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Original Article

METHOPRENE AND TEMPERATURE STIMULATION OF EMERGENCE AND LONGEVITY IN ADULTS OF *OSMIA RUFA* L. (MEGACHILIDAE; APOIDEA) DURING WINTERING PERIOD

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Abstract

In this study methoprene, a juvenile hormone analogues, was tested as a factor that stimulates the end of diapause, bee activation and emergence. In addition, the survival of bees was checked when combined with an application of methoprene. The experimental activation of wintering bees was carried out once a month between December and March, Three groups of bee cocoons were selected for each activation term: treated with either methoprene or acetone as vehicle control and untreated as controls. After the applications were finished the cocoons were incubated at temperature 26˚C. The emerged adult males and females were then kept in the laboratory and fed sucrose solution to evaluate the longevity of bees. The application of juvenile hormone analogue reduced the emergence time of adult bees in December, February and March. The rate of emergence presented in the form of cumulative percentage of emerged adult bees indicated that the bees treated with methoprene started to emerge 1-2 days earlier than bees from acetone and control groups and finished emergence 2-5 days earlier too. Methopren application did not reduce the longevity of the bees after emergence. Moreover, the median longevity of these females was higher than untreated in December and February.

Keywords: emergence, longevity, methoprene, *Osmia rufa*, temperature, wintering

INTRODUCTION

The red mason bee, *Osmia rufa*, a native European solitary bee from the megachillid family, is showing promise as a commercial manageable pollinator. This polilectic species has a strong preference for fruit trees and is an efficient pollinator in orchards (Wilkaniec & Radajewska, 1996; Wilkaniec & Maciejewska, 1998) This bee species is widely used by seed companies as well as plant growers for pollination, especially of onion, carrot, cabbage, turnip and lettuce (Schittenhelm, Giadis & Rao, 1997). Genus *Osmia* is also used in plant breeding, where plants are crossbred into isolators as to generate new varieties of plants (Kaminski, 2010).

In Poland, *O. rufa* is a native bee species managed for crop pollination. Strategies have been developed to manipulate the temperature conditions of keeping bees during the wintering period, which can be shortened or prolonged. This allows for the synchronisation of the bee emergence time with crop bloom period (Bosch & Kemp, 2000, 2004; Sedivy & Dorn, 2014).

Day length and temperature regulate diapause in many insects. Adult diapause is characterised by ovaries ceasing to develop and hypertrophy of the fat body (Hondelmann & Poehling, 2007). Accumulation of fat body glycogen can cause hypertrophy and is one of the mechanisms that enhances overwintering survival in some species (Mitchell & Briegel, 1989). Beyond the inducing factors, photoperiod and temperature, diapause is regulated by neuroendocrine processes (Denlinger, 2002). The endocrine regulation is important especially in reproductive diapause (i.e. a resting state with reduced metabolic activity) where juvenile hormone (JH) titers appear to play a crucial role (Simonet et al., 2004).

This species completes its development from

egg to the imago stage in the spring and summer seasons and bees winter in the nest as imagoes inside a cocoon (Tasei, 1973; Rust, Torchio & Trostle, 1989).The timing of entering diapauses in *O. rufa* is in November and termination occurs at the end of January (Wasielewski et al., 2011a) and during the next three months the adults are still cocooned. This time corresponds to a post-diapause quiescence (February–April), during which different metabolic rates are observed depending on tissue (generally high of fat body and low activity of biochemical compounds of haemolymph) (Sgolastra et al., 2010; Strachecka at al., 2017). During post-diapause in *O. rufa,* the protein concentrations rapidly changes with a decrease in fat body and increase in haemolymph and ovary (Wasielewski et al., 2011a; Strachecka et al., 2017).

The application of higher temperature at the end of diapause and post-diapause may cause bees to emerge. This process proceeds slowly which makes flights and blooming difficult to synchronize. Therefore, apart from the temperature we need to apply an additional hormonal factor that could accelerate the end of diapause. Methoprene, a juvenile hormone analogue, was the hormonal factor – applied during diapause which increased ovary growth and number of oocytes and mobilised fat body proteins and other reserve substances (Wasielewski et al., 2011b). Our primary goal was to test methoprene as a factor that stimulates the end of diapause, bee activation and emergence. In addition, importance was the observation of the condition of bees and their survival combined with an earlier application of methoprene.

Currently, the wintering period can be reduced with the use of temperature treatment and exogenous application of methoprene and synchronization of *O. rufa* bee flights with crop blooming only in the period from March to July. The development of a method of adult activation at any point of wintering allows the use of bees during autumn, winter and early spring for the pollination of greenhouse crops. Also, adults emergence acceleration has reduced the costs connected with bees incubation.

MATERIAL AND METHODS

Bees and experimental design

Experiments were conducted in 2010–2011 during the *O. rufa* wintering period . The bees were reared in artificial nests made of reed stalks following the method of Wójtowski and Wilkaniec (1978). The settled nests of the bees were left outdoors during wintering, and once a month they were moved to a laboratory, where nest tubes were dismantled and cocoons were removed from nest cells. The cocoons were sexed according to their size and position within the nest.

The experimental activation of wintering bees was carried out once a month between December and March (09.12.2010; 11.01.2011; 01.02.2011; 03.03.2011 to be precise). Three groups of bee cocoons, in each 50 males and 50 females, were selected for each activation term. The first group was treated with methoprene, the second with acetone, and the third untreated (intact, control group). After incubation and emergence we used 465 males (156 in methoprene group, 152 in acetone group, 157 in control group) and 437 females (149, 141, 147, respectively) over four months, normally more than 30 individuals in one group,

Bee treatments

The JH analogue, methoprene (kindly provided by Dr. Dalibor Kodrik, Institute of Entomology, Czech Republic) was topically applied on the cocoon surface once a day, for five consecutive days, with a Hamilton syringe (5 µl; Hamilton Co. Germany) in the first group. The experimental cocooned bees were treated everyday with 200 µg methoprene dissolved in 5 µl acetone (Sigma, USA) (Robinson et al., 1992; Malka, Katzav-Gozansky & Hefetz, 2009). The second group was treated 200 µg acetone and the third group was untreated with neither methoprene nor acetone. During application time, the cocoons of all groups were kept in the laboratory at temperature 22˚C. After the finish applications, the cocoons were transferred to Petri dishes (15 cm diameter), and placed in a climate chamber (model MLR-350H Sanyo) at temperature 26˚C.

The number of emerged *O. rufa* was checked every day until all live bees emerged. The sex of an adult was determined according to morphological characters, and they were settled into cuboid cages (25 cm x 40 cm x 25 cm). Adult males and females were kept in the laboratory at 22°C and 12L:12D photoperiod conditions. The bees were fed ad libitum sucrose solution (1:1) served in a Petri dish (5 cm diameter) with a floating circle of perforated comb foundation to prevent the insect from drowning. The beescages were checked every three days to determine the number of dead bees. This was done with the aim of evaluating the longevity of bees. Bee bodies were removed and sucrose solution feeds changed at that time.

Statistical analysis

The Kruskal-Wallis test (one-way ANOVA by ranks) was used for multiple comparison of emergence times and longevity in the groups (control, acetone, methoprene). The analysis was conducted at the significance level α= 0.05, separately for males and females using the Statistica v.12 software.

RESULTS

Time and rate of bee emergence

Bees after methoprene treatment emerged faster than control groups (acetone and untreated) in winter months (December, February, March). Median emergence times of the males in December were 28 days after treated with methoprene, 30.5 days for males treated with acetone and 30 days for untreated males (K-W test: H= 28.098; df – 2, 120; p < 0.001), and 31.5; 35; 35 days for females, respectively (K-W test: H= 16.890; df –2, 93; p<0.001) (Fig. 1A). There was no significant difference in the emergence time of adult bees among groups from various treatments in January. Most of the males in all experimental groups (K-W test: H = 1.642; df – 2, 114; p = 0.44) or females (K-W test: H = 3.059; df – 2, 101; p = 0.21) emerged in the same time (Fig. 1B). Despite no differences between groups, we noted that in methoprene treated females first bees were emerged 2-4 days earlier than acetone and control. In February the adults treated with methoprene emerged faster than those treated with acetone and untreated (K-W test; male: H = 11.312; df – 2, 117; p = 0.004; female: H = 25.335; df – 2, 122; p<0.001) (Fig. 1C). In March, similarly to December and February, bees after methoprene treatment emerged faster than after acetone and untreated (K-W test; male: H = 10.088; df – 2, 93; p = 0.006; female: H = 65.178; df – 2, 120; p < 0.001) (Fig. 4D).

In all terms and groups males emerged first before females (Fig. 2A-D). In the group of bees treated with methoprene, males started to emerge 1-2 days earlier than males from acetone and control groups and finished emergence 3-7 days earlier too. Females after methoprene application started to emerge 2-6 days earlier than females from acetone and untreated groups and finished emergence 2-5 days earlier. We also observed that at 14 days after emergence was started 90% of adult bees treated with methoprene, 75% treated with acetone and 77% untreated emerged in December; 88%, 78% 78% in January; 87%, 66%, 64% in February and 100%, 80%, 72% in March, respectively (Fig. 2A-D).

Adult longevity

In December, the median longevity in methoprene-treated males was 36 days, in acetone-treated males 31.5 and in untreated 27 days (K-W test; H = 2.641; df – 2; 97; p = 0.27) (Fig. 3A). Howevr, females treated with methoprene lived longer than females treated with acetone or were untreated (K-W test; H = 6.449; df – 2, 94; p = 0.04). There was no significant difference in the longevity of adult bees among the three groups in January (male: K-W test; H = 0.926; df – 2, 91; p = 0.63; female: K-W test; H = 3.483; df – 2, 93; p = 0.17) (Fig. 3B). The median longevity of bees activated in January was 36 days for males treated with methoprene, 33 days for males treated with acetone and 36 days for untreated males and 69, 63, 63 days for females, respectively.

The median longevity of the bees activated in February was 36 days for males treated with

methoprene, 31.5 days for males treated with acetone and 27 days for untreated males (K-W test; H = 0.130; df – 2, 96; p = 0.94) (Fig. 3C). In contrast, significant statistical differences in the longevity of adult females among the different experimental groups (K-W test; H = 15.454; df – 2; 93; p < 0.001) were detected in February The adult females treated with methoprene had a significantly longer median longevity (median: 66) than those with acetone (median: 48) and the control (median: 51) group. In the last month of our incubation experiments, we did not note any differences in the longevity of adult bees among the groups (male: K-W test; H = 2.36; df – 2, 93; p = 0. 31) (female: K-W test; H = 1.874; df – 2, 104; p = 0.39) (Fig. 3D). The median longevity of methoprene-treated males in March was 30 days, 27 days for acetone-treated males and 27 days for untreated males, and 66 61 57 days for females, respectively.

DISCUSSION

The availability of commercial pollinators is required to keep entomophilous crops in greenhouses except during insect flight. Our experiment shows the possibility of activating the red mason bee early in December, while retaining it high longevity .

Insect diapause in general is a dynamic process and consists of several successive phases (Koštál, 2006), but the precise terminology for the different phases in *O. rufa* has not been defined. *O. rufa* overwinters as an imago inside its cocoon in November, and diapause terminates towards the end of January, after the temperature rises. According to Koštál's (2006) model of diapause adapted for *O. lingaria* phenology by Sgolastra et al. (2010), the intensity of diapause increases during first 30 days of pre-wintering time and maintains for the next 30 days. After

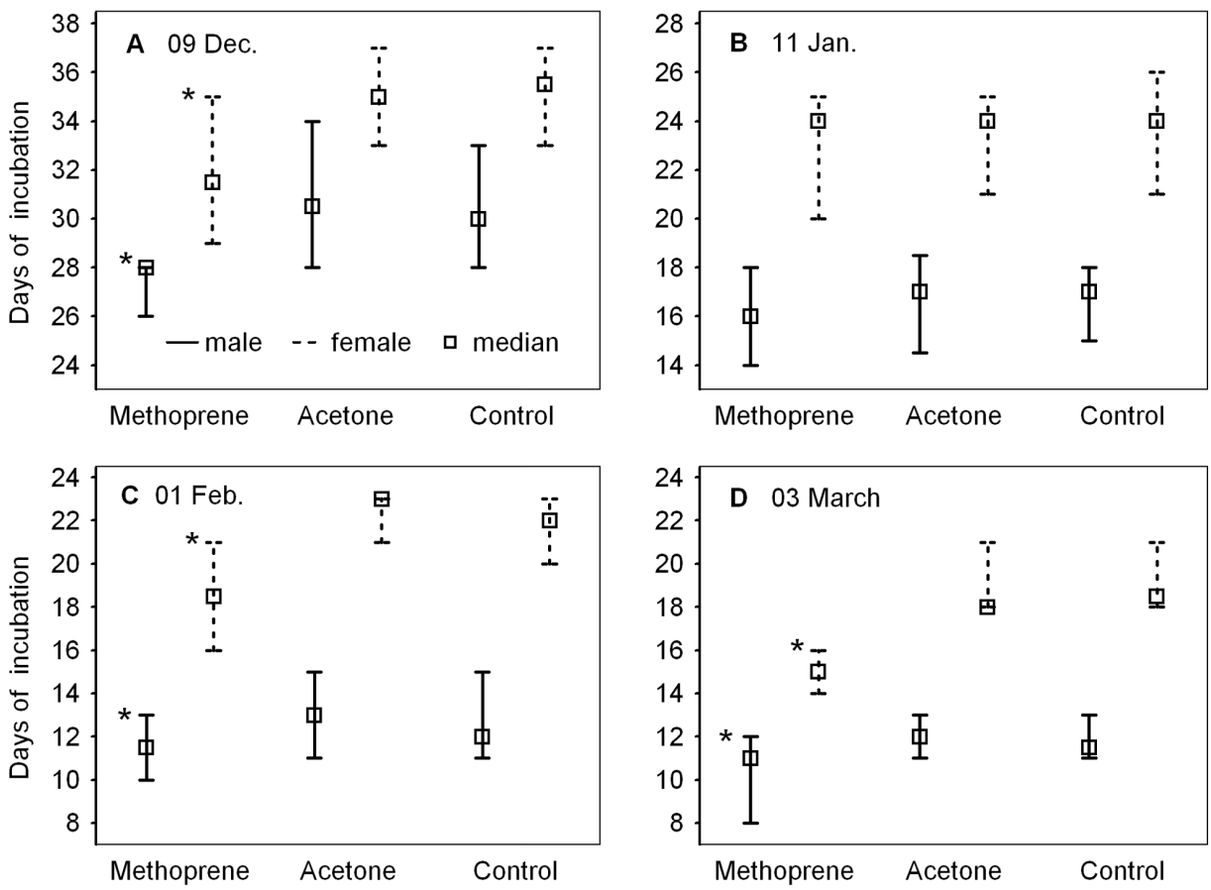

Fig. 1. Effect of methoprene treatment on the emergence time of bees of *O. rufa* activated in different terms. Data are presented in medians and quartiles (27-75). Significant differences ($p<0.05$) from the control (untreated and acetone treated) are indicated by asterisk

this time from 60 till 100 days of overwintering, when the intensity of diapause decreases gently, it is possible to activate adults. Our work also confirmed the possibility of activating *O. rufa* adults after 70 days of overwintering (Fig. 1).

In univoltine bees with imaginal diapause, the emergence rate is strongly dependent on overwintering temperature and duration (Bodch & Kemp, 2003; Sgolastra et al., 2010). When *O. ligniaria* has finished wintering within 30 days (in artificial condition), incubation time has prolonged to 75 days and survival of freshly eclosed bees is very low (Bosch & Kemp, 2003). Another species, *O. cornuta* emerged successfully after 75 days of overwintering and emergence periods were advanced and shortened as over-wintering periods were extended (range 75 – 120 days) (Bosch & Blas, 1994). In other experimental conditions after 90 days of overwintering, adult *O. cornuta* emerged after about fifteen days (Bosch & Kemp, 2004).

We planned the beginning of our experiments in December (70th day of overwintering) and our results indicated that adult bees without methoprene successfully emerged this winter after minimum 30 days of incubation (Fig. 1). Our experiments showed that in January half of male adults emerged after sixteen days and half of female adults after 24 days of incubation. Additionally, in the following months (February, March) the time of emergence was reduced. Based on the classification of the eco-physiological phases of insect diapause (Kostál 2006), we concluded that *O. rufa* enters diapause in November and diapause termination occurs

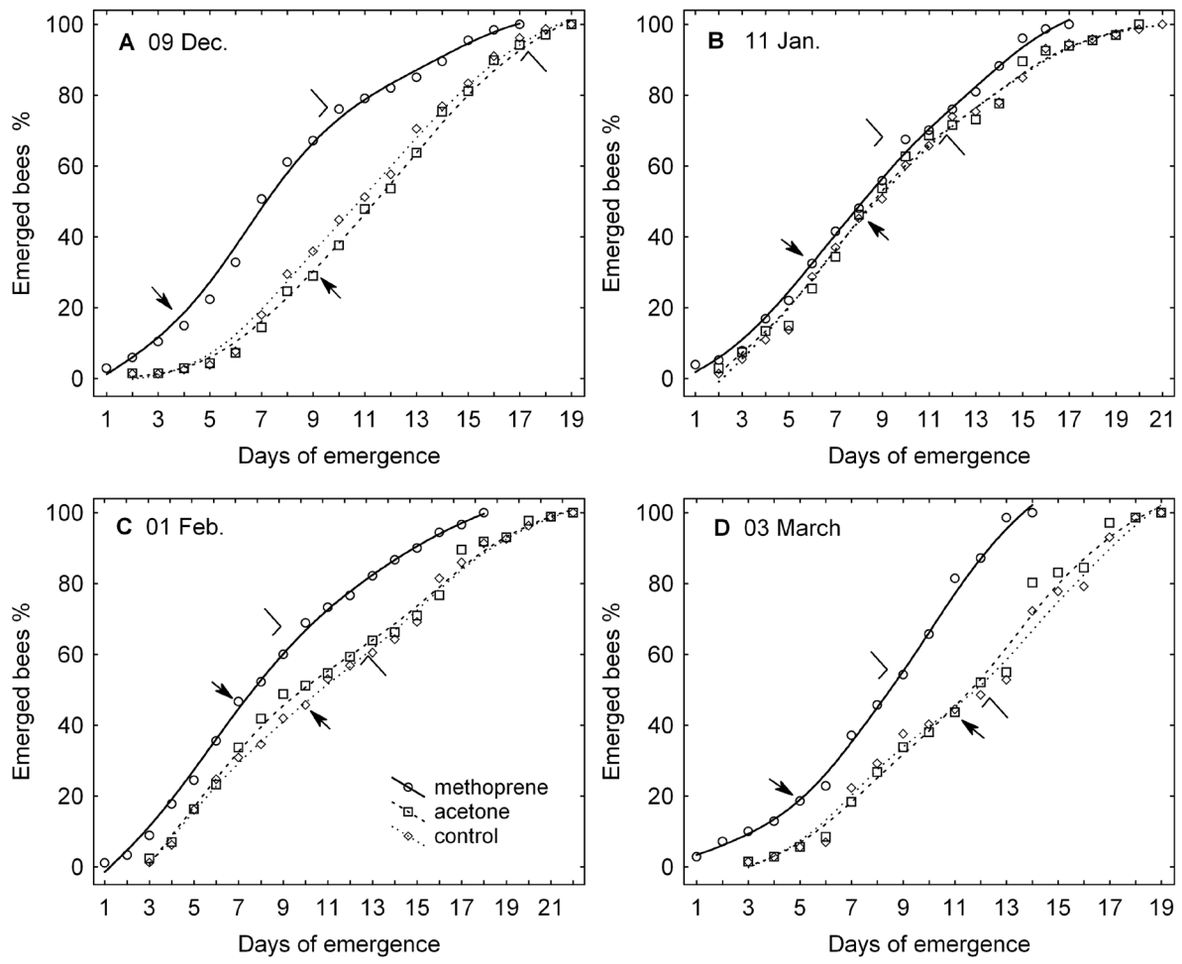

Fig. 2. Cumulative percentage of emerged bees of *O. rufa* treated with methoprene activated in different terms in wintering period.

→ female emergence start

¬ male emergence finish

at the end of January. Then, the unemerged adult remains in post-diapause quiescence till April (Wasielewski et al. 2011b). For this study, the entire overwintering period of *O. rufa* was classified as pre-wintering (September-October), diapause (November-January), and post-diapause quiescence (February-April). In our experiments, the overwintering time of *O. rufa* started at the beginning of October and the first period of activation started 70 days after that on December 9th.

The application of methoprene further reduced the emergence rate . In methoprene-administered groups all males and half of females emerged in December after 32 days of incubation (Fig. 2A). At the same time in the control group, we noted just over half of males and less than quarter of females. In February (> 120 days of overwintering) and March (>150 days of overwintering) the application of methoprene accelerated emergence especially in females. In January (> 100 days of overwintering) the emergence time was reduced compared to that in December. During this period ovary development resumed connected with fat body reserves consumption. The changes in protein content in the ovariesy and fat body tissue started after an increase of the outdoor temperature and bees demonstrated metabolic activity resumption gearing up towards emergence (Wasielewski et al., 2011a). Diapause in *O. rufa* terminates at the end of January with increasing temperatures. Therefore, it seems that temperature plays an important role in addition to hormonal regulation in the termination of diapauses (Fig. 1). The use of both these factors is crucial for achieving bees activation in the winter months (Wasielewski et al., 2011b).

Bees lose weight much more rapidly during incubation than during wintering. Because bees

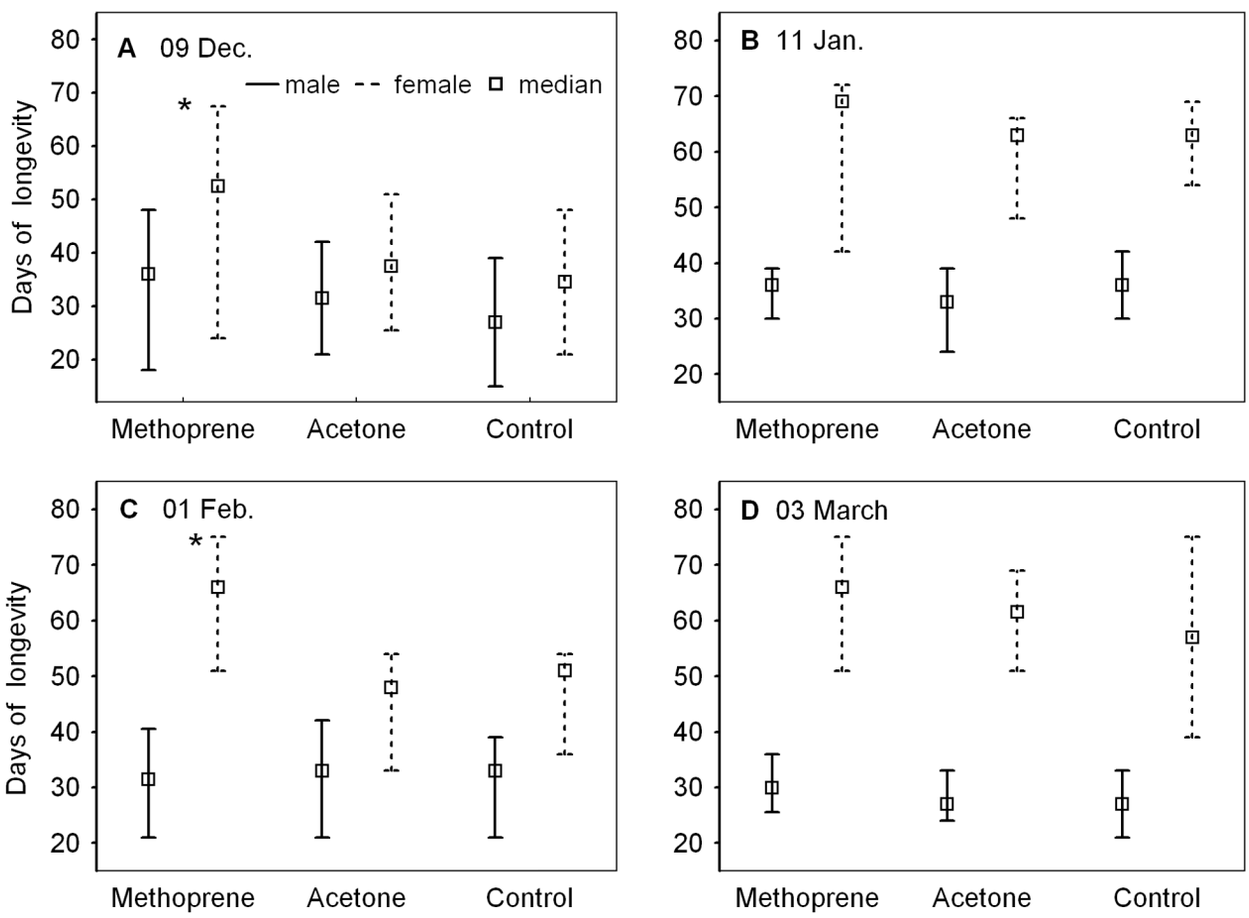

Fig. 3. The effect of methoprene treatment on the longevity of adult bees of *O. rufa* activated in different terms during diapause. Data are presented in medians and quartiles (27-75). Significant differences ($p<0.05$) from the control (untreated and acetone treated) are indicated by asterisk

wintered for shorter periods require longer incubation periods to emerge, fat body diminish more intensely and total body weight reduce (Sgolastra et al., 2010). When individual bees suffer great weight loss and fat body depletion, they have short post-winter longevity (Bosch, Sgolastra & Kemp, 2010). This explains the positive effect of methoprene application on the longevity of females in December and February, when the bees emerged faster. In our experiment, female bees with a shortened emergence time lived longer. Maximum survival and longevity were obtained with wintering durations of 90–150 days (Bosch & Kemp, 2004). Similarly, in our experiments bees incubated in January after 100 days of overwintering were characterized by longevity, independently from methoprene application (Fig. 3). Even though the bees treated with methoprene emerged faster in March, they did live longer than acetone-treated and untreated bees. Probably, the effect of methoprene did not appear in March, because emergence time was reduced mainly due to the wintering duration (Bosch & Kemp, 2003).

It seems that the diapause duration (or reduction) played a secondary role for longevity and primarily incubation at a higher temperature strongly affected bee vitality. For example, the survival of bees removed from cocoons (without earlier incubation) at 14 days of life cycle in either December, January or April was similar, although diapause reduction affected female fertilization (Fliszkiewicz, Giejdasz & Wilkaniec, 2011). Beyond the inducing factors, photoperiod and temperature, diapause is regulated by neuroendocrine processes (Denlinger, 2002). Endocrine regulation is especially important in reproductive diapause (i.e. a resting state with reduced metabolic activity) where juvenile hormone (JH) titers appear to play a crucial role (Simonet et al., 2004). A decrease in JH production in the corpus allatum (CA) induces cessation of reproduction, specifically, the arrest of vitellogenesis and regression of the ovaries (De Loof, 2008). Generally, administration juvenile hormone or its analogue methoprene into adult insects can exert both positive and negative effects on development and reproduction. The mobilizing effect of methoprene application was observed in mosquito adults (Klowden & Chambers, 1989). The glycogen content of sugar-fed female *Aedes aegypti* after methoprene treatment was significantly reduced, suggesting that treatment with juvenile hormone or its analogue (methoprene) induced vitellogenesis by mobilizing nutritional reserves or causing mortality when the reserves were depleted (Klowden & Chambers, 1989). Contrary, in our studies, there was no negative impact of metoprene on male and female vitality at any time of activation. Moreover, *O. rufa* females after methoprene treatment and winter months incubation were characterized by better vitality than the control group. However, Bosch, Sgolastra, and Kemp (2010) indicated that in bees of the *Osmia* family the reduction of fat body reserves caused higher temperature during the pre-wintering and wintering period which may affect emergence and post-emergence fitness.

It is worth mentioning that at the present time providing cocoons for different consumers is performed only in the spring period during normal physiological activity of bees and summer when wintering is prolonged artificially. Appropriate development control of this bee species will allow pollinators to be obtained during winter time. Normally the absence of pollinating insects during this period generates financial losses in companies which cultivate different plants even during the winter months.

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