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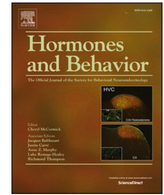

Juvenile hormone functions as a metabolic rate accelerator in bumble bees (*Bombus terrestris*)

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ABSTRACT

Juvenile hormone (JH) is a modulator of many physiological transitions in insects, including molting, metamorphosis, diapause, and reproduction. These processes often include metabolic changes. Here we show that JH accelerates metabolic rate in bumble bees (*Bombus terrestris*). We reduced JH levels in worker bumble bees by removing their corpora allata (allatectomy) and elevated JH levels in queens through a topical application of JH-III. Natural and high JH levels increased the metabolic rate in both workers and queens and triggered an increased protein turnover rate. Following the treatments, JH also caused an increase in food consumption and a reduction in lipid levels and flight muscle mass of queens, and a reduction in lipids levels in workers. Furthermore, the topical application of a JH analog to queens prior to their diapause caused a decline in their survival of diapause. These findings support the hypothesis that JH acts as a metabolic rate accelerator, initiating a resource shift in bumble bees, and thereby reducing diapause survival in queens. Based on previous studies on JH we suggest that, additional to its behavioral or physiological effects, JH's function as an accelerator of metabolic processes is conserved across different life stages and insect species.

1. Introduction

Juvenile hormone (JH) regulates many aspects of insect physiology. It has two prominent roles during an insect's life cycle: during the larval stage, JH controls larval growth, molting, and pupation timing, while in its second role, JH modulates the reproductive pathways (oogenesis) in adults (Jindra et al., 2013; Riddiford, 2012; Santos et al., 2019). Both functions involve substantial changes in morphology, development, behavior, and physiology at both the organismal and molecular levels (Riddiford, 2008).

The reproductive phase in insects involves behavioral and physiological changes. In females, certain behaviors, such as searching for and defending oviposition sites, mating, and building nests, lead to activation of the ovaries and the production of eggs (Alcock, 2001; Mathiron et al., 2019; Oi et al., 2020). These modifications occur in parallel to gene expression changes in multiple tissues (Benowitz et al., 2017; Shpigler et al., 2020), orchestrated by the endocrine system (Bellés and Maestro, 2005; Riddiford, 1980).

Bumble bees (*Bombus* spp.) offer an excellent model for studying changes related to reproduction in insects. Bumble bee colonies are established annually in the spring by a single queen and grow to a population of hundreds of workers. Towards the end of the summer the colony will produce gynes and then die. The new queens will emerge and enter a winter diapause until the following spring when they restart the cycle (Alford, 1975). In bumble bee queens and workers, JH is a gonadotropin that mediates ovarian development (Röseler and Röseler, 1986, 1988; Shpigler et al., 2014). Before enter diapause, the young mated queens possess low JH levels and undeveloped ovaries (Chen et al., 2021; Röseler and Röseler, 1986, 1988). When the queen exits diapause she enters the reproductive stage, her ovaries develop, and her JH levels rise. The queen then starts to lay eggs that develop into workers. The bumble bee queen suppresses the workers' ability to reproduce by means of her pheromones (Lopez-Vaamonde et al., 2007). However, workers separated from the presence of the queen are able to activate their ovaries (Röseler, 1974; Van Honk et al., 1981). The production of eggs in the ovaries requires allocating lipid reserves for protein synthesis, mostly synthesizing the yolk protein vitellogenin (Vg) into the developed oocytes (Tufail et al., 2014). Vg is synthesized in the

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bumble bee's fat body and is then transferred *via* the hemolymph to the ovaries, where it is embedded in the developing eggs (Hartfelder, 2000; Lockett et al., 2016; Shpigler et al., 2014). JH also affects other reproduction-related traits in bumble bees, such as wax production and honey pot construction (Shpigler et al., 2014). Beeswax is highly rich in long-chain fatty acids, and its production has a high energetic cost owing to its high energy content (Hepburn et al., 1991).

High JH levels in bumble bees upregulate genes related to major metabolic pathways, like glycolysis, the citric acid cycle, and oxidation phosphorylation in the fat body. Simultaneously, the expression of cytosolic ribosomal genes in the bee's brain is down-regulated, suggesting that this possible trade-off reflects the high protein synthesis in the fat body (Shpigler et al., 2020). These changes have been measured in workers but only at the mRNA level. Here we hypothesized that JH dictates the bee's metabolic rate and protein turnover in the fat body and brain (Shpigler et al., 2020). Furthermore, gene expression in queens in winter diapause, unlike that in reproductive queens, includes many JH-related genes, suggesting that JH is involved in regulating diapause in bumble bees (Amsalem et al., 2015; Chen et al., 2021). We thus hypothesized that the manipulation of JH levels would directly affect the bumble bees' basal metabolism.

Here, we tested the hypothesis that JH directly controls metabolism by measuring the effect of JH on the bumble bees' metabolism, employing several different approaches. We examined the effect of JH on resting metabolic rate and protein turnover in several tissues of workers and queens. We also tested the hypothesis that JH is involved in diapause physiology by measuring its effect on queen survival in diapause. Based on our findings and on previous studies on JH in other insects and at different life stages, we discuss the possibility that the function of JH as a metabolic rate accelerator in insects is conserved across species and life stages.

2. Material and methods

2.1. Bumble bees

Bombus terrestris colonies and queens were purchased from AgroBee, Ein Yahav, Israel, and Polyam Pollination Services, Yad-Mordechai, Israel. Each colony consisted in a queen, 5–10 workers, and brood at all developmental stages. Each colony was placed in a wooden nesting box (21 × 21 × 12 cm) with a transparent acrylic cover. The nesting boxes were housed in an environmentally controlled room (28 ± 1 °C; 50 ± 5% RH) at the I. Meier Segals Garden for Zoological Research at Tel Aviv University. The bees were kept in complete darkness and fed *ad libitum* with a 60% inverted sugar syrup and fresh pollen mixed with sugar syrup (Polyam Pollination Services, Israel).

2.2. JH manipulation in bumble bee workers

JH levels in bumble bee workers naturally increase when the workers are separated from the colony (Shpigler et al., 2014). JH levels were reduced by removing the corpora allata glands (allatectomy), the only JH source in bees, to prevent its production by workers. The procedure was performed as described by Shpigler et al. (2014). Briefly, newly emerged bumble bee workers were collected from ten source colonies and were anesthetized on ice for 5–30 min. The anesthetized bees were fixed with the dorsal side up on an ice-chilled metal stage and placed under a stereoscopic microscope (×50). The head was bent downwards to expose its posterior part. Using a fine scalpel, a latitudinal incision was made in the head capsule. The inner membrane and trachea were removed using fine forceps to expose the corpora allata (CA) glands. Each gland was gently detached, leaving the bee without CA glands. The entire procedure took between 2 and 5 min. The cuticle resumed its original shape, and the incision self-sealed within a few hours after the operation. For control of the dissection effect, we used sham-operated bees, which were handled and dissected similarly but the CA were only touched gently and not detached. Control bees were anesthetized and handled but were not operated. After the procedure, the bees were placed in small plastic cages (10 × 6 × 4 cm) with sugar syrup (60%) and pollen *ad libitum* together with similarly manipulated workers. The bees recovered overnight in an incubator (32 °C, 70% RH). Thirty-four percent of the allatecomized (n = 71), 50% of the sham-operated (n = 55) and 100% (n = 40) of the control bees survived the night. The surviving bees in each treatment were divided into groups of three or four and transferred to a new plastic cage. Bee survival was similar among the three experimental groups (allatectomy: 20/24, sham: 23/27, control: 36/40). The metabolic rate of workers was tested on day seven post-procedure (see below for details). At this age, the ovaries of the control workers were fully developed and they started to lay eggs (Shpigler et al., 2014). Following the metabolic rate analysis, all of the bees were frozen (−20 °C) for further analysis: ovarian development, lipid mass, and flight muscle mass, as detailed below.

2.3. JH manipulation in bumble bee queens

To determine the effect of JH on queens, mated queens were purchased from AgroBee, Ein Yahav, Israel, and from Polyam Pollination Services, Yad-Mordechai, Israel. The queens were from a pool of several colonies. They had been mated at the age of 7–10 days and sent together in a single box to Tel-Aviv University at the age of two weeks. Queens at this life stage have low levels of JH (Chen et al., 2021), and we artificially increased their JH levels using an application of JH (see below). The queens were randomly split into two treatment groups: a) treated with 100 μg JH-III (Sigma-Aldrich, cat #: J2000) dissolved in 5 μl of DMF (dimethylformamide, Sigma-Aldrich, cat #: 227056), and b) control queens treated with the solvent only, using topical application. We used this dose based on earlier studies in which 50–70 μg of JH-III had been used to treat workers. Since queens are larger, we increased the dose to suit the queen mass (Pandey et al., 2020; Shpigler et al., 2014). Prior to further analyses, the treated queens were kept for one week in an acclimation room (28 ± 1 °C; 50 ± 5% RH). We conducted three trials of this experiment. In the first trial, the queens were kept in groups of five following the treatment and used to analyze flight muscle mass, abdomen lipid levels, and diapause survival. For the other two trials, each queen was kept in a separate cage following the treatment and was analyzed for resting metabolic rate and protein turnover (for details, see below). In all trials, sugar-water and pollen were weighed on day one and at the end of the experiment to estimate the queen's food consumption (following our hypothesis that increased energy turnover would be followed by an increase in food consumption). At the end of the experiment, the queens were frozen at −20 °C and dissected for further analysis, as described below.

2.4. Metabolic rate analysis

Standard metabolic rate was measured using flow-through respirometry (following Lighton, 2018). We measured the metabolic rate for a total of 52 bees (32 workers and 20 queens). For each measurement, seven bees (deprived of food for 2 to 5 h before the test) were placed in individual metabolic chambers (30 ml) inside a temperature-controlled cabinet (Panasonic, Japan). The temperature was set at 28 °C for workers (the average temperature in an active colony; Kelemen and Dornhaus, 2018) and 20 °C for young queens (resembling the standard conditions of young queens outside the colony prior to diapause in Israel). Bees received dry CO_2-free air at a flow rate of 50 ml/s (V8, eight-channel flow mass control, Sable Systems, Las Vegas, Nevada). An empty cell was used as a baseline reference. Air from the chambers passed through eight-channel multiplexers (Sable Systems, Las Vegas, Nevada) to a Li-7000 CO_2/H_2O analyzer (Licor, Lincoln, Nebraska, USA) and through a column of magnesium perchlorate and Ascarite® (water and CO_2 adsorbents, respectively) into an O_2 analyzer (Oxzilla II, Sable Systems, Las Vegas, Nevada). We let the bees acclimate for 60 min and

then measured each worker for 20 min and each queen for 40 min. The measurement time was set to encompass at least five breathing cycles of the bees at rest. We used a longer period for the queens since their measurements were performed at a lower temperature, affecting the queens' breathing rate. A baseline of 5 min was run after every two samples. As respiratory quotient (RQ, ratio between VO_2 inhaled to VCO_2 exhaled) values for bumble bees are 1 (Levin et al., 2017a; Suarez et al., 2005), and as confirmed in our experiment, only CO_2 values were used to calculate the metabolic rate. VCO_2 was calculated using Eq. (10.1) in Lighton (2018). Bees were considered at rest when the gas exchange pattern was cyclic and stable.

2.5. Ovarian development

Bees were chilled on ice and fixed on a wax dissecting plate using entomological pins under a stereomicroscope. We opened the abdomen, immersed the internal organs in water, and dissected the ovaries using fine forceps. The length of the largest oocytes in all eight ovarioles was measured with an ocular ruler under the dissecting microscope (×10) to the nearest 0.1 mm. We used the mean length of the largest oocytes as an indicator of the ovarian developmental state for each bee. We compared the average ovarian development between the treatment groups.

2.6. Flight muscle weight

The bees' thorax was dissected and dried for three days at 60 °C (workers: n = 31, queens: n = 14). The dry thorax was weighed to the nearest 0.01 mg (Sartorius, Quintix, Germany) and placed in a new 5 ml plastic tube field with a strong base solution (KOH 2%). This solution dissolves any soft tissues, including muscles, but not the chitinized cuticle (Levin et al., 2016). After three days in the solution, the thorax was removed, rinsed with deionized water three times, and then redried for three days at 60 °C. The mass of the exoskeleton was again measured and deducted from the first measurement. Assuming that most of the thorax soft tissue mass is the flight muscle, we take the weight difference as representing each individual's flight muscle mass (Levin et al., 2016).

2.7. Lipid levels in the abdomen

The bee's abdomen was dissected and the ovaries and digestive system were removed (workers: n = 30, queens: n = 14). The dissected abdomen was dried for three days at 60 °C, and its mass was measured using an analytic scale (±0.001 mg). We used the gravimetric method (Meunier and Chapuisat, 2009) to extract the lipids from the abdomens. Abdomens were placed individually in 5 ml glass tubes with 4 ml petroleum ether (as lipid solvent) for three days. The solvent was replaced after two days. The abdomens were dried for three more days and weighed again. The difference between the first and second weight constitutes the total mass of lipids in the abdomen. The source of most of the lipids in the abdomen is assumed to be in the fat body.

2.8. Protein turnover

We supplemented the sugar-water fed to the bumble bees with $^{13}C_1$ labeled leucine (4 mg/1 ml sugar-water). The bees were not supplied with any alternative energy source during this time and consumed the supplemented leucine sugar syrup at the same rate as regular sugar syrup. Leucine is an essential amino acid and is used by bees for protein production in the body. Any modification of leucine involves removing carbon number 1 (labeled) so that any labeling observed in the tissues is related to incorporating leucine into proteins (Levin et al., 2017b; McCue, 2011). We fed the workers with the labeled syrup until the end of the experiment on Day 7 and froze them at −20 °C. In the queens' experiment, the isotopically labeled syrup was replaced with regular food one day before their collection to avoid labeled food in their crop. For each bee we dissected, the digestive system was removed and four tissues (brain, flight muscles, fat body, and ovaries) were collected for analysis (queens: n = 19, workers n = 31). We placed each tissue on a Petri dish (30 mm × 15 mm) and dried it for three days at 60 °C. One milligram from the dry sample was then placed into a tin capsule. Due to their small mass, we pooled worker brains in pairs and all eight of the undeveloped ovaries of the allatactomized workers' tissues together into a single sample. The DMF-treated ovaries of the queens were also pooled in pairs to reach a minimal weight of 1 mg. We measured the $\delta^{13}C$ of each sample using a Picarro (Santa Clara, CA) G2121-i cavity ring-down spectroscopy (CRDS) ^{13}C stable isotope analyzer with an A0201 combustion module (CM-CRDS), as described in Levin et al. (2016). All ^{13}C concentrations are expressed in δ^{13}CVPDB (Werner and Brand, 2001).

2.9. Survival of queens in winter diapause

Two-week-old mated queens were kept in plastic cages (10 × 6 × 4 cm) in groups of five individuals. Cages were stored in a climate-controlled room (28 °C ± 1 °C, RH = 60% ± 10%). We treated half of the queens with 100 μg of JH-III dissolved in 5 μl of DMF, and the other half with 5 μl of DMF as described above. Three queens from each group were killed on Day 7 to determine the effect of JH treatment on ovarian development. The remaining queens – 15 JH-treated (two had died earlier) and 17 DMF-treated queens – were placed in cardboard egg cartons. We placed each queen in an individual cup in the carton, covered with a transparent plastic lid, and placed the egg cartons in an incubator in complete darkness (4 °C ± 0.5°, RH = 60% ± 5%), mimicking winter diapause conditions. We examined the hibernating queens once a week for 5–10 min to monitor survival during the 12 weeks of the experiment.

2.10. Statistical analysis

Data analysis was performed using the Real Statistics Resource Pack software (Release 7.2). Data analysis of the worker data, which comprised three experimental groups, was carried out using ANOVA followed by Tukey's post-hoc test. For small sample sizes, or if the data distribution was not normal, we analyzed the data using a Kruskal-Wallis test followed by a Conover test. The queen data analysis was carried out using a two-tailed *t*-test and Mann-Whitney test. We report the effect size for each statistical test, Cohen's d for t-test and η^2 for ANOVA, Kruskal-Wallis test, and Mann-Whitney test. The survival data analysis was carried out using the Kaplan-Meier procedure with the log-rank test.

3. Results

3.1. Ovarian development

In the allatectomized workers (CA^-), the largest oocytes were smaller than those of the sham and the control groups (Fig. 1a, Med = 0.24 mm, 2.36 mm, 2.00 mm for the CA^-, sham, and control respectively; Kruskal-Wallis test $\chi^2 = 21.6$, $p < 0.001$, $\eta^2 = 0.61$). JH-treated queens had larger oocytes compared to those of the DMF-treated bees. This was true both when the queens were held in groups of five (Fig. 1b, Med = 0.38 mm, 2.9 mm for DMF and JH-treated queens, Mann-Whitney test, U = 49, N = 7 (DMF), 7 (JH), $p < 0.001$, $\eta^2 = 0.7$), and when they were kept individually (Fig. 1c, Med = 0.20 mm, 1.29 mm for DMF and JH-treated queens, respectively, Mann-Whitney test, U = 98.5, $p < 0.001$, $\eta^2 = 0.67$). The effect of JH treatment on the queens' ovarian development was less when the queen was kept individually than when kept in groups. Moreover, we found eggs in four out of five cages of the grouped JH-treated queens. None of the queens kept individually laid eggs.

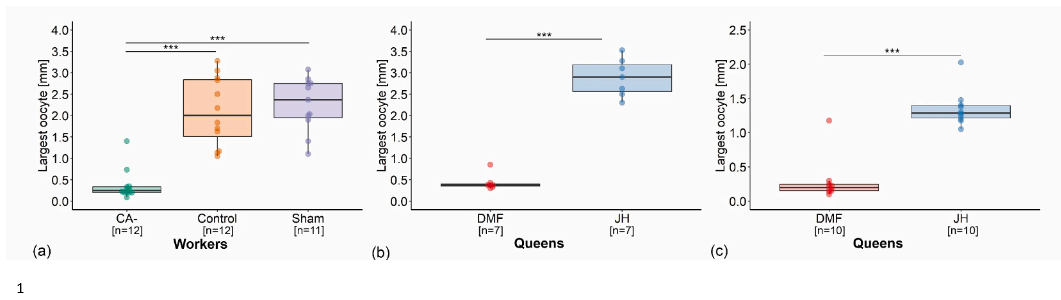

Fig. 1. Mean of the largest oocyte length [mm] in workers and queens under different experimental treatments. In workers (a), treatments comprised allatectomy (CA-), sham-operated, and control; queens were treated with DMF or with JH and were kept either in groups (b) or individually (c). Kruskal-Wallis tests and Mann-Whitney Test were conducted to test for differences among the treatments in workers and queens, respectively. Significance levels are noted with an asterisk, as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

3.2. Food consumption

Queens treated with JH consumed more pollen and sugar water than DMF-treated queens. JH-treated queens consumed 0.17 ± 0.05 g (n = 10) of pollen over seven days, significantly more than the DMF-treated queens, which consumed only 0.12 ± 0.04 g (n = 9) during the same period (Fig. 2a, Two-tailed *t*-test, t = 2.63, p = 0.017, d = 1.21). The sugar-water consumption for the same period was also higher in the JH-treated queens compared with the DMF treated queens (0.55 ± 0.22 g (n = 9) and 0.31 ± 0.06 g (n = 10), respectively; Fig. 2b, Two-tailed t-test, t = 3.28, p = 0.004, d = 1.51).

3.3. Metabolic rate

The metabolic rate of the CA-bees was 0.021 ml O_2 g^{-1} h^{-1} ± 0.001 (n = 11), being slower in comparison to the control and sham-operated bees, which exhibited a metabolic rate of 0.036 ml O_2 g^{-1} h^{-1} ± 0.004 (n = 12) and 0.036 ml O_2 g^{-1} h^{-1} ± 0.006 (n = 11), respectively (Fig. 3a, Kruskal-Wallis Test, $\chi^2 = 11.0$, p = 0.004, $\eta^2 = 0.29$, followed by Conover test, p < 0.05 for CA- *vs* control and sham). Similarly, the metabolic rate of the JH-treated queens was 0.014 ml O_2 g^{-1} h^{-1} ± 0.008 (n = 10), higher than that of the DMF-treated queens, which was 0.006 ml O_2 g^{-1} h^{-1} ± 0.003 (n = 9) (Fig. 3b, Mann-Whitney Test, U = 11, p = 0.006, η^2 = 0.406).

3.4. Lipid content in the abdomen

Bumble bee workers without JH (CA^-) had a higher lipid content in their abdomen than the sham and the control bees. The CA^- bees had 8.15 mg ± 2.3 (mean ± SE, n = 11) of lipids on average, significantly higher than the sham and control group, which had 1.17 mg ± 0.11 (n = 9) and 1.21 mg ± 0.16 (n = 10) of lipids, respectively (Fig. 4a, One-way ANOVA, $F_{2,27} = 7.0$, p = 0.003, $\eta^2 = 0.34$). The CA^- group had a significantly higher mass of lipids than the two control groups (Tukey post-hoc test, p = 0.01). The JH-treated queens had 43.4 mg ± 5.5 lipid mass in the abdomen (n = 7), and a lower lipid mass than the DMF-treated queens, 64.7 mg ± 4.5 (n = 7) (Fig. 4b, two-tailed *t*-test: t = 2.96; p = 0.011, d = 1.58).

3.5. Flight muscle mass

The flight muscle mass of the CA^- workers was 13.0 ± 0.42 mg (mean ± 1 SD; n = 12), similar to the sham and the control groups, which had 13.9 ± 0.47 mg and 12.9 ± 0.49 mg, respectively (Fig. 5a, One-way ANOVA, $F_{2,28} = 1.4$, p = 0.26, $\eta^2 = 0.09$). However, queens treated with JH had lighter flight muscles than the DMF-treated queens. JH-treated queen flight muscle mass was 52.5 ± 1.1 mg (n = 7), lower than the DMF-treated queen flight muscle, which was 61.9 ± 3.25 mg (n = 7) (Fig. 5b, two-tailed t-test: t = 2.72; p = 0.018, d = 1.45).

3.6. Protein turnover

$\delta^{13}C$ label level of the CA^- workers was lower in the flight muscles and the fat body compared to the control and the sham groups (Fig. 6b, c; fat body: F = 47.8, p < 0.001, $\eta^2 = 0.77$; muscles: $F_{2,29} = 4.7$, p = 0.016, $\eta^2 = 0.25$). Because worker brains are small, we pooled two brains per one sample. The brain $\delta^{13}C$ values were also low in the CA^- bees compared to the control but not compared to the sham group

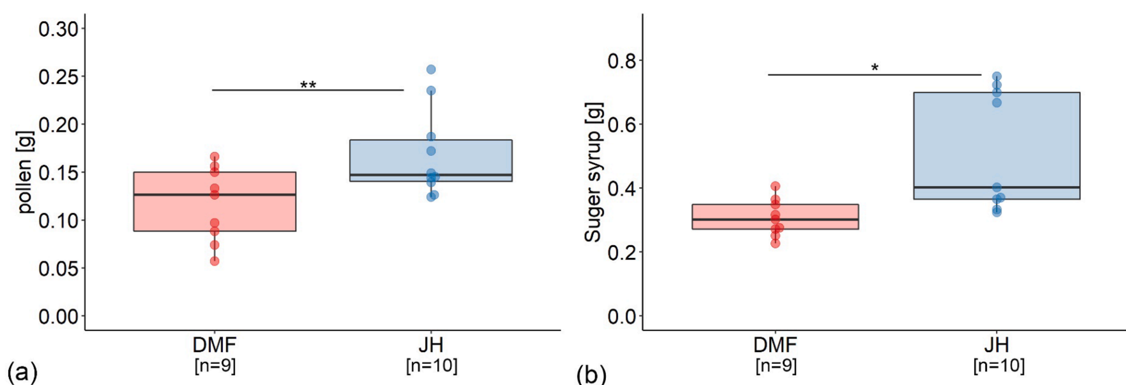

Fig. 2. Food consumption [g] in queens under the different experimental treatments. We measured pollen consumption (a) and sugar syrup consumption (b) in queens treated with DMF or JH. Two-tailed *t*-tests were conducted to test for differences among the treatments. Significance levels are noted with an asterisk, as follows: *: $p < 0.05$, **: $p < 0.01$. Mean is shown with the first and third quartiles and 95% confidence interval of median.

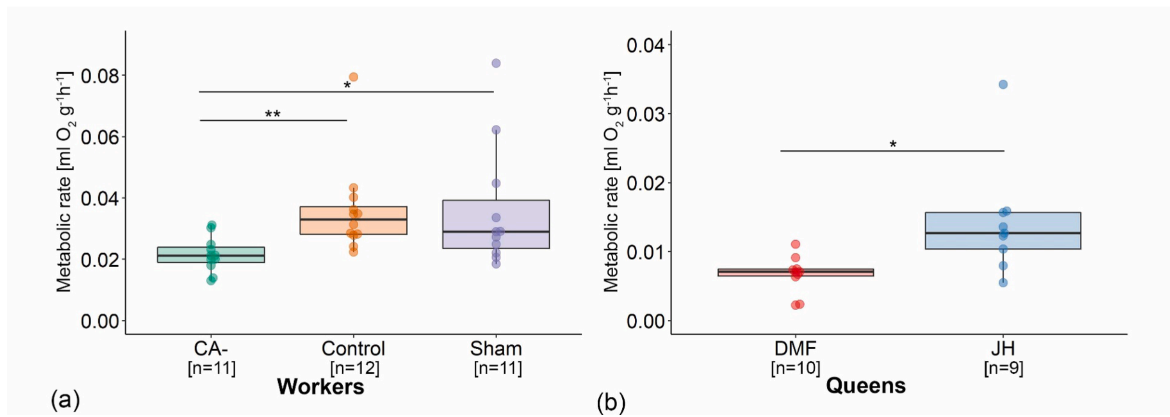

Fig. 3. Metabolic rate [ml O_2 g^{-1} h^{-1}] in workers and queens under different experimental treatments. In workers (a), treatments comprised allatectomy (CA-), sham-operated, and control; queens were treated with DMF or with JH. Kruskal-Wallis tests and Mann-Whitney Test were conducted to test for differences among the treatments in workers and queens, respectively. Significance levels are noted with an asterisk, as follows: *: $p < 0.05$, **: $p < 0.01$. Mean is shown with the first and third quartiles and 95% confidence interval of median.

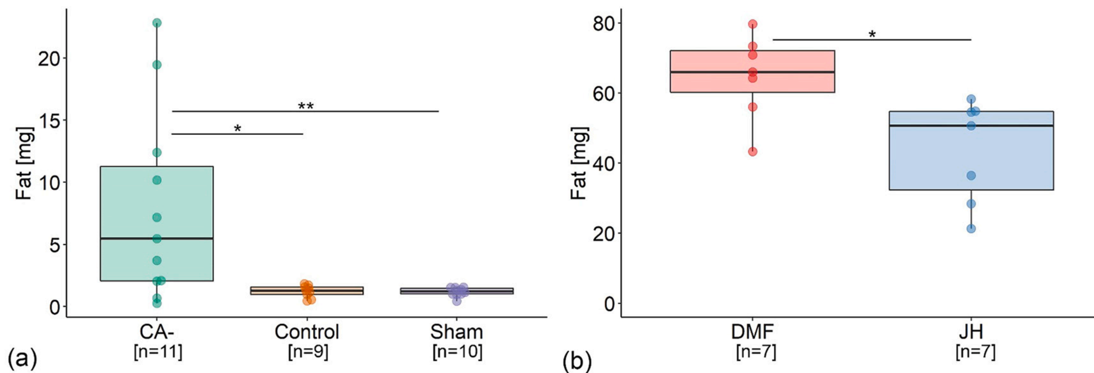

Fig. 4. Lipid content in the abdomen [mg] in workers and queens under the different experimental treatments. In workers (a), treatments comprised allatectomy (CA-), sham-operated, and control. In queens (b) treatments comprised either DMF or JH. A one-way ANOVA test and a two-tailed *t*-test were conducted to test for differences among the treatments in workers and queens, respectively. Significance levels are noted with an asterisk, as follows: *: $p < 0.05$, **: $p < 0.01$. Mean is shown with the first and third quartiles and 95% confidence interval of median.

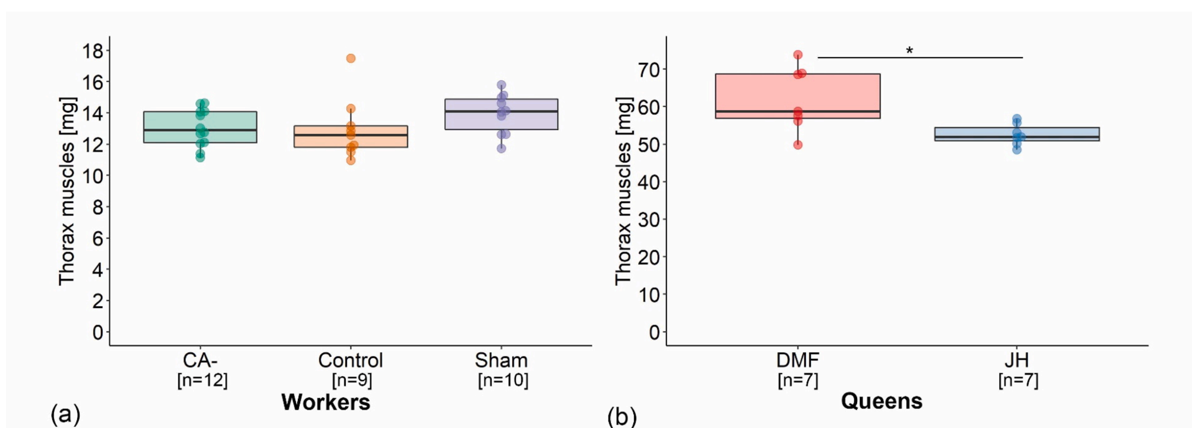

Fig. 5. Flight muscle weight [mg] in workers and queens under the different experimental treatments. In workers (a), treatments comprised allatectomy (CA-), sham-operated, and control. In queens (b), they were treated with either DMF or JH. One-way ANOVA tests and a two-tailed t-test were conducted to test for differences among the treatments in workers and queens, respectively. Significance levels are noted with an asterisk, as follows: *: $p < 0.05$. Mean is shown with the first and third quartiles and 95% confidence interval of median.

(Fig. 6a, Kruskal-Wallis Test, $\chi^2 = 6.26$ $p = 0.043$, $\eta^2 = 0.3$, followed by Conover test: $p = 0.03$ for the control and $p = 0.051$ for the sham operated). The ovaries of the CA^- bees were undeveloped and were pooled into a single sample, preventing our running valid statistics, but the δ13C of the CA^- was lower than in the other groups. For the brains, muscles, and fat bodies $\delta^{13}C$ values were higher in the JH-treated queens

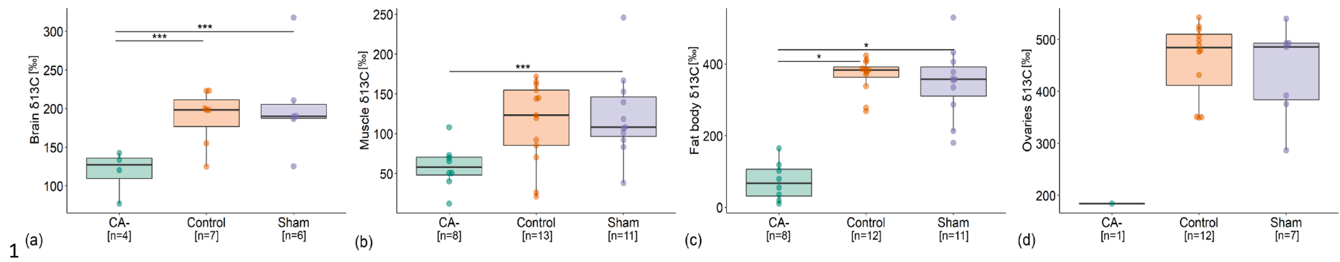

Fig. 6. Protein turnover [δ^{13}C %] in the different tissues (the brain (a), muscle (b), fat body (c), and ovaries (d)) in workers under the different experimental treatments. Treatments comprised allatectomy (CA^{-}), sham-operated, and control, Kruskal-Wallis Test tests were conducted to test for differences among the treatments in the brain, muscle, and fat body. Significance levels are noted with an asterisk, as follows: *: $p < 0.05$, ***: $p < 0.001$. Mean is shown with the first and third quartiles and 95% confidence interval of median.

compared to the DMF-treated queens (N = 9) in all four tissues (Fig. 7a–d two-tailed *t*-test brain: $t = 4.33$; $p < 0.001$, $d = 1.9$; fat body: $t = 2.49$ $p = 0.02$, $d = 1.1$; ovaries: $t = 3.85$ $p = 0.002$, $d = 2.1$; muscles: $t = 4.02$; $p = 0.001$, $d = 1.8$).

3.7. Queen survival during diapause

The JH-treated queens survived for a shorter period under conditions mimicking winter diapause compared to the DMF-treated queens. All the queens treated with JH (n = 15) died within the first ten weeks of diapause, while most of the queens (12 out of 17) treated with DMF survived the entire twelve weeks of the experiment (Fig. 8, Kaplan-Meier log-rank test, $\chi^2 = 17.3$, $p < 0.001$).

4. Discussion

We have demonstrated here that a topical application of JH to queen bumble bees increased their food consumption, standard metabolic rate, and protein turnover, accelerated the depletion of lipid storage and flight muscle mass, and increased ovarian development. Removing the corpora allata in workers (with the consequent reduction in JH levels) reduced their resting metabolic rate and protein turnover but increased their accumulation of lipids. These findings suggest that JH is an essential factor in shifting metabolism from energy accumulation to energy expenditure, high protein turnover, and allocation to reproduction. This metabolic shift led to a dramatic decrease in the queens' survival during winter diapause.

The allatectomized bumble bee workers, with the expected consequent low JH levels, accumulated lipids, possessed undeveloped ovaries, and revealed lower standard metabolic rate, and low protein turnover. We suggest that the physiology of allatectomized workers resembles that of young queens before the winter diapause, leading such queens to accumulate reserves (*e.g.*, lipids) and reduce their metabolic rate. In contrast, non-allatectomized workers in a queenless colony will transition into a reproductive state, and their JH levels will dramatically increase (Amsalem and Hefetz, 2011; Shpigler et al., 2014; Shpigler et al., 2016). Consequently, we suggest that the workers' metabolism

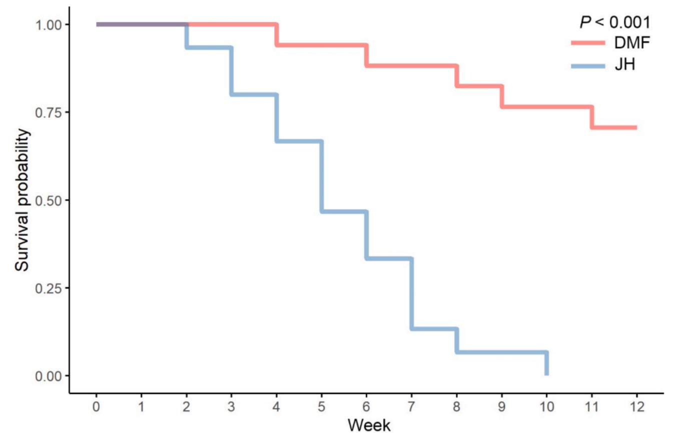

Fig. 8. Queen survival rate during diapause under JH and DMF treatments. The difference between queens treated with DMF (red line) or JH (blue line) was tested using Kaplan-Meier log-rank test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

resembles that of the queens in the reproductive phase. The survival of queens in diapause is strongly correlated with their body mass, age, and the lipids stored in their fat bodies (Treanore and Amsalem, 2020). It has been previously shown that relatively small queens with a body mass under 600 mg cannot survive the winter diapause (Beekman et al., 1998). Since workers are much smaller than queens (100–300 mg), their chance of surviving the winter diapause is thus substantially lower, even if they accumulate lipids. Worker fitness can therefore only be achieved by their helping the queen raise their sisters and their own laying of unfertile male-eggs in the colony. Such a powerful selective force can explain the lack of change in pre-diapause physiology in workers under natural conditions (in contrast to queens). Hunt (2007) suggested that JH-related changes in diapause physiology are part of the mechanism that underlies the evolution of queens and of worker phenotypes in

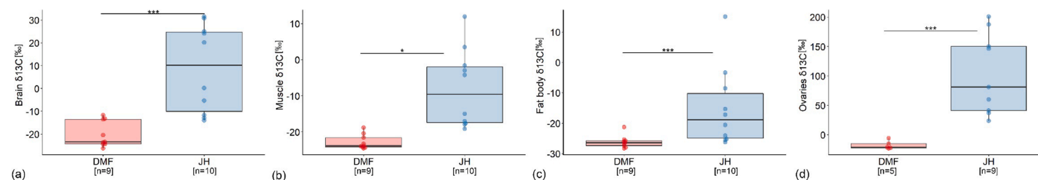

Fig. 7. Protein turnover [δ^{13}C %] in the different tissues (the brain (a), muscle (b), fat body (c), and ovaries (d)) in queens under the different experimental treatments Queens were treated with either DMF or JH, and two-tailed *t*-tests were conducted to test for differences among the treatments. Significance levels are noted with an asterisk, as follows: *: $p < 0.05$, ***: $p < 0.001$. Mean is shown with the first and third quartiles and 95% confidence interval of median.

paper wasps, known as the 'diapause ground plan hypothesis' (Hunt, 2007). Here we show that we were able to produce a queen-like diapause physiology in workers by manipulating JH levels, indicating that a similar process (as suggested by Hunt) might also be involved in the worker caste evolution in bumble bees.

In addition to its role as a gonadotropin, we have shown that JH also functions as a metabolic rate accelerator in both workers and queens, as the allatectomized workers displayed a lower metabolic rate, while the topical application of JH to young mated queens increased their resting metabolic rate. One might argue that this metabolic rate increase was due to ovary activation, but JH has also been directly connected to elevated metabolic rates in other insect species in non-reproductive life stages. For example, in the larvae of the dermestid beetle, *Dermestes maculatus*, synthetic JH analog treatment caused a dramatic increase in the beetle's metabolic rate, correlated with the dose of JH provided in the treatment. This high metabolism was partly associated with the uncoupling of oxidative phosphorylation, which produces heat (Sláma and Kryspin-Sørensen, 1979). In pupae of the flesh fly, *Sarcophaga crassipalpis*, cycles of high oxygen consumption were correlated with JH activity during diapause (Denlinger et al., 1984). JH was also shown to increase metabolic rate in the hemimetabola insects *Blattela germanica* and *Oncopeltus fasciatus* (Garcera et al., 1989). Isolated mitochondria of the Indian meal moth (*Plodia interpunctella*) and fruit fly (*Drosophila melanogaster*) showed increased activity in response to an *in-vivo* concentration of JH (Firstenberg and Silhacek, 1973; Stepien et al., 1988). In more recent studies in the honey bee (*Apis mellifera*), JH-analog treatment increased both the metabolic rate during flight and the expression of genes related to oxidative phosphorylation pathways (Sullivan et al., 2003; Whitfield et al., 2006). In honey bees, in contrast to our findings in bumble bees, the worker high JH levels are related to foraging behavior and not to reproduction. The increase in metabolic rate in honey bees can be explained by the fact that foraging activity involves a high energy turnover in this species. However, bumble bee foraging behavior is not associated with high JH levels (Cameron and Robinson, 1990; Shpigler et al., 2016). Based on these studies, we suggest that the role of JH as a metabolic rate accelerator is conserved among both young and adult insects and is not exclusively associated with reproduction or foraging activity.

The high JH levels in our studied bumble bees were also followed by a high turnover of protein in various tissues, most significantly the fat body and the ovaries. This high protein turnover observed under high JH can be partly explained by the synthesis of vitellogenin (Shpigler et al., 2014; Shpigler et al., 2020; Tufail et al., 2014). Moreover, the correlation between a high metabolic rate and high protein turnover might be related to oxidative damage. Increased aerobic metabolism can cause oxidative damage to proteins, resulting in their high turnover, since it is more "cost-effective" to replace damaged proteins than repair them (Levin et al., 2017a). This mechanism can explain the negative effect of JH on the survival of queens in diapause. Whatever the explanation, it is clear that low JH levels before and during diapause are critical for the insects' survival of prolonged diapause (Wyatt and Davey, 1996).

We found that a high JH level was correlated with a high resting metabolic rate in both worker and queen bumble bees. In a meta-analysis of over 70 studies, Mathot et al. (2019) found that a high resting metabolic rate was correlated with dominance and boldness in various taxa (Mathot et al., 2019). High levels of JH have also been suggested to be associated with aggression and social conflicts in insects (Bloch et al., 2000; Kou et al., 2009; Pandey et al., 2020). Following these correlations, we suggest that the accelerated metabolism caused by JH can be linked to aggressiveness. In honey bees, mature workers with high JH levels are also more aggressive (Pearce et al., 2001). The whole-brain, intact mitochondrial respiration was found to be significantly higher for older forager honey bees than for two-day-old adults (Rittschof et al., 2018). The association between JH and increased metabolic rate can therefore also explain the behavioral change to aggression. These findings suggest that high JH incurs not only a physiological cost but also a behavioral one in the form of increased aggressiveness. These findings support the "challenge hypothesis" in insects that perceives JH as a beneficial factor for reproduction and dominancy, while negatively affecting survival and health when its levels are high (Tibbetts and Huang, 2010; Tibbetts et al., 2020), similar to the effect of androgen hormones in mammals.

JH has many functions in an insect's life cycle, including controlling molting and pupation timing at the larval stage, diapause, and reproduction in adults in most insects, and the division of labor in advanced eusocial Hymenoptera (Amsalem et al., 2014). The shared feature of all these processes is that of a dramatic change in metabolic rate. While JH can initiate many different functions, we suggest that its functions as an accelerator of metabolism and energy expenditure is conserved across all insect life stages and taxa.

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