Bulletin of Insectology **73** *(2): 303-311, 2020*
ISSN 1721-8861 eISSN 2283-0332

Effect of a topical treatment with juvenile hormone analogues on dominance, ovarian development and corpora allata size in *Bombus terrestris* workers

Laura Bortolotti[1], Gherardo Bogo[2], Filip Pošćić[3], Marie José Duchateau[4]
[1] *Council for Agricultural Research and Economics - Research Centre for Agriculture and Environment (CREA-AA), Bologna, Italy*
[2] *Grupo de Ecología de la Polinización, INIBIOMA (Universidad Nacional del Comahue-CONICET), San Carlos de Bariloche, Argentina*
[3] *Department of Applied Science, Institute for Adriatic Crops and Karst Reclamation, Split, Croatia*
[4] *Faculty of Biology, Animal Ecology, Utrecht University, Utrecht, The Netherlands*

Abstract

Juvenile hormone analogues (JHAs) are insecticides that mimic the effect of juvenile hormone (JH), one of the main regulatory hormones in insects. Negative effects of JHAs on preimaginal stages are well known, while little is known of their effects on adults. In this study we assessed the effect of three JHAs on newly emerged workers of *Bombus terrestris* L. kept in queenless groups. Reproductive dominance status, oocyte length and corpora allata volume were assessed 4 and 7 days after treatment. Contrary to expectations, none of the treatments had a significant stimulating effect on ovarian development in reproductive dominant workers. Conversely, most treatments caused a significant reduction in oocyte size and in corpora allata volumes of seven-day-old reproductive subordinates, indicating a feedback loop of exogenous JHAs on these glands. The study suggests that besides JH, multiple factors influence worker ovarian activity, which makes it difficult to predict the effect of the insecticides in wild colonies.

Key words: Apidae, *Bombus terrestris*, corpora allata, dominance, Hymenoptera, juvenile hormone analogues, ovarian development.

Introduction

Bumble bees form annual, primitively eusocial colonies with a single queen that starts in spring alone, foraging for pollen and feeding the larvae that develop into workers. The emerged workers, morphologically similar as the queen, will take over the duties of foraging and feeding. This worker caste have undeveloped ovaries and do not lay eggs, leaving the queen as the only egg layer. This social equilibrium was thought to be maintained by a non-volatile pheromone produced by the queen's mandibular glands, that inhibits oogenesis in worker ovaries (van Honk *et al*., 1980; 1981; Röseler *et al*., 1981). However, recently the role and mode of action of queen pheromone in worker reproduction has been questioned (Bloch and Hefetz, 1999a; Alaux *et al*., 2004; 2006). At certain moment in the colony development or following the death of the queen, the ovaries of some workers start to develop and produce eggs. Older workers are usually the ones that achieve reproductive dominance (Ayasse *et al*., 1995; Bloch and Hefetz, 1999b; Bloch *et al*., 2000; Alaux *et al*., 2007; Lopez-Vaamonde *et al*., 2007). This social instable phase is characterized by aggressive behaviour, egg robbery and impairment of the colony (Duchateau and Velthuis, 1989).

When young workers are confined in small queenless groups, a clear dominance hierarchy is observed, in which dominant workers with developed ovaries suppress ovarian development and reproduction in subordinate workers (Van Doorn, 1987; Amsalem and Hefetz, 2010; 2011; Sibbald and Plowright, 2013).

Dominance and reproduction in bumble bees are closely linked to rates of juvenile hormone (JH) biosynthesis (Röseler, 1977; Larrere and Couillaud, 1993; Bloch *et al*., 2000; Shpigler *et al*., 2014). In many insect species juvenile hormone, secreted by corpora allata, acts as a gonadotropic hormone, promoting production of the yolk protein vitellogenin in the fat body and its accumulation in developing oocytes, thus regulating female fertility (Riddiford, 2008; 2012). In the bumble bee *Bombus terrestris* L., higher JH synthesis in queenless workers is associated with enlargement of the corpora allata (Röseler *et al*., 1981) and oocyte development (Shpigler *et al*., 2010; 2014; Amsalem *et al*., 2014). Dominant workers show a higher JH titre in haemolymph and faster ovarian development than subordinate queenless workers, and queenright workers, indicating that there is a strong positive correlation between JH titre in haemolymph, degree of ovarian development and onset of reproduction in queenless workers (Röseler and Röseler, 1978; Larrere and Couillaud, 1993; Bloch *et al*., 1996). Exogenous JH applications stimulate ovarian development of workers and increase their probability of becoming dominant in a dose-dependent way (Röseler, 1977; van Doorn, 1989), although Pandey *et al*. (2020) found that multiple factors, including JH, body size, and previous experience affect dominance and aggression queenless workers.

Juvenile hormone analogues (JHAs) are insecticides belonging to the group of insect growth regulators that interfere with insect metabolism by mimicking the effect of JH (Dhadialla *et al*., 2005). They can be broadly classified into two groups, according to their chemical structure: the terpenoid JHAs, as methoprene and kinoprene, and the phenoxy JHAs, such as fenoxycarb and pyriproxyfen. As insecticides and biocides, they are mainly used for their ovicide and larvicide effects, since they disrupt egg development and larval metamorphosis, preventing the emergence of adult insects (Bortolotti *et al.*, 2000; El-Sheikh *et al.*, 2016). On the contrary they do not have a direct lethal effect on adults, but can cause sterility in both sexes (Pener and Dhadialla, 2012).

Methoprene is the first and probably the best known JHA developed for pest control. It is not approved as insecticides in the European Union (EU), but is widely used as biocide to control mosquito larvae in the USA and the EU, and its use has gained increasing interest after the spread of mosquito-borne West Nile virus infection (Bellini *et al.*, 2014; Lawler, 2017). It is considered harmless to non-target insects, but a recent study showed that it can affect behavioural development in honey bees (Huang *et al.*, 2016).

Fenoxycarb was the first phenoxy JHA found to have a juvenilising effect. Although it belongs to the carbamate pesticide group, it has no carbamate-like neurotoxic effects. It is nevertheless slightly toxic to non-target species and can be very dangerous for beneficial insects such as honey bees, silkworms and neuropterans (Cappellozza *et al.*, 1995; Bortolotti *et al.*, 2005; Aupinel *et al.*, 2007; Milchreit *et al.*, 2016). It is authorized as spray insecticide in many European countries against Lepidoptera Tortricidae in apple and pear orchards, but for its high risk to bees and non-target arthropods, its use is subject to precautions and mitigation measures (EU, 2011).

Pyriproxyfen is a phenoxy analogue similar to fenoxycarb and one of the most potent JHAs currently available. In Europe it is used as insecticide against the greenhouse and cotton whitefly -*Trialeurodes vaporariorum* (Westwood) and *Bemisia tabaci* (Gennadius)- on tomato and eggplant in greenhouses, and in the field against cotton whitefly on cotton crops (EU, 2010). Pyriproxyfen show potential risks to aquatic organisms; it is classified as non-toxic to adult honey bees, although from experimental studies it has been found to affect honey bee larval and pupal development (Bitondi *et al.*, 1998; Zufelato *et al.*, 2000; Chen *et al.*, 2016).

Several studies have shown that treatment of adult insects with JHAs induces vitellogenin synthesis and oocyte development, similar to application of JH. At gene expression level, JH and JHA both activate transcription of vitellogenin genes in the fat body and stimulate protein synthesis in various ovarian tissues (Wyatt and Davey, 1996). Application of the JHA methoprene accelerates ovarian development of diapausing females of the parasitic fly *Melinda pusilla* (Villeneuve) and the blow fly *Protophormia terraenovae* Robineau-Desvoidy (Agui *et al.*, 1991; Matsuo *et al.*, 1997) and overwintering females of *Osmia rufa* (L.) (Wasielewski *et al.*, 2011).

Adult bees may be exposed to pesticides via bodily contact with dust generated during the planting of treated seeds, foliar sprays, or residues on various substrates. The most probable contamination route is the direct contact with sprays for workers that leave the colony to forage. Since bumble bees nest are located underground, another possible exposure route for workers is the presence of residues in the soil surrounding the nest, that can contaminate both foragers and non-foragers (Gradish *et al.*, 2019).

Most studies on JHA on bumble bees regard effects on brood, while they are usually considered harmless to adults (Tasei, 2001; Thompson, 2001). Relatively few studies have investigated the sub-lethal effects of JHA on adult workers, namely anomalous ovarian development and consequent disruption of social balance, due to disturbance of JH metabolism. Mommaerts *et al.* (2006) found that topical application of an aqueous solution with 65 $\mu g\ L^{-1}$ of the JHA kinoprene led to a significant increase in ovary length and oocyte number of dominant workers, with a stimulatory effect on their reproduction.

The aim of the present study was to evaluate the impact of topical applications of several JHAs in different concentrations to queenless workers of the bumble bee *B. terrestris*, assessing the effects on ovarian and corpora allata development. We also evaluated their effect on the dominance position of queenless workers and a possible effect of JHA on social equilibrium. Based on the results of previous studies with JHA and the physiological role of JH in bumble bees, we expected applied JHAs to exert a stimulating effect on ovarian development, promoting the achieving of reproductive dominance, and a feedback inhibitory effect on corpora allata volume.

Materials and methods

Experimental insects

Colonies of *B. terrestris* were obtained from Koppert Biological Systems, The Netherlands, and the study was performed in Italy. Each colony contained a queen, 30-50 workers, larvae and pupae at different stages of development. The colonies were maintained in a climate room at 28 ± 1 °C and 60 ± 10% RH, in constant darkness. Commercial sugar syrup and fresh frozen pollen, collected by honey bees, were supplied to the colonies *ad libitum*.

Newly emerged workers, distinguishable by their grey hairs, were taken from queenright colonies. Groups of three queenless workers were confined in cylindrical plastic boxes (20 cm diameter, 15 cm high) covered by a transparent plastic plate. The boxes were equipped with a male pupa in his cocoon, fixed to the bottom of a Petri dish with a drop of honeybee wax, as well as pollen and sugar syrup *ad libitum*. The choice to work on small queenless groups responded to the need to obtain many replicas and to exclude the effect of the queen's pheromones. Male pupa was added to stimulate egg laying behaviour (Kwon et al. 2003). The boxes were kept in a dark climate room at 28 ± 1 °C, 60 ± 10% RH. All experimental operations were carried out under red light.

Juvenile hormone analogue treatment

A one to three hours after confinement, the groups of three queenless workers were randomly assigned to the different treatments; they were anaesthetised with a single dose of CO_2, as recommended in the official guidelines for bumble bee testing (OECD, 2017). It has been documented that the administration of CO_2 to bumble bee queens has an effect on ovarian activation (Amsalem and Grozinger, 2017), but there is no evidence that it has the same effect on bumble bee workers. As CO_2 in our study was used for narcosis both on treated and untreated workers, a possible effect would have been equally present in both groups and thus we did not consider further CO_2 influence.

After narcosis the three workers were randomly marked with three different water-based colours (pink, blue and green) and one worker out of three (the pink one), was treated topically with 5 µl JHA solution on the ventral abdomen. Three JHAs were applied at different concentrations of active ingredient (a.i.): methoprene (Altosid® 5% emulsifiable concentrate, Zoecon) at 25, 62.5 and 100 µg a.i./bee; fenoxycarb (Insegar® 25% water dispersible granules, Syngenta) at 25 and 62.5 µg a.i./bee; pyriproxyfen (Admiral® 10% emulsifiable concentrate, Sumitomo) at 5 µg a.i./bee. All JHAs were dispersed in water. The control (untreated) groups were only anaesthetised with CO_2.

In bee toxicity tests aimed at the registration of plant protection products, chemicals are commonly solved in acetone, to favour the penetration through the hydrophobic cuticle. Nevertheless, we chose to dilute the JHAs in water to better simulate the condition occurring in the field, where they are dissolved in aqueous solution before spraying. In long term test insecticides are applied as aqueous solutions at their field recommended concentration (Mommaerts *et al.* 2006). Since adult insects are not targeted by JHAs, the doses were intentionally higher (50 to 200 times) than recommended field concentrations, so as to reveal hidden effects.

Workers were sacrificed 4 and 7 days later, when queenless workers start and complete, respectively, their sexual development (Gosterit *et al.*, 2016). A total of 97 groups (289 workers) were used, 44 of them (131 workers) were sacrificed at 4th day and 53 (158 workers) at 7th day. The number of groups and workers undergoing each treatment and sacrificed at the two ages are indicated in table 1.

Reproductive dominance

The workers of each group were classified as "reproductive dominants" (RD) or "reproductive subordinates" (RS) on the basis of their ovarian development rank (Duchateau and Velthuis, 1989; van Doorn, 1989; Bloch *et al.*, 1996; 2000; Alaux *et al.*, 2004; Amsalem and Hefetz, 2010; 2011). In most boxes, only one of the three workers become RD, except in three boxes, where two workers were classified as RD because they had a similar stage of ovarian development.

Oocytes and corpora allata size measurements

At the end of the test period, workers were anaesthetised with CO_2 and sacrificed by freezing. Their wing span was measured on millimetre paper and their abdomen and head were dissected under a stereo microscope. For the determination of ovarian development, the ovaries were removed, the length of eight terminal oocytes was measured with an ocular micrometer and their mean value calculated. The two corpora allata were removed surgically through an opening in the ventral part of the head and placed under a microscope. To calculate the volume of each (subspherical) gland, two measures of the diameter were taken at right angles using an ocular micrometre and the mean was recorded. The mean value of the two glands was used for statistical analysis. Unfortunately, two and six samples were unsuitable for oocytes and corpora allata measurements, respectively, and they were excluded from the analyses.

Statistical analysis

Previous studies showed that JH administered in acetone was able to circulate among the workers of a colony and affect also the untreated individuals (Bortolotti *et al.*, 2001). Since JHA in aqueous solution has a low cuticle penetration, as demonstrated by other author (Mommaerts *et al.*, 2006), we assume that in our study the amount of JHA which did not penetrate the cuticle was dispersed inside the cage, possibly resulting in oral intake of JHA by all the three workers, including the two untreated ones. For this reason we studied the effects of JHA considering the whole group of workers as treated workers.

There was no significant effect of mortality caused by the initial CO_2 narcosis or by treatment with JHAs. None of the workers died after the initial CO_2 narcosis necessary to apply JHAs. Among all the workers of the treated groups, only two died before the end of the test: one after fenoxycarb treatment at 62.5 µg in the 4-day group, and the other after methoprene treatment at 62.5 µg in the 7-day group. Only the former was the insect actually treated. They were both excluded from the statistical analysis.

Table 1. Number of groups (and the corresponding worker counts) for the different juvenile hormone analogue treatments, separated and otherwise by experimental duration.

Treatment Product	Dose (µg a.i./bee)	Number of groups (and corresponding cumulative number of workers) 4 days	7 days	Total number of groups (and corresponding cumulative number of workers)
Untreated	-[a]	7 (21)	11 (33)	18 (54)
Methoprene	25	6 (18)	7 (21)	13 (39)
Methoprene	62.5	6 (18)	7 (20)[b]	13 (38)
Methoprene	100	6 (18)	6 (18)	12 (36)
Fenoxycarb	25	6 (18)	10 (30)	16 (48)
Fenoxycarb	62.5	7 (20)[b]	6 (18)	13 (38)
Pyriproxyfen	5	6 (18)	6 (18)	12 (36)
Total	-	44 (131)	53 (158)	97 (289)

[a] Not applicable. [b] In one group out of seven one worker died during the test.

Analysis of proportions was performed to test the odds of treated and untreated workers developing into RD bumble bees (Sokal and Rohlf, 2012). Three-way analysis of variance (ANOVA) was performed on wing span data using dominance status, age and product at the chosen concentration as fixed factors.

Since we expected a strong influence of body size on oocyte and corpora allata dimensions (Röseler, 1977; van Doorn, 1989), two or three-way analysis of covariance (ANCOVA) was performed (Sokal and Rohlf, 2012). Wing span, a measure of body size, was taken as a covariate, while age and product were fixed factors in ANCOVA on oocyte dimensions, and dominance status, age and product were fixed factors in ANCOVA on corpora allata dimensions. Before ANCOVA, we tested slope homogeneity of the different treatments between the covariate and the dependent response variables. Slope homogeneity proved not to be significantly different as required by the ANCOVA assumptions. The non-significant covariate interaction term was removed before running ANCOVA. The dependent response variables were significantly ($p \leq 0.05$) correlated with the covariate as required by ANCOVA assumptions.

Effect size of the independent variables (factors) and their interactions in ANOVA and ANCOVA was estimated with Cohen's ω^2 (an unbiased estimate of η^2). *A posteriori* comparison of individual means was based on the minimum significant difference (MSD) method obtained by Hochberg's GT2 statistic (Sokal and Rohlf, 2012). Other multiple comparisons of interests were also done by the GT2-method (Sokal and Rohlf, 2012).

The data were log transformed before analysis, which effectively homogenized the variances (Levene's test) and produced normal distributions (Shapiro-Wilk test) (Sokal and Rohlf, 2012). We therefore report geometric mean values instead of arithmetic means.

Results

Effect on reproductive dominance

The percentage of treated workers that became RD with different products and doses, and the corresponding odds ratio are shown in table 2. At four days of age, only workers treated with fenoxycarb at both doses and with pyriproxyfen showed a higher probability of becoming RD than untreated workers. At seven days this probability was only significant for workers treated with pyriproxyfen (table 2).

Wing span and reproductive dominance

The mean wing spans were significantly different ($F_{S[1,261]} = 16, p < 0.0001, \omega^2 = 0.04$) between RD and RS workers at 4 days but not at 7 days. Furthermore, the means were significantly different ($F_{S[1,261]} = 14$, $p = 0.0002$, $\omega^2 = 0.04$) at 4 days and 7 days for RS workers but not for RD workers. Mean wing spans with their 95% confidence intervals in brackets, irrespective of treatment, were 2.63 (2.56-2.70) cm for 4-day RD workers (n = 45), 2.50 (2.45-2.54) cm for 4-day RS workers (n = 86), 2.72 (2.65-2.78) cm for 7-day RD (n = 55) and 2.62 (2.58-2.67) cm for 7-day RS workers (n = 103).

Analysis of covariance on ovarian development

Irrespectively on reproductive dominance, analysis of covariance on oocyte length showed a significant influence of both main effects, age and product, and their interactions (table 3). Age had large effect ($\omega^2 \geq 0.14$) while product and the interactions term had low effects ($\omega^2 \leq 0.01$) on oocyte length (table 3).

There was a significant increase in the size of oocytes with age for both RD and RS workers, except for RS workers treated with methoprene 62.5 and 100 µg (figure 1). Treatments with different JHAs and different concentrations did not significantly influence ovarian development in 4-day RD and RS workers except for RD workers treated with fenoxycarb 25 µg that had significantly longer oocytes than those under untreated controls. Moreover, ovarian development in 7-day RD workers were not significantly affected by different JHAs as well. On the contrary, all treatments, except fenoxycarb 25 µg and methoprene 25 µg, significantly reduced ovarian development in 7-day RS workers with respect to untreated controls.

Analysis of covariance on corpora allata volume

Analysis of covariance on corpora allata volume showed a significant influence of all main effects and of age × product, and reproductive dominance × product interactions (table 4). Only reproductive dominance had a large effect ($\omega^2 \geq 0.14$); the other significant sources of variation had moderate effects ($0.01 \leq \omega^2 \geq 0.14$) on corpora allata volume (table 4).

Table 2. Percentages of treated workers became reproductive dominant (with a higher ovarian development rank) in groups of three queenless workers and the corresponding odds ratio and 95% confidence interval (CI).

Treatment		At 4 days		At 7 days	
Product	Dose (µg a.i./bee)	Percentage	Odds (95% CI)	Percentage	Odds (95% CI)
Fenoxycarb	25	66.7	10 (1.3-79)*	50.0	2.3 (0.52-10)^{ns}
Fenoxycarb	62.5	66.7	7.3 (1.0-52)*	50.0	3.0 (0.44-20)^{ns}
Methoprene	25	16.7	0.28 (0.03-2.3)^{ns}	28.6	0.72 (0.12-4.5)^{ns}
Methoprene	62.5	50.0	2.0 (0.31-13)^{ns}	42.9	1.7 (0.28-10)^{ns}
Methoprene	100	33.3	1.0 (0.15-6.8)^{ns}	16.7	0.28 (0.03-2.3)^{ns}
Pyriproxyfen	5	66.7	10 (1.3-79)*	66.7	10 (1.3-79)*

* $p \leq 0.05$. ^{ns} not significant.

Table 3. Analysis of covariance (ANCOVA), separately for reproductive dominant and reproductive subordinates, on oocyte lengths adjusted for wing span.

Source of variation	Reproductive dominants						Reproductive subordinates					
	SS	*df*	*MS*	F_S	*p*	ω^2	*SS*	*df*	*MS*	F_S	*p*	ω^2
Age	12.6	1	12.6	1050	**<0.0001**	0.77	12.4	1	12.4	246	**<0.0001**	0.36
Product	0.17	6	0.03	2.3	**0.0391**	0.01	4.03	6	0.67	13	**<0.0001**	0.11
Age × Product	0.21	6	0.03	2.8	**0.0141**	0.01	3.35	6	0.56	11	**<0.0001**	0.09
Error	1.02	85	0.01				8.66	172	0.05			
Total	16.4	99					34.2	186				

Significant p-values are highlighted in bold.

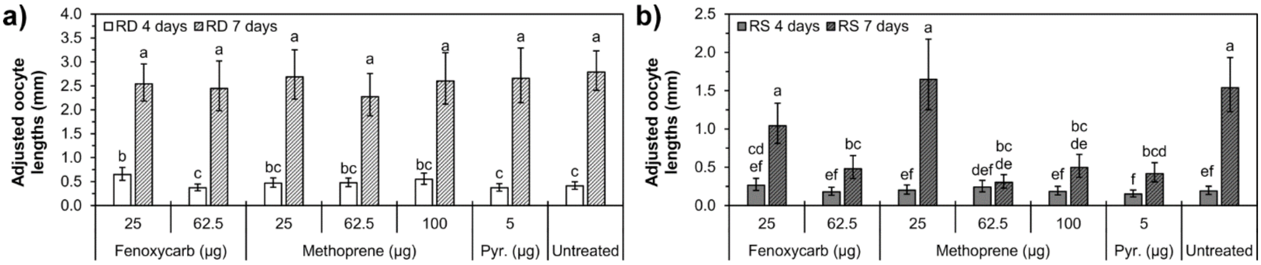

Figure 1. Adjusted mean oocyte lengths of bumble bees after ANCOVA (see table 3), and their 95% confidence intervals calculated by the GT2-method, separately for (**a**) reproductive dominant (RD) and (**b**) reproductive subordinates (RS), 4 or 7 days after different treatments. Different letters indicate significant differences between treatments separately for RD and RS ($p \leq 0.05$). Oocyte lengths were adjusted for RD and RS mean wing span, respectively, 2.68 and 2.57 cm. Pyr. = Pyriproxyfen.

Table 4. Analysis of covariance (ANCOVA) on corpora allata volumes (adjusted for wing span).

Source of variation	*SS*	*df*	*MS*	F_S	*p*	ω^2
Age	0.20	1	0.20	11	**0.001**	0.02
Reproductive dominance (RD)	1.28	1	1.28	72	**<0.0001**	0.14
Product	0.24	6	0.04	2.3	**0.038**	0.02
Age × RD	0.01	1	0.01	0.46	0.497	0.00
Age × Product	0.51	6	0.08	4.7	**0.0001**	0.04
RD × Product	0.25	6	0.04	2.4	**0.029**	0.02
Age × RD × Product	0.06	6	0.01	0.56	0.760	0.00
Error	4.51	254	0.02			
Total	9.23	282				

Significant p-values are highlighted in bold.

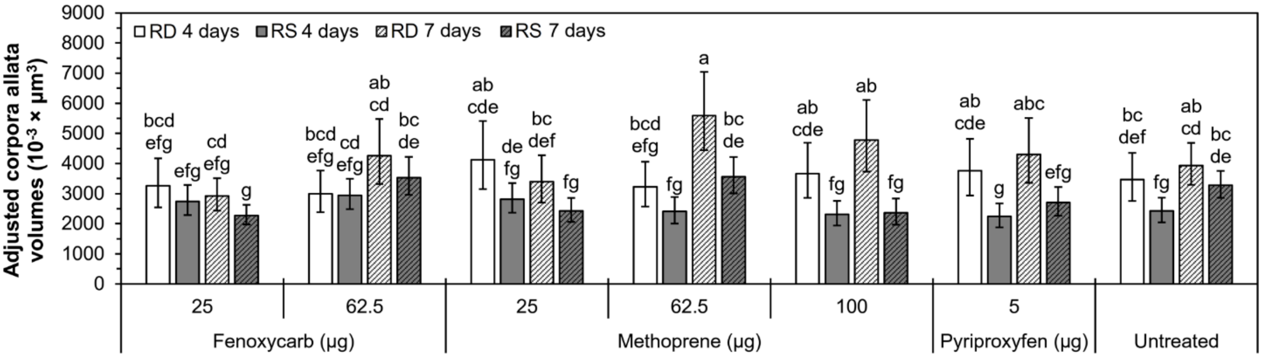

Figure 2. Adjusted mean corpora allata volumes of bumble bees after ANCOVA (see table 4) and their 95% confidence intervals calculated by the GT2-method for reproductive dominant (RD) and reproductive subordinates (RS) 4 or 7 days after different treatments. Different letters indicate significant differences between treatments ($p \leq 0.05$). Corpora allata volumes were adjusted for overall mean wing span (2.61 cm).

Except for fenoxycarb 25 µg and methoprene 25 µg, the volume of corpora allata increased with age for all treatments including control, and was higher in RD than RS workers, although not always significantly (figure 2). Only RD workers treated with methoprene 62.5 µg, and methoprene 100 µg and pyriproxyfen 5 µg, showed significantly higher corpora allata volume at 7 days and at 4 and 7 days, respectively, than the corresponding RS workers. However, only workers treated with methoprene 62.5 µg showed significantly different corpora allata volumes at the two ages.

Compared to the untreated control, the volume of corpora allata was generally increased in RD workers 7 days after treatments, although not significantly. On the contrary, RS workers 7 days after the treatments had lower corpora allata volumes than the untreated control, except under fenoxycarb and methoprene 62.5 µg treatments.

Discussion

In this study we assessed the effect of three juvenile hormone analogues (JHAs) on reproductive dominance, and oocyte and corpora allata development, applied topically to queenless bumble bee workers. JHA could have sublethal effects since in adult bumble bees juvenile hormone rates are strongly related to reproduction and dominance (Bloch *et al.*, 2000; Amsalem *et al.*, 2014; Shpigler *et al.*, 2014; Pandey *et al.*, 2020).

The topical treatment with active ingredient solved in water was chosen because it resemble much the possible routes of contamination of the studied JHAs. Methoprene-based biocides are dispersed in water for the treatment of mosquitoes larvae, and through the water they can contaminate the surrounding soil, where the bumblebees nest and walk. Another possible way of contamination for bees is through food, both directly by contaminated water and through vegetation, since residues of methoprene have been found in bees and pollen (Hakme *et al.*, 2017). However an oral treatment would have been less suitable for testing the effects of the other two JHAs. Fenoxycarb and pyriproxyfen are used as spray on crops and therefore the most likely way of contamination of adult bumble bees is by direct bodily contact (Gradish *et al.*, 2019). The JHA doses were 50 to 200 times higher than recommended field concentrations, in order to reveal sublethal effects, and to compensate for the scarce penetrability of the JHA dissolved in aqueous solution.

Due to this low penetrability, the JHAs administered to the treated worker was probably exchanged among the three workers through the physical contract and oral intake during auto- and allogrooming, as shown by previous studies (Bortolotti *et al.*, 2001). For this reason we have decided to consider all the workers of the treated groups as they were treated, although this could have flattened the effect of the treatment. The effects of JHAs on corpora allata and ovaries were thus estimated not on single treated worker, but on the whole exposed group, distinguishing between RD and RS workers.

Our treatments did not show any lethal or any toxic effect of JHAs in adult bumble bee workers, in line with other tests on bumble bees in laboratories (Mommaerts *et al.*, 2006) and caged (Gretenkord and Drescher, 1996).

Irrespective of the type of JHA treatment in our groups of each three queenless workers, one worker became reproductive dominant (RD), meaning that this worker had the greatest ovarian development (except three cases in which two workers were equally reproductively dominant) while the other two workers were reproductive subordinate (RS). On average, the single treated worker of the three did not have a greater chance of becoming the RD worker (50% and 59% in 4 and 7 old workers respectively), although workers treated with fenoxycarb and pyriproxyfen seemed slightly more favoured for reproductive dominance at 4 days, and those treated with pyriproxyfen seemed more favoured at 7 days (table 2). Furthermore, a significant difference in the body size of RD and RS workers was observed at 4 days, but not at 7 days. These results suggest, in accordance with other authors, that body size has more influence on achieving reproductive dominance in queenless groups than treatment with JHAs, and that worker size can be decisive for acquiring reproductive dominance in queenless workers (Pandey *et al.*, 2020), especially in the first days of confinement (van Doorn, 1989), less in the following days.

As oocyte lengths was used to discriminate RD and RS, we could not include dominance as a fixed factor in ANCOVA of oocyte lengths. Nevertheless, the ovarian development in 4-day RD and RS workers was very similar, both in treated and untreated groups, indicating that JHAs potentially do not have an accelerating effect in queenless 4-day workers (figure 1). The difference in ovary development between RD and RS workers was more apparent in 7-days workers (figure 1). Indeed, by the age of 7 days dominant workers have completed their oogenesis (Duchateau and Velthuis, 1989; Gosterit *et al.*, 2016), while subordinate workers still have small ovaries, since dominant workers suppress ovarian development and hence reproduction in subordinate bees (Van Doorn, 1987; Amsalem and Hefetz, 2010; 2011; Sibbald and Plowright, 2013). Our treatments inhibited oocyte growth in RS workers and this effect became visible 7 days after treatment, probably due to the combined effect of treatment and dominant workers. This is further confirmed by significantly lower oocyte growth in RS workers when compared to control RS workers for almost all treatment (figure 1), except those with the lower concentrations of fenoxycarb and methoprene. The ovaries of RD workers were generally neither inhibited nor stimulated by JHA treatments, suggesting that dominant status prevailed over the effect of treatment.

In previous studies with bumble bees an injection of 50 µg of exogenous JH was sufficient to induce complete oogenesis in as little as 5 days (Röseler, 1977; van Doorn, 1989) and a topical application of 70 µg JH diluted in Dimethylformamide (DMF) led to developed ovaries in 7-day-old queenright workers (Shpigler *et al.*, 2010). The lack of stimulating effect in our study could be due to the fact that JHAs do not exert the same effect as the JH used in other studies, or that injection and application with DMF are more effective. On the other hand, the inhibiting effect on RS workers, which seems opposite to our expectation, is similar to those found with JHA in honeybees, where the application of methoprene ranging between 150 and 200 µg inhibited ovarian development in queenless workers (Robinson *et al.*, 1992; Malka *et al.*, 2009). Although the role of JH in honeybee is different from bumble bees, having lost its gonadotropic role and rather acting in modulating worker behavioural development (Hartfelder, 2000; Sullivan *et al.*, 2000), this results could indicate a differential effect of JH and JHA in subordinate workers.

Another possible explanation lies in the applied dose: Rutz *et al.* (1976) found that vitellogenin production and ovarian development in honeybees workers are stimulated by the application of low doses (0.1 µg) of JH III, but suppressed by higher doses (10 µg); similarly, the effect of the JHA pyriproxyfen on queenless honeybee workers depends on the dose: 1 µg induces a slight increase of vitellogenin (Bitondi *et al.*, 1994), higher doses (1.25, 2.5 and 10 µg) prevent its synthesis in the fat body and its accumulation in the haemolymph, and lower doses (0.001, 0.01 and 0.1 µg) are ineffective (Pinto *et al.*, 2000). In our study it was not possible to determine the exact dose affecting each worker, but the unexpected effect in suppressing ovarian development of RS workers and the lack of effect on RD workers could depend on a suboptimal dose of JHA.

Corpora allata volume was always greater in RD than in RS workers, though not always significantly (figure 2). The correlation between body size and corpora allata volume, suggest a role of corpora allata in the reproductive dominance hierarchy of queenless workers, as also found by van Doorn (1989). Juvenile hormone, secreted by corpora allata, acts as a gonadotropic hormone, an effect clearly shown by Shpigler *et al.* (2014) and Amsalem *et al.* (2014) in queenless workers, where allatectomy or treatment with precocene-I suppressed ovarian development, except when workers were treated with JH-III, the natural bumble bee JH. Ovary development is also associated with dominance and this is especially seen in small groups of queenless workers, where dominant workers with faster ovarian development had higher JH concentrations in haemolymph than non-dominant workers (Röseler and Röseler, 1978; Larrere and Couillaud, 1993; Bloch *et al.*, 1996).

In many of our JHA treatments, 7-day RS workers showed a lower corpora allata volume than control bees (figure 2). In bumble bee workers, corpora allata volume is correlated with JH synthesis (Röseler and Röseler, 1978) and JH exerts negative feedback on the gland size (van Doorn, 1989). Our results suggest that in RS workers exogenous JHAs can take over the role of endogenous JH, exerting negative feedback on the size of corpora allata. In RD workers this effect is presumably counteracted by development of corpora allata due to dominant status (Van Doorn, 1989).

It is difficult to predict what the effect of JHA will be on queenright workers in a colony. Since from our results JHA affected mainly RS workers and in a queenright colony the majority of workers have undeveloped ovaries, we can hypothesize that the application of exogenous JHA could interfere with the inhibitory effect of the queen, enhancing or hindering it. This could result in instability of the reproductive equilibrium and changes in the colony development parameters described in the literature, such as the production of males ('switch point') and onset of worker reproduction ('competition point') (Duchateau and Velthuis, 1988; Gosterit, 2011; Bogo *et al.*, 2018). We feel that this topic deserves further instigation, and our results suggest the need to extend research into the effects of JHA to whole colony parameters, in order to highlight alterations in worker reproductive development.

Acknowledgements

We thank three anonymous reviewers for their comments, the late Giovanni Sbrenna and Anna Micciarelli Sbrenna for their support and Bioplanet srl (Cesena, Italy) for supplying the bumblebee colonies.

References

AGUI N., MINORU MIHARA M., KURAHASHI H., 1991.- Effect of juvenile hormone analogue on ovarian development of the reproductive-diapausing parasitic fly, *Melinda pusilla*.- *Medical Entomology and Zoology*, 42: 311-317.

ALAUX C., JAISSON P., HEFETZ A., 2004.- Queen influence on worker reproduction in bumblebees (*Bombus terrestris*) colonies.- *Insectes Sociaux*, 51: 287-293.

ALAUX C., JAISSON P., HEFETZ A., 2006.- Regulation of worker reproduction in bumblebees (*Bombus terrestris*): workers eavesdrop on a queen signal.- *Behavioral Ecology and Sociobiology*, 60: 439-446.

ALAUX C., BOUTOT M., JAISSON P., HEFETZ A., 2007.- Reproductive plasticity in bumblebee workers (*Bombus terrestris*) - reversion from fertility to sterility under queen influence.- *Behavioral Ecology and Sociobiology*, 62: 213-222.

AMSALEM E., GROZINGER C. M., 2017.- Evaluating the molecular, physiological and behavioral impacts of CO narcosis in bumble bees (*Bombus impatiens*).- *Journal of Insect Physiology*, 101: 57-65.

AMSALEM E., HEFETZ A., 2010.- The appeasement effect of sterility signaling in dominance contests among *Bombus terrestris*.- *Behavioral Ecology and Sociobiology*, 64: 1685-1694.

AMSALEM E., HEFETZ A., 2011.- The effect of group size on the interplay between dominance and reproduction in *Bombus terrestris*.- *PloS ONE*, 6: e18238.

AMSALEM E., TEAL P., GROZINGER C. M., HEFETZ A., 2014.- Precocene-I inhibits juvenile hormone biosynthesis, ovarian activation, aggression and alters sterility signal production in bumble bee (*Bombus terrestris*) workers.- *Journal of Experimental Biology*, 217: 3178-3185.

AUPINEL P., FORTINI D., MICHAUD B., MAROLLEAU F., TASEI J. N., ODOUX J. F., 2007.- Toxicity of dimethoate and fenoxycarb to honey bee brood (*Apis mellifera*), using a new in vitro standardized feeding method.- *Pest Management Science*, 63: 1090-1094.

AYASSE M., MARLOVITS T., TENGÖ J., TAGHIZADEH T., FRANCKE W., 1995.- Are there pheromonal dominance signals in the bumblebee *Bombus hypnorum* L. (Hymenoptera, Apidae)?- *Apidologie*, 26:163-180.

BELLINI R., ZELLER H., VAN BORTEL W., 2014.- A review of the vector management methods to prevent and control outbreaks of West Nile virus infection and the challenge for Europe.- *Parasites & Vectors*, 7: 323.

Bitondi M. M. G., Simões Z. L. P., Nascimento A. M., Garcia S. L., 1994.- Variation in the hemolymph protein composition of confined *Apis mellifera* and partial restoration of vitellogenin titre by juvenile hormone analogue treatment.- *Journal of Hymenoptera Research*, 3: 107-117.

Bitondi M. M., Mora I. M., Simoes Z. L., Figueiredo V. L., 1998.- The *Apis mellifera* pupal melanization program is affected by treatment with a juvenile hormone analogue.- *Journal of Insect Physiology*, 44: 499-507.

Bloch G., Hefetz A., 1999a.- Reevaluation of the role of mandibular glands in regulation of reproduction in bumblebee colonies.- *Journal of Chemical Ecology*, 25: 881-896.

Bloch G., Hefetz A., 1999b.- Regulation of reproduction by dominant workers in bumblebee (*Bombus terrestris*) queenright colonies.- *Behavioral Ecology and Sociobiology*, 45: 125-135.

Bloch G., Borst D. W., Huang Z. Y., Robinson G. E., Hefetz A., 1996.- Effect of social conditions on Juvenile Hormone mediated reproductive development in *Bombus terrestris* workers.- *Physiological Entomology*, 21:257-267.

Bloch G., Borst D. W., Huang Z. Y., Robinson G. E., Cnaani J., Hefetz A., 2000.- Juvenile hormone titers, juvenile hormone biosynthesis, ovarian development and social environment in *Bombus terrestris*.- *Journal of Insect Physiology*, 46: 47-57.

Bogo G., de Manincor N., Fisogni A., Galloni M., Zavatta L., Bortolotti L., 2018.- Different reproductive strategies and their possible relation to inbreeding risk in the bumble bee *Bombus terrestris*.- *Insectes Sociaux*, 65: 289-295.

Bortolotti L., Porrini C., Micciarelli Sbrenna A., Sbrenna G., 2000.- Ovicidal action of fenoxycarb on a predator, *Chrysoperla carnea* (Neuroptera: Chrysopidae).- *Applied Entomology and Zoology*, 35: 265-270.

Bortolotti L., Duchateau M. J., Sbrenna G., 2001.- Effect of juvenile hormone on caste determination and colony processes in the bumblebee *Bombus terrestris*.- *Entomologia Experimentalis et Applicata*, 101: 143-158.

Bortolotti L., Micciarelli Sbrenna A., Sbrenna G., 2005.- Action of fenoxycarb on metamorphosis and cocoon spinning in *Chrysoperla carnea* (Neuroptera: Chrysopidae): identification of the JHA-sensitive period.- *European Journal of Entomology*, 102: 27-32.

Cappellozza L., Cappellozza S., Sbrenna G., 1995.- Changes in the developmental programme of *Bombyx mori* caused by oral treatment with fenoxycarb during the last larval instar.- *Sericologia*, 35:427-436.

Chen Y.-W., Wu P.-S., Yang E.-C., Nai Y.-S., Huang Z. Y., 2016.- The impact of pyriproxyfen on the development of honey bee (*Apis mellifera* L.) colony in field.- *Journal of Asia-Pacific Entomology*, 19: 589-594.

Dhadialla T. S., Retnakaran A., Smagghe G., 2005.- Insect growth- and development-disrupting insecticides, pp. 55-115. In: *Comprehensive insect molecular science, vol. 6: control* (Gilbert L. I., Kostas I., Gill S. S., Eds).- Pergamon Elsevier, Oxford, UK.

Duchateau M. J., Velthuis H. H. W., 1988.- Development and reproductive strategies in *Bombus terrestris* colonies.- *Behaviour*, 107: 186-207.

Duchateau M. J., Velthuis H. H. W., 1989.- Ovarian development and egg laying in workers of *Bombus terrestris*.- *Entomologia Experimentalis et Applicata*, 51: 199-213.

El-Sheik E. S. A., Kamita S. G., Hammock B. D., 2016.- Effects of juvenile hormone (JH) analog insecticides on larval development and JH esterase activity in two spodopterans.- *Pesticide Biochemistry and Physiology*, 128: 30-36.

EU, 2010.- Review report for the active substance fenoxycarb finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on 14 March 2008 in view of the inclusion of pyriproxyfen in Annex I of Directive 91/414/EEC.- European Commission, Health & Consumers Directorate General.

EU, 2011.- Review report for the active substance fenoxycarb finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on 28 January 2011 in view of the inclusion of fenoxycarb in Annex I of Directive 91/414/EEC.- European Commission, Health & Consumers Directorate General.

Gosterit A., 2011.- Effect of different reproductive strategies on colony development characteristics in *Bombus terrestris*.- *Journal of Apicultural Science*, 55: 45-51.

Gosterit A., Koskan O., Gurel F., 2016.- The relationship of weight and ovarian development in *Bombus terrestris* L. workers under different social conditions.- *Journal of Apicultural Science*, 60: 51-58.

Gradish A. E., Van Der Steen J., Scott-Dupree C. D., Cabrera A. R., Cutler G. C., Goulson D., Klein O., Lehman D. M., Lückmann J., O'Neill B., Raine N. E., Sharma B., Thompson H., 2019.- Comparison of pesticide exposure in honey bees (Hymenoptera: Apidae) and bumble bees (Hymenoptera: Apidae): implications for risk assessments.- *Environmental Entomology*, 48 (1): 12-21.

Gretenkord C., Drescher W., 1996.- Laboratory and cage test methods for the evaluation of insect growth regulators (Insegar®, Dimilin®) on the brood of *Bombus terrestris* L., pp. 34-38. In: *Proceedings of the 6th international symposium hazard of pesticides to bees*, Germany, 17-19 September 1996, Intern Commission Plant-Bee Relationships, Braunschweig.

Hakme E., Lozano A., Gómez-Ramos M. M., Hernando M. D., Fernández-Alba A. R., 2017.- Non-target evaluation of contaminants in honey bees and pollen samples by gas chromatography time-of-flight mass spectrometry.- *Chemosphere*, 184: 1310-1319.

Hartfelder K., 2000.- Insect juvenile hormone: from "status quo" to high society.- *Brazilian Journal of Medical and Biological Research*, 33: 157-177.

Huang Z. Y., Lin S., Ahn K., 2016.- Methoprene does not affect juvenile hormone titers in honey bee (*Apis mellifera*) workers.- *Insect Science*, 25: 235-240.

Kwon Y. J., Saeed S., Duchateau M. J., 2003.- Stimulation of colony initiation and colony development in *Bombus terrestris* by adding a male pupa: the influence of age and orientation.- *Apidologie*, 34: 429-437.

Larrere M., Couillaud F., 1993.- Role of juvenile hormone release in dominance status and reproduction of the bumblebee, *Bombus terrestris*.- *Behavioral Ecology and Sociobiology*, 33: 335-338.

Lawler S. P., 2017.- Environmental safety review of methoprene and bacterially-derived pesticides commonly used for sustained mosquito control.- *Ecotoxicology and Environmental Safety*, 139: 335-343.

Lopez-Vaamonde C., Brown R. M., Lucas E. R., Pereboom J. J. M., Jordan W. C., Bourke A. F. G., 2007.- Effect of the queen on worker reproduction and new queen production in the bumble bee *Bombus terrestris*.- *Apidologie*, 38: 171-180.

Malka O., Katzav-Gozansky T., Hefetz A., 2009.- Uncoupling fertility from fertility-associated pheromones in worker honeybees (*Apis mellifera*).- *Journal of Insect Physiology*, 55: 205-209.

Matsuo J., Nakayama S., Numata H., 1997.- Role of the corpus allatum in the control of adult diapause in the blow fly, *Protophormia terraenovae*.- *Journal of Insect Physiology*, 43: 211-216.

Milchreit K., Ruhnke H., Wegener J., Bienefeld K., 2016.- Effects of an insect growth regulator and a solvent on honeybee (*Apis mellifera* L.) brood development and queen viability.- *Ecotoxicology*, 25: 530-537.

Mommaerts V., Sterk G., Smagghe G., 2006.- Bumblebees can be used in combination with juvenile hormone analogues and ecdysone agonists.- *Ecotoxicology*, 15: 513-521.

OECD, 2017.- *Test No. 246: Bumblebee, acute contact toxicity test. OECD guidelines for the testing of chemicals, Section 2*.- OECD Publishing, Paris, France.

PANDEY A., MOTRO U., BLOCH G., 2020.- Juvenile hormone interacts with multiple factors to modulate aggression and dominance in groups of orphan bumble bee (*Bombus terrestris*) workers.- *Hormones and Behavior*, 117: 104602.

PENER M. P., DHADIALLA T. S., 2012.- An overview of insect growth disruptors; applied aspects.- *Advances in Insect Physiology*, 43: 1-162.

PINTO L. Z., BITONDI M. M. G., SIMÕES Z. L. P., 2000.- Inhibition of vitellogenin synthesis in *Apis mellifera* workers by a juvenile hormone analogue, pyriproxyfen.- *Journal of Insect Physiology*, 46: 153-160.

RIDDIFORD L. M., 2008.- Juvenile hormone action: a 2007 perspective.- *Journal of Insect Physiology*, 54: 895-901.

RIDDIFORD L. M. 2012.- How does juvenile hormone regulate insect metamorphosis and reproduction?- *Integrative and Comparative Biology*, 179: 477-484.

ROBINSON G. E., STRAMBI C., STRAMBI A., HUANG Z.-Y., 1992.- Reproduction in worker honey bees is associated with low juvenile hormone titers and rates of biosynthesis.- *General and Comparative Endocrinology*, 87: 471-480.

RÖSELER P. F., 1977.- Juvenile hormone control of oogenesis in bumblebee workers, *Bombus terrestris*.- *Journal of Insect Physiology*, 23: 985-992.

RÖSELER P. F., RÖSELER I., 1978.- Studies on the regulation of the juvenile hormone titer in bumblebee workers, *Bombus terrestris*.- *Journal of Insect Physiology*, 24: 707-713.

RÖSELER P. F., RÖSELER I., VAN HONK C. G. J., 1981.- Evidence of inhibition of corpora allata activity in workers of *Bombus terrestris* by a pheromone from the queen's mandibular glands.- *Experientia*, 37: 348-351.

RUTZ W., GERIG L., WILLE H., LÜSCHER M., 1976.- The function of juvenile hormone in adult worker honeybees, *Apis mellifera*.- *Journal of Insect Physiology*, 22: 1485-1491.

SHPIGLER H., PATCH H. M., COHEN M., FAN Y., GROZINGER C. M., BLOCH G., 2010.- The transcription factor *Krüppel homolog 1* is linked to hormone mediated social organization in bees.- *BMC Evolutionary Biology*, 10: 120.

SHPIGLER H., AMSALEM E., HUANG Z.-Y., COHEN M., SIEGEL A. J., HEFETZ A., BLOCH G., 2014.- Gonadotropic and physiological functions of juvenile hormone in bumblebee (*Bombus terrestris*) workers.- *PLoS ONE*, 9: e100650.

SIBBALD E. D., PLOWRIGHT C. M. S., 2013.- On the relationship between aggression and reproduction in pairs of orphaned worker bumblebees (*Bombus impatiens*).- *Insectes Sociaux*, 60: 23-30.

SOKAL R. R., ROHLF F. J., 2012.- *Biometry. The principles and practice of statistics in biological research*.- Freeman and Co, New York, USA.

SULLIVAN J. P., JASSIM O., FAHRBACH S. E., ROBINSON G. E., 2000.- Juvenile hormone paces behavioral development in the adult worker honey bee.- *Hormones and Behavior*, 37: 1-14.

TASEI J. N., 2001.- Effects of insect growth regulators on honey bees and non-*Apis* bees. A review.- *Apidologie*, 32: 527-545.

THOMPSON H. M., 2001.- Assessing the exposure and toxicity of pesticides to bumblebees (*Bombus* sp.).- *Apidologie*, 32: 305-321.

VAN DOORN A., 1987.- Investigation into the regulation of dominance behaviour and of the division of labor in bumblebee colonies (*Bombus terrestris*).- *Netherlands Journal of Zoology*, 37: 255-276.

VAN DOORN A., 1989.- Factors influencing dominance behaviour in queenless bumblebee workers (*Bombus terrestris*).- *Physiological Entomology*, 14: 211-221.

VAN HONK C. G. J., VELTHUIS H. H. W., RÔSELER P. F., MALOTAUX M. E., 1980.- The mandibular glands of *Bombus terrestris* queens as a source of queen pheromones.- *Entomologia Experimentalis et Applicata*, 28: 191-198.

VAN HONK C. G. J., RÖSELER P. F., VELTHUIS H. H. W., HOOGEVEEN J. C., 1981.- Factors influencing the egg laying of workers in a captive *Bombus terrestris* colony.- *Behavioral Ecology and Sociobiology*, 9: 9-14.

WASIELEWSKI O., WOJCIECHOWICZ T., GIEJDASZ K., KRISHNAN N., 2011.- Influence of methoprene and temperature on diapause termination in adult females of the over-wintering solitary bee, *Osmia rufa* L.- *Journal of Insect Physiology*, 57: 1682-1688.

WYATT G. R., DAVEY K. G., 1996.- Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects.- *Advances in Insect Physiology*, 26: 1-155.

ZUFELATO M. S., BITONDI M. M., SIMOES Z. L., HARTFELDER K., 2000.- The juvenile hormone analog pyriproxyfen affects ecdysteroid-dependent cuticle melanization and shifts the pupal ecdysteroid peak in the honey bee (*Apis mellifera*).- *Arthropod Structure and Development*, 29: 111-119.

Authors' addresses: Gherardo BOGO (corresponding author: gherardo1985@hotmail.com), Grupo de Ecología de la Polinización, INIBIOMA, Universidad Nacional del Comahue-CONICET, San Carlos de Bariloche, 8400, Argentina; Laura BORTOLOTTI, Council for Agricultural Research and Economics - Research Centre for Agriculture and Environment (CREA-AA), via di Saliceto 80, 40128 Bologna, Italy; Filip POŠĆIĆ, Department of Applied Science, Institute for Adriatic Crops and Karst Reclamation, Put Duilova 11, 21000 Split, Croatia; Marie José DUCHATEAU, Faculty of Biology, Animal Ecology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

Received March 21, 2020. Accepted August 28, 2020.