Bull Environ Contam Toxicol
DOI 10.1007/s00128-017-2144-0

Impact of Thiamethoxam on Honey Bee Queen (*Apis mellifera carnica*) Reproductive Morphology and Physiology

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Received: 4 February 2017 / Accepted: 19 July 2017

Abstract High honey bee losses around the world have been linked in part by the regular use of neonicotinoids in agriculture. In light of the current situation, the aim of this study was to investigate the effects of thiamethoxam on the development of the reproductive system and physiology in the honey bee queen. Two experimental groups of honey bee queen larvae were treated with thiamethoxam during artificial rearing, applied via artificial feed in two cycles. In the first rearing cycle, honey bee larvae received a single treatment dose (4.28 ng thiamethoxam/queen larva on the 4th day after larvae grafting in artificial queen cells), while the second honey bee queen rearing cycle received a double treatment dose (total of 8.56 ng thiamethoxam/queen larva on the 4th and 5th day after larvae grafting in artificial queen cells). After emerging, queens were anesthetized and weighed, and after mating with drones were anesthetized, weighed, and sectioned. Ovary mass and number of stored sperm were determined. Body weight differed between untreated and treated honey bee queens. The results also show a decrease in the number of sperm within honey bee queen spermathecae that received the double thiamethoxam dose.

Keywords Thiamethoxam · Honey bee queen · Ovaries · Spermathecae-stored sperm · Artificial queen rearing

The influences of multiple environmental stressors have been recognized as a possible cause of the decreased strength or increased mortality of honey bee colonies (Goulson et al. 2015). The major deleterious factors affecting colonies individually or simultaneously include parasites, pathogens (Genersch et al. 2010), malnutrition, and exposure to pesticides (Sandrock et al. 2014). Systemic pesticides present in the environment can have an adverse effect on non-target organisms, including insect pollinators (Bonmatin et al. 2015). Special concern has been given to thiamethoxam, which has long-term residual effects (Meinfisch et al. 2001) that can accumulate in hive products, especially in comb wax, thus chronically exposing young emerging honey bees to sublethal doses (Pilling et al. 2013; Wu et al. 2011). Thiamethoxam acts as a neurotoxin by binding to the nicotinic acetylcholine receptors in the insect's nervous system, causing behavioural, motor and social disorders, and also potentially impacting the physiological development of individual honey bees (Gill et al. 2012; Henry et al. 2012; Renzi et al. 2016). Despite considerable attention focused on neonicotinoids, which are applied to crops for pest control (Simon-Delso et al. 2015; Chagnon et al. 2015), little research has been conducted specifically on their effects on honey bee queens, their organs and tissues. Research performed in laboratory and/or in field conditions has demonstrated the impacts of neonicotinoids on individual worker bees or effects at the honey bee colony level (Blacquiere et al. 2012; Costa et al. 2014; Fairbrother et al. 2014), as well as effects on adult bumblebees (Whitehorn et al. 2012). The safe use of these compounds in the ecosystem is a subject of concern, and

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Published online: 26 July 2017

the European Union has regulated their use (EFSA 2013, 2015).

There is a lack of information on the impacts of neonicotinoid pesticides on the development, survival, mating and artificial rearing success of honey bee queens. Therefore, this study was aimed at determining the effects of known doses of thiamethoxam on developing honey bee queen larvae during the rearing process. Thiamethoxam, which is widely used in agricultural settings (Simon-Delso et al. 2015), is characterized by high acute toxicity for bees, with known sublethal effects (Mullin et al. 2010; Pilling et al. 2013). For the European honey bee, the LD50 for the oral administration of thiamethoxam over 48 h is 5 ng/bee, and the LD50 for contact administration over 24 h is 29 ng/bee (Decourtye et al. 2005). For the Carniolan honey bee, the hour LD50 of thiamethoxam was 7.86 ng/bee (Gregorc et al. 2016).

Lethal or sublethal effects are often provoked by the ingestion of neonicotinoid concentrations that may impair queen quality (Williams et al. 2015). Knowledge on the effects of pesticides, including thiamethoxam, within hives and especially on queen development and performance is crucial for understanding individual bee or colony mortality, which has appeared in recent years (Cornman et al. 2012). Therefore, we decided to study the effects of thiamethoxam on queen honey bee development and early stages after emerging and mating. The main objectives of this study were: (1) to establish the effects of administering two different thiamethoxam doses (4.28 and 8.56 ng/bee) to honey bee queens during development, (2) to determine the effect of thiamethoxam administration in the queen larval stage on survival, body mass, ovaries weight and sperm stored in spermathecae, and (3) to evaluate the mating success of queens receiving thiamethoxam during larval development.

Materials and Methods

The experiment was performed at the testing apiary of the Agricultural Institute in Slovenia, during the queen rearing season in 2014. Three cell builder honey bee colonies (*Apis mellifera carnica*) were established for queen larval development in early June. Additionally, a starter, queenless colony was used, where grafted larvae were incubated for the first 24 h. One-day old queen larvae were grafted from the selected colony from the same apiary. The standard rearing method was modified with specific feeding of queen larvae, using thiamethoxam (analytical grade with 98% purity, Sigma-Aldrich) added to the queen diet prepared according to Aupinel et al. (2005). The larval diet served as the carrier for thiamethoxam offered to larval treatment groups. Queen larvae were randomly assigned to one treatment group and one control (untreated) group for each concentration; in each group were 50 larvae. The treatment diets were freshly prepared for each experiment and stored in the freezer at −20°C. Prior to feeding, the defrosted diet was warmed to 35°C for immediate use.

One-day old larvae from the selected colonies were grafted into artificial cell cups and placed in a starter colony overnight. The following day, the frames with the grafted larvae were transferred to a cell builder colony for sealing the grafted brood and rearing quality queen cells. During the first treatment protocol, on the 4th day after grafting, larvae in artificial cell cups were taken from the cell builder colony and transferred to the laboratory, where they were treated with 4.28 ng thiamethoxam per queen bee larvae added to 12.5 µL artificial feed (340 ppb). A manual automatic pipette was used for feed pipetting. The treated larvae were kept approximately 4 h in the incubator [34.5°C, 95% relative humidity (RH)] to ensure feed consumption, and then were returned to the original cell builder colony. In the second treatment protocol, queen larvae were fed two doses of thiamethoxam, i.e., one on the 4th and one on the 5th day after grafting. Therefore, these larvae in the artificial queen cell cups were treated with a double pesticide dose, i.e., a total of 8.56 ng thiamethoxam/queen bee larva. The respective single dose of 4.28 ng thiamethoxam/queen bee is below LC_{50} value known for the adult Carnolian honey bee (Gregorc et al. 2016), which is more sensitive to neonicotinoids then larvae (Gregorc and Ellis 2011; Blacquiere et al. 2012). The two control queen larvae groups, one group as a control to the first and one group to the second treatment protocol, were fed with the same amounts of artificial feed without the added pesticide, i.e., one group receiving the feed on the 4th day while the second group received the untreated diet on both the 4th and 5th days after grafting.

After treated or untreated larvae were sealed in queen cells, approximately on the 9th day after grafting, all queen cells were transferred and separately inserted into emerging queen cages supplied with a drop of honey on the bottom. Honey was derived from colonies which were previously not exposed to any pesticide from the environment or to any acaricide. In the laboratory, capped queen cells were maintained in the dark at 34.5°C and 60% RH. Queen cells were observed twice per day, at 9:00 a.m. and 3:00 p.m. Emerged queens were taken from the incubator, visually inspected, briefly anaesthetized with carbon dioxide to allow individual weighing, and then re-caged with five attendant young workers and provided with sugar candy in cages. Queens were marked with a queen marker dorsally on the thorax and transported to the apiary where they were placed in the mating nuclei. Each mating nucleus with a volume of 3.3 L was populated with workers and one virgin queen. Worker bees were a mixture of young bees

from three clinical examined healthy colonies. In total, 15 queens were populated in the mating nuclei for each treatment group (single and double dose thiamethoxam) and 19 queens were tested for each control group. Mating nuclei were visually inspected for the presence of queens, and for the presence of brood in combs on the 15th day after nuclei establishment. Final observation was performed on the 28th day, when surviving queens were removed from their mating nuclei and the nuclei were visually inspected for the presence of brood. Queens were transported to the laboratory where they were anaesthetized using carbon dioxide and weighed a second time. Dissection was performed using a stereo microscope (Zeiss Stereo Discovery V12). Spermathecae were dissected and ovaries were removed and weighed. Spermathecae were fixed in insect ringer (Hayes solution) and stored in the refrigerator for 48 h. The number of spermatozoa in spermathecae was determined using the Bürker Türk haemocytometer and compound microscope (Zeiss Axioskop 2plus light microscope), at 400× magnification (Harizanis 1983).

Statistical analyses were performed using GraphPad Prism software (SD, CA, USA). Values of queen body mass, ovaries and spermathecae, as well as number of spermatozoa were expressed as mean values ± standard error (M ± SE). The Shapiro–Wilk test was used for the determination of data distribution, analysis of variance to compare mean values and the non-parametric *t*-test student or Tukey multiple comparisons test for determination of differences between the mass and/or number of reproductive parameters samples. Statistically significant differences are expressed at the $p \leq 0.05$, 0.01 and 0.001 levels.

Results and Discussion

The success rate of queen rearing from grafting larvae to the emergence young queens receiving a single dose of thiamethoxam was 73.5% and in corresponding control group was 86.0%. Also, queens emerging rates in the brood receiving the double doses of thiamethoxam was 60.5% and 70.0% in its control group. The difference in mortalities of queens between both control groups was not significant ($p > 0.05$), but lower survival in double dose treated queen larvae may be due to the presence of intoxicated larvae in the same nurse colony. The difference in queen development and survival from grafting to emergence differed significantly between each thiamethoxam treated group and its control ($F = 1.01$; $df = 4$; $p < 0.05$). Queen body mass, measured just after emergence, indicated differences ($F = 10.33$; $df = 7$) between double dose treated larvae in comparison with its control group ($p < 0.001$). Differences in weight were also found between larvae from both treatment groups and their controls ($p < 0.01$). Significant differences in body weights at the time following emergence ($p < 0.001$), and body weights after mating ($p < 0.05$) were also observed in queens receiving the double dose of thiamethoxam. Such a difference in body weights after emergence was not found in queens receiving the single dose of thiamethoxam ($p > 0.05$), but after mating queens from this treatment group weighed less than untreated queens ($p < 0.05$). Figure 1 shows the mass of queens after emergence and after mating. These results demonstrate that queens fed a double dose of thiamethoxam during larval development achieved a lower mass than untreated queens. It is supposed that queens with a lower body weight at emergence had a smaller spermatheca size, and therefore heavier queens at emergence were able to pass a higher number of spermatozoa into the spermatheca compared to a lighter queen (Akyol et al. 2008; Bieńkowska et al. 2009). Therefore, it can be considered that in mass queen rearing technology, queen quality is largely determined by body weight (Hatjina et al. 2014). Furthermore, the weight of the queen is dependent on its reproductive activity at the time of removal from the mating nucleus (Nelson and Gary 1983) and potentially reduced reproductive activity could thus be impaired by thiamethoxam feeding during larval development. The impact of thiamethoxam in the diet seems to be important for queen mating and further reproductive development. It appears that the administration of thiamethoxam

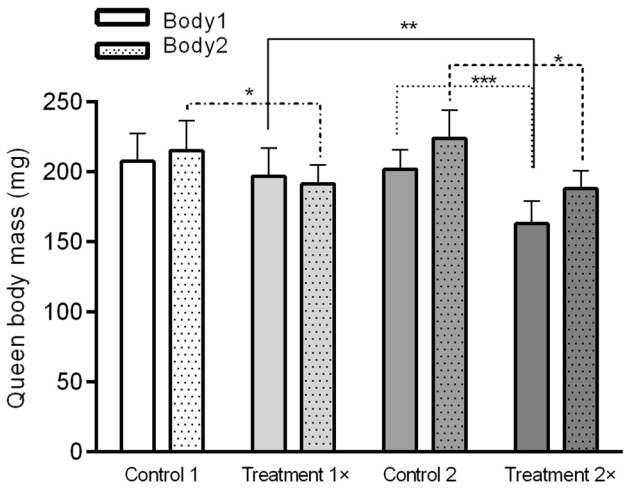

Fig. 1 Overview of mean values (M ± SE) of queen body mass after emergence from queen cells (*un-stippled bars*), and after mating (*stippled bars*). Single dose treatment, *Treatment 1*× 12.5 µL artificial diet + 4.28 ng thiamethoxam, 4th day after grafting larvae; double dose treatment, *Treatment 2*× 2 × 12.5 µL artificial diet + 4.28 ng thiamethoxam (total consumption 8.56 ng thiamethoxam), 4th and 5th day after grafting larvae; *Control 1* 12.5 µL uncontaminated diet; *Control 2* 2 × 12.5 µL uncontaminated diet; *body 1* queen body weight (mg) after emergence; *body 2* queen body weight (mg) after mating. Note statistical differences in queen body mass between treatment groups and controls; *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$

to larvae reduced larval survival. The results of the study demonstrate that the intake of field-realistic thiamethoxam concentrations during the larval stage of native bee species *Scaptotrigona* aff. *depilis* impaired both survival and development (Rosa et al. 2016).

In the mating nuclei examination 28 days after introduction of the queen, 70.59% of queens were from the single dose thiamethoxam treatment group and 46.16% queens were from the double dose thiamethoxam treatment group. In all mating nuclei where queens were found, eggs and larvae were also in comb cells, indicating the queens had mated. In the control mating nuclei in the single dose treatment group, 81.80% of mated queens were found, as opposed to the double dose control group where 75.00% of mated queens were found. In comb cells, eggs and developing larvae were also present. The relative numbers of mated queens between both treatment groups differed ($F=1.2$; $df=4$; $p<0.05$). Differences were also found in the percentage of queens successfully mated from both treatment groups and the control queens for each group ($F=1$; $df=2$; $p<0.05$). The mating success of queens depends on race characteristics, climatic conditions and ethological factors within the mating nucleus, in addition to the physiological characteristics of the queen. The success rate can range from 82% and 100% in warmer weather, or be as low as 59% in colder conditions (Jung 1981). The normal mating success found in a previous study was 80% (Gregorc et al. 2008). This is similar to the mating success rate of control queens in the present study developed from untreated queen larvae. On the other hand, the observed reductions in queen mating success rates with sublethal exposures to thiamethoxam may have potentially negative effects on overall queen performance.

After mating, queens were dissected, ovaries and spermathecae weighed, and sperm in spermathecae counted. Ovary masses of queens receiving the single or double dose of thiamethoxam differed significantly to their corresponding control untreated larvae ($F=19.98$; $df=3.78$; $p<0.05$) that were developing in two separate cell builder colonies. Differences were also found between queens receiving the double dose of thiamethoxam and the corresponding untreated control group ($p<0.01$). Ovary mass of thiamethoxam-treated queens and the corresponding untreated controls are shown in Fig. 2.

No differences were found in spermathecae mass between the treated queens and their corresponding untreated controls. However, the numbers of spermatozoa stored in spermathecae differed ($p<0.05$) between queens receiving the single or the double dose of thiamethoxam (Fig. 3). In comparative experiments, thiamethoxam treatment triggered a reduction of 20% in the number of stored spermatozoa and resulted in a 9% decrease in the proportion of live versus dead sperm (Williams et al. 2015).

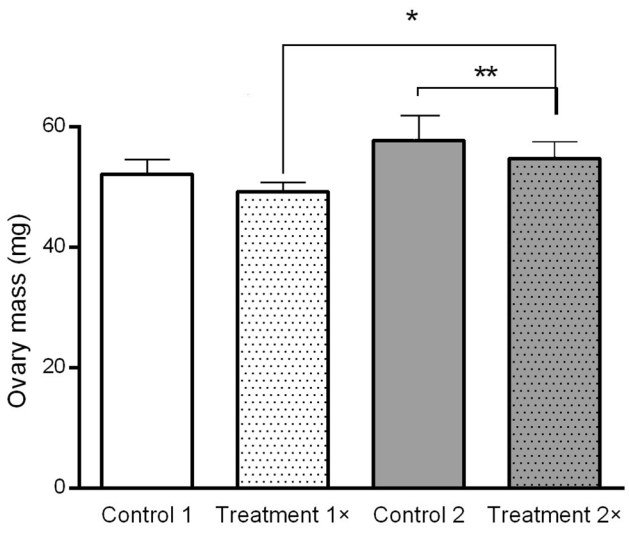

Fig. 2 Ovary masses after mating queens treated with thiamethoxam during larval development (M ± SE). Single dose treatment, *Treatment 1*× 12.5 µL artificial diet + 4.28 ng thiamethoxam, 4th day after grafting larvae; double dose treatment, *Treatment 2*× 2 × 12.5 µL artificial diet + 4.28 ng thiamethoxam (total consumption 8.56 ng thiamethoxam), 4th and 5th day after grafting larvae (*stippled bars*); *Control 1* 12.5 µL artificial diet; *Control 2* 2 × 12.5 µL artificial diet (*un-stippled bars*); *$p<0.005$; **$p<0.05$

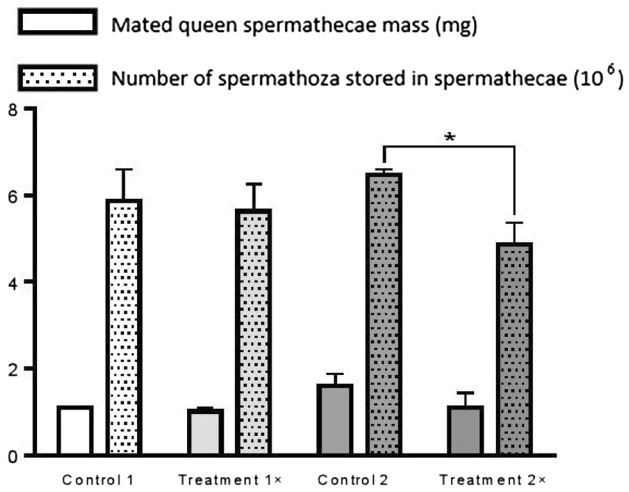

Fig. 3 Mated queen spermathecae masses (*unstippled bars*) and number of spermatozoa (*stippled bars*) (M ± SE) in mated queens treated with thiamethoxam during larval development. Two treatment schedules: single dose treatment, *Treatment 1*× 12.5 µL artificial diet + 4.28 ng thiamethoxam, 4th day after grafting larvae; double dose treatment, *Treatment 2*× 2 × 12.5 µL artificial diet + 4.28 ng thiamethoxam (total consumption 8.56 ng thiamethoxam), 4th and 5th day after grafting larvae; *Control 1* 12.5 µL artificial feed; *Control 2* – 2 × 12.5 µL artificial feed; *$p<0.005$

This study demonstrated the sublethal effects of thiamethoxam doses found in agricultural settings on the characteristics of honey bee queens. The use of standard,

high-quality queens is a prerequisite for any research on colony development and behaviour, and economically successful beekeeping. In a previous study, the LD50 of thiamethoxam was determined for the adult Carniolan honey bee at 7.86 ng/bee (Gregorc at al. 2016), with the indication that Carniolan bees can resist higher doses of thiamethoxam than the Africanized honey bee (AHB), which has a LD50 of 4.28 ng/bee (Oliveira et al. 2014). It was assumed that Carniolan bees are less sensitive to thiamethoxam than AHB due to their larger body weight. In this respect, queen bees may be even less sensitive to thiamethoxam in comparison to worker bees. This study shows that low thiamethoxam doses affect queen development and reproductive characteristics. Examining the success rate of queen rearing, mating success, body weights and sperm counts induced by sublethal thiamethoxam treatments in Carniolan bees, there were also similarities in the body weights after emergence when queens received the single dose of thiamethoxam in their larval diet. These data provide novel insights into the sublethal effects of low doses of thiamethoxam on different physiological characteristics of queens. Reduced emerging queen body weights, reduced ovary weight, and lower sperm counts are the main observed effects at the individual queen bee level or the organ level. A better understanding of the mechanisms induced by thiamethoxam is needed to elucidate the effects observed on increased reduction of queen reproductive performance or even premature queen mortality or other dysfunctions in honey bee colonies.

Acknowledgements The study was supported by the Erasmus Placement exchange student Program 2013/2014; as well as financial support from the Slovenian Research Agency, Research Programme P4-133 and FP7 Project CropSustaIn grant agreement FP7 – REG-POT – CT2012-316205.

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