **Avaliação da toxicidade de (bio)pesticidas de risco reduzido em abelhas não-*Apis***. Orientadores: Raul Narciso Carvalho Guedes e Guy Smagghe. Coorientadores: Gustavo Ferreira Martins e Maria Augusta Lima Siqueira.

Já tem sido dito que a raça humana e a natureza seriam seriamente impactadas se não houvesse mais abelhas no mundo. De fato, abelhas são organismos cruciais para polinização sem a qual a manutenção e conservação de diferentes biomas através do mundo seriam prejudicadas. Para nós, seres humanos, tal relevância ecológica é complementada ainda pela importância econômica e social desses organismos através da polinização de diversas plantas cultivadas e produção de especiarias tais como, mel, própolis e cera. Nas últimas décadas, no entanto, o receio da perda de tais polinizadores tem se tornado uma realidade com diversos declínios na diversidade e abundância de abelhas sendo detectadas pelo mundo. Pesados esforços têm sido feitos para investigar as causas e consequentemente conceber medidas mitigatórias para o problema de declínio de abelhas. Um dos principais fatores de risco identificados e relacionados ao declínio de colônias tem sido o envenenamento de abelhas por pesticidas na agricultura, particularmente inseticidas. Apesar da importância de uma enorme variedade de espécies de abelhas espalhadas pelo mundo, a grande maioria dos estudos ecotoxicológicos e de risco, no entanto, tem ficado circunscrita a uma única espécie modelo, a abelha melífera, *Apis melífera*. Isto se deve ao seu valor incontestável, principalmente em regiões do hemisfério norte, onde a polinização comercial por essa espécie é intensamente utilizada. Adicionalmente, uma segunda e grande deficiência de tais estudos é sua circunscrição a poucos inseticidas, tais como neonicotinoides, o que acaba por negligenciar os riscos potenciais de outros compostos em abelhas. Compostos conhecidos como inseticidas de risco reduzidos e bioinsecticides têm sido apresentados como produtos alternativos para o manejo de culturas possuindo supostamente baixa toxicidade a abelhas polinizadoras. No entanto, devido à exclusividade dos estudos regulatórios com abelhas melíferas, e a crença de que a origem natural especificamente de bioinseticidas determina baixo potencial de risco a organismos benéficos, a rotulagem de tais compostos pode ser mal interpretada e sua segurança a espécies de abelhas não-*Apis* é não ser passível de avaliação e questionamentos devidos. O presente trabalho buscou o preenchimento de algumas dessas lacunas nas avaliações de risco através de estudos de laboratório explorando potenciais impactos de um seleto grupo de inseticidas de risco de reduzido e bioinseticidas em abelhas nativas, cuja importância ecológica e econômica pode até mesmo ultrapassar *A. mellifera*. Como principais resultados, no primeiro alvo das investigações, o contato e a ingestão de espinosade demonstrou alta toxicidade em operárias de duas espécies de abelhas sem ferrão, *Partamona helleri e Scaptotrigona xanthotrica*, importantes polinizadores de plantas nativas e cultivadas na América Neotropical. Azadiractina e chlorantraniliprole, no entanto, exibiram baixa toxicidade a essas espécies, com sobrevivência em torno de 100% após o contato ou ingestão com suas concentrações recomendadas. Contudo, ambos os compostos comprometeram a atividade de vôo vertical livre desses insetos. No segundo alvo das investigações, os efeitos letais e subletais da azadiractina e espinosade foram avaliados através da contaminação da dieta larval da abelha sem ferrão *Melipona quadrifasciata anthidioides*, outro importante polinizador na região Neotropical. Nesse estudo, a sobrevivência das larvas e o peso das pupas sobreviventes diminuíram com aumento da dose de ambos os compostos. Além disso, tanto azadiractina quanto espinosade produziram pupas e adultos deformados. Adultos sobreviventes à exposição durante a fase larval tiveram ainda sua atividade de caminhamento comprometida mediante exposição ao espinosade. Finalmente, no terceiro estudo, a ingestão crônica de azadiractina comprometeu a sobrevivência e a reprodução de microcolônias de espécie de mamangaba *Bombus terrestris*, uma importante espécie polinizadora intensamente utilizada para polinização comercial em casas-de-vegetação e que prevalece no Hemisfério Norte. Além disso, em um ensaio de laboratório onde as operárias de *B. terrestris* necessitaram forragear, os efeitos letais e subletais na reprodução foram ainda mais fortes sob o efeito da ingestão de azadiractina em relação ao ensaio sem forrageamento. Tais evidências desafiam a noção de segurança de inseticidas de risco reduzido e bioinseticidas quando espécies não-*Apis* são utilizadas em estudos toxicológicos, o que merece melhor atenção. Como futuras perspectivas, recomendamos a complementariedade de nossos estudos com avaliações de semi-campo e campo, onde por um lado, a degradação dos compostos ou a

pequena quantia deles que normalmente chegam ao pollen e néctar podem atenuar os potenciais efeitos deletérios, mas por outro lado a necessidade de forrageamento pode exarcerbar o risco advindo de exposição inseticida em contextos mais realísticos.

SAMEVATTING

BARBOSA, Wagner Faria, D.Sc., Universidade Federal de Viçosa, januari 2015. **Bepaling van de toxiciteit van (bio)verminderd risico van pesticiden in niet-*Apis* bijen**. Adviseurs: Raul Narciso Carvalho Guedes en Guy Smagghe. Co-adviseurs: Gustavo Ferreira Martins en Maria Augusta Lima Siqueira.

Men kan gerust stellen dat de natuur en de mens een serieuze impact zouden ondervinden als bijen zouden uitsterven, want bijen zijn cruciale organismen voor de bestuiving. Zonder hen zou het voortbestaan en behoud van verschillende biomen in het gedrang komen. Hun ecologisch belang wordt verder onderstreept door de bestuiving van vele wilde en gecultiveerde plantensoorten alsook de productie van honing, koninginnenbrij, was en propolis, wat hen een extra economische en sociale waarde geeft. De voorbije jaren is de bezorgdheid rond het verlies van bestuivende insecten toegenomen door verschillende rapporten over het verlies aan bijenpopulaties wereldwijd. Er zijn bijgevolg grote inspanningen geleverd om de oorzaken hiervan te onderzoeken en manieren te bedenken om het verlies aan bijen tegen te gaan. Een lijst van mogelijke oorzaken is daarbij opgesteld waaronder vergiftiging door pesticiden, in het bijzonder insecticiden, waarvan wordt aangenomen dat ze één van de hoofdoorzaken zijn. De meerderheid van de ecotoxicologische risicostudies is echter gebaseerd op één specifieke modelsoort, namelijk de honingbij *Apis mellifera* en dit ondanks het belang van een grote verscheidenheid aan bijensoorten wereldwijd. Honingbijen zijn dan wel bestuivers van onbetwistbare waarde, in het bijzonder in de Noordelijke hemisfeer waar men intensief gebruikt maakt van honingbijen voor de bestuiving van commerciële gewassen. Daarom verdienen ze ook de nodige aandacht. Een tweede tekortkoming van deze studies is dat ze zich meestal beperken tot een beperkt aantal stoffen zoals de neonicotinoïde insecticides, waardoor het potentiële risico van andere stoffen genegeerd wordt. De insecticiden met een laag risico en bio-insecticiden worden vaak voorgesteld als alternatief voor de bescherming van gewassen tegen schadelijke insecten, omdat ze beweren weinig tot niet toxisch te zijn voor de bestuivers. Door de nadruk voor honingbijen in de regelgeving tot erkenning van het gebruik van een pesticide en de perceptie dat de bio-insecticiden veilig zijn voor nuttige organismen omdat ze van natuurlijke oorsprong zijn, bestaat er een grote

noodzaak om de veiligheid van dergelijke stoffen ten aanzien van niet-doelsoorten, zoals wilde bestuivers, kritisch te onderzoeken. Dit onderzoekswerk kwam tot stand om een aantal gebreken in de risico-analyse van een aantal insecticiden met een laag risico en bio-insecticiden aan te pakken waarbij door laboratoriumonderzoek de potentiële impact van een aantal van deze producten op inheemse bijensoorten werd bestudeerd. De ecologische en economish waarde van deze soorten kan in bepaalde omstandigheden deze van de honingbij *A. mellifera* zelfs overstijgen. Een eerste studie naar de inname van of contact met spinosad toonde aan dat deze stof zeer toxisch is voor de werkbijen van twee angelloze bijensoorten: *Partamona helleri* en *Scaptotrigona xanthotrica*. Dit zijn twee belangrijke bestuivers van wilde planten en landbouwgewassen in Neotropisch Amerika. Azadirachtin en chlorantraniliprol daarentegen waren weinig toxisch voor deze soorten met een overlevingskans van 100% na contact met de concentraties zoals aanbevolen voor gebruik in het veld. Beide stoffen hadden echter wel een negatieve impact op de vertikale vlucht van deze insecten. In een tweede studie werden de letale en subletale effecten van azadirachtin en spinosad onderzocht. Hierbij werd het product toegevoegd aan het larvendieet van de angelloze bij *Melipona quadrifasciata anthidioides*. Deze bijensoort is eveneens een belangrijke bestuiver in het Neotropisch gebied. In deze studie werden een dalende overlevingskans van de larven en een verminderd gewicht van de poppen vastgesteld bij toenemende hoeveelheden van het product. Zowel azadirachtin als spinosad veroorzaakten misvormingen bij de poppen en volwassen dieren. Daarenboven beïnvloedde spinosad de wandelactiviteit van de volwassen werkerbijen die als larve aan de stof waren blootgesteld. Dit was niet het geval voor azadirachtin. Ten slotte toonde een derde studie aan dat de chronische inname van azadirachtin de overlevingskansen en voortplanting van microkolonies van de hommel *Bombus terrestris* negatief beïnvloedde. Dit is een belangrijke bestuiver die in het Noordelijk halfrond vaak als commerciële bestuiver wordt gebruikt in serres. Daarenboven waren de letale en subletale effecten van azadirachtin in laboratoriumbiotoetsen waar de hommels moeten foerageren sterker dan in biotoetsen zonder foerageren. Deze bevindingen betwisten de gerapporteerde veiligheid van insecticide met een laag risico en bio-insecticiden voor niet-*Apis* bijen. Daarom raden we aan om in de toekomst bijkomende studies uit te voeren waarbij de werkelijke praktijkomstandigheden beter worden benaderd. Hierbij worden dan enerzijds de afbraak van de producten en de aanwezigheid van residu’s in de pollen en nectar in rekening gebracht, en anderzijds ook de foerageeractiviteit van de bijen om naar de bloemen te vliegen. Dit moet leiden tot een betere inschatting van het risico van het gebruik van deze producten in combinatie met bestuivende insecten zoals de bijen.

ABSTRACT

BARBOSA, Wagner Faria, D.Sc., Universidade Federal de Viçosa, January, 2015. **Toxicity assessment of reduced-risk (bio)pesticides on non-*Apis* bees**. Advisers: Raul Narciso Carvalho Guedes and Guy Smagghe. Co-advisers: Gustavo Ferreira Martins and Maria Augusta Lima Siqueira.

It has been said that the human beings and nature would be seriously impacted if bees were wiped out from the world. Indeed, bees are crucial organisms for pollination without which the maintenance and conservation of different biomes would be compromised. Such ecological relevance is accompanied by their economic and social importance through the pollination of numerous wild and cultivated plant species, besides of producing of honey, royal jelly, wax and propolis. In recent decades, the concern with the bee pollinator loss has become increasingly real with many reports of bee declines. Substantial efforts have been made to investigate the causes and consequently to conceive mitigation actions against bee decline. A long list of causes of such decline has been recognized including poisoning by pesticides, particularly insecticides, which has been reported as one of the main factors involved. However, the majority of ecotoxicological risk studies have been restricted to a single model species, the honeybee *Apis mellifera*, despite the importance of a wide variety of bee species throughout the world. Indeed, honeybees are pollinators of irrefutable value, particularly in the Northern Hemisphere where their commercial pollination is intensively used, and therefore, they deserve attention. Furthermore, as a second shortcoming, these studies have been circumscribed to a few compounds such as neonicotinoid insecticides, which may neglect the potential risks of other compounds to bees. The well-regarded reduced-risk insecticides and bioinsecticides have been presented as alternative compounds for crop management with allegedly low toxicity to bee pollinators. However, because the emphasis of regulatory studies on honeybees, and specifically the perception that the natural origin of bioinsecticides determines their low potential risk to beneficial organisms, the perceived safety of such compounds to non-target species, particularly wild pollinator species, is questionable and should be the target of scrutiny. This work comes to light in order to fill some of these gaps in risk assessments exploring, through laboratory studies, the potential impacts of a select group of reduced-risk insecticides and bioinsecticides on native bees, whose ecological

and economic importance can even surpass the honey bee *A. mellifera*. In the first study performed, the contact and ingestion of spinosad showed high toxicity to workers of two stingless bee species, *Partamona helleri* and *Scaptotrigona xanthotrica*, which are important pollinators of wild and cultivated plants in the Neotropical America. However, azadirachtin and chlorantraniliprole exhibited low toxicity to these species with around 100% of surviving individuals after the contact or ingestion at their respective field recommended concentration. Both compounds, however, impaired the vertical free-flight activity of these insects. In the second study performed, lethal and sublethal effects of azadirachtin and spinosad were evaluated through contaminated larval diet of the stingless bee *Melipona quadrifasciata anthidioides*, another important pollinator in the Neotropics. In this study, the larval survival and pupal weight decreased with increasing doses of both compounds. Both azadirachtin and spinosad also produced deformed pupae and adults. In addition, workers that survived the larval exposure to insecticides had their walking activity compromised only by spinosad, not by azadirachtin. Finally, in a third study, the chronic ingestion of azadirachtin impaired the survival and reproduction of microcolonies of the bumblebee *Bombus terrestris*, an important bee pollinator species prevailing in the Northern Hemisphere, which is extensively used for commercial pollination in greenhouses. Furthermore, in a laboratory bioassay where the bumblebee workers needed to forage, the lethal and sublethal effects on their reproduction were stronger than the bioassay without foraging under the effect of azadirachtin ingestion. Such findings may challenge the safety notion of reduced-risk insecticides and bioinsecticides when non-*Apis* bee species are used in toxicology studies, which deserve attention. As future perspectives, we recommend complementary studies with assessments in field and semi-field conditions, where on the one hand, the degradation and the small amount of insecticides that usually reach the pollen and/or nectar can mitigate potential harmful effects, but on the other hand, the need for foraging in more realistic environments may exacerbate the risk of insecticide exposure.

GENERAL INTRODUCTION

PESTICIDES AND REDUCED-RISK INSECTICIDES, NATIVE BEES AND PANTROPICAL STINGLESS BEES: PITFALLS AND PERSPECTIVES

Public perception on honey bee decline and pest control

Invertebrates are generally not particularly liked or praised in Western society, with a few exceptions - the European honey bee, *Apis mellifera* L., is one of them (Kellert 1993, Barua et al. 2012). The reasons for this are deeply ingrained and intuitive. Bugs" (i.e., insects), in general, are subject to a dominionistic and negativist views due to the perception that they are pests (Kellert 1993). This is exemplified by Aesop's view of ants as "thieves" in his *fables* ("*Zeus and The Ant*"). However honey bees are the target of a more naturalistic and utilitarian views, extending even to aesthetics if their social characteristics are considered (Kellert 1993, Barua et al. 2012). Again, Aesop comes to mind in his fable "*The Bear and The Bees*" (*Aesop's Fables*). Therefore, it comes as no surprise that there is still a raging debate over honey bee decline that has moved beyond beekeepers, academia, industry and regulatory agencies, extending to non-governmental organizations (NGOs), mass media, fiction writers and general public.

The earlier suspicion that the involvement of pesticides was leading to the reported honey bee colony collapse disorder (CCD) added further fuel to the debate, which shifted from the CCD phenomenon (detected mainly in the US between 2006-2008) to honey bee colony decline, particularly in the US and European Union (vanEngelsdorp et al. 2007, 2008, Kluser et al. 2010). Such heated debate proved invaluable in recognizing knowledge gaps and led to the mobilization of resources for the scientific research focusing on the spread, amplitude and causes of honey bee colony decline (Kleinman and Suryanarayanan 2013, vanEngelsdorp andMeixner 2010, Chauzat et al. 2014). The end result of the ongoing effort to settle this debate show some points of congruence, which include the following: 1) the recognition of honey bee decline in different areas and countries, but not in every area of every country; 2) the multifactorial nature of the phenomenon; and 3) the apparent lack of evidence for a direct association between the honey bee decline and neonicotinoid use (Blacquière et al. 2012, Creswell et al. 2012, Cutler et al. 2014b, Staveley et al. 2014, van der Zee et al. 2014). This is not saying that pesticides, particularly neonicotinoid insecticides, lack importance in this debate, as they are most likely important components in this scenario, potentiating colony decline in a period where there is a high demand for pollination services (Breeze et al. 2014, Sanchez-Bayo and Goka 2014).

The concern surrounding the potential impact of pesticides, particularly insecticides, on the honey bee and its products and ecological services is justifiable, not only because of the importance of such products and services of the increased demand for pollinators in current agriculture production (Anonymous 2013, Breeze et al. 2014, Oliveira et al. 2014). High yield agriculture systems and middle to high income countries continue the heavy use of pesticides, with evidences of overuse reflected in average pesticide amounts: above 2.0 Kg/ha in the US, Canada, and several European countries, and over 10 Kg/ha in countries such as Brazil and China, among others (Anonymous 2013, Ghimire and Woodward 2013, Oliveira et al. 2014). Among agriculture pesticides, insecticides are also without noticeable reduction in use, with some having an actual increase in use, even under the intense adoption of genetically modified crops (Ghimire and Woodward 2013, Oliveira et al. 2014). The challenge remains, as always, the effective management of insect pests with minimum non-target impacts.

Pesticides, reduced-risk insecticides and the honey bee

Pesticide use has remained the basis of crop protection for decades, and the routine use of these compounds remains largely irreplaceable by other pest management practices (Metcalf 1980, Cooper and Dobson 2007, Oliveira et al. 2014), despite the recognized risks and controversies surrounding them (Edwards-Jones 2008, Van Maele-Fabry et al. 2012, Köhler and Triebskorn 2013). However, it can not be denied that there is a progressive change in attitudes regarding pesticide use, which favors the search for new compounds with better toxicological and ecotoxicological profiles (Gilbert and Gill 2010, Krämer et al. 2012). The end-result is the current prevalence of a far broader range of pesticidal compounds. These compounds have a greater potency against the target pest, requiring lower field application rates and affording higher levels of harmlessness for non-target organisms, but they are used at a higher frequency, particularly for agriculture production in middle to high income countries (Gilbert and Gill 2010, Krämer et al. 2012, Ghimire and Woodward 2013). This scenario has allowed for the burgeoning of neologisms and pleonasms in coining alternative references to pesticides, including some fallacious ones, which vary greatly from country to country and include "agricultural protectants", "plant protection agents", "phytosanitary products", “agrochemicals”, “agrotoxics”, “biological pesticides”, “biopesticides”, “biorational pesticides”, and “reduced-risk pesticides”, among others. This semantic exuberance frequently exhibits little scientific or technical value and, worst of all, conveys subliminal and equivocated notions such as the intrinsically higher (or lower) level of harmlessness of a pesticidal compound.

The myriad of pesticide groups currently available and the present societal perception of pesticides create new regulatory challenges, as new toxicological tests and endpoints seem necessary. The honey bee provides an interesting paradox because, on one hand, this species is needed throughout the world for basic toxicological assessments aimed at pesticide registration for agricultural use. On another hand, it is reported to be suffering from pesticide-influenced decline in different countries. The later circumstance led the restriction or even downright ban of some compounds, notably neonicotinoid insecticides and particularly in Europe and the US (EFSA 2013a, Gross 2013, Tirado et al. 2013). Although a few other insecticides are also considered, including organophosphates of old, pyrethroids and fipronil (Tirado et al. 2013), the general concern is largely focused on neonicotinoid insecticides (EFSA 2013a, Gross 2013, Tirado et al. 2013, Sanchez-Bayo and Goka 2014). The plant systemicity of neonicotinoids and the broad scale of their use, with high lethal and pronounced sublethal toxicity to honey bees, are the key reasons for the concern and attention to this group of insecticides, a group that still exhibits potential for increased use against agricultural arthropod pest species.

The recent expansion and incentives towards the development and use of the so-called reduced-risk pesticides, particularly biopesticides, are reactions to the environmental harmlessness concerns sparked by the Western society, and the neonicotinoid risk to the honey bee decline illustrates this fact. The increased demand for organically produced food items (i.e., where only natural insecticides are allowed) also reinforces the demand for reduced-risk (bio)pesticides, although current levels of (conventional) pesticide residues on foodstuffs does not appear to be of significance to human health, and pesticide residues are also frequently detected on organically produced food (Winter 2012). The US Environmental Protection Agency defines reduced-risk pesticides as those exhibiting at least one trait of the following six advantageous traits over existing pesticides: 1) low impact on human health, 2) low toxicity to non-target organisms, 3) low potential for groundwater contamination, 4) lower use rates, 5) low pest resistance potential, and 6) compatibility with integrated pest management (IPM) (USEPA 2014b). Therefore, the concept is not particularly stringent and is likely to fit the majority of insecticides developed and used since the 1970's, even if they are not safe for non-target organisms, such as plant pollinators.

The concept of biopesticides, which may also be reduced-risk pesticides, is another potential pitfall playing with public perception. Although some authors reserve the term biopesticide for living organisms (Glare et al. 2012), the more frequently used concept gives a broader definition of biopesticides (or biological pesticides), encompassing all molecules of biological origin (USEPA 2014c, Villaverde et al. 2014). The problem with this is the common assumption that biopesticides (or biological pesticides, or natural pesticides) pose a lower risk than synthetic insecticides, which is aligned with the public perception and the supporters of organic production. The deception lies in the fact that the stated assumption is not necessarily true because the origin (either natural or synthetic) is not a determinant of toxicity, which is a function of the chemical structure and the derived physico-chemical properties of the compound (Coats 1994, Bahlai et al. 2010, Isman and Grieneisen 2014). In the context, biopesticides and/or reduced-risk insecticides may exhibit significant lethal and/or sublethal toxicity to the honey bee and other pollinator bees, even showing toxicity as high as that attributed to the neonicotinoids, a possibility usually neglected despite some available evidence (Besard et al. 2011, Biondi et al. 2012b).

Native bees: extended concerns with pesticides

The significant decline in honey bee colonies observed in the US and parts of Europe drew attention to wild pollinator communities and their importance (Winfree et al. 2007, Brosi and Briggs 2013, Garibaldi et al. 2013). Wild pollinators can perform equally well, or even better pollination than the honey bee in some crops and wild plants (Winfree et al. 2007, Garibaldi et al. 2013). Furthermore, wild pollinators are important in maintaining plant diversity in natural landscapes (Brosi and Briggs 2013), but they are also likely affected by pesticide use, and, again the primary concern has been with neonicotinoids and their potentially higher toxicity to wild bees (Arena and Sgolastra 2014, Roubik 2014, van der Valk and Koomen 2013). The honey bee is routinely used as surrogate bee pollinator in pesticide risk assessments, but recent meta-analysis indicates the need for more comparative information between the honey bee and non-*Apis* bees, and a range 10-fold in pesticide sensitivity exists between both bee groups (Arena and Sgolastra 2014). Such concern and need has also been expressed in different global surveys and studies (Roubik 2014, van der Valk and Koomen 2013), and some progress has been achieved.

Higher insecticide use compromising pollinator diversity as well as differences between insecticide susceptibility of honey bees and wild bees were further recognized (Brittain et al. 2010, Biddinger et al. 2013). Neonicotinoids were again the focus of attention, but although there has been an increased number of in studies with solitary bees and other wild bee pollinators, bumble bees have been the center of attention (Cameron et al. 2011, Biddinger et al. 2013, Sandrock et al. 2014). Bumblebees prevail in the Northern Hemisphere, although some species do exist in South America, and they have become increasingly important in agriculture as pollinators of cultivated crops, such as greenhouse tomatoes and strawberry (Velthuis and van Doorn 2006). Concerns about bumble bee decline have also risen and there is an accumulated evidence on bee's vulnerability to neonicotinoids in particular (Gels et al. 2002, Goulson et al. 2008, Mommaerts et al. 2010, Cameron et al. 2011). However, little information is available regarding the potential impact of reduced-risk insecticides to wild bees and even to bumble bees, but the few studies available indicate the potential for the substantial impact of some such pesticidal compounds, which deserves further attention (Besard et al. 2011, Biondi et al. 2012b).

Pesticides and bees in the tropics: beyond honey bees and bumble bees

The Neotropics deal with scenario and challenge that are different from the US and Europe, even though similar concerns regarding the possible correlation between honey bee decline and neonicotinoid exist. Brazil for instance is the world's 2nd largest consumer of pesticides in agriculture, with an average consumption of 10 kg/ha/year and an intensive use of neonicotinoid insecticides (SINDAG 2013, Oliveira et al. 2014). The call for the injunctive suspension of the aerial application was issued in 2012 by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA), from the Brazilian Ministry of the Environment, and was subsequently reviewed with a call for additional studies with the honey bee (DOU no. 192 of October 3, 2012, Ofício Circular/12/CGASQ/DIQUA of November 2012, and DOU no. 3 of January 4, 2013). An important shortcoming is that no records of honey bee decline exist in Brazil, or Latin America, and elsewhere. There are a few exceptions, such as South Africa, where nearly 30% of colony losses were registered as being due to a social parasite, suggesting a different set of causes than those experienced in the Northern Hemisphere (Pirk et al. 2014, van der Valk and Koomen 2013).

Another important issue to consider is that the honey bee subspecies and hybrids prevailing in Europe and North America are distinct from those of occuring in the tropics. The latter may exhibit different behaviour and physiology which can lead to differences in susceptibility to pesticides and pathogens (Suchail et al. 2000, Laurino et al. 2013). In Latin America, European honey bees were introduced and flourished for several years. The European subspecies were subsequently replaced by hybrids from a Brazilian honey bee breeding effort after the escape of some swarms of the African honey bee subspecies *A. mellifera scutellata*. These Africanized honey bees proved to be a dominant, outcompeting their European counterparts in Latin America and quickly spreading throughout the region and becoming the prevailing honey bee genotype in one of the most successful biological invasions currently recorded (Winston 1993, Caron 2002, Kaplan 2004). In addition, some typical traits that are prevalent in Africanized honey bees, such as the high level of defensiveness, area under foraging behaviour, trends in swarm, colony hygiene, etc, may minimize their likelihood of decline as observed with European honey bees in the US and parts of Europe, remain to be assessed.

The large-scale agricultural use of pesticides and the resource competition imposed by the Africanized honey bee are threats to native bees in Neotropical America, potentially more important than the decline of (Africanized) honey bees in the region (Wilms et al. 1996, Goulson 2003, Del Sarto et al. 2014), a status which largely remains unconfirmed. It is not only the Neotropics, but the whole Pantropical region that houses hundreds of wild bee species that are vulnerable to agricultural pesticides (Del Sarto et al. 2005, Brown and Oliveira 2014). Among these wild bee species, the rather diverse and perennially active eusocial stingless bees (Apidae: Meliponini) encompass a variety of pollinators that are very important for wild and cultivated plant species where honey bees exhibit marginal performance (Del Sarto et al. 2005, Kremen et al. 2002). The sparse information currently available indicates that Pantropical stingless bees are more susceptible to pesticides than the honey bee, but such information is based mainly on dose-response (acute) toxicity bioassays with only recent and scant information on sublethal effects of pesticides (Arena and Sgolastra 2014, Del Sarto et al. 2014). Once more, neonicotinoids, in addition to fipronil and a few older insecticides, are the focus of attention and no information is available regarding the potential impact of the over 150 active ingredients of agricultural pesticides in use in the tropics today (Lourenço et al. 2012, Jacob et al. 2013).

The commercial importance of the honey bee products is easy to recognize, as is the potential economic impact of their decline, even in the tropics. However, the concern about the ecosystem services (namely pollination) provided by (Africanized) honey bees in the Neotropical America, seems disputable because wild stingless bees are more important in the region and vulnerable not only to pesticide use but also to habitat destruction and competition from the invasive Africanized honey bee (Wilms et al. 1996, Cortopassi-Laurino et al. 2006, Poderoso et al. 2012, Brown and Oliveira 2014, Del Sarto et al. 2014). Until recently, a representative of the stingless bees was included in the red list of endangered species of the International Union for the Conservation of Nature and Natural Resources (IUCN 2014), and remains recognized as such by the Brazilian Ministry of Environment (MMA 2014). Attention to the group is therefore necessary and long overdue.

Concluding remarks

The apparent concern with colony decline of the main species used worldwide as the surrogate pollinator species for basic toxicological studies for the use registration of agricultural pesticides is not difficult to understand in the light of the knowledge gaps that are likely created precisely by regulatory requirements (Kleinman and Suryanarayanan 2013). The regulatory reliance on dose-mortality bioassays, i.e. lethal acute and/or chronic effect of pesticides, on a particular species – the honey bee, leads two shortcomings: 1) knowledge gaps exploring sublethal insecticide effects, and 2) the non-provision of necessary stimuli to pursue the potential indirect effects of pesticides on other potentially more important species in certain scenarios. This second shortcoming also deters initiatives of studies exploring higher levels of hierarchical impact, including impacts at the population and community levels.

The gaps in regulatory knowledge about bee-pesticide interactions have been subjected to subsequent attention since the onset of CCD in the US and realization of the potential extent of the honey bee colony decline in the US and parts of Europe. However, the attention remains focused on honey bees, as observed in the main regulatory guidelines for risk assessments on pollinators (OECD 1998a, 1998b, 2007, 2014a, 2014b, EPPO 2010, EFSA 2013b, USEPA 2014a). Only the European Food Safety Authority (EFSA) and the US Environmental Protection Agency (USEPA), the last in a joint effort with Health Canada's Pest Management Regulatory Agency and the California Department of Pesticide Regulation, refer to tiered assessments on other important pollinators such as bumble bees and solitary bees, but with all protocols based on honey bees (EPPO 2010). Nonetheless, several of the existing gaps in knowledge regarding honey bees have been scrutinized, and the level of knowledge has improved allowing some congruence in guiding the regulatory decision-making process. Even the initial and extensive focus on a single group of insecticides has improved and attention has been shifting, encompassing other groups of insecticides, fungicides, and pesticide mixtures, which seems paramount in the whole pollinator-pesticide risk assessment scenario. Nonetheless, misleading semantics of pesticide references and concepts, such as that of biopesticides and reduced-risk pesticides, convey questionable perception of the environmental safety of these compounds, potentially discouraging studies exploring their environmental impact in general and their potential impact to pollinators in particular. This notion deserves revision.

The focus on honey bees also invites careful consideration, particularly where this species is invasive and its benefits (e.g., production of honey, propolis, royal jelly, beewax, etc.) are outweighed by its potential threat to more important local pollinators. This is potentially the case with tropical stingless bees, particularly in Neotropical America. The potentially higher pesticide susceptibility and vulnerability of stingless bee species in the tropics should not be neglected. Considerable effort has been exerted to meet some of the shortcomings pointed out here, with increasing success. However several pitfalls and shortcomings remain to be faced when configuring appealing research perspectives that are potentially worth pursuing.

CHAPTER I

IMPACT OF REDUCED-RISK (BIO)INSECTICIDES ON SURVIVAL AND BEHAVIOR OF TWO NEOTROPICAL STINGLESS BEE SPECIES

1.1 Introduction

The much-debated association between honeybee colony decline and neonicotinoids use is still going on among academics, politicians, regulators, beekeepers, non-governmental organizations (NGOs) and the general public in a myriad of venues from scientific journal articles to regulations and guidelines, media article pieces, and even popular fiction (Rollins 2009, Schacker 2009, Blacquière et al. 2012, Kleinman and Suryanarayanan 2012, Gross 2013, Tirado et al. 2013, Chauzat et al. 2014, Godfray et al. 2014, Roubik 2014). Such exchange seems to be providing some points of congruence, including the recognition of honeybee decline in different areas and countries, the multifactorial nature of the phenomenon, and the absence of evidence for a direct association between honeybee decline and neonicotinoids (Kluser et al. 2010, Neumman and Carreck 2010, Potts et al. 2010, Creswell 2011, Blacquière et al. 2012, Creswell et al. 2012, Vanbergen et al. 2013, Cutler et al. 2014b, Fairbrother et al. 2014, Staveley et al. 2014). Insecticides, and particularly neonicotinoids, are most likely important components in this scenario, setting fire on such debate (Johnson et al. 2013, Breeze et al. 2014, Chauzat et al. 2014, Godfray et al. 2014, Zhu et al. 2014).

The honey bee is perceived as very sensitive to insecticides (Porrini et al. 2003, Schacker 2009, Tirado et al. 2013) and because is globally available and inexpensive to use, it has been the representative surrogate pollinator for the environmental bioindication of insecticide pollution (Porrini et al. 2003, Klein et al. 2007, Tomé et al. 2012). Although a recent meta-analysis study supports such use of honeybees, a 10-fold sensitivity rate correction seems necessary for the extrapolation of insecticide toxicity results from the honeybee to other bee species (Arena and Sgolastra 2014). This recommendation is largely based on acute toxicity data and Pantropical stingless bees (Meliponini) are generally more susceptible to insecticide exposure than the honey bee based on such toxicity dataset (Arena and Sgolastra 2014).

Despite the fact that stingless bees appear to be more sensitive to insecticides than honeybees, there is little research undertaken on this topic (Tomé et al. 2012, van der Valk and Koomen 2013, Del Sarto et al. 2014, Arena and Sgolastra 2014). However, because stingless bees are the primary pollinators of wild and cultivated plants in Neotropical America (Slaa et al. 2006, Palma et al. 2008, Bispo dos Santos et al. 2009, Roubik 2014), they demand more attention regarding the potential effects of pesticides in this particular geographic region. The contribution to pollination by stingless bees is important even in the presence of the honeybee, due to their higher efficiency as pollinators of several native and cultivated plant species, and production of specialty honey (Slaa et al. 2006, Arena and Sgolastra 2014, Roubik 2014). Furthermore, the reliance on the honeybee for insecticide toxicity assessments may compromise more susceptible pollinator species as stingless bees and thus impair agricultural production and plant diversity in the neotropics (Klein et al. 2007, Winfree et al. 2007, Brosi and Briggs 2013). In addition, the presence of some stingless bees species in the Brazilian endangered species list (MMA 2014) further emphasize the need to assess pesticide impact in this group of pollinators.

The general focus on the impact of neonicotinoids on pollinators, particularly honeybees, has led to an expansion and incentives of reduced-risk pesticides and particularly of biopesticides (USEPA 2014b Villaverde et al. 2014, Gerwick and Sparks 2014). The encouragement for using such compounds is illustrated by European Pesticide Regulation No. 1107/2009/EC and Directive 2009/128/EC of the European Parliament and of the Council in addition to similar regulatory efforts in Canada, the USA, and elsewhere (AAFC 2003, Jones 2004, Villaverde et al. 2014). Nonetheless, reduced-risk insecticides may still be highly toxic and represent a high risk to non-target beneficial insects such as stingless bees, which are completely neglected in ecotoxicology and risk assessment studies. Furthermore, biopesticides are not necessarily safer than synthetic pesticides, because origin is not a determinant of toxicity or risk (Bahlai et al. 2010, Biondi et al. 2012ab, Isman and Grieneisen 2014).

Azadirachtin, the main biopesticide in use today, exemplifies the stated concerns as its perceived safety to non-target arthropods has been challenged (Qi et al. 2001, Medina et al. 2004, Cordeiro et al. 2010, Barbosa et al. 2015). Reduced-risk insecticides have been equally challenged (Bahlai et al. 2010, Biondi et al. 2012ab, Tomé et al. 2015). Here, we hypothesized that the oral and contact (acute) toxicity of the recommended label rates of a reduced-risk insecticide (chlorantraniliprole), a bioinsecticide (azadirachtin), and a reduced-risk bioinsecticide (spinosad) might compromise the survival of two species of stingless bees, *Partamona helleri* (Friese) and *Scaptotrigona xanthotrica*

(Moure) (Hymenoptera: Apidae: Meliponini), which are important native pollinators in Neotropical America (Slaa et al. 2006, Winfree et al. 2007, Palma et al. 2008, Bispo dos Santos et al. 2009, Brosi and Briggs 2013). The insecticide concentrations used simulate a worst case scenario in which maximum residue expose would take place via plant surface contamination or pollen and nectar contamination (either through direct surface exposure or eventual translocation, which is low for the tested compounds). We further assessed group activity and flight take-off of adult workers of both bee species exposed to azadirachtin or chlorantraniliprole for impact prediction on behavior.

1.2 Material and Methods

1.2.1 Insects and insecticides

Three colonies of each of the stingless bee species *P. helleri* (ca. 1,000-3,000 individuals/colony) and *S. xanthotrica* (over 10,000 individuals/colony) 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, away from field crops and at the edge of a secondary forest. The adult workers of each bee species were collected as group of 10 individuals per colony at the hive entrance of their respective colonies using glass jars. They were subsequently taken to the laboratory and maintained without food inside wooden cages covered with organza (35 x 35 x 35 cm) for 1 h at 25 ± 2°C, 70 ± 10% RH, and total darkness until the bioassays were initiated. The waiting period before exposure was necessary to standardize the feeding condition of the tested workers.

Three insecticides were used in their respective commercial formulations as follows: azadirachtin (emulsifiable concentrate at 12 g/L, DVA Agro Brasil, Campinas, SP, Brazil), chlorantraniliprole (suspension concentrate at 200 g/L, DuPont do Brasil, Barueri, SP, Brazil), and spinosad (suspension concentrate at 480 g/L, Dow AgroSciences, Santo Amaro, SP, Brazil). In addition, the neonicotinoid imidacloprid (water dispersible granules at 700 g/Kg, Bayer CropScience, São Paulo, SP, Brazil) was used as positive control due to its high and widely recognized toxicity to bee pollinators (e.g., Blacquière et al. 2012, Tomé et al. 2012, Arena and Sgolastra 2014). The insecticides were used at rates calculated based on the spray volume per hectare (azadirachtin: 1000 L/ha, chlorantraniliprole: 1000L/ha, spinosad: 400L/ha, imidacloprid: 333 L/ha) for the control of the white fly *Bemisia tabaci* (Gennadius) (Hemiptera: Sternorrhyncha: Aleyrodidae) and the tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in accordance with the recommendations of the Brazilian Ministry of Agriculture (MAPA 2014). Such insecticide label rates are in the range usually sprayed in the same crops and against the same target species in different countries. The insecticide formulations were diluted either in distilled and deionized water (contact exposure bioassays) or in a 50% (w/w) aqueous sucrose solution (oral exposure bioassays) at the following concentrations based on the maximum field label rates registered for each insecticide (MAPA 2014): azadirachtin at 30 mg/L, chlorantraniliprole at 3 mg/L, imidacloprid at 42 mg/L, and spinosad at 20.4 mg/L.

1.2.2 Time-mortality residual contact bioassays

Inner walls of transparent (low-density polyethylene) plastic containers (250 mL; with negligible sorption and resistant to organic chemicals under short-term exposure (Topp and Smith 1992, Nerin et al. 1996)) were treated with 500 μL of insecticide solution (or water, in the case of the control) using an artist's air brush (Sagyma SW440A, Yamar Brasil, São Paulo, SP, Brazil) coupled with an air pump (Prismatec 131A Tipo 2 VC, Itu, SP, Brazil) at a pressure of 6.9 x 10^4 Pa. These containers were allowed to dry for 2 h under a fume hood at 25 ± 3°C without incidence of direct light, after which 10 adult workers were released within each container and retained by covering the top with organza fabric. Three containers (replicates), one per colony of each species, were used. Untreated sucrose solution was provided in a feeder to the bees through a hole in the plastic containers. After a 3-h exposure, the insects were transferred to untreated containers with 1 mL of 50% w/w sucrose solution. Bee survival was recorded hourly for 24 h from the beginning of the residual contact exposure. The insects were considered dead when they were unable to walk the length of their body and no insect recognized as dead by such criteria was able to recover in the study.

1.2.3 Time-mortality ingestion bioassays

Low-density plastic containers (250 mL) were again used as experimental units containing 10 worker bees fed on 500 μL of insecticide-contaminated sucrose solution (except for untreated controls) in longitudinally cut Eppendorf tubes used as plastic feeders and inserted through a hole in the plastic container. The insecticide dose ingested was obtained by weighting the feeders before and after the experiment. The oral ingestion of insecticide-contaminated 50% w/w sucrose solution by each 1-h starved bee species (between 0.69 and 1.12 µL/adult worker of *P. helleri,* and between 0.52 and 0.77 µL/adult worker of *S. xanthotrica*) led to the following ingested doses of insecticide by workers: *P. helleri* - 25.80 ng/bee of azadirachtin, 2.84 ng/bee of chlorantraniliprole, 28.90 ng/bee imidacloprid, and 22.79 ng/bee of spinosad*;* and *S. xanthotrica* - 15.48 ng/bee of azadirachtin, 2.06 ng/bee of chlorantraniliprole, 25.28 ng/bee imidacloprid, and 15.82 ng/bee of spinosad. Three containers (replicates), one per colony of each species, were used. Bee survival was recorded as previously described for the contact bioassays.

1.2.4 Group activity

Bioassays of the group activity of workers of both stingless bee species were performed 24 h after the period of exposure (contact and ingestion) to azadirachtin and chlorantraniliprole, in addition to the distilled water-treated control. Imidacloprid and spinosad were not used in the sublethal (behavior) bioassays, due to 100% mortality by both contact and oral exposure. The insects were exposed either by contact or ingestion, as previously described, and subsequently transferred to plastic Petri dishes (9.0 cm diameter) in groups of 10 workers bees from the same colony and three different colonies (i.e., replicates) of each species. 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. Activity recording was performed after a 1 h acclimation to the Petri dish arena to prevent confounding effects derived from insect handling. The overall insect activity were recorded for 10 min and digitally transferred to a video-tracking system equipped with a digital CCD camera (ViewPoint LifeSciences, Montreal, QC, Canada) (Fig. 1). The overall insect activity was recorded as changes in pixels between two subsequent pictures of the insect group, which were registered every 10^{-2} s. The changes of quantified pixels between the subsequent pictures represented all movements within the arena (including walking, body part movements, and conspecific interactions) that were captured by the system every 10^{-2} s. The bioassays were performed at 25 ± 2°C and under artificial fluorescent light between 2:00 and 6:00 p.m.

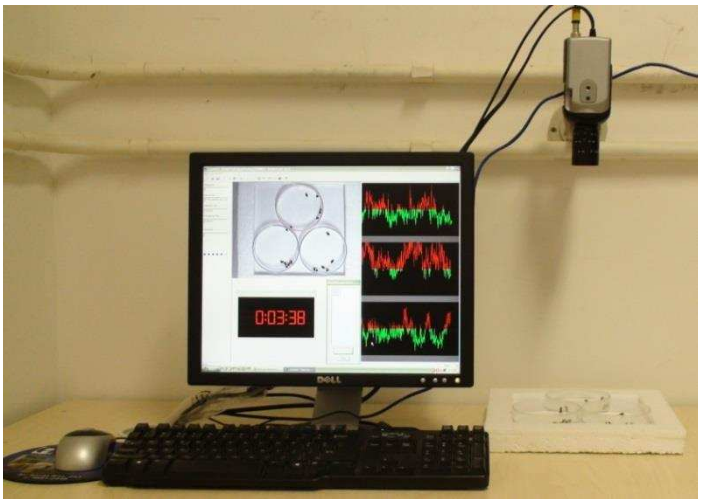

Fig. 1 A videotracking system for the group activity bioassay. Moviments of a group of 10 bees were recorded and expressed in terms of Δ pixesl/ 100^{-2} s.

1.2.5 Flight take-off bioassay

The workers subjected to the group activity bioassays were subsequently subjected to flight take-off bioassays 25 h after the period of exposure, as described by Tomé et al. 2015. The same number of workers was used per replicate (i.e., 10) in three replicates (i.e., colonies) per treatment. A 105 cm tall tower was formed with three stacked wooden cages (35 x 35 x 35 cm each) opened in their interior to allow free insect flight through them. A fluorescent lamp was placed 15 cm above the top of the tower in a dark room. The flight take-off bioassay explored the vertical bee flight towards the light source after the insect was released at the center bottom of the tower. The flight take-off was recorded within 1 min of worker release and was designated 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 36 and 70 cm high, IV) flight between 71 and 105 cm high, and V) flight reaching the light source at a height of 120 cm.

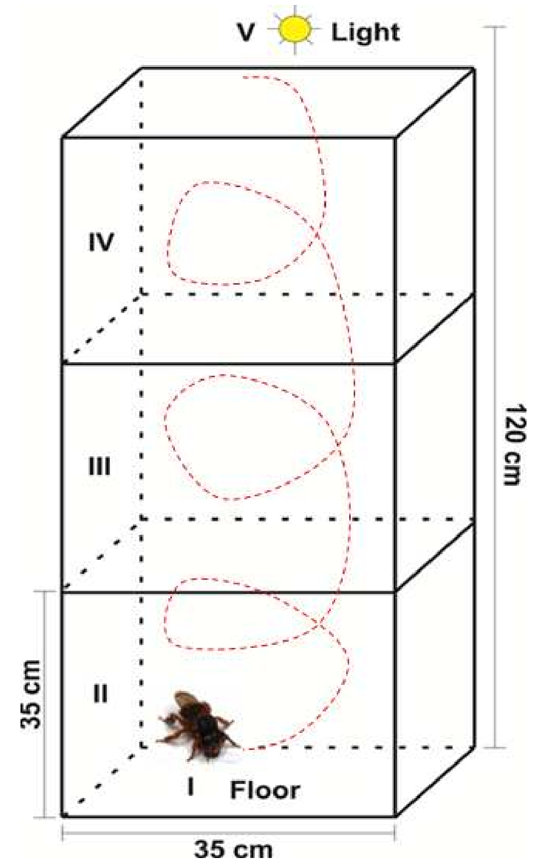

Fig. 2 Scheme of the flight take-off bioassay. A 105 cm tall tower formed with three stacked wooden cages and a fluorescent lamp placed 15 cm above the top of the tower. The insects were realead from the center bottom of the tower and their flight take-offs were scored.

1.2.6 Statistical analyses

The data from the time-mortality (survival) bioassays were subjected to survival analyses using Kaplan-Meier estimators to obtain the survival curves and estimates of the median survival time (LT_{50}) (PROC LIFETEST in SAS) (SAS Institute 2008). The insects still alive at the end of the bioassays were treated as censored data. The overall similarity among survival curves (and estimated LT_{50}s) was tested by the χ^2 Log-Rank test, and the pairwise comparisons between curves were tested using the Bonferroni method. The data from the overall group activity were subjected to analyses of variance after being checked for normality and homoscedasticity (PROC UNIVARIATE from SAS) (SAS Institute 2008), which were satisfied. The results of flight take-off were subjected to the (non-parametric) Kruskal-Wallis test ($p < 0.05$) (PROC NPAR1WAY from SAS) (SAS Institute 2008).

1.3 Results

1.3.1 Time-mortality by contact exposure

The survival of *P. helleri* and *S. xanthotrica* after insecticide contact exposure exhibited a significant difference among the treatments (*P. helleri*: Log-rank χ^2 = 229.42, df = 4, p < 0.001; *S. xanthotrica*: Log-rank χ^2 = 215.57, df = 4, p < 0.001) (Figs. 3A, 3C). Azadirachtin and chlorantraniliprole did not cause any mortality within 24 h among adult workers of *P. helleri*, resembling the control (with only water application). However, imidacloprid and spinosad caused 100% mortality within 5 h with median lethal times (LT_{50} ± SE) of 0.25 ± 0.00 h and 1.00 ± 0.14 h, respectively (Fig. 3B). A similar trend was also observed for *S. xanthotrica* with azadirachtin and chlorantraniliprole exhibiting negligible mortality with 24 h exposure, and imidacloprid and spinosad leading to 100% mortality within 5 h of exposure (LT_{50} ± SE of 0.25 ± 0.00 h for imidacloprid and 4.00 ± 0.00 h for spinosad) (Fig. 3D). LT_{50}'s for azadirachtin, chlorantraniliprole and untreated control were not shown because the mortality did not exceed 50%, which is the minimum value that need to be reached throughout the time for estimation of such parameter.

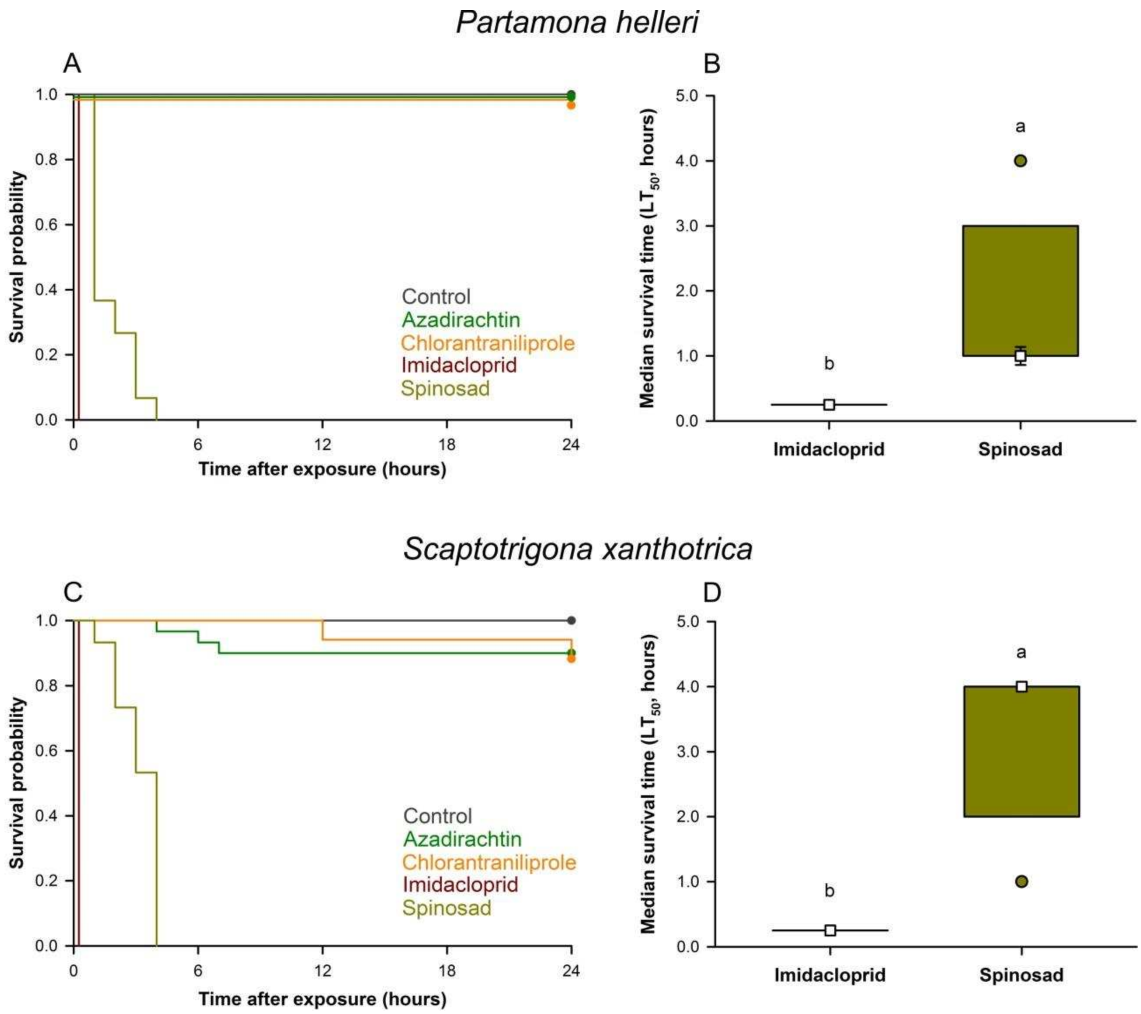

Fig. 3 Survival curves (**A, C**) and box plots of the median survival times (LT_{50}'s) (**B, D**) of workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) contact-exposed to the field rates of commercial insecticides. *Box plots* indicate the median (line within the box), mean (open square with standard error bars) and range of dispersion (lower and upper quartiles, represented as the limits of the box, and outliers (symbol)) of the LT_{50}s. The box plots with different lower case letters are significantly different by Bonferroni's method ($p < 0.05$).

1.3.2 Time-mortality by oral exposure

The survival curves of adult workers exposed to the insecticides by ingestion also exhibited trends similar to those obtained by contact exposure. The insecticides led to significant differences in the mortality profile of both *P. helleri* (Log-rank $\chi^2 = 189.24$, df = 4, $p < 0.001$) and *S. xanthotrica* (Log-rank $\chi^2 = 209.60$, df = 4, $p < 0.001$) (Figs. 4A, 4C). Azadirachtin and chlorantraniliprole led to negligible mortality for both stingless bee species, once again resembling the control. By contrast, imidacloprid and spinosad led quickly to 100% mortality of adult workers of *P. helleri* (LT_{50}'s ± SE of 0.25 ± 0.03 h for imidacloprid and

2.00 ± 0.00 h for spinosad) (Fig. 4B) and *S. xanthotrica* (LT_{50}'s ± SE of 0.25 ± 0.00 h for imidacloprid and 2.00 ± 0.00 h for spinosad) (Fig. 4D).

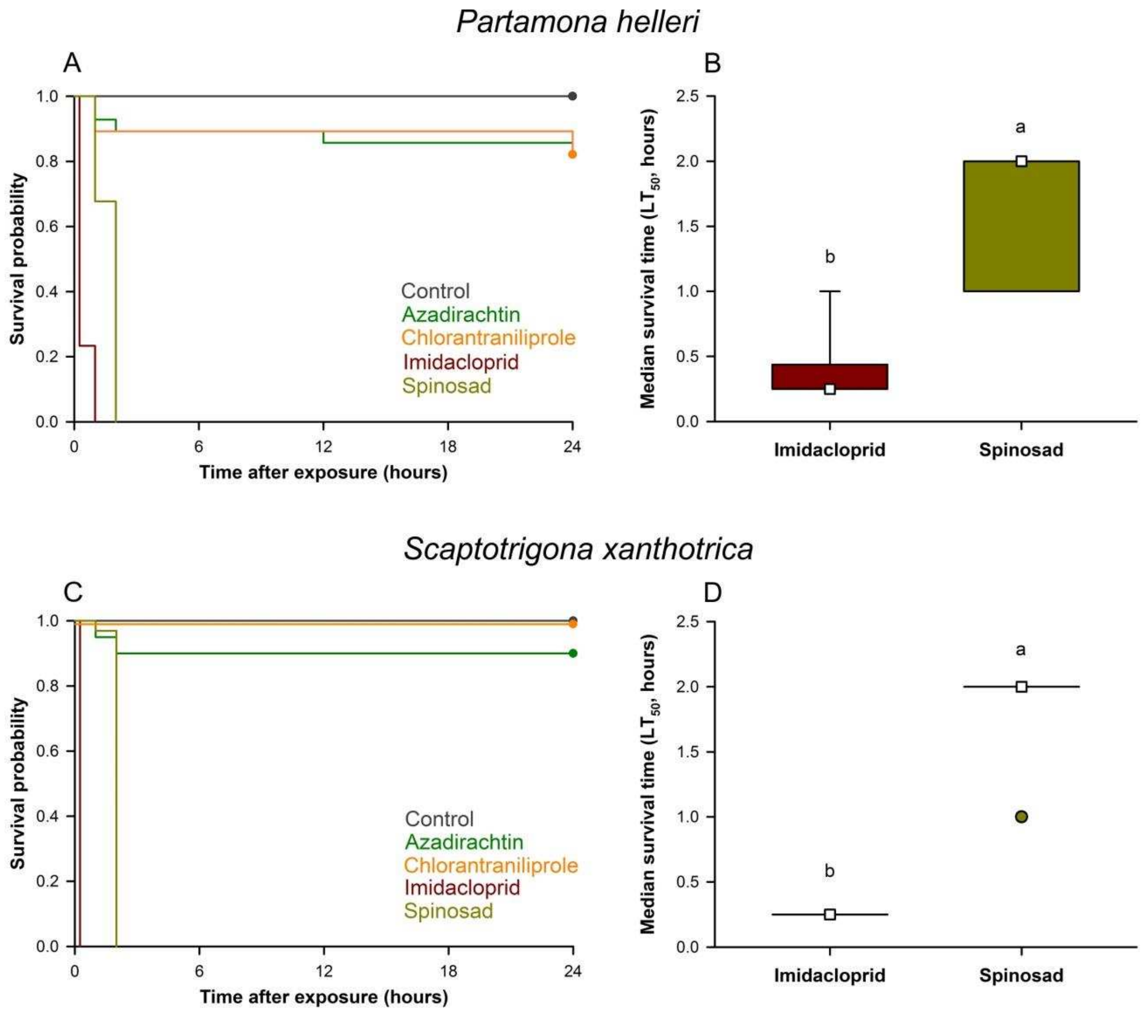

Fig. 4 Survival curves (**A, C**) and box plots of the median survival times (LT_{50}'s) (**B, D**) of workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) orally-exposed to the field rates of commercial insecticides. *Box plots* indicate the median (line within the box), mean (open square with standard error bars) and range of dispersion (lower and upper quartiles, represented as the limits of the box, and outliers (symbol)) of the LT_{50}s. The box plots with different lower case letters are significantly different by Bonferroni's method ($p < 0.05$).

1.3.3 Group activity

The group activity was assessed for azadirachtin- and chlorantraniliprole-exposed insects and unexposed insects (control), but no significant effect was detected ($F_{2,7} < 1.45$ $p > 0.31$). The mean overall activity (± SE) was 46.70 ± 13.56 Δ pixels/s x 10^{-2} and 66.98 ± 16.76 Δ pixels/s x 10^{-2} for *P. helleri* among the treatments with contact and oral exposure, respectively, and 206.01 ± 31.80

Δ pixels/s x 10^{-2} and 302.35 ± 23.33 Δ pixels/s x 10^{-2} for *S. xanthotrica* among the treatments with contact and oral exposure, respectively.

1.3.4 Flight take-off activity

Contact exposure to azadirachtin did not affect the take-off flight of *P. helleri* (H = 0.40, df = 1, p = 0.53) (Fig. 5A), whereas chlorantraniliprole significantly impaired such flight preventing bees from reaching the light source (H = 4.50, df = 1, p = 0.03) (Fig. 5B). By contrast, both insecticides impaired flight take-off of *S. xanthotrica* (H > 13.40, df = 1, p < 0.001) (Figs. 5C, 5D).

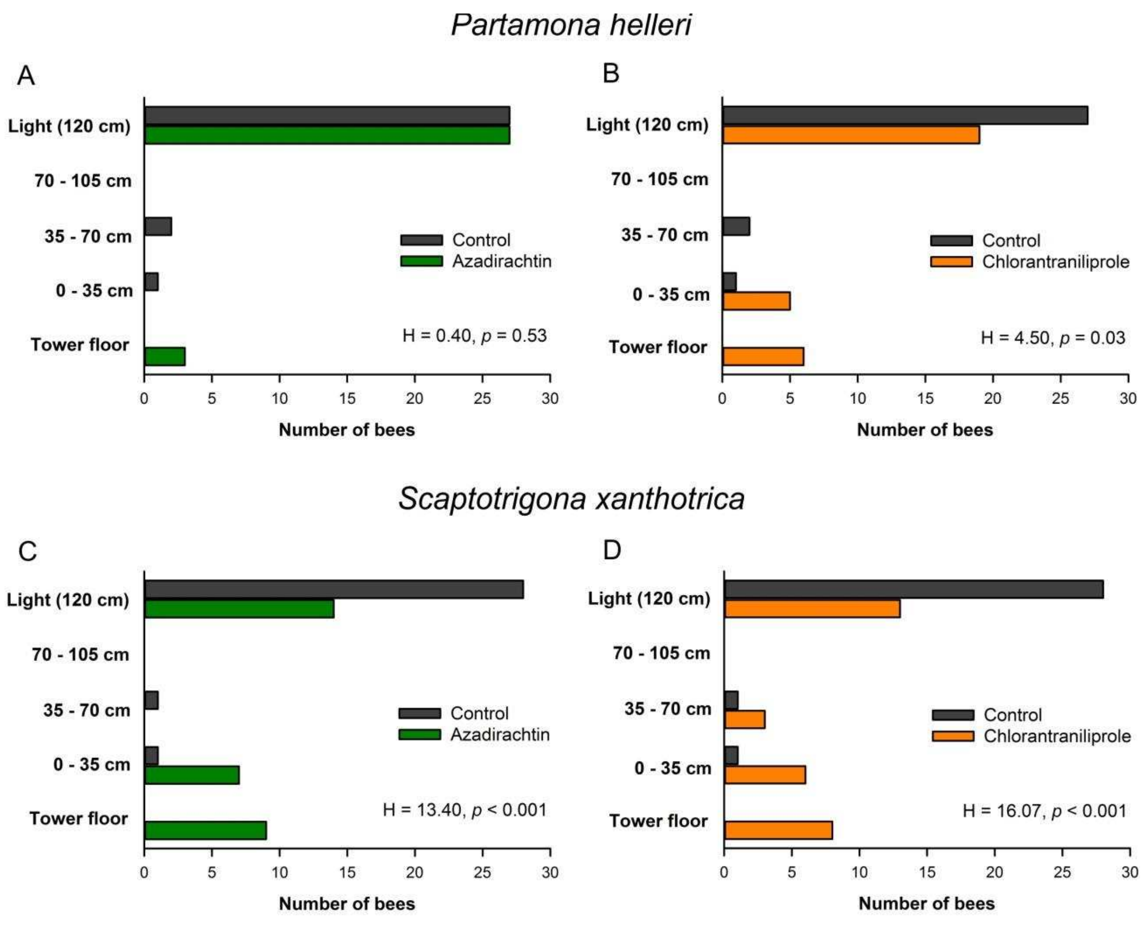

Fig. 5 Flight take-off activity of adult workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) contact-exposed to the field rates of the commercial insecticides azadirachtin (**A, C**) and chlorantraniliprole (**B, D**). The results of the (non-parametric) Kruskal-Wallis test (p < 0.05) used to test the differences between untreated and insecticide-treated insects are indicated.

Oral ingestion of either azadirachtin or chlorantraniliprole impaired flight take-off by *P. helleri* (H > 4.98, df = 1, p ≤ 0.02), reducing the number of individuals taking-off for flight and the number reaching the light source (Figs. 6A, 6B). By contrast, there was no significant effect of azadirachtin and chlorantraniliprole on *S. xanthotrica* regarding their flight take-off activity (H ≤ 1.16, df = 1, $p \geq 0.28$) (Figs. 6C, 6D).

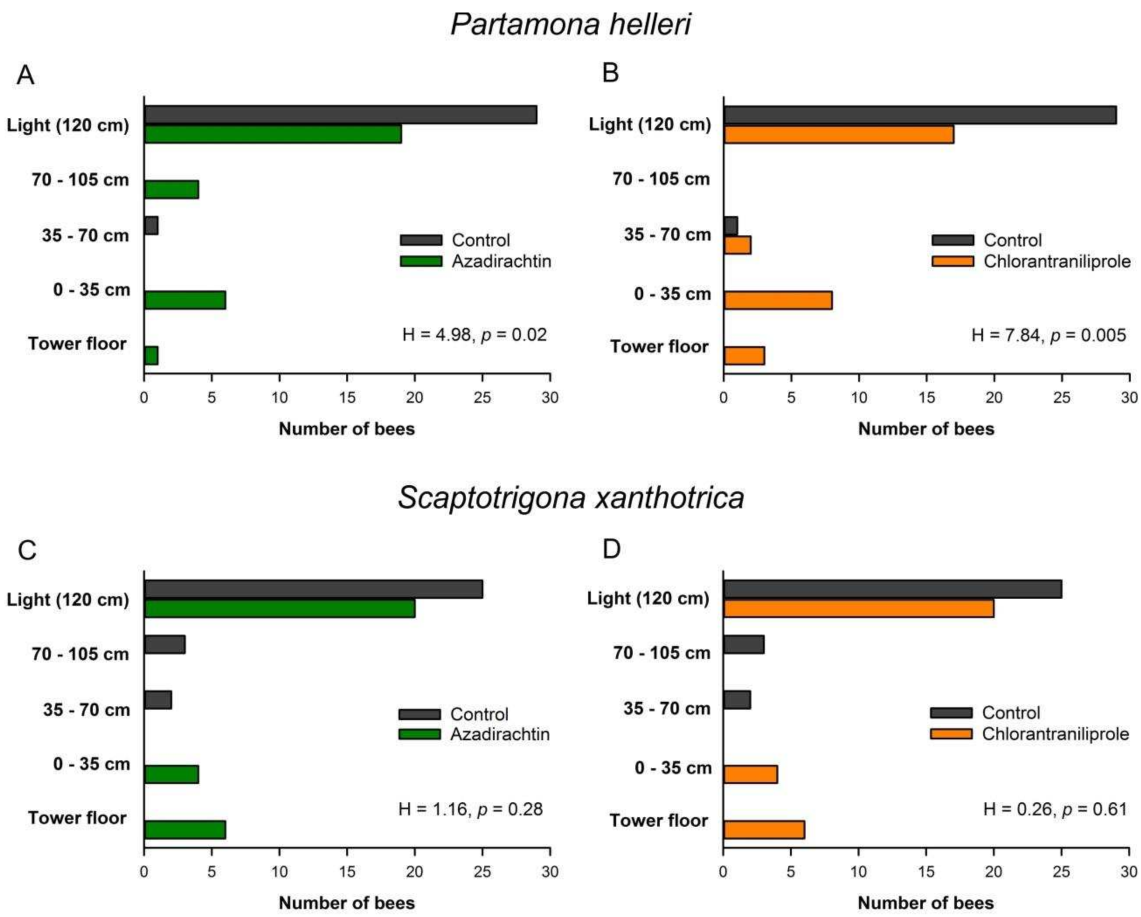

Fig. 6 Flight take-off activity of adult workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) orally-exposed to the field rates of the commercial insecticides azadirachtin (**A, C**) and chlorantraniliprole (**B, D**). The results of the (non-parametric) Kruskal-Wallis test ($p < 0.05$) used to test the differences between untreated and insecticide-treated insects are indicated.

1.4 Discussion

The currently debated decline of bee populations and consequent impairment of their pollination services is a major target of attention, although largely limited to honeybees and neonicotinoid insecticides (Johnson et al. 2013, Breeze et al. 2014, Chauzat et al. 2014, Godfray et al. 2014, Zhu et al. 2014). However, native pollinators frequently surpass the honeybees in ecological importance, due to their pollination services for native and cultivated plants, particularly in regions subjected to artificial introduction of the latter and where Africanized honeybees prevail, as in the Neotropics (Slaa et al. 2006, Bispo dos Santos et al. 2009, Tomé et al. 2012, van der Valk and Koomen 2013, Arena and Sgolastra 2014, Del Sarto et al. 2014, MAPA 2014, Roubik 2014). Furthermore, assuming that insecticide susceptibility is similar between the honeybee and native stingless bees is questionable based on the few studies available with the latter group (Tomé et al. 2012, Arena and Sgolastra 2014, Del Sarto et al. 2014). Such studies are restricted to a few insecticidal compounds with emphasis on neonicotinoid insecticides and fipronil (Lourenço et al. 2012, Sánchez et al. 2012, Tomé et al. 2012, Jacob et al. 2013, Arena and Sgolastra 2014).

The susceptibility of stingless bees to modern substances defined as reduced-risk insecticides, including bioinsecticides, has received little attention. Spinosad for instance, a reduced-risk bioinsecticide made of spinosyns generated as a fermentation product from the actinomycete species *Saccharapolyspora spinosa* (Mertz & Yao) (Sparks et al. 2001), was deemed harmless for the stingless bee species *Plebeia moureana* (Ayala) at up to 80 mg/L (Sánchez et al. 2012), but exhibited deleterious effects in honeybees and bumblebees at concentrations as low as 1 mg/L (Milles 2003, Morandin et al. 2005, Biondi et al. 2012a, Besard et al. 2011). Here we observed that spinosad is highly toxic at 20.4 mg/L to both stingless bee species tested, *P. helleri* and *S. xanthotrica*, causing quick and complete mortality of the worker bees within 5 h of either contact or oral exposure. Only imidacloprid exhibited more rapid mortality of workers than spinosad, regardless of the exposure method.

The terpenoid bioinsecticide azadirachtin, extracted from the seeds of the Indian neem tree (*Azadirachta indica* A. Juss (Meliacea)), is the most widely used botanical pesticide since the introduction of organosynthetic pesticides (Isman and Grieneisen 2014). It caused negligible adult mortality in both species of stingless bees used in this study, similar to the reduced-risk diamide insecticide chlorantraniliprole. The low acute mortality caused by azadirachtin and chlorantraniliprole was expected, because the former usually requires very high doses to achieve repellence and impair development in Hymenoptera (Mordue (Luntz) and Nisbet 2000), and the latter exhibits insecticidal activity limited to caterpillars, flies and beetles (Cordova et al. 2006, Brugger et al. 2010), with low toxicity against honeybees and bumblebees at the recommended field label rate (Gradish et al. 2010, Larson et al. 2013). The differential ryanodine receptor sensitivity to chlorantraniliprole in bee pollinators is the likely reason for the low acute toxicity of this insecticide to bee species (Yang et al. 2008, Brugger et al. 2010), whereas the reasons for the low azadirachtin acute toxicity to pollinators have not yet been studied.

The assessment of sublethal insecticide effect, although frequently neglected, is also very important because field rates target few pest species at their lethal levels exhibiting sublethal exposure to most of the non-target species. This allows for a supposed sublethal exposure of a much larger species assembly, which includes native bee pollinators. In addition, the lethal dose initially applied is subjected to environmental degradation, extending the sublethal exposure to much longer periods than the lethal exposure. As sublethal exposure may also compromise insect survival and reproduction, the sublethal responses of *P. helleri* and *S. xanthotrica* to azadirachtin and chlorantraniliprole should also be assessed. Therefore, the impact of azadirachtin and chlorantraniliprole in the overall group activity and flight take-off of adult workers was assessed.

Azadirachtin and chlorantraniliprole did not affect overall group activity of workers, which is an important trait since represents insect-insect interactions and individual activity within a group of social bees. However, flight take-off of *P. helleri* was impaired by chlorantraniliprole, and the flight take-off of *S. xanthotrica* was impaired by azadirachtin and chlorantraniliprole, regardless of the route of exposure. Neither compound has been reported to impair pollinator activity, unlike neonicotinoids in honeybees (Schneider et al. 2012, Fischer et al. 2014), and nenicotinoids and pyrethroids in bumblebees (Gill et al. 2012, Gill and Raine 2014). However, these compounds have not been subjected to such studies, which is likely due to their perceived (although questionable) overall environmental safety. Nonetheless, the azadirachtin interference with the availability of brain neurosecretory peptides and the chlorantraniliprole interference with muscle activity allow for the flight take-off impairment (Mordue (Luntz) and Nisbet 2000, Cordova et al. 2006).

Our findings partially support the perceived notion of the environmental safety of azadirachtin and chlorantraniliprole at their recommended field rates in a worst case scenario, which is reinforced by their recognition as reduced-risk insecticides (or bioinsecticide, in the case of azadirachtin). However, such a perception is not valid for spinosad, another reduced-risk (bio)insecticide, which exhibited high acute lethality to the two stingless bee species tested, resembling the drastic and broadly recognized toxicity of imidacloprid to pollinators (Johnson et al. 2013, Chauzat et al. 2014, Godfray et al. 2014, Zhu et al 2014). Furthermore, azadirachtin and chlorantraniliprole impaired the flight take-off of stingless bees, potentially impairing foraging and compromising colony survival, as may happen with honeybees under sublethal impact of neonicotinoids (Yang et al. 2008, Henry et al. 2012). Therefore, the perceived notion of pollinator safety associated with reduced-risk insecticides is misleading; although the term is accurate for its purpose (i.e., it is relative and has its roots in comparison to insecticides of old), low toxicity to non-target species is only one of the alternative requirements (which are fairly broad) (USEPA 2014). Regarding bioinsecticides, origin is not a determinant of toxicity, and the perceived safety of such compounds may be a misinterpretation. The proper assessment of such compounds should not be neglected by being labeled as reduced-risk insecticides and/or as bioinsecticides before a proper assessment has been performed. In order to assure a more comprehensive and realistic risk assessment, we further recommended the assessment of the reduced-risk and bioinsecticides under semi-field and field conditions, where the degradation of the insecticidal compounds or the small amount of insecticides that usually reach in the pollen and/or nectar (potential routes of contamination by ingestion) (USEPA 2012, Cutler et al. 2014a) may minimize the potential harmful effects of such compounds to the native stingless bees.

CHAPTER II

BIOPESTICIDE-INDUCED BEHAVIORAL AND MORPHOLOGICAL ALTERATIONS IN THE STINGLESS BEE *MELIPONA QUADRIFASCIATA*

2.1 Introduction

Several evidences place pesticides as major stressors contributing to bee decline in recent years (Valdovinos-Núñez et al. 2009, Brittain et al. 2010, Johnson et al. 2010, Bryden et al. 2013). Even with the consensus that other factors such as parasites, pathogens, poor nutrition and habitat fragmentation may also decrease bee populations (Goulson et al. 2008, Freitas et al. 2009, Ratnieks and Carreck 2010, Vanbergen et al. 2013), both scientific and political opinions have converged to the use of the insecticides fipronil and neonicotinoids as the primary stressors (Blacquière et al. 2012, EFSA 2013a, Gross 2013).

Mortality is the main explored effect of pesticides on bees, although recently more studies have also devoted efforts to sublethal effects. Sublethal toxicity may lead to decreased individual fitness and subsequent colony loss (Desneux et al. 2007, Bryden et al. 2013). Some of the sublethal effects may include immune system weakness (Nazzi et al. 2012, Di Prisco et al. 2013), developmental impairment (Wu et al. 2011), neural and locomotor disorders (Tomé et al. 2012, Palmer et al. 2013), and impaired learning, memory and foraging (Decourtye et al. 2004a, Yang et al. 2008, Henry et al. 2012). Most of these sublethal impacts on bee species have been recorded for synthetic pesticides. As alternative, molecules of natural origin have been assumed to be less toxic to non-target arthropods, including bees (Gerwick and Sparks 2014, Villaverde et al. 2014). Such compounds have received considerable attention and labelled as biopesticides or biorational pesticides (Isman 2006, Rosell et al. 2008).

Spinosad for instance is a prominent bioinsecticide derived from the fermentation of the actinomycete *Saccharopolyspora spinosa* (Biondi et al. 2012a). It shows reduced spectrum of toxicity and low potential risk to mammals compared to synthetic compounds, and thus it has been recognized by the US Environmental Protection Agency as a reduced-risk insecticide (Thompson et al. 2000, Williams et al. 2003). As a compound of natural origin, spinosad was originally considered less harmful for non-target arthropods when compared to old compounds, and thus its use for plant protection and insect vectors quickly expanded (Milles 2003, Sarfraz et al. 2005, Tomé et al. 2014b). However, its selectivity to natural enemies and pollinators has been questioned (Morandin et al. 2005, Biondi et al. 2012a).

Azadirachtin is another bioinsecticide which has been widely used in crop protection. This compound is derived from the Indian plant *Azadirachta indica* and shows high efficacy (Mordue et al., 2010). Azadirachtin may cause feeding deterrence, behavioral changes, incomplete ecdysis altered developmental time, morphological deformities and sterility due to hormonal disruption (Schmutterer 1990, Mordue (Luntz) and Blackwell 1993, Mordue et al. 2010, Tomé et al. 2013). However, opinions regarding its selective action in non-target organisms have been also questioned due to divergent research outcomes (Gordon and Gimme 2001, Medina et al. 2004, Charleston et al. 2006, Mordue et al. 2010, Cordeiro et al. 2010, Biondi et al. 2012a).

Faced with the alleged fact that the origin of the molecule drives the potential for toxicity on non-target organisms, more studies regarding non-target effect of natural insecticides are necessary. In the present study we investigated the toxicity of spinosad and azadirachtin on workers of the stingless bee species *Melipona quadrifasciata anthidioides* Lepeletier, which is one of the most important native bees for pollination in the Neotropics (Kremen et al. 2002, Slaa et al. 2006, Bispo dos Santos et al. 2009). *M. quadrifasciata anthidioides* is also closely related to *M. capixaba* Moure & Camargo, another Neotropical stingless bee species formally recognized as an endangered species (IUCN 2014). These factors emphasize the importance of *M. quadrifasciata* as a model pollinator in insecticide risk assessments. Using an *in vitro* rearing technique (Tomé et al. 2012), we hypothesised that bee larvae exposed to contaminated diet might have their development and survival impaired. Furthermore, we assessed the emerged workers of the stingless bees to confirm whether sublethal impacts might appear on their external morphology and locomotory behavior.

2.2 Material and methods

2.2.1 Stingless bee colonies

Colonies of *M. quadrifasciata anthidioides* were collected in Viçosa County (MG, Brazil; 20° 45’ S and 42° 52’ W) and maintained at the Experimental Meliponary of the Federal University of Viçosa (Viçosa, state of Minas Gerais, Brazil) for subsequent use in the bioassays. All colonies were originally collected at different fields in order to guarantee a representative genetic variability.

2.2.2 Insecticides

Commercial formulations of the bioinsecticide azadirachtin (Azamax EC, 12 g/L, DVA Agro Brasil, Campinas, SP) and spinosad (Tracer SC, 480 g/L, Dow AgroScience, Santo Amaro, SP) available and registered for agriculture use in Brazil were used in the bioassays. Both compounds were used at doses which were calculated based on their maximum field recommended concentration (MFRC) for the control of the tomato borer *Tuta absoluta* in accordance to the Brazilian Ministry of Agriculture (MAPA 2014).

2.2.3 *In vitro* rearing bees

Stingless bee workers were reared as described in Tomé et al. (2012) by transferring egg-containing brood combs to artificial honeybee wax-made cells filled with 130 µL-diet (containing 10 µL-distilled water), which provided food for full larval development (Figs. 1A, 1B, 1C and 1D). The artificial cells were placed into the wells of polyethylene microplates (24-well plates) and individually closed with honeybee wax caps (Fig. 1C). Each microplate received 24 eggs (i.e., an egg/artificial cell) from a single field colony and was maintained at 28±1°C temperature, 95±5% relative humidity (RH) and continuous darkness until the end of the feeding period. The artificial brood combs were removed at the end of the larval period and transferred to new artificial brood combs, which were maintained at 28±1°C, 70±5% (RH) and continuous darkness, similar to the natural conditions. Upon the emergence, the adult workers were marked with different colors using a non-toxic water soluble paint (Acrilex, São Bernardo do Campo, SP, Brazil) for monitoring age. These were maintained in Petri dishes (9 cm in diameter and 2 cm high), where they were supplied daily with a 50% honey and 50% pollen-based diet. Each Petri dish received the emerged bees from the same microplate and was kept under 28±1°C, 70±5% (RH) and continuous darkness.

2.2.4 Larval exposure to the bioinsecticides

The stingless bee larvae (i.e., 24 larvae/colony) were exposed to azadirachtin or spinosad via contaminated diet and four different colonies (i.e., replicates) were used for each bioinsecticide (i.e., 96 larvae/bioinsecticide). Each compound was independently mixed into the 10 µL of water added to the 130 µL diet provided for each larva in the artificial brood chamber. Azadirachtin was applied at the increasing doses of 42, 210, 420 and 840 ng/bee which corresponded the MFRC-based dilutions of 1/100, 1/20, 1/10 and 1/5, respectively. Azadirachtin at its MFRC-based dose (4200 ng/bee) was not considered for the bioassays because at this concentration the insecticide solution floats to the diet surface preventing the eggs from standing upright. Spinosad was applied at the increasing doses of 0.57, 1.14, 2.29, 11.4, 22.9, 114, 228, 1142 and 11424 ng/bee which represented the MFRC-based dilutions of 1/20000, 1/10000, 1/5000, 1/1000, 1/500, 1/100, 1/50, 1/10 and 1/1, respectively. Distilled water was used as control. The full doses of both azadirachtin and spinosad were known because each larva ingested the entire amount of food provided.

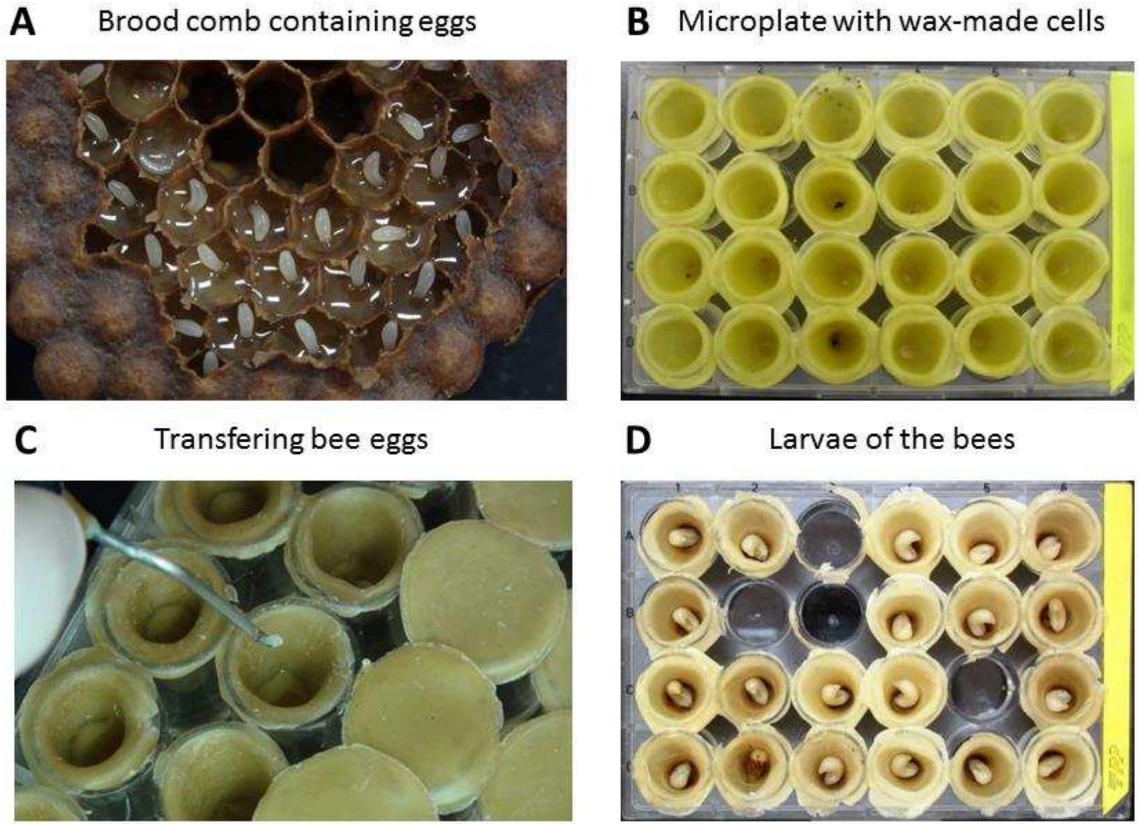

Fig. 1 A summariazed scheme of the in vitro rearing setup of the stingless bee Melipona *quadrifasciat.* A: a brood comb of the stingless bee with eggs. B: an artificial comb formed with a 24-well microplate with honey bee wax cells. C: transfer of the stingless bee eggs. D: An artificial comb with stingless bee larvae; empty wells indicate the removal of dead larvae.

2.2.5 Insect survival, developmental time, fresh body mass and external morphology

The survival of stingless bee larvae was recorded by daily monitoring the age of each individual throughout development, which encompassed the period between egg hatching until death or adult emergence. Dead individuals were identified by the absence of spiracle movement, and then removed. Each dose of azadirachtin or spinosad was represented by 96 larvae and sets of 24 larvae from four different colonies were used for each compound. The developmental time (days) from egg hatching until adult emergence was also recorded for each insect. All insects that survived to azadirachtin or spinosad exposure were weighed on an analytical scale (Sartorius BP 210D, Götingen, Germany) to determine their fresh body mass when reaching the white-eyed pupa stage (until four days after pupation). Deformed insects were counted at the pupal and adult stages and their external morphology (i.e. wings, legs, head and/or any part of the insect with different shape of a healthy bee) was evaluated. The insects identified as queens or drones were removed from the subsequent data analysis.

2.2.6 Walking behavior

Surviving adult workers of 0, 3, 6, 9 and 12 days old, which were previously exposed to azadirachtin or spinosad via contaminated diet were subjected to walking bioassays. Here, no deformed worker, drone or queen was used. Each insect was individually transferred to an arena consisting of an open Petri dish (9 cm in diameter and 2 cm high) lined at the bottom with filter paper (Whatman no. 1) and with inner walls coated with Teflon PTFE (DuPont, Wilmington, DE) to prevent escape. The movement of each insect within the arena was recorded for 10 min and digitally transferred to a computer using an automated video tracking system equipped with a CCD camera (ViewPoint LifeSciences, Montreal, Canada). The variables recorded in each arena were numbers of stops, resting time (s), distance walked (cm) and velocity (cm/s). The behavioral bioassays were carried out in a room with artificial fluorescent light at 25±2°C and 70±5% (RH). Five workers were used from each of the four different colonies per dose (i.e. 20 insects/dose). The average of the five individual bees from a single colony was considered as a replicate. It is worth mentioning that since the workers of a certain age were placed back to a Petri dish with up to 12 bees after being submitted to the behavioral test, it was not possible to determine whether the same bees would be subjected to behavioural test after a subsequent age (i.e., 3 days latter).

2.2.7 Statistical analyses

The data from the survival bioassays were subjected to survival analyses in which survival curves were obtained using Kaplan-Meier estimators (PROC LIFETEST in SAS) (SAS 2008). As the workers emerged at different developemental times (39 to 45 days), the survival curves were standardized by censuring the data when the insects completed 45 days old (counted from egg hatching). The overall similarity among survival curves was tested by the χ^2 Log-Rank test and the pairwise comparisons between curves were tested using the Bonferroni method. Insect body mass, developmental time and number of deformed individuals were subjected to regression analysis in the software TableCurve 2D v5.01 with bioinsecticide dose (azadirachtin or spinosad) as independent variable. The data from walking activity was submitted to multiple regression analyses in the software TableCurve 3D v4.0 with bioinsecticide dose and age after emergence as independent variables. The models used to describe the effect of both azadirachtin and spinosad on the measured variables were selected based on parsimony, high F values and steep increase of R^2 with model complexity and plotted using the software SigmaPlot 12.0 (Systat, San Jose, CA).

2.3 Results

2.3.1 Survival of the stingless bee larvae

The larval survival of *M. quadrifasciata anthidioides* was significantly impaired after ingestion of increasing doses of azadirachtin (Log-rank χ^2 = 112.44, df = 4, p < 0.001) (Fig. 2). Azadirachtin at 42 ng/bee resembled the untreated control (with only water addition) (Fig. 2). Conversely, azadirachtin at the highest dose of 840 ng/bee caused high mortality throughout development, reaching nearly 80% at the end of the bioassay. The median lethal time (LT_{50} ± SE) estimated was 24.0 ± 2.5 days.

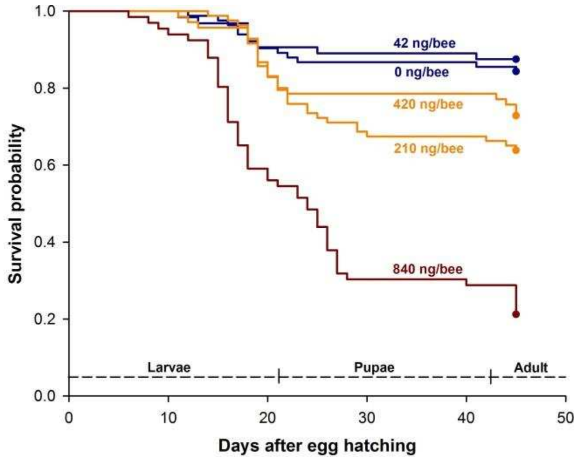

Fig. 2 Survival plots of stingless bees larvae (*Melipona quadrifasciatha*) reared on azadirachtin contaminated-diet. Survival curves coded with the same color were not significantly different by the Bonferroni method ($p > 0.05$). Closed circle indicates censured data.

With ingestion of spinosad, the survival of the larvae was also impaired in a dose-dependent manner (Log-rank $\chi^2 = 369.57$, df = 9, $p < 0.001$) (Fig 3). Spinosad at 0.57 to 22.85 ng/bee did not cause significant mortality in the immature stingless bees resembling the untreated control (Fig. 3). However, spinosad significantly reduced the survival of larvae when they were exposed to 114 ng/bee and higher doses. A negative relationship between the insecticide dose and the median survival time was also noticed (Figs. 3A, 3B). More than 80% of the individuals died before reaching the pupal stage at doses of 1142 and 11424 ng/bee (Fig. 3).

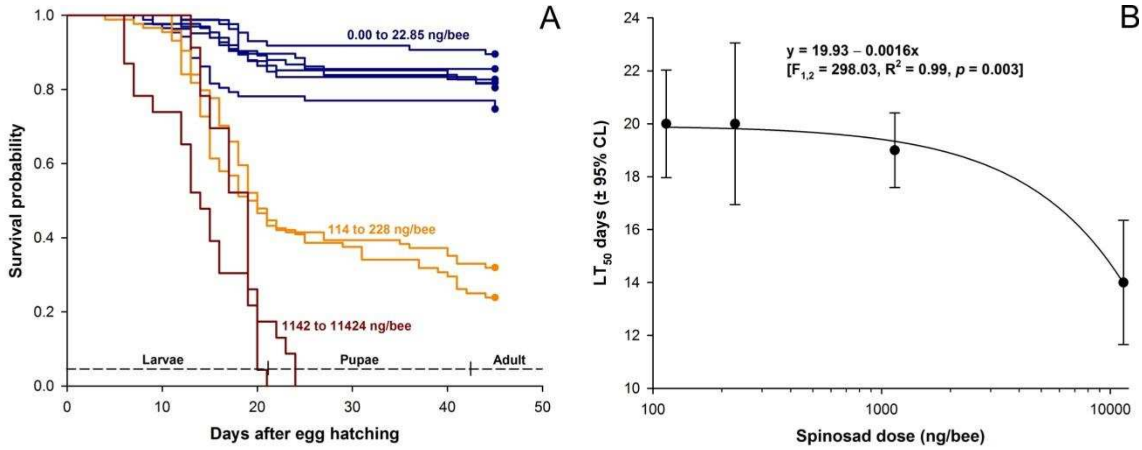

Fig. 3 Survival plots (**A**) and median lethal times (LT_{50}'s) (**B**) of stingless bees larvae (*Melipona quadrifasciatha*) reared on spinosad contaminated-diet. (**A**) Survival curves coded with the same color were not significantly different by the Bonferroni method ($p > 0.05$). Closed circle indicates censured data. (**B**) Vertical bars indicate 95% of confidence intervals.

2.3.2 Developmental time of the stingless bee larvae

The ingestion of azadirachtin ($F_{1,15}$ = 3.98, p = 0.06) or spinosad ($F_{1,29}$ = 2.28, p = 0.14) during the larval stage did not affect significantly the developmental time of the insects (period from the egg hatching to emergence of the workers). Therefore, the average among the doses and control of the larva-adult period (± SE) was 42.24 ± 0.30 and 41.15 ± 0.35 for azadirachtin and spinosad treated larvae, respectively.

2.3.3 Fresh body mass of the stingless bee pupae

Azadirachtin ($F_{1,17}$ = 13.34, p = 0.002) and spinosad ($F_{1,27}$ = 27.93, p < 0.001) significantly reduced the body mass of the pupae (Fig. 4A and 4B), which is reflected in the regression analysis for each insecticide. Such reduction in the body mass was not caused by rejection of the food since the whole diet was ingested by the larvae, regardless the treatment.

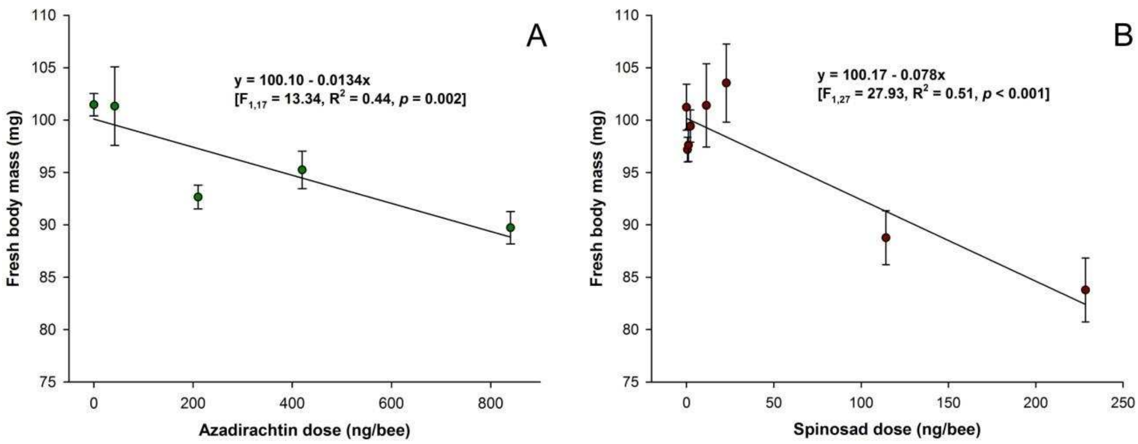

Fig. 4 Fresh body mass (± SE) of the white-eye pupae of the stingless bee (*Melipona quadrifasciatha*) reared on azadirachtin (**A**) or spinosad (**B**) contaminated-diet. The symbols represent the mean of adult workers from four independent replicates (i.e., colonies).

2.3.4 External morphology in pupae and adult bees

The ingestion of both azadirachtin and spinosad produced deformed pupae (Fig. 5) which resulted in adults with external deformities (Fig. 6). Deformed pupae showed contracted appendages of antennae, legs, wings and mouth parts (Fig. 5). All deformed adults derived from deformed pupae. This indicates that the larva-pupa molt (but not pupa-adult molt) was crucial for the appearance of morphological abnormalities in adult bees. Deformed workers showed no stretched wings appearing as pupal wing pads, contorted legs or antennae and color tegumentary pattern different from control bees (Fig. 6).

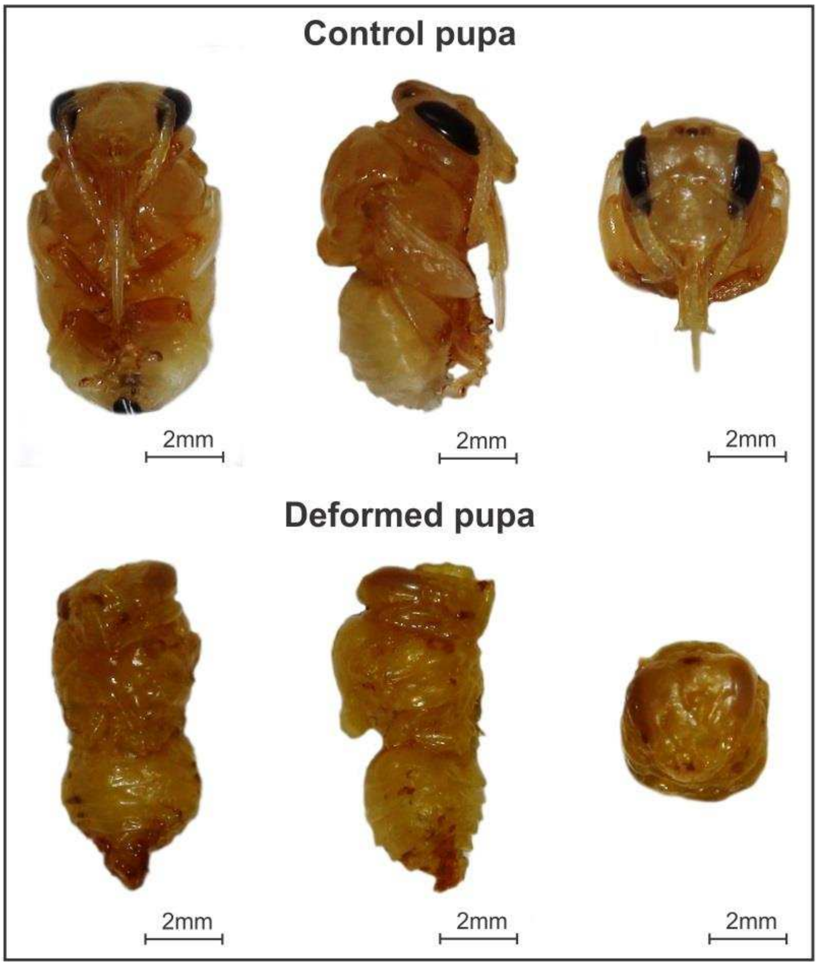

Fig. 5 Overview of the stingless bee (*Melipona quadrifasciatha*) at the pupal stage after larval exposure to control (Top) or azadirachtin treated-diet (Bottom).

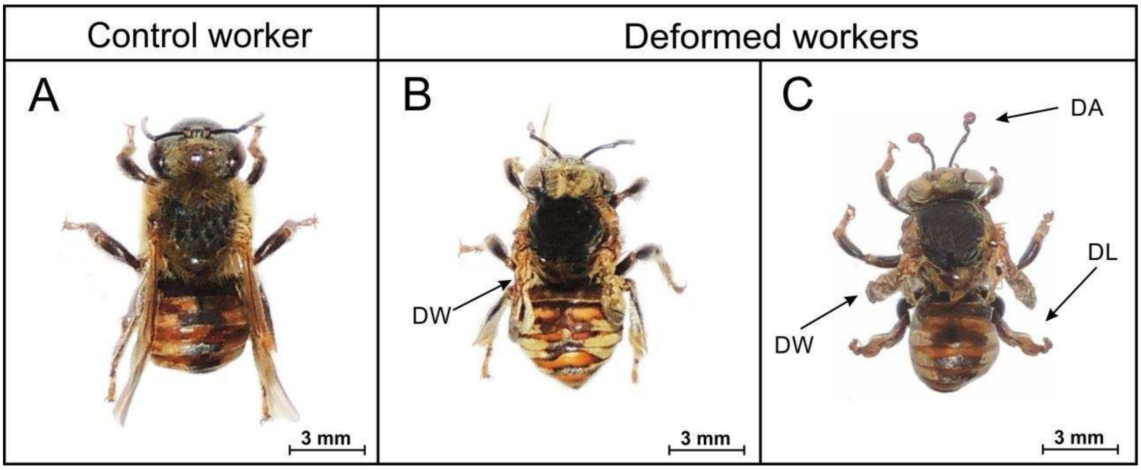

Fig. 6 Overview of the stingless bee (*Melipona quadrifasciatha*) at the adult stages after larval exposure to the control- (A), azadirachtin- (B) or spinosad- (C) treated diet. Arrows indicate deformities. *DW*: deformed wings; *DL*: deformed legs; *DA*: Deformed antenna.

Eighteen out of 205 deformed pupae were found under azadirachtin exposure, and only two of these reached the adult stage. These numbers from azadirachtin treatments allowed the adjustment of a linear model for the appearance of deformed pupae in terms of percentage [% deformed individual = (no. deformed insects/total no. insect)*100] (Fig. 7A, Pupae: $F_{1,17} = 5.71$, $p = 0.03$), but no significant regression was adjusted to the percentage of deformed adults (Fig. 7A, $F_{1,15} = 0.21$, $p = 0.65$). For spinosad, the number of deformed pupae was 51 out of 426 and adults 23 out of 387. The appearance of deformed individuals increased in a dose-dependent manner both at the pupal (Fig. 7B, $F_{2,30} = 42.13$, $p < 0.001$) and adult stage (Fig. 7B, $F_{1,19} = 48.75$, $p < 0.001$).

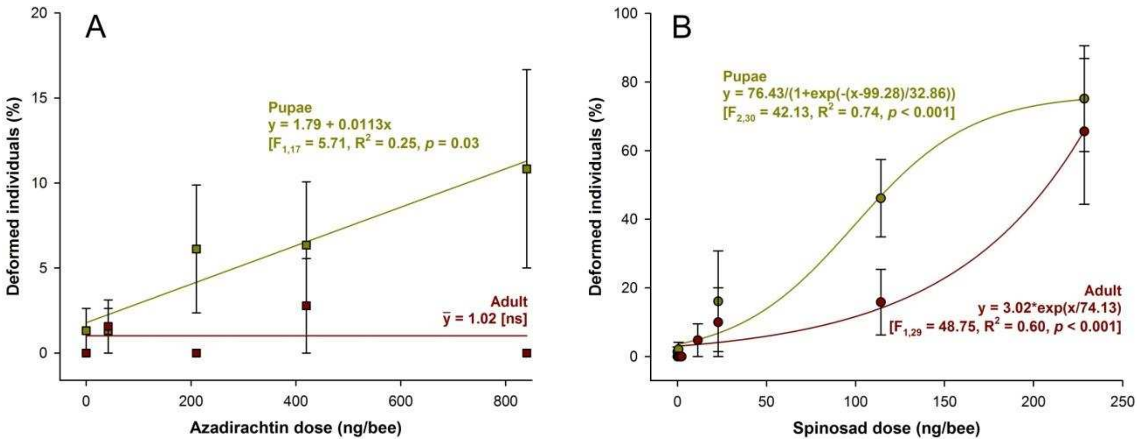

Fig. 7 Percentage of deformed individuals (± SE) of the stingless bee (*Melipona quadrifasciatha*) at their pupal and adult stages after reared on azadirachtin (**A**) or spinosad (**B**) contaminated-diet.

2.3.5 Walking behavior of adult workers

The trends in the walking behavior dependent variables (numbers of stops, resting time, distance walked and walking velocity) of young adult workers originated from larvae orally exposed to azadirachtin, were described by multiple regression models (Table 1) which were plotted in Figure 8. Azadirachtin dose did not have any contribution for the best adjustment in 3 out of 4 equations; this is shown by the absence of the independent variable that represents the dose (variable "x") in the equations (Table 1). In addition, even for the resting time equation (Table 1), the azadirachtin dose slightly deformed the shape of the response surface curve, while the age of the workers (independent variable "y") caused a substantial drop in the resting time (Table

1, Fig. 8B). Therefore, the plotted equations showed that the worker age (represented by the independent variable “y”) was the prime source for shape variation in the response surface curves (Table 1, Fig. 8). For the walking variables, the increasing worker age caused a straight decrease in the number of stops and a curvilinear increase in the distance walked and walking velocity with stabilization tendency of the dependent variable “z”.

Table 1 Summary of the regression analyses for the walking activity variables (Fig. 8) of the workers of the stingless bee *Melipona quadrifasciata* after the exposure to increasing doses of azadirachtin during the larval stage. All estimated parameters (a, b, c and d) of the equations were significant at $p < 0.05$ by Student's t-test.

Walking variables	Model*	Parameter estimates (± SE)				df_{error}	F	p	Adjusted R^2
		a	b	c	d				
Number of stops	$z=a+by^3+cy^{0.5}\ln y$	545.62 ± 27.90	0.12 ± 0.05	-54.99 ± 10.73		78	26.03	<0.001	0.38
Resting time	$z=a+bx\ln x+cx^2+de^{-y}$	72.03 ± 13.10	0.04 ± 0.02	-0.0004 ± 0.0002	298.65 ± 20.62	77	71.93	<0.001	0.72
Distance walked	$z=a+by+cy\ln y$	134.86 ± 65.53	256.64 ± 41.91	-75.15± 16.42		78	53.05	<0.001	0.56
Walking velocity	$z=a+by+cy^{1.5}$	0.60 ± 0.09	0.35 ± 0.06	-0.07 ± 0.02		78	46.78	<0.001	0.52

*In the regression models, z indicates the dependent variable (walking variables), x indicates the first independent variable (azadirachtin dose) and y indicates the second independent variable (age after emergence).

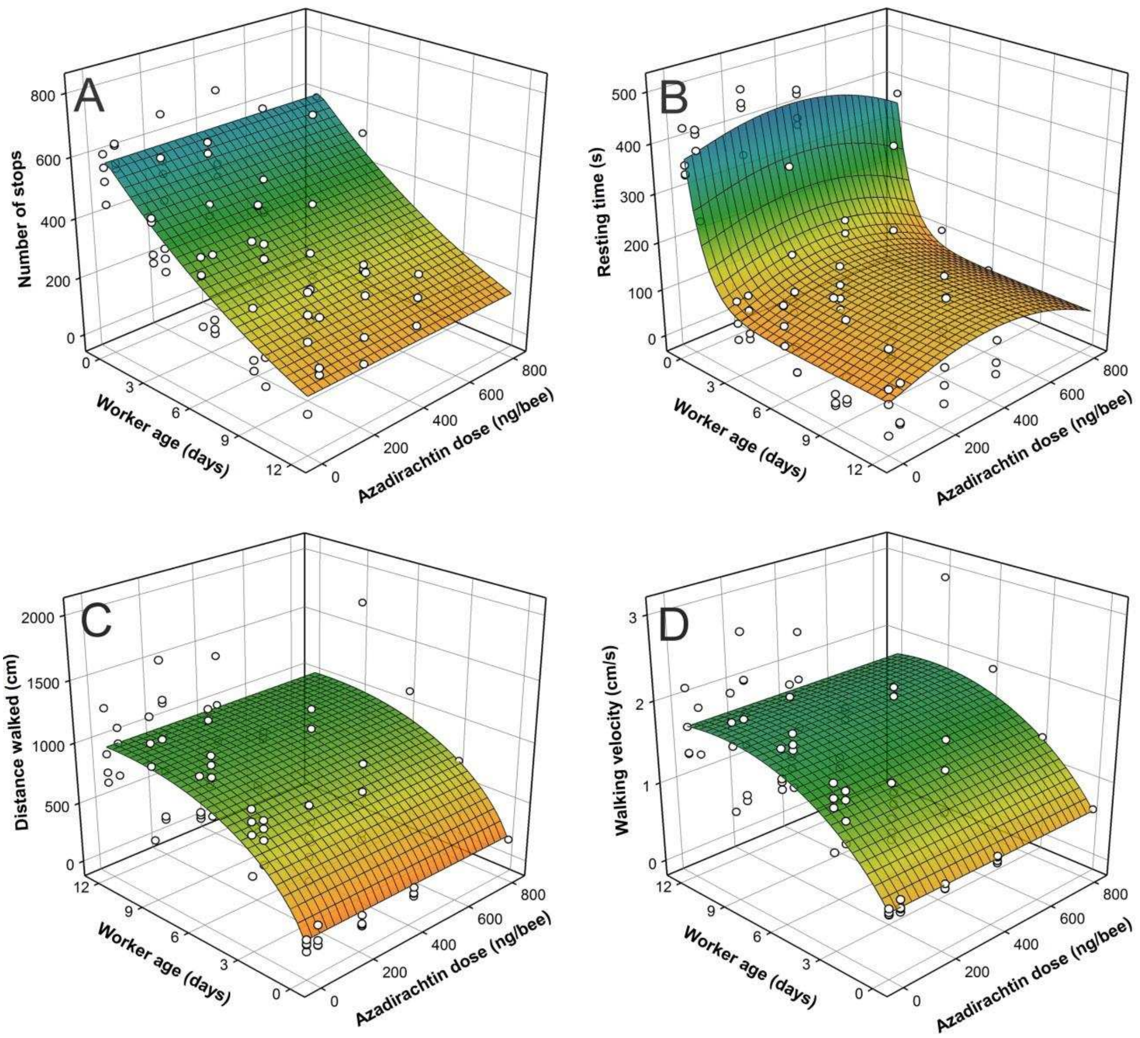

Fig. 8 Walking behaviour plots of workers of the stingless bee *Melipona quadrifasciata* after larval exposure to azadirachtin. (**A**) number of stops, (**B**) resting time, (**C**) distance walked and (**D**) walking velocity. Changes in the color pattern of the surface curve indicate changes in the range of values in the walking variables (i.e., the dependent variable in the z-axis of each plot).

By contrast, walking behaviour of adults engendered from spinosad-treated larve depended on insect age and insecticide dose (Table 2, Fig 9). The highest doses of spinosad (1142 and 11424 ng/bee) were not represented because they killed all the individuals before they reached the adult stage. The response surface curves for spinosad showed that increasing the age (in days) decreased the numbers of stops (Fig. 9A) and resting time (Fig. 9B), but it increased the distance walked (Fig. 9C) and walking velocity (Fig. 9D). The overall shape of each response surface curve reflected the interaction between the independent variables (age x dose) on the walking parameters measured with high spinosad doses compromising walking activity (Fig. 9).

Table 2 Summary of the regression analyses for the walking activity variables (Fig. 9) of workers of the stingless bee *Melipona quadrifasciata* after the exposure to increasing doses of spinosad during the larval stage. All estimated parameters (a, b and c) of the equations were significant at $p < 0.05$ by Student's t-test.

Walking variables	Model*	Parameter estimates (± SE)			df_{error}	F	p	Adjusted R^2
		a	b	c				
Number of stops	$z=a+bx/\ln x+cy^3$	442.28 ± 21.98	4.83 ± 1.60	-0.06 ± 0.02	124	9.51	<0.001	0.11
Resting time	$z=a+bx^{0.5}+ce^{-y}$	129.26 ± 12.03	13.21 ± 2.39	208.27 ± 20.68	124	74.86	<0.001	0.54
Distance walked	$\ln z=a+bx^{0.5}+ce^{-y}$	6.94 ± 0.05	-0.14 ± 0.02	-0.91 ± 0.30	124	45.17	<0.001	0.41
Walking velocity	$\ln z=a+bx^{0.5}+ce^{-y}$	0.54 ± 0.03	-0.08 ± 0.01	-0.69 ± 0.12	124	53.97	<0.001	0.45

*In the regression models, z indicates the dependent variable (walking variables), x indicates the first independent variable (spinosad dose) and y indicates the second independent variable (age after emergence).

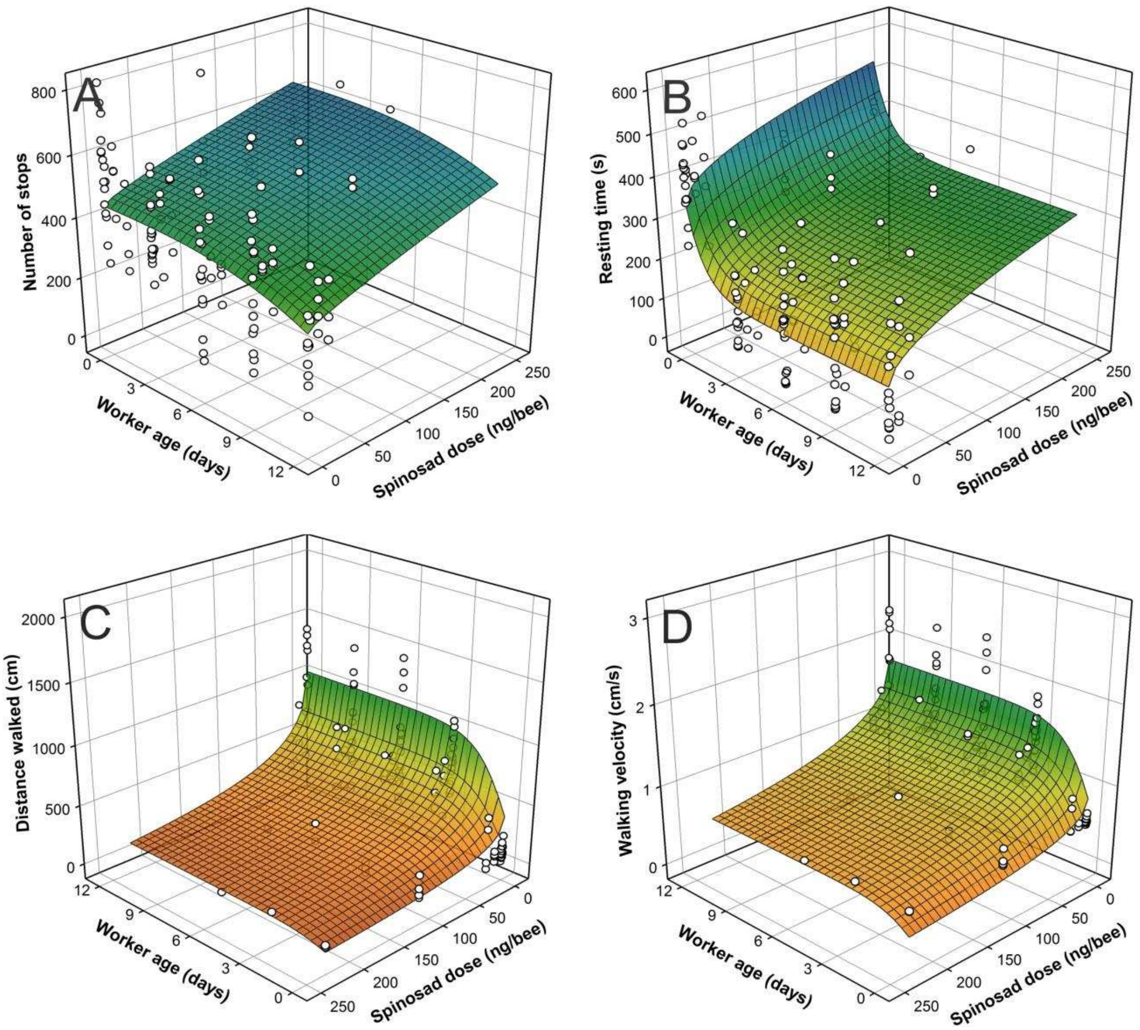

Fig. 9 Walking behaviour plots of workers of the stingless bee *Melipona quadrifasciata* after larval exposure to spinosad. (**A**) number of stops, (**B**) resting time, (**C**) distance walked and (**D**) walking velocity. Changes in the color pattern of the surface curve indicate changes in the range of values in the walking variables (i.e., the dependent variable in the z-axis of each plot).

2.4 Discussion

Over decades, the the impact of pesticides remains as one of the possible causes for the decline in bee abundance and diversity (Potts et al. 2013, Vanbergen and Insect Pollinators Initiative 2013). The very heated debates concerning bee decline are however frequently restricted to a few synthetic insecticides such as neonicotinoids and fipronil and to a limited group of pollinators, with great emphasis to the honey bee *Apis mellifera* (vanEngelsdorp and Meixner 2010, Gross 2013). This skewed focus diverts the attention from other pollinators, such as stingless bees, which are key pollinator species in wild and cultivated crops in the Neotropics (Slaa et al. 2006, Palma et al. 2008, Bispo et al. 2009), and to biopesticides, which are still poorly understood concerning their risk imposed on pollinators (Milles 2003, Morandin et al. 2005, Sánchez et al. 2012). It is well known that pesticide residues are found in bee colonies (Koch and Weiber 1997, Rortais et al. 2005, Mullin et al. 2010) and have potential to impair the brood with lethal and sublethal effects (Thompson 2003, Desneux et al. 2007, Wu et al. 2011, Tomé et al. 2012, Bryden et al. 2013). Nonetheless, most of the studies addressing the effects of pesticides on bees are directed only to the adult stage, therefore neglecting the immature larval stages. Considering this above-mentioned lack of information in the risk assessment of pesticides on pollinators, we carried out a successful rearing *in vitro* of the stingless bee *M. quadrifasciata anthidioides* and assessed the lethal and sublethal effects on the brood and surviving adults after larval exposure to the bioinsecticides azadirachtin and spinosad.

In the present study, the overall survival and median survival time of the larvae were used as first signs of susceptibility of the stingless bees to both azadirachtin and spinosad. The chronic ingestion of these bioinsecticides impaired the survival of the larvae in a dose-dependent manner but showed significant toxicity only for doses higher than 210 ng/bee for azadirachtin and 114 ng/bee for spinosad. Afterwards, appearing as sublethal effect, the fresh body mass of the pupae was negatively correlated to the ingested dose of each bioinsecticide (azadirachtin and spinosad). Such decrease in the pupal body mass is not an effect of a feeding behavioral impairment since no residual diet was observed after the end of the feeding period within the artificial cells, which contained enough food (140 µL of diet) for the full development of the workers. Therefore, neither azadirachtin nor spinosad stopped the feeding behavior of the exposed stingless bee larvae; not even azadirachtin which is well known by its antifeedant activity (Mordue (Luntz) and Nisbet 2000, Morgan 2009).

Altered developmental time was not observed with both bioinsecticides but, as expected, azadirachtin caused deformities in the pupal stage, which also resulted in deformed adults after molting. This effect is probably due to disturbances in the juvenile hormone and/or ecdysteroids titers which are related to the insect growth regulator (IGR) property of azadirachtin (Mordue (Luntz) and Nisbet 2000, Morgan 2009). Surprisingly though, deformed pupae and adults also appeared as consequence of spinosad exposure and at proportions higher than those found with azadirachtin. Such response is certainty not due to the well-established spinosad cause-effect relationship, which is to hyperexcite neurons leading to an eventual insect paralysis with subsequent death (Salgado 1998, Sparks et al. 2001), but to another physiological impairment possibly involving hormonal disturbances as described for IGRs (Mordue (Luntz) and Nisbet 2000, Arthur 2001, Senthil Nathan et al. 2007). However, there is no mechanism in the literature to describe how spinosad may physiologically trigger hormonal disorders in insects and consequently leads to deformities. It is obvious that deformed workers are less able or even useless to perform the variety of tasks, which the colonies depends on to survive since essential activities related to locomotion, foraging, feeding and olfactory perception and communication were compromised.

As sublethal effects, behavioral abnormalities on the walking locomotion of the workers of the stingless bee *M. quadrifasciata* were also noted. Newly emerged adults were virtually inactive regardless of the bioinsecticides, because their locomotor activity increases with age (Tomé et al. 2014a). However, adults of 3 days old or older showed distinct walking activity when exposed to azadirachtin or spinosad during their larval stage. The walking activity of azadirachtin-exposed workers increased with age similar to the untreated insects. Nonetheless, spinosad at doses as low as 2.29 ng/bee compromised the gradual increase in the walking activity. Such divergence on walking response for azadirachtin and spinosad is explained probably by difference between the modes of action of these bioinsecticides. While azadirachtin acts primarily as IGR by causing hormonal disturbances and remotely by interfering with the insect nervous system through the impairment of brain neuropeptides (Sayah et al. 1998, Mordue (Luntz) and Nisbet 2000, Morgan 2009), spinosad acts primarily in the insect nervous system as agonist primarily of nAChR and secondarily of γ-aminobutyric acid (GABA) receptors (Salgado 1998, Sparks et al. 2001).

The behavioral impairment caused by spinosad is corroborated by a similar effect caused by the synthetic insecticide imidacloprid in the walking activity of workers of *M. quadrifasciata anthidioides* reared at the same way as in this study (Tomé et al. 2012). Like spinosad, imidacloprid interferes agonistically with nACh receptors in the insect nervous system, but in a different target site (Goldberg et al. 1999, Armengaud et al. 2000, Déglise et al. 2002). In addition, morphometric analyses revealed that imidacloprid impaired the development of the mushroom bodies in the brain of *M. quadrifasciata anthidioides* (Tomé et al. 2012) and this activity was also confirmed with

Kenyon cell cultures from other pollinators as bumblebees (Wilson et al. 2013). The mushroom bodies are brain structures related to the processing and integration of multisensory information, learning and memory in bees (Heisenberg 1998, Mobbs 1998, Menzel 1999, Giurga 2003). These structures show volumetric plasticity dependent on age and experience in adult workers (Durst et al. 1994, Farris et al. 2001, Fahrbach 2006, Tomé et al. 2014a).

We hypothesize that the impairment in the walking activity of the workers may be also related to a restriction in the development of the mushroom bodies because (1) spinosad and imidacloprid share similar target receptor in the insect neuron system, and (2) the mushroom bodies include motor control as part of their sensory integration. A direct effect on the locomotor activity of the workers by spinosad also can be possible, since GABA receptors are found in neuromotor neuron as observed in cockroaches (Sattelle et al. 1988). As future perspective, an even worse impairment would be expected in the flight behavior of the stingless bees with spinosad after compromising walking activities. Therefore, every task of the stingless bees played throughout their lives for the maintenance and survival of their colony could be impaired, for instance, hygienic behavior, comb production, food storing, larvae feeding, guarding and foraging (Waldschmidt and Campos 1997).

The difference in magnitude of the observed effects between the bioinsecticides places spinosad as a more serious threat for stingless bees than azadirachtin. However, it is worth mentioning that for both compounds the results reaffirm that the origin is not a relevant determinant of toxicity (unlike the compound structure and relative physico-chemical properties) and therefore their assessment and potential impact should not be neglected in native pollinators such as *M. quadrifasciata*. In order to confirm the differences observed here with bioinsecticide-exposed stingless bees, more complex experimental setups, including semi-field and field studies need to be performed. This is so because such setups will allow assessment of more complex behaviors, like foraging, which are very important for colony survival and follows recent regulatory guidance (e.g., EFSA 2012). In addition, potential reduced persistence of bioinsecticides may minimize potentially harmful effects detected under laboratory conditions emphasizing the need of semi-field and field studies for a more comprehensive and realistic assessment of the risk imposed by bioinsecticides to native stingless bees.

CHAPTER III

LETHAL AND SUBLETHAL EFFECTS OF AZADIRACHTIN ON THE BUMBLEBEE *BOMBUS TERRESTRIS* (HYMENOPTERA: APIDAE)

Adapted from

Wagner F. Barbosa, Laurens De Meyer, Raul Narciso C. Guedes, Guy Smagghe. 2015. Lethal and sublethal effects of azadirachtin on the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). *Ecotoxicology* 24: 130-142. DOI 10.1007/s10646-014-1365-9

3.1 Introduction

Bees are crucial organisms for the ecology, stability and conservation of terrestrial ecosystems. They are also economically important providing a high valued pollination service for food production in agricultural systems (Klein et al. 2007, Gallai et al. 2009). Recently, some reports have given rise to concerns for the declining of bee populations (Goulson et al. 2008, Brown and Paxton 2009). In the light of recent studies, many factors are attributed to bee declines encompassing parasites, diseases, habitat losses, climate changes and pesticides, which have led to the development of a multifactorial hypothesis for the bee decline problem (Freitas et al. 2009, Van Engelsdorp and Meixner 2010). However, no definitive consensus about the causes of bee decline has been established, but so far pesticides are considered a very important component due to their range of detrimental effects caused in insects (Brittain and Potts 2011).

The use of pesticides has been the major approach in crop protection for decades (Metcalf 1980, Cooper and Dobson 2007) and many insecticide compounds with different modes of action are marketed every year (Lamberth et al. 2013). Consequently, the concern regarding the risk of pesticide exposure to bee species has been increasing (Gill et al. 2012, Johnson et al. 2013). In addition, pesticides may cause a variety of sublethal effects such as impaired development, reproduction and behavior, which are as harmful as the lethal effect for the survival of colonies (Wu et al. 2011, Blacquiere et al. 2012, EFSA 2012, Bryden et al. 2013, Smagghe et al. 2013). Faced with potential risks of pesticides, a new challenge lies in the search for new compounds that are considered less harmfull for the environment (Villaverde et al. 2014). In this context, insecticides of natural origin, also called biorational insecticides or bioinsecticides, have received considerable acceptance (Cantrell et al. 2012).

Currently, azadirachtin, a complex tetranortriterpenoid limonoid extracted from seeds of the Indian neem tree *Azadirachta indica* (Meliaceae) is one of the most prominent bioinsecticides available (Boeke et al. 2004). Because of its natural origin, low mammalian toxicity and fast degradation, the utilization of azadirachtin has been widely encouraged for crop protection (Isman 2006). However, azadirachtin is known to possess strong biological properties as feeding deterrent and insect growth regulator (IGR) (Morgan 2009) which may warrant assessments of the potential risks against beneficial arthropods, especially bees.

The bumblebee species *Bombus terrestris* is a well-known pollinator of wild flowers and has also become economically important since it has been utilized for over decades in the commercial pollination of agricultural crops like tomato and strawberry (Velthuis and van Doorn 2006). Until now, there is no study related to the effects of azadirachtin on bumblebees, and the few existing studies were exclusively carried out with *Apis mellifera* (Melathopoulos et al. 2000, Thompson et al. 2005). In this sense, more information about the potential impairments of azadirachtin on bumblebees needs to be generated. In this study we hypothetised that azadirachtin could lead to lethal and sublethal effects on feeding behavior, morphology, reproduction and foraging behavior of *B. terrestris*. First, bumblebees were exposed to azadirachtin at individual level in the laboratory where effects of food repellence were investigated. Second, microcolonies were exposed to the compound and we scored effects against bumblebee survival and nest reproduction. Third, we tested for risks when bumblebees needed to forage for their food in a behavior laboratory setup. In the experiments, the compound was tested at different concentrations above and below the maximum concentration that is used in the field (32 mg/L). We believe these results may help to better understand the potential risks of azadirachtin for bumblebees and bees in general.

3.2 Materials and methods

3.2.1 Insects

All bumblebees were obtained from a continuous mass-rearing (Biobest, Westerlo, Belgium) and maintained in a room at 30 °C, 60 % of relative humidity (RH) and continuous darkness. The insects were fed with commercial sugar water (Biogluc, Biobest) and honeybee-collected pollen (Soc. Coop. Apihurdes, Pinofranqueado-Cáceres, Spain) as energy and protein source, respectively (Mommaerts et al. 2010).

3.2.2 Chemicals

Commercial formulations of azadirachtin (Insecticida Natural Neem, BioFlower, Tàrrega, Spain) and imidacloprid (Confidor 200 SL, Bayer CropScience, Machelen, Belgium) were used. Azadirachtin was tested in a series of concentrations above and below the maximum field recommended concentration (MFRC): 32 mg/L. Imidacloprid was added as reference of drastic effects and tested at 0.02 mg/L because this concentration was reported to affect foraging behavior in bumblebees (Mommaerts et al. 2010). All insecticide solutions were prepared using commercial sugar water (Biogluc) as used in the colony rearing.

3.2.3 Repellence bioassay with individual workers

A laboratory bioassay was performed to assess the repellent effect of azadirachtin on bumblebee workers via treated sugar water. Prior to exposure, workers were randomly collected from four queen-right colonies of *B. terrestris* and starved for 2 h in a plastic box (15 × 15 × 10 cm). The selected workers were individually exposed to 5 µL of azadirachtin-treated sugar water inside a glass tube (2 cm diameter × 10 cm length). The 5 µL-drop of treated sugar water was placed on the bottom of each glass tube maintained in a horizontal position. A light source was provided at the bottom of the glass tubes to attract the workers nearby to the treated solution. The tubes were closed with a Styrofoam cork and the exposure was maintained for 5 min. After this period, azadirachtin-exposed workers were placed in clean glass tubes with 5 µL of untreated sugar water during 5 min in the same way as described above. Bees were considered repelled when they did not or only partially fed on the treated sugar water drop and subsequently fed on the untreated sugar water. Bumblebees that rejected both the treated and subsequently the untreated sugar water were not considered properly starved and did not enter as data. Five concentrations of azadirachtin (32, 160, 320, 640 and 1600 mg/L) were used; plain sugar water was used as control treatment. A total of 80 bumblebees were used per concentration; i.e. 20 bees from each queen-right colony. The average repellence among the bees of given queen-right colony was considered as a replicate. The experiment was carried out in a red-lightened room at 30 °C temperature and 60 % RH.

3.2.4 Chronic bioassay with microcolonies not including foraging behavior

A laboratory bioassay was carried out to quantify the lethal effect and reproduction fitness of bumblebee's microcolonies under chronic oral exposure.

The microcolonies were made by placing five newly-emerged workers into an artificial plastic nest box (15 × 15 × 10 cm). The microcolonies were fed with plain sugar water via a container of 500 mL under the nest box and pollen inside the nest (Mommaerts et al. 2010). After 1 week one worker bumblebee became dominant and started to lay unfertilized eggs that produce only male offspring (Michener 1974).

Immediately after the 1-week period, the workers were orally exposed to a range of azadirachtin concentrations via treated sugar water that was placed in a container (500 mL) beneath the artificial nests. Azadirachtin was diluted at 320, 64, 32, 16, 6.4 and 3.2 mg/L, corresponding to 10/1, 2/1, 1/1, 1/2, 1/5 and 1/10 times of the MFRC. The exposure lasted 11 weeks. Plain sugar water was used as control treatment. Imidacloprid was used at 0.02 mg/L (Mommaerts et al. 2010). Pollen was replaced twice a week. Eight artificial nests with five workers were used per treatment.

The mortality was assessed every two days and used to estimate survival curves. The sublethal effect on reproduction was monitored on a weekly basis by removing the emerged drones from the microcolonies and counting them. As a measure of sublethal effect, the body mass of the male progeny was also scored by weighting the drones after they had been killed by freezing during 1 hour. The amount of the consumed sugar water was followed by weighting the containers every week; the impact of evaporation was subtracted from the weight loss by assessing the weight of sugar water containers coupled with artificial nests without workers that were placed in parallel with the bioassay under the same environmental conditions.

To verify the effect of azadirachtin on the ovarian development, a dominant worker from each concentration of 0.0, 3.2, 6.4, 16, 32 and 64 mg/L was taken in microcolonies with at least four individuals at 45 days after the beginning of the exposure. The ovaries were dissected in a phosphate buffer solution, photographed and their length measured using the software Image Pro Plus™ (www.mediacy.com/index.aspx?page=IPP, MediaCybernetics, Bethesda, MD). The dominant worker was chosen based on the threatening behavior and overt aggression described for bumblebees (van Honk et al. 1981, van Doom and Heringa 1986).

3.2.5 Chronic bioassay with microcolonies including foraging behavior

A laboratory bioassay was carried out to assess the impact of lethal and sublethal concentrations of azadirachtin on the performance of bumblebee microcolonies which included foraging behavior under laboratory conditions. This was performed following an adapted foraging behavior bioassay as described by Mommaerts et al. (2010). Briefly, two artificial plastic nest boxes A and B (15 × 15 × 10 cm) were connected by a plastic tube (20 cm length and 2 cm of diameter). Five newly emerged workers were placed in box A where they received pollen placed in the box and sugar water via a container (500 mL) placed beneath the box. After eight days, when egg-laying started in box A, the sugar water was removed from box A and replaced underneath box B. The workers were then allowed two days to adapt to this new situation. Subsequently, plain sugar water in box B was replaced with treated sugar water.

Azadirachtin was diluted at 32, 3.2, 0.64, 0.32, 0.16 and 0.064 mg/L, corresponding to 1/1, 1/10, 1/50, 1/100, 1/200, 1/500 times of the MFRC. The exposure lasted 11 weeks. Plain sugar water was used as control treatment. Imidacloprid was used at 0.02 mg/L (Mommaerts et al. 2010). Pollen was replaced twice a week to avoid unattractive reactions. Eight experimental units (connected boxes A and B with five workers) were used per treatment.

The mortality was assessed every two days and used to estimate survival curves. The sublethal effect on the reproduction was monitored on a weekly basis by counting the number of emerged drones. The body mass of the male progeny was also scored as a measure of sublethal effect. The male offspring was photographed in order to characterize morphology disturbances. The amount of consumed sugar water was followed by weighting the containers on a weekly basis as already described.

3.2.6 Male progeny survival and sperm length

A laboratory bioassay was carried out to verify whether azadirachtin interferes with the survival and reproductive features of the adult male progeny exposed to the compound in earlier larval stages. For that purpose, microcolonies with five newly-emerged workers were setup as described in the chronic bioassay with microcolonies not including foraging behavior. Here, azadirachtin was used at 0.64, 0.32 and 0.064 mg/L, corresponding to 1/50, 1/100, 1/500 times of the MFRC. Plain sugar water was used as control treatment. Imidacloprid was used at 0.02 mg/L (Mommaerts et al. 2010). Five microcolonies were used per treatment. When the male offspring started to emerge, the first fifteen new born drones were collected from each microcolony and individually placed in horizontally positioned Falcon tubes. Pollen and plain sugar water were provided to the drones as food source and replaced twice a week. The drones were kept in the tubes for two weeks and the mortality was recorded daily. Both microcolonies and Falcon tubes were maintained in a room under complete darkness at 30 °C, and 60 % RH.

At the age of 14 days, fifteen drones of each treatment (three drones per microcolony) were taken from the Falcon tubes and prepared for dissection. The drones were dissected and the male accessory testis were opened in 200 μL of PBS and homogenized. An aliquot of 10 μL was dispensed onto a glass slide, air dried, fixed in ethanol 70 % at −20 °C for 30 min and washed in PBS for 1 min. The sperm on the glass slides was photographed under light microscope coupled with a camera and 10 non-coiled spermatozoids of each collected drone were measured using the software Image Plus™ (www. mediacy.com/index.aspx?page=IPP; MediaCybernetics, Bethesda, MD).

3.2.7 Statistical analysis

The repellence data was subjected to probit analysis (PROC PROBIT) using SAS (SAS Institute, Cary, NC). The worker's and drone's survival data were subjected to survival analysis using the procedure Survival LogRank in SigmaPlot 12.0 (Systat, San Jose, CA). The survival curves were obtained by Kaplan–Meier estimators and all were pairwise compared using the Bonferroni method. The insects that survived until the end of the experiment or insects collected for ovarian comparison were treated as censored data (Allison 1998). The median survival times (LT_{50}) were subjected to regression analysis considering azadirachtin concentration as the independent variable or pairwise compared using the 95 % confidential intervals when necessary. Logistic regression was carried out to the cumulative number of drones using the curve-fitting procedure from SigmaPlot 12.0. Model selection was based on parsimony, high F values (and mean squares), and steep increase in R^2 with model complexity. Insect body mass was also subjected either to analysis of variance or regression analysis in SAS. The assumptions of normality and homoscedasticity were checked before data analysis (PROC UNIVARIATE, SAS Institute).

3.3 Results

3.3.1 Repellence bioassay with individual workers

Nearly 83 % of repellence was scored with the highest concentration of azadirachtin tested (1600 mg/L), while only 7 % was scored at the lowest concentration (32 mg/L). Four out of five azadirachtin concentrations (160, 320, 640 and 1600 mg/L) were suitably adjusted to the probit model ($\chi^2 = 3.96$, $p = 0.14$) (Fig. 1) which allowed to estimate the median repellence concentration (RC_{50}) as 504 mg/L (95 % fiducial limits = 417–606 mg/L) (Fig. 1).

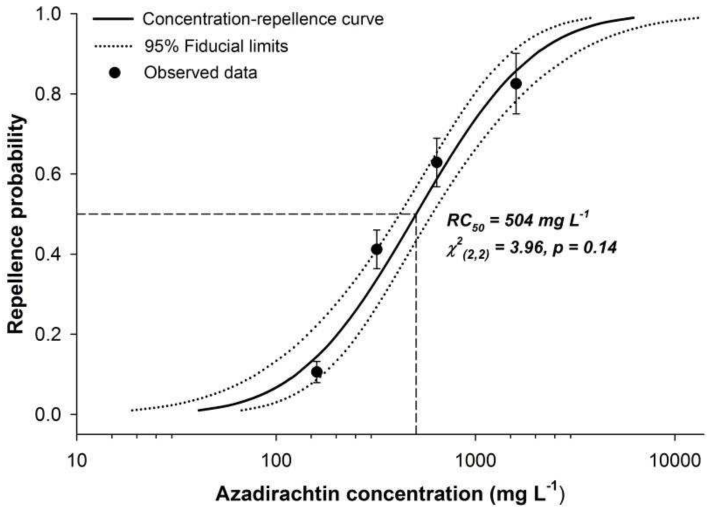

Fig. 1 Repellence of *Bombus terrestris* after oral exposure to increasing concentrations of azadirachtin via treated sugar-water. A probit curve is shown with upper and lower 95 % fiducial limits. *Closed circles* and *verticalbars* represent the repellence mean value and standard errors, respectively, among individuals from four different colonies. The value of the median repellence concentration is indicated in RC_{50}

3.3.2 Chronic bioassay with microcolonies not including foraging behavior

Survival of bumblebee workers was significantly different among azadirachtin concentrations (Log-Rank test: $\chi^2 = 369.28$, d.f. = 7, $p < 0.001$). The survival curve of azadirachtin at 3.2 mg/L was similar to both control ($p = 0.43$) and imidacloprid at 0.02 mg/L ($p = 0.15$) curves. A strong effect was observed for insects exposed to azadirachtin at 320 mg/L with complete mortality (100 %) around 2 weeks (15 days) of exposure (Fig. 2A). After

11 weeks of exposure, survival rates were below 30 % for insects exposed to azadirachtin concentrations between 6.4 and 320 mg/L. Survival rates were above 50 % only for insects exposed to the lowest concentration of azadirachtin (3.2 mg/L), imidacloprid at 0.02 mg/L and control treatment (Fig. 2A). In addition, a negative relationship was observed between azadirachtin concentration and median survival time (LT_{50}) (Fig. 2B).

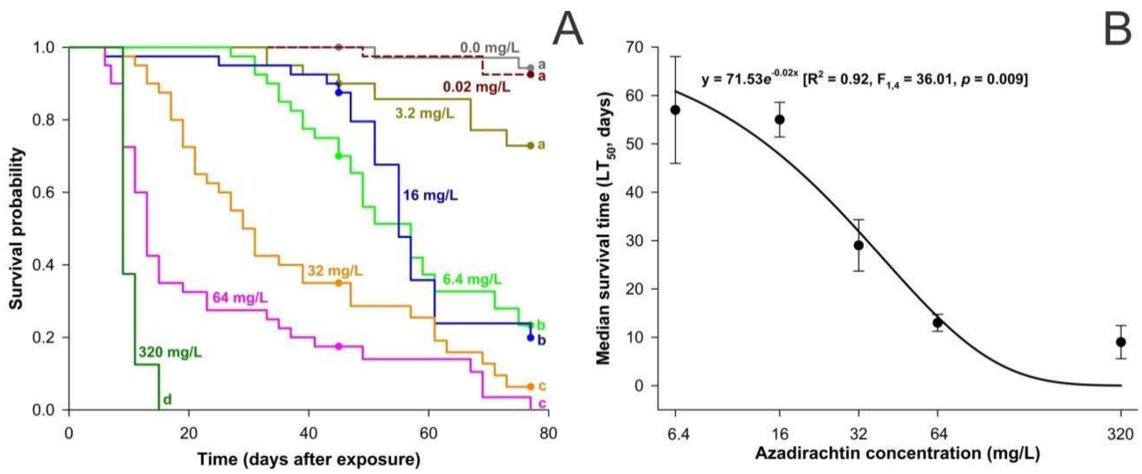

Fig. 2 Survival plots (**A**) and median survival times (LT50) (**B**) of bumblebee workers (*Bombus terrestris*) chronically exposed to a series of azadirachtin concentrations via treated sugar water. Data originated from chronic bioassay without foraging behavior. Untreated sugar water (control) is represented by a *grey solid curve*; imidacloprid at 0.02 mg/L is represented by a *red dashed curve*. **A** Same letters at the end of survival curves indicate no significant difference by Bonferroni method ($p > 0.05$). *Closed circle* indicates censured data. **B** *Vertical bars* indicate 95 % of confidence intervals.

We also observed a negative effect of azadirachtin on bumblebee reproduction. No male offspring was produced in the microcolonies exposed to azadirachtin concentrations above 6.4 mg/L during the 11 weeks of assessment. Drone production was only observed in microcolonies exposed to the control treatment, imidacloprid at 0.02 mg/L and azadirachtin at 3.2 mg/L. However, the number of drones produced with imidacloprid at 0.02 mg/L (42.9 ± 4.7) and azadirachtin at 3.2 mg/L (2.2 ± 1.0) was lower than the control (58.6 ± 3.3). Azadirachtin at 3.2 mg/L also inhibited the appearance of the male progeny in 6 weeks (Fig. 3). Azadirachtin at 3.2 mg/L reduced the body weight of the male progeny (0.17 g ± 0.01) when compared to the control (0.25 g ± 0.01) ($p < 0.001$) (Fig. 4).

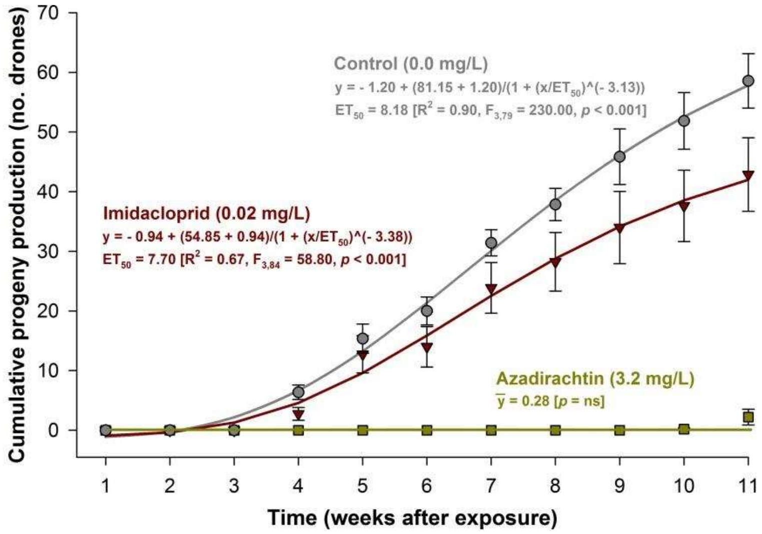

Fig. 3 Reproduction of bumblebee (*Bombus terrestris*) chronically exposed to azadirachtin via treated sugar water. Untreated sugar water (control) is represented by a *grey solid curve*; imidacloprid at 0.02 mg/L is represented by a *red solid curve*. ET_{50} represents median effective time and *vertical bars* represent standard errors.

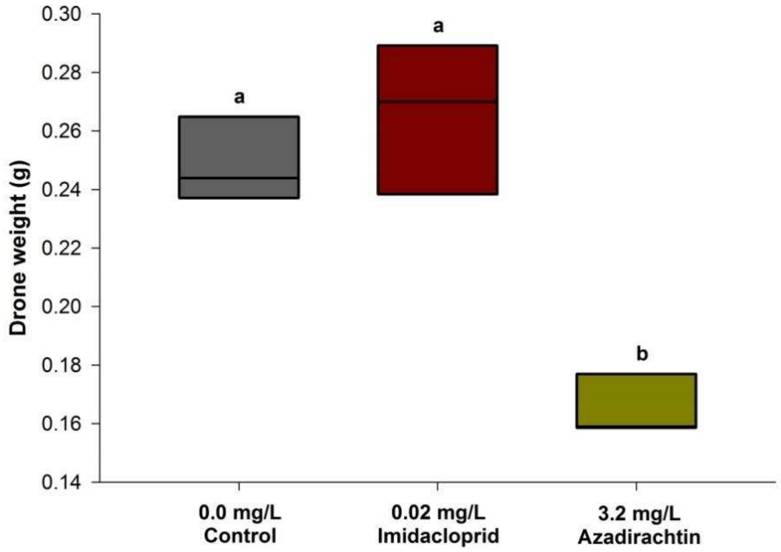

Fig. 4 Body mass of the male progeny produced in microcolonies of bumblebees (*Bombus terrestris*) chronically exposed to azadirachtin via treated sugar water. Data originated from chronic bioassay without foraging behavior. Untreated sugar water (control) is represented by a *grey box blot*; imidacloprid at 0.02 mg/L is represented by a *red box blot*. Boxes followed by the same letter indicate that means (line within the box) were not significantly different by Tukey's HSD test (p < 0.05)

As shown in Fig. 5, the length of ovaries of the dominant workers decreased responding to the increase of the azadirachtin concentration and was 44.9 ± 0.9, 41.6 ± 0.0, 38.1 ± 2.1, 32.0 ± 0.3, 18.4 ± 0.1 and 11.5 ± 1.6 mm for the negative control and azadirachtin at 3.2, 6.4, 16.0, 32.0 and 64.0 mg/L, respectively. Moreover, azadirachtin at 16, 32 and 64 mg/L strongly impaired the ovaries which resulted in the absence of oocytes (Fig. 5).

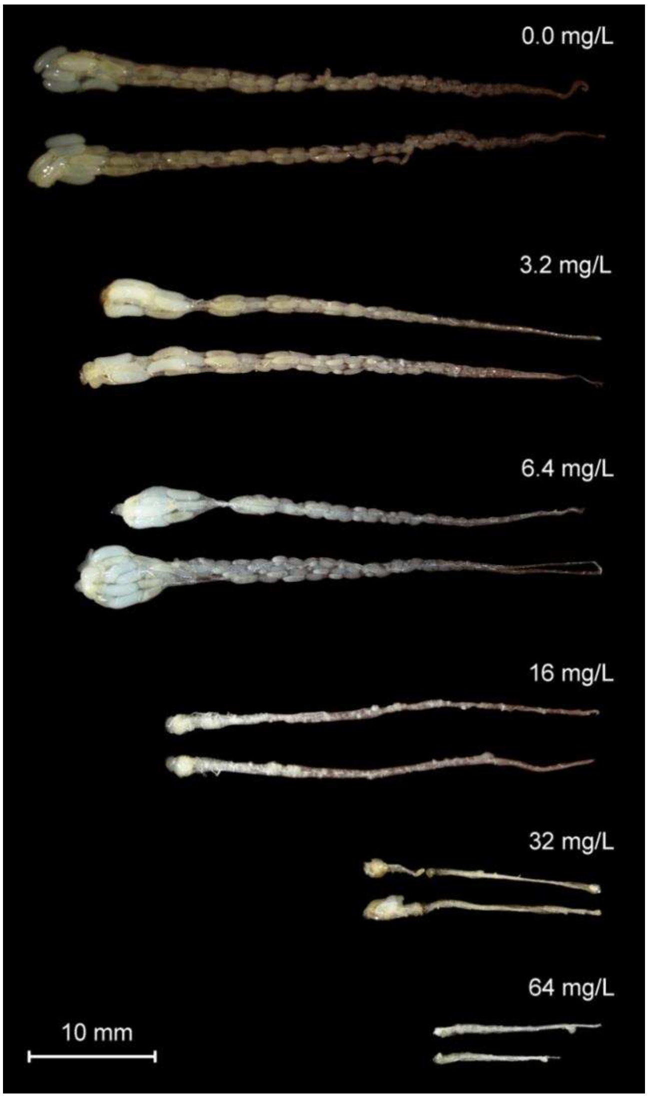

Fig. 5 Overview from ovaries of dominant bumblebee workers (*Bombus terrestris*) chronically exposed to increasing concentrations of azadirachtin. Data originated from chronic bioassay without foraging behavior. *White bar* 10 mm

The sugar water consumption by the bumblebee workers in the control group started with 1.85 ± 0.06 mL per worker and exhibited a peak of 3.21 ± 0.12 mL per worker at the 4th week after exposure, matching the peak in reproduction. In contrast, the consumption of sugar water solution contaminated with azadirachtin at 3.2 mg/L remained stable throughout the experiment (1.53 ± 0.08 mL per worker), while for higher azadirachtin concentrations (i.e., above 6.4 mg/L) there was a steady decrease in consumption. Such decline was larger for azadirachtin concentrations of 16, 32 and 64 mg/L, which started with 1.44 ± 0.15 mL per worker and ended with 0.17 ± 0.09 mL per worker, thus reaching nearly 88 % of decrease throughout the weeks until the end of the experiment. For azadirachtin at 320 mg/L, the sugar water consumption was restricted to 0.36 ± 0.00 mL per worker at the first two weeks after exposure when workers were still alive.

3.3.3 Chronic bioassay with microcolonies including foraging behavior

A significant impaired effect occurred on the survival when bumblebee workers were exposed to increasing concentrations of azadirachtin in the experiment exploring foraging behavior (Log-Rank test: $\chi^2 = 411.447$, d.f. = 7, $p < 0.001$). At this time, the survival curve of azadirachtin at 3.2 mg/L was significantly lower ($p < 0.001$) than the control curve but was similar ($p = 0.09$) to imidacloprid at 0.02 mg/L. The survival curves of azadirachtin concentrations between 0.064 and 0.64 mg/L were also similar ($p > 0.05$) to the control treatment (Fig. 6A). Estimates of median survival time (LT_{50}) were reached only for insects exposed to azadirachtin at 3.2 (75.0 days, 95 % CI = 70.0–80.1 days) and 32 mg/L (31.0 days, 95 % CI = 23.9–38.1 days) and were significantly different ($p < 0.05$) (Fig. 6B).

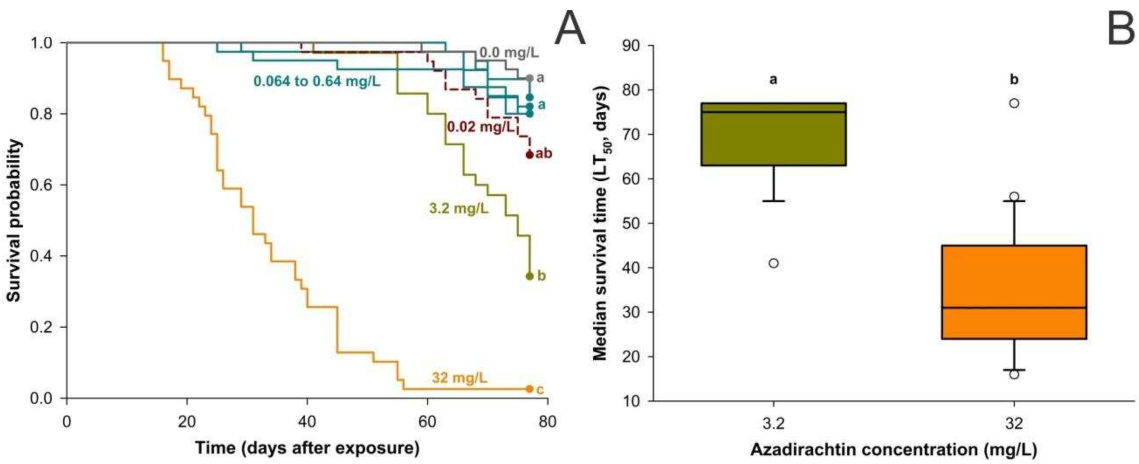

Fig. 6 Survival plots (**A**) and median survival times (LT50) (**B**) of bumblebee workers (*Bombus terrestris*) exposed to azadirachtin via treated sugar water. Data originated from chronic bioassay with foraging behavior. (**A**) Untreated sugar water (control) is represented by a *grey solid curve*; imidacloprid at 0.02 mg/L is represented by a *red dashed curve*. Same letters at the end of survival curves indicate no significant difference by Bonferroni method ($p > 0.05$). *Closed circle* indicates censured data. (**B**) *Box plots* indicate the median (line within the box), mean (open square with standard error bars) and range of dispersion (lower and upper quartiles, represented as the limits of the box, and outliers (symbol)) of the LT_{50}s. The box plots with different lower case letters indicates significant difference between treatments by pairwise comparison of their confidence intervals ($p < 0.05$)

Sublethal effect on the bumblebee reproduction appeared as an absence or reduction in the number of the male progeny when microcolonies were exposed to even the lowest azadirachtin concentrations in the bioassay exploring foraging behavior. A Gaussian regression model was estimated in order to show the pattern of the male progeny production using azadirachtin concentration and time as the independent variables ($F_{4,556} = 1067.11$, $p < 0.001$) (Fig. 7A). The number of drones produced varied slightly throughout the weeks for azadirachtin concentrations between 0.0 (control) to 0.32 mg/L (Fig. 7A). At the concentration of 0.64 mg/L, the male progeny production throughout the weeks was lower than the control treatment (Fig. 7B). For azadirachtin at 3.2 and 32 mg/L, no drone production was observed (Fig. 7A); also poorly developed broods with only few and incomplete sugar pots were observed in these concentrations (Fig. 7D), which contrasts with the control nests (Fig. 7C).

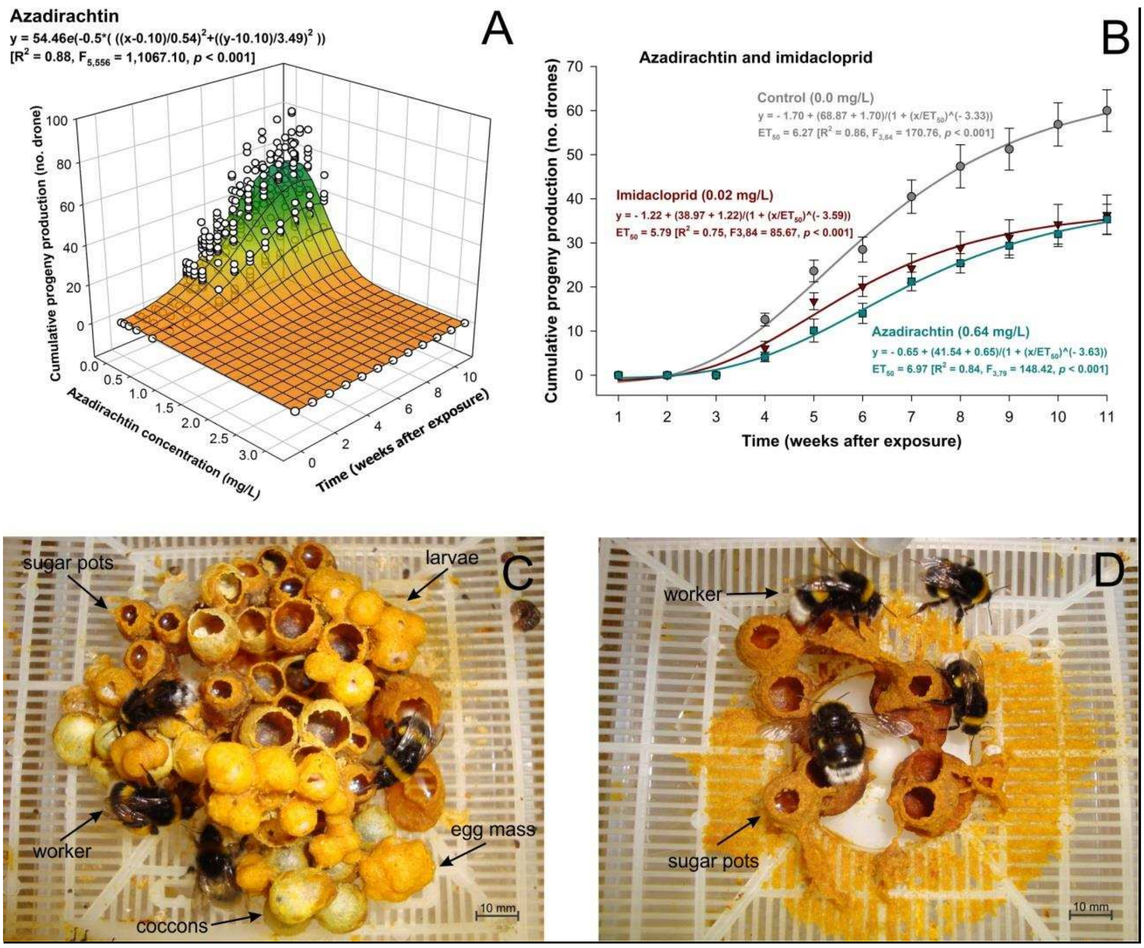

Fig. 7 Reproduction and overview of the nests of bumblebees (*Bombus terrestris*) chronically exposed to a series of azadirachtin concentrations via treated sugar water. Data originated from chronic bioassay with foraging behavior. (**A**) *A Gaussian regression model* representing the progeny production during azadirachtin exposure. (**B**) *Logistic regression models* representing the progeny production during exposure to azadirachtin at 0.64 mg/L. Untreated sugar water (control) is represented by a *grey solid curve*; imidacloprid at 0.02 mg/L is represented by a *red solid curve*. *ET_{50}* represents the median effective time and *vertical bars* represent standard errors. (**C**) A well-constructed bumblebee nest from the control treatment with sugar pots and all immature phases of the male progeny. (**D**) A badly-constructed bumblebee nest from the treatment with azadirachtin at 3.2 mg/L where only few sugar pots were constructed and no eggs were laid. Nests were photographed 7 weeks after the exposure.

Body mass of the male progeny was also negatively affected by azadirachtin ($F_{3,36} = 27.49$, $p < 0.001$) (Fig. 8). Imidacloprid at 0.02 mg/L also impaired the body mass of the male progeny compared to the control treatment ($p < 0.05$) (Fig. 8). Exposure to azadirachtin also caused deformities on the adult appendages as wings, legs, mouth parts and antennae of the drones (Fig. 9). Eight deformed drones appeared over three concentrations of azadirachtin (0.064, 0.32 and 0.64 mg/L). As shown in Fig. 9B and 9C, we observed incomplete wing development in which these appeared as the wing pads of the pupae or a constriction at the end of the wings, legs with undeveloped tarsi, head integument injuries, and deformed mouth parts and antennae.

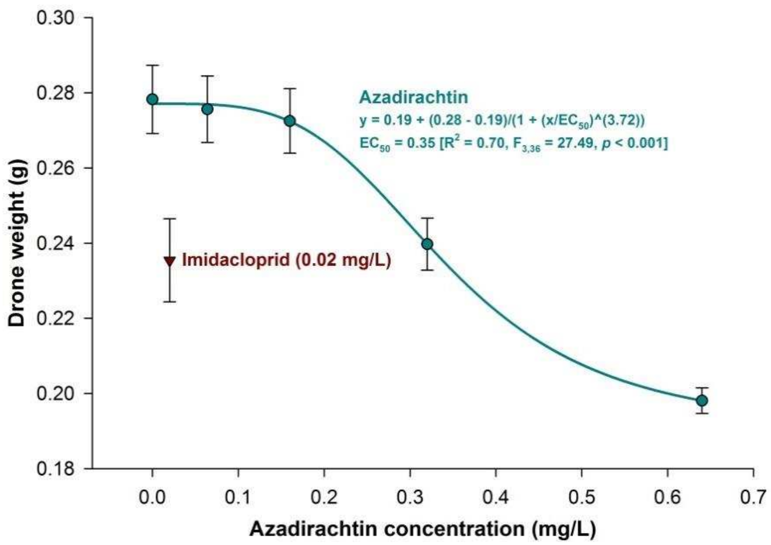

Fig. 8 Body mass of the male progeny produced in microcolonies of bumblebees (*Bombus terrestris*) exposed to increasing concentrations of azadirachtin via treated sugar water. Data originated from chronic bioassay with foraging behavior

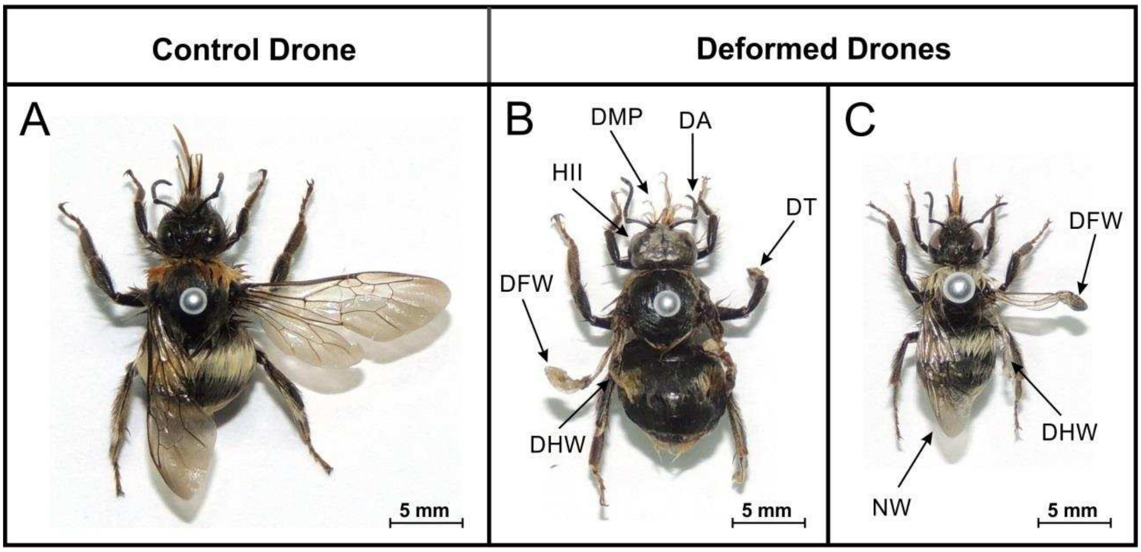

Fig. 9 Overview of external deformities of the male progeny from microcolonies of bumblebees (*Bombus terrestris*) chronically exposed to azadirachtin via treated sugar water. **A** Healthy drone from control treatment, **B** deformed drone from azadirachtin at 0.064 mg/L and **C** deformed drone from azadirachtin at 0.64 mg/L. *DHW* deformed hindwing, *DFW* deformed forewing, *HII* head integument injury, *DMP* deformed mouth parts, *DA* deformed antenna, *DT* deformed tarsus, *NW* normal wings.

The sugar water consumption by the bumblebee workers started with 2.68 ± 0.03 mL per worker and exhibited a peak of 4.10 ± 0.13 mL per worker at the 4th week after exposure, matching the peak in reproduction, when the solution was uncontaminated or contaminated with azadirachtin at concentrations lower than 3.2 mg/L. In contrast, the consumption of sugar water solution contaminated with azadirachtin at 3.2 mg/L started with 2.58 ± 0.08 mL per worker and ended with 0.76 ± 0.19 mL per worker, thus reaching nearly 71 % of decrease throughout the weeks until the end of the experiment. For azadirachtin at 32 mg/L, the consumption of sugar water solution started with 2.00 ± 0.10 mL per worker but at the end of the experiment it was only 0.02 ± 0.00 mL per worker.

3.3.4 Male progeny survival and sperm length

Drones from microcolonies exposed to azadirachtin at 0.64 mg/L had their survival impaired compared to the control treatment (Log-Rank test: χ^2 = 15.99, d.f. = 4, *p* = 0.003). No significant difference was found in the sperm length among the treatments ($F_{4,19}$ = 1.02, *p* = 0.43).

3.4 Discussion

Bumblebees are important pollinators of wild and cultivated plants around the world (Velthuis and van Doorn 2006). As many bee species, bumblebees are in danger of poisoning by pesticides (Gels et al. 2002, Brittain and Potts 2011, Gill et al. 2012). Azadirachtin is one of the most prominent biorational insecticides nowadays (Boeke et al. 2004) and has been considered safe for most beneficial insects (Isman 2006). However, studies addressing the effects of azadirachtin on bees are scarce with focus on a single species namely, the honeybee *Apis mellifera* (Melathopoulos et al. 2000, Thompson et al. 2005). Consequently, a research demand to generate information about risks of azadirachtin to native bee pollinators is needed. Here, at firsthand, a deep assessment was performed in order to reveal the potential impact of azadirachtin on the bumblebee *B. terrestris*. For that purpose, different laboratory bioassays were performed to test the properties of azadirachtin as repellent, IGR and sterilizing compound.

Our first experiment revealed that bumblebees were not very sensitive to the well-known repellent effect triggered by azadirachtin (Mordue (Luntz) and

Nisbet 2000). Azadirachtin used at its MFRC (32 mg/L) caused nearly 7 % of repellence in the bumblebees and the estimated median repellence concentration (RC_{50}) was about 16 times more than the MFRC. The RC_{50} of the bumblebee workers was found in the same range to those reported for other hymenoptera (about 100–500 ppm) and it was higher than those found to other insect orders (Mordue (Luntz) and Nisbet 2000). The repellence may be attributed to the ability of the taste receptors, located in the bumblebee's mouthparts, to recognize azadirachtin. This recognition triggered the primary antifeedant effect of azadirachtin which is also referred as the gustatory antifeedant effect (Mordue (Luntz) et al. 1998). In this case, the stimulation of specific deterrent cells or blockage of the firing of sugar receptor cells in chemoreceptors stops the feeding behavior (Blaney et al. 1990, Simmonds et al. 1995). Considering the MFRC of azadirachtin, the repellence results shown here have a significant relevance for oral exposure in bumblebees, since the gustatory antifeedant effect will probably not be triggered and, thereby, azadirachtin-contaminated food sources (i.e. pollen and nectar) may be collected and transferred to the bumblebee nests and become part of the larval diet. This has an implication for the brood development of bumblebees due to the IGR properties of azadirachtin and indeed, azadirachtin affected the bumblebee larval progeny according to our results. So far there is no evidence of translocation of azadirachtin to pollen or nectar when this compound is sprayed onto leaves (Naumann and Isman 1996). When applied to the soil, azadirachtin has been able to translocate in plants and control phytophagous insects (Thoeming and Poehling 2006), but the compound and its derivatives have not been quantified on nectar or pollen.

Azadirachtin also may cause a secondary antifeedant effect in insects which comprises a reduction of food intake and digestive efficiency (Mordue (Luntz) and Nisbet 2000). This secondary effect is called physiological antifeedancy because it is triggered by hormonal disturbances that suppress the gut peristalsis and/or the synthesis or release of digestive enzymes (Timmins and Reynolds 1992, Trumm and Dorn 2000). The physiological antifeedancy is activated post-ingestion and therefore it is unlikely to have contributed to the repellent effect in bumblebee workers that tasted only a small amount of azadirachtin-treated sugar water (5 µL) in our previous repellence experiment.

However, this secondary antifeedancy may have contributed for the other effects measured in this study.

The survival of adult bumblebees was negatively correlated to the azadirachtin concentration in both experiments with and without foraging behavior. This change in the workers' survival profile can be explained by an exchange between the gustatory and physiological antifeedant effects of azadirachtin on insects. The gustatory antifeedancy immediately stops the utilization of the energy source (sugar water) because it blocks the food intake, while the physiological antifeedancy has a palliative impact reducing the food intake and/or uptake. Therefore, the fast decline of the survival in worker bumblebees exposed to high concentrations of azadirachtin may be due to the gustatory antifeedancy that blocked the food intake. Indeed, workers were more sensitive to the gustatory antifeedancy only in high concentrations as showed in the repellence test. Without sugar water, worker bumblebees cannot survive more than 2 days after starvation as we observed in a small extra experiment. However, individuals exposed to high concentrations of azadirachtin (for instance, 64 and 320 mg/L) started to die nearly 10 days after exposure. This was probably because workers were able to feed on the untreated sugar water as stored in the nest pots before the exposure allowing them to survive more than 2 days. On the other hand, individuals that showed a prolonged survival when exposed to the other concentrations may have had a better use of the energy source because they were sensitive only to the secondary antifeedant effect of azadirachtin. Apart from the antifeedant effects, azadirachtin has a range of cytotoxic effects such as interference with cell division, vacuolization of the cytoplasm and breakdown of protein synthesis in a variety of insect tissues (Salehzadeh et al. 2002, Sayah 2002), which may have contributed to impair the integrity of the workers' living body.

Additionaly to the lethality, sublethal effects were also recorded on bumblebee microcolonies. The impact on reproduction, for instance, was quite severe when microcolonies were chronically exposed to azadirachtin. First, azadirachtin was able to reduce or completely block the production of drones depending on the concentration used. Azadirachtin has sterilizing activity among different insect species (Sayah et al. 1998, Arno and Gabarra 2011). This effect is generally attributed to disturbances in the synthesis or release of hormones or neurohormones involved in the insect reproduction (Dorn et al. 1986, Barnby and Klocke 1990). In bumblebees, as well as in distinct insect species, juvenile hormone (JH) and ecdysteroids are the main hormones linked to the behavioral and physiological aspects of the reproduction (Bloch et al. 2000a, 2000b). Such hormonal disturbances caused by azadirachtin are in general linked to damages on ovarian development or related processes (Sayah et al. 1996, Lucantoni et al. 2006). These impairments include blockage of oogenesis, disruption of vitellogenesis and vitelline envelope formation, degeneration of follicle cells and breakdown of yolk protein production (Sayah et al. 1996, 1998).

As perceived even with a crude visual analysis, azadirachtin impaired the oogenesis of the bumblebee ovaries and the severity of the effects depended on the concentration used. However, since azadirachtin has antifeedant effects against insects, the impact on ovarian development and consequently reproduction may be additionally attributed to a low food intake or uptake. In our experiments, for instance the consumption of sugar water per worker decreased nearly 90 % throughout the weeks with azadirachtin at 16, 32 and 64 mg/L, where not only egg-laying was blocked but also no oocyte was observed in the ovaries. At the concentrations of 3.2 and 6.4 mg/L, azadirachtin only slightly affected the ovarian length and the consumption of sugar water per worker remained constant throughout the weeks. Therefore, these effects on ovaries indicate that the antifeedant effect possibly also contributed to the increased severity of the impairment caused by azadirachtin on bumblebee reproduction.

It is worth to mention that the support, given by the subordinate workers to the dominant worker in order to reproduce, became much lower as the survival was impaired over time by azadirachtin. The reduced number of workers impaired the construction of the nest and in turn this may affect the egg-laying of the dominant worker. However, in our experiments the egg-laying was immediately blocked after the oral exposure to concentrations of azadirachtin above of 3.2 mg/L; this indicates that the impairment on reproduction was mainly due to physiological effects trigged by azadirachtin on the dominant worker, but not due to the lack of subordinated workers to support them.

We observed that egg-laying was restored in the treatment with azadirachtin at 3.2 mg/L in the laboratory experiment without foraging behavior. At this concentration, larvae were also able to complete their development and drones emerged. In this case, the recovery of reproduction measured as male progeny production, is probably related to the degradation of azadirachtin through time, which may have reduced poisoning allowing egg-laying and ensuring survival of the dominant workers and larvae, respectively. Azadirachtin kept into aqueous solutions and under low ultra-violet (UV) condition shows much less degradation when compared with dry surfaces and under high UV condition (Thompson et al. 2002, Kumar and Poehling 2006). This probably contributed to the delay in 6 weeks of the egg-laying of the dominant worker exposed to azadirachtin at 3.2 mg/L in the laboratory bioassay without foraging behavior. Second, reproduction probably was also restored due to the production of detoxifying enzymes and/or excretion of the compound allowing the recovery of the impaired physiological systems associated with the oviposition.

Sublethal effects of azadirachtin were also expressed as a reduction in the body mass of the adult male offspring. This is probably because the progeny underwent the physiological antifeedant effect of azadirachtin during its larval stages. Therefore, treated larvae may have eaten less than larvae from the control. In insects, bad nutrition, starvation, or restriction of food during larval stages may force pupation before the achievement of an ideal species-specific weight given rise to smaller adult individuals (Munyiri et al. 2003, Chen and Ruberson 2008). For imidacloprid, we believe that reduction of body mass of male progeny was due to impairment of the foraging behavior. This was because imidacloprid reduced the weight of the drones only in the chronic bioassay including foraging behavior. Impact on foraging probably led to an indirect effect in the care of the offspring because the collection of food was reduced and consequently the supplying to the larvae. Imidacloprid is a well-known neonicotinoid insecticide that acts as agonist of nicotinic acetylcholine receptors (nAChR) leading to hyperexcitation of neurons (Casida and Quistad 2004, Jeschke and Nauen 2008). Due to its neurotoxic character, imidacloprid may impair learning, memory and foraging behavior of bee species (Decourtye et al. 2004a, Yang et al. 2008). Thus, the impact on the drone body mass could only be observed in the laboratory experiment that included the possibility to perform foraging behavior. For many insects body mass or size of males may interfere with the mating dynamics, sexual selection, reproductive potential and/or progeny production (Zanuncio et al. 2002, Schluns et al. 2003, Amin et al. 2012). Therefore, measures on body mass or size in the male progeny of bumblebees become an important sublethal effect.

As expected, azadirachtin induced deformities on the adult male progeny which appeared mainly on wings, but also on legs and antennae. These effects are likely related to defective metamorphosis which may be a result of disturbances of ecdysteroids or JH titers (Lowery et al. 1996, Senthil Nathan et al. 2007). Similar deformities caused by azadirachtin on wings and legs were also reported in *Nilaparvata lugens* (Homoptera: Delphacidae) (Senthil Nathan et al. 2007). For a better understanding, the relevance of these sublethal impacts should be also investigated under more field related conditions.

Putting the data together from both chronic toxicity bioassays with and without foraging behavior, we can infer that the inclusion of foraging behavior in the experimental setup increases the overall lethal and sublethal effects of the compound tested. For instance, the survival of bumblebee workers was lower with azadirachtin at 3.2 mg/L and imidacloprid at 0.02 mg/L when foraging behavior was included in the setup. In addition, egg-laying was completely blocked during 11 weeks of exposure to azadirachtin at 3.2 mg/L when foraging behavior was included in the setup. For imidacloprid, the body mass of the male progeny was impaired only when foraging was included in the setup. With the same laboratory behavioral setup for chronic toxicity, Mommaerts et al. (2010) also found that the impairment by imidacloprid on lethal and sublethal traits was higher when foraging behavior was included. Therefore, the results as shown here reinforce the need to increase the complexity of the experimental setup with foraging behavior in order to ensure better outcomes in studies of risk assessment in *B. terrestris* what is also in accordance with the new guidance document of the European Food Safety Authority (EFSA 2012). It is constantly stated in the literature that azadirachtin is safe for beneficial arthropods (Boeke et al. 2004); although our results have shown that the compound may affect *B. terrestris* with a range of sublethal effects, which are very important for the development and survival of the colonies. Here it should be remarked that, although the effects of this study were found under laboratory conditions with long-term chronic exposure which are unexpected under semi-field or field conditions with the low residual potential persistence of azadirachtin in these situations (Kumar and Poehling 2006, Kovacova et al. 2013), Africanized honeybees (*A. mellifera*) have been found to undergo lethal and sublethal effects on adult and larval individuals in their colonies when the foragers start to pollinate the Indian neem tree (*A. indica*), the plant from which azadirachtin is obtained (Alves 2010). The latter findings may indicate that the effects as observed upon chronic exposure to azadirachtin are conserved among bee pollinators and thus should not be neglected. For a better understanding of the effects caused by azadirachtin, we suggest that future semi-field and field studies should be performed considering situations that may include acute and chronic exposure in the risk assessment setup.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Potential impacts of pesticides, particularly insecticides, are a major concern among a multifactorial list of causes related to honey bee decline (Johnson et al. 2013, Breeze et al. 2014, Godfray et al. 2014, Zhu et al. 2014). This is not surprising, since insecticide use has remained the basis for crop protection for decades (Metcalf 1980, Pimentel 2005, Cooper and Dobson 2007, Sexton et al. 2007), despite the controversies surrounding them, which include the risks of exposure to beneficial organisms such as pollinators (Diamand 2003, Edwards-Jones 2008). Indeed, hundreds of insecticidal compounds have been detected in bee hives (Johnson et al. 2010, Mullin et al. 2010), which can directly affect or synergistically act with other factors (eg: viruses, bacterial diseases and malnutrition) to impact the individuals of a colony (Johnson et al. 2013, Breeze et al. 2014, Godfray et al. 2014, Zhu et al. 2014).

Besides the more severe effect, i.e. the mortality, most of the insecticides may cause sublethal effects on bees. Most of them are related to neuronal disruptions (Casida and Quistad 2004, Jeschke and Nauen 2008), which can lead to abnormal behaviors performed either within or outside the colonies (Guez et al. 2001, Decourtye et al. 2003, 2004ab, Yang et al. 2008, Aliouane et al. 2009). For some insecticides, such as neonicotinoids, their plant systemic property facilitates the bee exposure by ingestion of contaminated nectar and pollen with more harmful consequences to bees (Stoner and Eitzer 2012). Such damage potential has brought this group of insecticides to the center of discussions regarding risks of pesticides on bees already culminating with their ban in Europe (Blacquière et al. 2012, EFSA 2013a, Gross 2013), and use restrictions in other countries (IBAMA 2012).

The changes in attitudes and demands regarding insecticides have favored the search for new compounds with better toxicological and ecotoxicological profiles (Nauen and Bretschneider 2002, Price and Watkins 2003, Matsumura 2004, Matthews 2008, Gilbert and Gill 2010, Krämer et al. 2012, Casida and Durkin 2013). The so-called reduced-risk insecticides, and particularly bioinsecticides, have come as a response to the need for environmental harmlessness. Such compounds are generally perceived as exhibiting low toxicity to non-target organisms including bees (US Environmental Protection Agency 2014a, US Environmental Protection Agency 2014b, Gerwick and Sparks 2014, Villaverde et al. 2014). However, their use in sustainable agriculture may hide some pitfalls: public perception of their safety due to their natural origin, and toxicity results are based on a single pollinator species, the honey bee *Apis mellifera* (Coats 1994, Bahlai et al. 2010, Isman and Grieneisen 2014).

The extrapolations of the toxicological and ecotoxicological studies with honey bees and the poorly conceived perceptions regarding the safety of reduced-risk insecticides or bioinsecticides can compromise the understanding and characterization of the real risks that they may impose to bees in a global agricultural context. Recent meta-analysis has indicated that some non-*Apis* bee species may be significantly more vulnerable to insecticides than honey bees (Arena and Sgolastra 2014). Furthermore, recent progress in ecotoxicological studies with native bee species have challenged the notion of safety of both reduced risk insecticides and/or bioinsecticides by showing contradictory results (Besard et al. 2011, Biondi et al. 2012b, Del Sarto et al. 2014). However, such studies still need attention requiring larger surveys to mitigate some of the shortcomings generated by risk assessments with skewed focus towards honey bees and neonicotinoid insecticides.

The scenario reported above led to the present work exploring the potential lethal and sublethal effects of reduced-risk insecticides and/or bioinsecticides on important native bee pollinator species. The expectation was to provide early diagnoses of potential impacts of these insecticidal compounds to native pollinators that still remain largely neglected as study subjects. All of the non-*Apis* bee species used in our study were chosen based on their economic and ecological relevance in the regions where they exist and perform pollination services that are complementary to or that even frequently surpass the services of the honey bee. In addition, all compounds were selected based on their appeal for use and popularity, which generally reflects on their frequency of use and therefore greater likelihood of bee pollinator exposure.

The first target of our studies was the oral and contact (acute) toxicity of the recommended label rates of three reduced-risk insecticides and/or bioinsecticides (azadirachtin, chlorantraniliprole, and spinosad) against workers of two species of stingless bees, *Partamona helleri* and *Scaptotrigona xanthotrica*, which are important native pollinators of wild and cultivated crops in Neotropical America (Slaa et al. 2006, Winfree et al. 2007, Brosi and Briggs 2013). As most important results, spinosad exhibited high oral and contact toxicities in workers of both species at the recommended label rates, with median survival times (LT_{50}s) ranging from 1 to 4 h. Azadirachtin and chlorantraniliprole, however, exhibited low toxicity at the recommended label rates, with negligible mortality that did not allow LT_{50} estimation. However when sublethal behavioral assessments with azadirachtin and spinosad were performed, both compounds were able to impair the individual flight take-off of *P. helleri* and *S. xanthotrica* worker bees.

As larval exposure is still largely unexplored in ecotoxicology assessments on bees in general, our second study targeted the lethal and sublethal effects of azadirachtin and spinosad provided via contaminated diet for larvae of the stingless bee *Melipona quadrifasciata anthidioides*, another important pollinator in the Neotropics (Slaa et al. 2006, Bispo dos Santos et al 2009). As most important results, the survival of the stingless bee larvae was significantly compromised with doses above 210 ng/bee for azadirachtin and 114 ng/bee for spinosad, which are doses considerably reduced compared to the recommended label rates of these compounds. When sublethal effects were investigated on the bee larvae, no effect was observed in their developmental time, but both azadirachtin and spinosad negatively affected their pupal body mass. In addition, azadirachtin also produced deformed pupae and adult individuals as expected due to its insect growth regulator (IGR) property. Curiously though, spinosad, which has a different mode of action by impairing the insect neuron system, was more harmful and produced more deformed individuals than azadirachtin. Only the sublethal effects expressed under spinosad exposure significantly compromised the bees impairing their walking activity at doses higher than 2.29 ng/bee, which is 5000 times lower than the recommended label rate of this compound.

Finally, in the third study, lethal and sublethal effects on feeding behavior, morphology, reproduction and foraging behavior of the bioinsecticide azadirachtin were investigated in an increasingly important bee species that prevails in the Northern Hemisphere, the bumblebee *Bombus terrestris*. Such pollinator has been intensively used for commercial pollination in greenhouse cultivated crops like tomatoes and strawberry (Velthuis and van Doorn 2006), and its relevance for risk assessments has inevitably increased since strong declines on its colonies have been detected (Gels et al. 2002, Mommaerts et al. 2010, Cameron et al. 2011). As most important results, azadirachtin repelled bumblebee workers in a concentration-dependent manner with a median repellence concentration (RC_{50}) at around 16 times higher (504 mg/L) than the recommend concentration (32 mg/L). When bumblebee microcolonies were chronically exposed to azadirachtin via treated sugar water, high mortality ranging from 32 to 100% were recorded with concentrations ranging between 3.2 and 320 mg/L after 11 weeks of exposure. Moreover, no reproduction was scored when concentrations were higher than 3.2 mg/L and at this concentration azadirachtin significantly inhibited egg-laying and, consequently, drone production during 6 weeks. Such effect on reproduction was related to a decrease in ovarian length of the dominant workers exposed to azadirachtin. In addition, with a more complex laboratory setup where bumblebees were required to forage for their food, the sublethal effects of azadirachtin were stronger as the numbers of drones were reduced at concentrations as low as 0.64 mg/L. A negative correlation was found between body mass of the male offspring and azadirachtin concentration.

The scrutiny of all these results found along the entire content of the present work certainly challenges the common perception of the non-target safety of reduced-risk insecticides and bioinsecticides when non-*Apis* bee species are considered. Some important inferences can be drawn from the findings presented here. First, reduced-risk insecticides may be highly toxic to both adult and immature stages of native bee species and even when such compounds do not show any lethal effect since they may compromise important behaviors when in sublethal levels, which themselves compromise the colony health. Second, bee larvae seem to be much more sensitivity to insecticide exposure than the adults with the occurrence of strong lethal and sublethal effects. Third, even when no sublethal effects are detected on larvae in response the diet contamination, the surviving adults may still exhibit negative insecticide effects impairing their behavior. Fourth, when foraging behavior is included in the experimental setup, bees are more vulnerable to the toxicity of reduced-risk insecticides expressed both in terms of lethal and sublethal effects. Therefore, the proper assessment of insecticidal compounds, reduced-risk insecticides and bioinsecticides included, should not be neglected in ecotoxicology studies with non-target species.

As observed here, significant lethal and sublethal effects were trigged on native stingless bees and bumblebees under exposure of the reduced-risk insecticides or bioinsecticides indicating that the effects of these compounds should be also investigated under semi-field and field conditions. This is because under such circumstances the potential effects of the reduced risk-insecticides or bioinsecticides may be ameliorated by their environmental breakdown, but they may be yet comparable with the laboratory assessments. In addition, under more realistic scenarios, where bees may perform complex tasks important for colony survival such as foraging, insecticide impact may be higher. Furthermore, such semi-field and field studies will also meet with the demands of recent regulatory guidances (e.g., EFSA, 2012). In view of these demands, the pespectives are: to intensify the approach with these compounds in risk assessments, especially addressing non-*Apis* bee species, such as the stingless bee species from Neotropical regions, and other important bee species, such as bumblebees, from European regions, where toxicological and ecotoxicological studies on these species are still very scarce. With this, it is expected to mitigate the negligence against the so-called reduced risk (bio)pesticides, created from the misinterpretation of its term; and to generate a better diagnosis of the potential risks of pesticides to these pollinators. In addition, the challenge in doing this will also further improve the experimental setups of the toxicological and ecoltoxicological bioassays for risk assessments on non-*Apis* bee species that will be more efficient for a better understanding of the potential harmfull pesticides on these pollinators.

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