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# Honeybee queen exposure to a widely used fungicide disrupts reproduction and colony dynamic<sup>☆</sup>

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## ABSTRACT

Pollinators have to cope with a wide range of stressful, not necessarily lethal factors limiting their performance and the ecological services they provide. Among these stressors are pesticides, chemicals that are originally designed to target crop-harming organisms but that also disrupt various functions in pollinators, including flight, communication, orientation and memory. Although all these functions are crucial for reproductive individuals when searching for mates or nesting places, it remains poorly understood how pesticides affect reproduction in pollinators. In this study, we investigated how a widely used fungicide, boscalid, affects reproduction in honey bees (*Apis mellifera*), an eusocial insect in which a single individual, the queen, fulfills the reproductive functions of the whole colony. Boscalid is a succinate dehydrogenase inhibitor (SDHI) fungicide mainly used on rapeseed flowers to target mitochondrial respiration in fungi but it is also suspected to disrupt foraging-linked functions in bees. We found that immature queen exposure to sublethal, field relevant doses of boscalid disrupted reproduction, as indicated by a dramatic increase in queen mortality during and shortly after the nuptial flights period and a decreased number of spermatozoa stored in the spermatheca of surviving queens. However, we did not observe a decreased paternity frequency in exposed queens that successfully established a colony. Queen exposure to boscalid had detrimental consequences on the colonies they later established regarding brood production, *Varroa destructor* infection and pollen storage but not nectar storage and population size. These perturbations at the colony-level correspond to nutritional stress conditions, and may have resulted from queen reduced energy provisioning to the eggs. Accordingly, we found that exposed queens had decreased gene expression levels of vitellogenin, a protein involved in egg-yolk formation. Overall, our results indicate that boscalid decreases honey bee queen reproductive quality, thus supporting the need to include reproduction in the traits measured during pesticide risk assessment procedures.

## 1. Introduction

Insect pollinators have steeply declined worldwide in the last decades (Zattara and Aizen, 2021; Hallmann et al., 2017; Potts et al., 2010; Cameron et al., 2011). Given that most wild and crop flowering plant species depend on pollination services provided by insects, this ongoing decline poses serious ecological and economical threats (Gallai et al., 2009; Ollerton et al., 2011). Multiple stressors are suggested to disrupt pollination, including climate changes, invasive species, pathogens and large-scale intensive agriculture (Vanbergen and the Insect Pollinator Initiative, 2013; Belsky and Joshi, 2019). Intensive agriculture

management negatively impacts pollinators by decreasing the diversity of feeding and nesting resources available, but also more directly through exposure to pesticides (Godfray et al., 2015; Braak et al., 2018; Belsky and Joshi, 2020). These substances are used to protect crops against target organisms, including plants, fungi or insects, but they simultaneously harm a wide range of non-target organisms.

Pesticide toxicity is assessed before registration but current procedures can underestimate negative effects on non-target organisms (Barascou et al., 2021). First, tests are mostly conducted in laboratory conditions, thereby underestimating the toxicity of pesticides in natural, more unpredictable, conditions (Whitehorn et al., 2012; Henry et al.,

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2012). Second, assays conducted to evaluate pesticide toxicity last up to 10 days while some pollinators live several years, thus excluding investigations of mid- to long-term effects (Simon-Delso et al., 2018). Third, protocols are largely restricted to the monitoring of mortality while evidence of sublethal effects of pesticides on pollinators and detrimental impacts on their associated services have alarmingly accumulated in the last decade (Braak et al., 2018; Barascou et al., 2021; Desneux et al., 2007). Disrupted functions following pesticide exposure include immunity (Collison et al., 2016; Pamminger et al., 2018; James and Xu, 2012), development (Yang et al., 2020; Basley and Goulson, 2018), social communication (Tison et al., 2016; Walsh et al., 2020; Otesbelgue et al., 2018), foraging (Henry et al., 2012; Gill et al., 2012) and associated cognitive abilities such as memory, learning or orientation (Tison et al., 2016; Papach et al., 2017; Siviter et al., 2018; Artz and Pitts-Singer, 2015). Although all these functions are crucial for reproductive individuals when searching for mates or nesting places, few studies investigated the sublethal effects of pesticides on pollinator reproduction until recently (Walsh et al., 2020; Milone and Tarpy, 2021; Forfert et al., 2017).

The vast majority of current pesticide toxicity assessments on pollinators concern honeybee workers (More et al., 2021), thereby precluding the investigation of pesticides' detrimental effects on reproduction before marketing authorization. In honeybees and other monogynous insects, a single female, the queen, fulfills the reproductive functions of the whole colony. Given the key role of queens within colonies, suboptimal queen reproductive quality is frequently suggested as one of the main drivers of honeybee colony losses (Amiri et al., 2017; Pettis et al., 2016). Honeybee queens perform nuptial flights during the first days of their life to mate with multiple males. They store spermatozoa in a spermatheca and will not mate again during their life (Winston, 1987). Recent studies found that queens reared in colonies exposed to pesticides through food or wax had reduced mating number (Forfert et al., 2017) and reduced quantity and quality of spermatozoa stored in their spermatheca (Milone and Tarpy, 2021; Williams et al., 2015; Rangel and Tarpy, 2015). A decrease in queen mating number could result from disruption of nuptial flights, possibly because pesticides induce poor flight performance or orientation, as found in workers (Henry et al., 2012; but see Liao et al., 2019; Williams et al., 2015), and/or changes in pheromones involved in mate attraction (Walsh et al., 2020). Nuptial flight success is mandatory as it determines genetic diversity within colony, which is a crucial determinant of colony development, health and longevity (Mattila and Seeley, 2007; Delaplane et al., 2015; Tarpy and Seeley, 2006). Established queens also suffer from pesticide exposure as suggested by the rapid brood reduction observed in colonies fed with neonicotinoid-treated pollen (Sandrock et al., 2014; Wu-Smart and Spivak, 2016). Brood reduction following colony exposure to pesticides may result from decreased queen fecundity (Wu-Smart and Spivak, 2016), reduced egg hatchability (Fine, 2020) and/or increased larva mortality (Walsh et al., 2020; Traynor et al., 2021). While these brood disorders may be explained through a direct effect of pesticides on queen reproductive quality (i.e., polyandry (Tarpy et al., 2012), fecundity (Woyke, 1962) and/or fertility (Fine et al., 2018)) they may also result from the poisoning of the brood itself or from the reduced quantity and/or quality of nursing bees (Walsh et al., 2020; Wu-Smart and Spivak, 2016). Two recent studies found that queens reared in colonies exposed to pesticides through food or wax had reduced fecundity (Walsh et al., 2020) and later established colonies with increased brood mortality (Milone and Tarpy, 2021), thus indicating that pesticides directly impact the quality of queens and their offspring.

While investigations have focused on insecticides or pesticide cocktails, no study has explored the long-term consequences of queen exposure to fungicides. Fungicides are the most common pesticides found in wax comb and pollen stored in honeybee colonies, probably because they are applied at high concentrations during bloom when bee foraging is at its peak (Böhme et al., 2018; Mullin et al., 2010; Pettis

et al., 2013). Fungicides are generally considered safe to pollinators given the expected selectivity between fungi and animals and based on short-term mortality assays in laboratory conditions but recent evidence indicate the contrary (Belsky and Joshi, 2020; Cullen et al., 2019; Main et al., 2020). Among fungicides suspected to be harmful to pollinators are those targeting mitochondrial respiration in fungi by inhibiting succinate dehydrogenase, which is part of the adenosine triphosphate (ATP) energy production pathway. This pathway is widely shared among organisms and evidence suggest that succinate dehydrogenase inhibitor (SDHI) fungicides also disrupt energy production in invertebrates (Lebrun et al., 2021), including honeybees (Degrandi-Hoffman et al., 2015), possibly via disruption of detoxification mechanisms (Mao et al., 2017). These detoxification issues may explain why honeybees exposed to SDHIs are more susceptible to other pesticides (Tsvetkov et al., 2017) and pathogens (DeGrandi-Hoffman et al., 2013). SDHIs also provoke protein absorption disorders (Degrandi-Hoffman et al., 2015), as further suggested by increased pollen consumption and collection in field studies (Fisher et al., 2021a; Fisher et al., 2021b). Overall, SDHIs are not acutely toxic to honeybee workers but observations lasting longer than the standard 10-days test (Simon-Delso et al., 2018) or under field conditions (Fisher et al., 2021a; Fisher et al., 2021b) revealed increased mortality following exposure to field-relevant concentrations of SDHIs. Whether SDHIs adversely impact honeybee queen reproduction has not yet been investigated but it might be expected because queen extended longevity is likely to favor cumulative toxicity of SDHIs and given recent findings indicating that SDHIs reduce flight performance and learning in worker honeybees (Liao et al., 2019; DesJardins et al., 2021) and disrupt nest recognition in solitary bees (Artz and Pitts-Singer, 2015).

Here, we investigated whether exposure of emerging honeybee queens to boscalid, a widely used SDHI fungicide, impacts queen reproductive health and the growth of their future colony. Boscalid is the active ingredient of dozens of commercial solutions used to control pathogenic fungal development during blooming in rapeseed cultures and orchards (ANSES, 2022). Given its extensive, wide-spectrum, use on flowers, boscalid is one of the most ubiquitous pesticides found in cultures and beehives (Böhme et al., 2018; Simon-Delso et al., 2014; David et al., 2016; Simon-Delso et al., 2017). Previous studies on bees found that boscalid, either alone or in association with another chemical (e.g. the fungicide pyraclostrobin in the commercial solution Pristine®) decreases energy production (Degrandi-Hoffman et al., 2015), flight performance (Liao et al., 2019), learning (DesJardins et al., 2021) and orientation (Artz and Pitts-Singer, 2015). Pristine may also decrease queen emergence but contradictory results were found (DeGrandi-Hoffman et al., 2013; Johnson and Percel, 2013). Here, we exposed emerging queens to a sub-lethal dose of boscalid, either as a pure form or in the commercial solution Pictor Pro® (BASF) which contains 50% boscalid and is mostly used on blooming rapeseed. We investigated whether exposed queens successfully reproduced and if so, we followed the development of their colonies over four months and then conducted physiological and genetic analyses to explore underlying mechanisms of queen performance.

## 2. Materials and methods

### 2.1. Queen rearing and pesticide exposure

We raised honey bee queens in spring 2021 using two queenright colonies headed by naturally mated sister queens and located at the INRAe Le Magneraud research center (46°8'59.104"N, 0°41'28.609"W), France. We introduced small NicotPlast® plastic cups (1 cm<sup>3</sup>) in both queenright colonies for queens to lay eggs. Three days later, we transferred plastic cups containing one-day-old larvae in a section of the hive that excluded queen access (i.e., to simulate supersedure conditions), thereby allowing workers to raise the young larvae as future queens. A week after, we transferred capped queen cells to an incubator (34.5 °C,

60% humidity, in darkness). One day prior to emergence, each capped queen cell was placed in an individual plastic box ( $16 \times 10 \times 10$  cm) with ten one-day-old workers from the same mother colony. Bees were fed 50% sucrose/water solutions with or without the addition of a treatment (see below for more details) *ad libitum*. At emergence, queens were weighted using a digital scale to the nearest 0.01 mg and paint-marked (Posca®). Each queen was kept with workers exposed to treatment for 48 h after her emergence. The queens were then introduced individually, as caged adults, in Langstroth® hives each containing ~5000 bees, a frame of honey and pollen, a frame of capped brood and three frames with a thin strip of wax foundation. We opened hives three days after introduction to confirm that queens were accepted by workers. We repeated the whole rearing process twice, at an interval of two weeks in late April ( $n = 31$  queens) and early May ( $n = 20$  queens).

## 2.2. Pesticide treatments

Sucrose/water solutions used to feed workers, and therefore queens, for 48 h after emergence were either kept (a) untreated ( $n = 11$  queens) or mixed separately with (b) boscalid (Cluzeau Info Lab, France) at a concentration of 0.25 ng/ $\mu$ l, diluted in 0.1% acetone ( $n = 14$  queens), or (c) 0.1% acetone ( $n = 12$  queens), or (d) Pictor Pro®, a commercial solution containing 50% boscalid (BASF, France), at a concentration of 0.5 ng/ $\mu$ l ( $n = 14$  queens). Acetone was necessary to obtain a homogeneous boscalid solution. Fungicide exposure was calculated based on a pesticide residue survey conducted in southern Germany (Böhme et al., 2018). This survey found boscalid at concentrations up to 1500 ng per gram of pollen (1500 ppb). We based our calculations on worker feeding rate: 1) given that young worker bees consume approximately 0.005 g of pollen per day (Pernal and Currie, 2000; Alaux et al., 2010), we estimated that a young bee consumes  $0.005 \times 2 \times 1500 = 15$  ng of boscalid in two days (0.0015% of the oral LD50; European Commission, 2008), and 2) given that a bee consumes 30  $\mu$ l of sucrose solution per day (Renzi et al., 2016), we prepared sucrose solutions at a concentration of 15 ng/(30  $\mu$ l  $\times$  2) = 0.25 ng/ $\mu$ l. Concentration of the Pictor Pro® sucrose solution was two-times higher (i.e., 0.5 ng/ $\mu$ l) because Pictor Pro® contains 50% boscalid. We confirmed these concentrations using gas chromatography with mass spectrometry detection (GC-MS/MS; Anses, Avignon). There was no significant difference between control queens and queens exposed to acetone in any of the parameters investigated in this study (see Supplementary material), thus we pooled these two groups for statistical analyses (i.e. named control group hereafter).

## 2.3. Queen survival and colony growth measurements

To assess the impact of queen exposure to treatments on colony performance, we took monthly measurements from May to September to record the surface occupied by open brood (i.e. eggs and larvae), capped worker brood, pollen, honey and adult workers on both sides of all frames (see Table S1 for a detailed chronology of colony checks). A single observer used a gridded frame consisting of fifty,  $5 \times 5$  cm squares to help in the visual estimation of these parameters. In addition, we checked for queen presence and royal cells. In early August, during sunflower flowering, a single observer estimated foraging frequency of each colony ( $n = 17$  control, 8 boscalid and 5 Pictor Pro®) by counting the number of workers returning to hive per minute and discriminating whether they carried pollen or not (Mattila and Seeley, 2007). In late August, we estimated varroa (*Varroa destructor*) intensity within each colony ( $n = 17$  control, 8 boscalid and 5 Pictor Pro®) by using the icing sugar technique, i.e. placing a given mass of bees in a box filled with icing sugar and shaking it to detach mites from their host (Dietmann et al., 2013). We fed all hives once with 1 L of Butiforce® syrup (Cargill, Baupre, France) in mid-June when natural resources were scarce.

## 2.4. Statistical analyses

We tested whether queen exposure to pesticide impaired their survival over four months using a Cox proportional hazard model built in the *survival* package (Therneau, 2021) in R 4.0.1 (R Core Team, 2021). We checked for proportionality of hazard ratios using a score test in the “cox.zph” function of the *survival* package. We also checked visually for outliers using the “ggcoxdiagnostics” function in the *survminer* package (Kassambara et al., 2021). We assessed significance of the treatment variable by the change in deviance after removal of that variable (Likelihood-Ratio Test [LRT]) using a chi-square test. Significant differences between treatments were tested using the *pairwise\_survdiff* function in the R package *survival* (Therneau, 2021), with *fdr* p-value corrections for multiple comparisons.

The impact of queen exposure to pesticide treatment on colony growth was tested using linear mixed models (LMMs) built in the *lme4* R package (Bates et al., 2015). Colony growth parameters were frame surface occupied with either worker bees, nectar, pollen, open brood or capped brood. We also tested the effects of treatments on the proportion of open brood surface over total brood surface using a general linear mixed model (GLMM) with a binomial distribution built in the *lme4* R package. This ratio should remain constant over time once the colony is established and otherwise could be an indicator of brood production issues. Predictor variables included treatment, time (in months) and their interaction. We included the square of time to test whether the development of colony parameters over time better fitted a quadratic relation. Queen ID was also included in models as a random effect to consider repeated measures over time. Normal distribution of residuals and homoscedasticity were checked in LMMs, as well as normal distribution of random effects in all models. Model selection followed a backward-stepwise approach: we assessed significance of predictor variables, starting with the interaction, with an LRT using a chi-square test. The interaction was eliminated from the model if  $P > 0.05$  and when significant, post hoc comparisons with *fdr* P-value corrections were performed using the *emmeans* R package (Lenth, 2022) to identify when control colonies differed from colonies headed by treated queens.

We also investigated whether treatment was associated with varroa intensity using a general linear model (GLM) with a Poisson distribution built in the *stats* R package. However, the model was overdispersed and we thus used a negative binomial distribution, which gave a better fit according to AICs. Predictor variables included treatment, the surface occupied with brood at the time of varroa sampling and the mass of bees sampled for varroa counting. In addition, to test whether treatment was associated with foraging activity, i.e. the proportion of workers returning to hive with pollen over total number of workers returning to hive in 1 min, we performed a GLM with a binomial distribution built in the *stats* R package. Predictor variables included treatment and frame surface occupied with adult workers when observations were conducted. The significance of predictor variables was assessed in both models using the LRT method described above and post hoc comparisons were done using *emmeans*.

## 2.5. Paternity frequency

To estimate paternity frequency, capped brood from successfully established colonies ( $n = 17$  control,  $n = 8$  boscalid and  $n = 6$  Pictor Pro®) was collected at mid-June and immediately frozen in dry ice before storage at  $-20^\circ$ . Total DNA was extracted individually from 30 nymphs per colony by placing a leg in 150  $\mu$ l of 10% Chelex and 6  $\mu$ l 10 mg/ml proteinase K (Walsh et al., 1991). Each sample was placed in a thermocycler for 1 h at  $55^\circ\text{C}$ , 15 min at  $99^\circ\text{C}$ , 1 min at  $37^\circ\text{C}$ , and 15 min at  $99^\circ\text{C}$  and stored at  $-20^\circ\text{C}$  until microsatellite genotyping. Each nymph was analyzed at seven microsatellites separated in two multiplexes (Multiplex 1: Am125, Am059, Am043 and Am046; Multiplex 2: Am098, Am052, Am061; Estoup et al., 1995; Solignac et al., 2003). Each 10  $\mu$ l PCR reactions contained 5  $\mu$ l multiplex PCR Qiagen Master Mix, 2

× 1 µL tagged primers, and 3 µL DNASE/RNASE free H<sub>2</sub>O. All reactions were amplified at 95 °C for one 15 min, 30 cycles of 95 °C for 30 s, 58 °C for 90 s, 72 °C for 60 s, and a final extension at 60 °C for 30 min. PCR products were run on an ABI 3730XL automated capillary DNA sequencer by Genoscreen (Lille, France). Microsatellite fragment size was scored using GeneMapper 3.7 software (Applied Biosystems) and microsatellite data were then analyzed in the program COLONY (Jones and Wang, 2010) to compute the total number of different marker sets within a colony (i.e. mating number). We then used the proportion of each subfamily within a colony to calculate queen effective paternity frequency, an unbiased measure corresponding to queen mating number if all males are represented equally within her offspring (Nielsen et al., 2003).

## 2.6. Statistical analyses

To test for the association between pesticide treatments and queen mating number we performed a GLM with a Poisson distribution using the *stats* R package. We checked for overdispersion and outliers. Regarding effective paternity frequency, we used a linear model (LM) and we checked for normality of residuals, homoscedasticity and outliers. We assessed the significance of the treatment variable in both models using an LRT.

## 2.7. Queen physiology and gene expression

In September, five months after introduction, queens were collected on dry ice and stored at −80 °C. The head was separated from the body and kept at −80 °C in a separate vial until gene expression analyses (see below). Body dissections were performed in RNAlater (Qiagen, France) using a binocular microscope. Ovaries were dissected and separated, while fat bodies were removed from the cuticle. All tissues were stored at −80 °C in separate vials until gene expression analyses. The spermathecae was removed and stored in 100 µl modified Kiev buffer (D + glucose, 0.3 g; potassium chloride, 0.41 g; sodium bicarbonate, 0.21 g; sodium citrate-2 hydrate, 2.43 g; in 100 ml of deionized, sterilized water; see Collins, 2004) at −20° until sperm counting.

### 2.7.1. Sperm count

Spermathecae were ruptured and contents were diluted in 4 ml of modified Kiev buffer (Collins, 2004). Spermatozoa were immediately counted on a hemocytometer (KOVA®) using a x400 microscope (Zeiss®). The average sperm count from five fields of view was corrected for dilution to calculate the total number of stored sperm.

### 2.7.2. Gene expression

Total RNA was isolated from left ovaries using a RNAeasy plus extraction kit (Qiagen, France) and from fat bodies and heads using a RNA Universal kit (Qiagen, France). Tissues were prepared using bead beating lysis (FastPrep-24®, MP Biomedicals). Ovary wet weight was determined after immersion in protective RLT buffer using a digital scale to the nearest 0.01 mg, subtracting the exact mass of the tube. cDNA was synthesized from 250 ng RNA for ovaries and heads and 125 ng RNA for fat bodies using reverse transcriptase (SuperScript® IV cDNA synthesis kit; Invitrogen, France). Expression levels of eleven target genes involved in storage, antioxidant, immunity, mitochondrial respiration and orientation functions (Table S2) were measured using quantitative real-time RT-PCR (qRT-PCR) with a LightCycler® 480 SYBR Green I Master (Roche, France). Samples were amplified at 95 °C for one 10 min, 35 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s, followed by a melt curve from 65 to 97 °C in 0.5 °C/5 s increments to confirm each amplicon. Ribosomal protein S5 and β-Actin were used as housekeeping genes, i.e. genes which expression levels remain unchanged in bees irrespective of conditions (Jeon et al., 2020). The relative gene expression was calculated using the 2<sup>−ΔΔCt</sup> method (Livak and Schmittgen, 2001). For each sample, triplicate qRT-PCR reactions were performed

and averaged.

### 2.7.3. Statistical analyses

Only three queens exposed to Pictor Pro® were alive in September. Thus, we excluded the Pictor Pro® treatment from all statistical analyses on queens' physiology. We tested the effects of boscalid on sperm number stored in spermatheca and left ovary weight using t-tests. Ovary weight was corrected by queen weight at emergence. We also used t-tests to investigate whether boscalid changed gene expression in ovaries, fat bodies and head. We checked for normality of residuals, homoscedasticity and outliers. Fold changes in *CYP9Q3* and *Apidaecin* expression in fat bodies and in *CytC* expression in ovaries were log transformed to reach normality of residuals.

## 3. Results

### 3.1. Queen survival

Among the 51 queens introduced in hives, 25 died in the following four-months. Queen survival was significantly associated with pesticide treatment ( $X^2_1 = 8.35$ ,  $P = 0.015$ ), with control queens being significantly more likely to survive than queens exposed to Pictor Pro® (48.1% decreased survival; estimate ± SE: 1.39 ± 0.49;  $P = 0.004$ ). Control queens had also a 19.6% increased survival rate compared to queens exposed to boscalid, although the difference was not significant (estimate ± SE: 0.63 ± 0.59;  $P = 0.15$ ). Most of the mortality events (80%) occurred during the first month after queen introduction in colonies (Fig. 1). Seven queens disappeared before the first check (i.e. before reaching 17–20 days old) and 13 queens disappeared before the second check (i.e. before reaching 31–34 days old). There was no evidence of laying in these 20 colonies, meaning that these queens might have failed nuptial flights.

### 3.2. Colony growth

Pollen surface was significantly associated with the interaction between treatment and time ( $X^2_1 = 6.11$ ,  $P = 0.047$ ; Fig. 2a). In July, after sunflower started flowering, control colonies stored significantly less pollen (mean ± SD: 83.2 ± 42.9 cm<sup>2</sup>) than colonies headed by queens

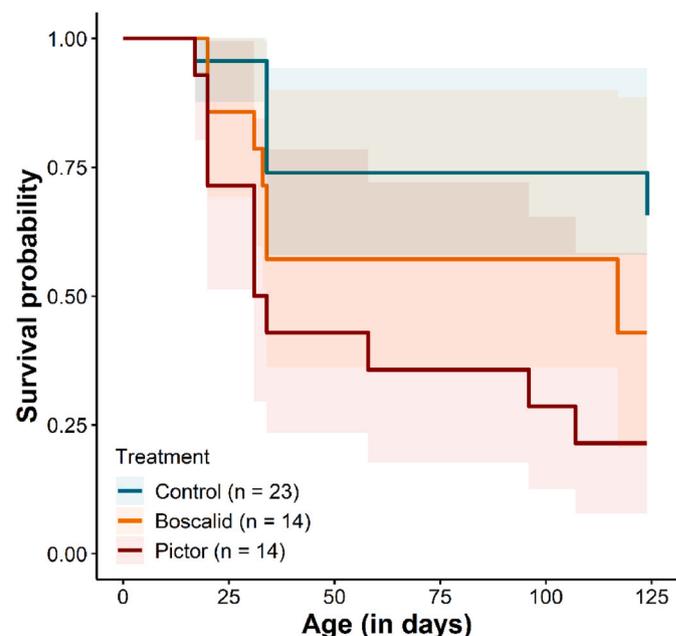
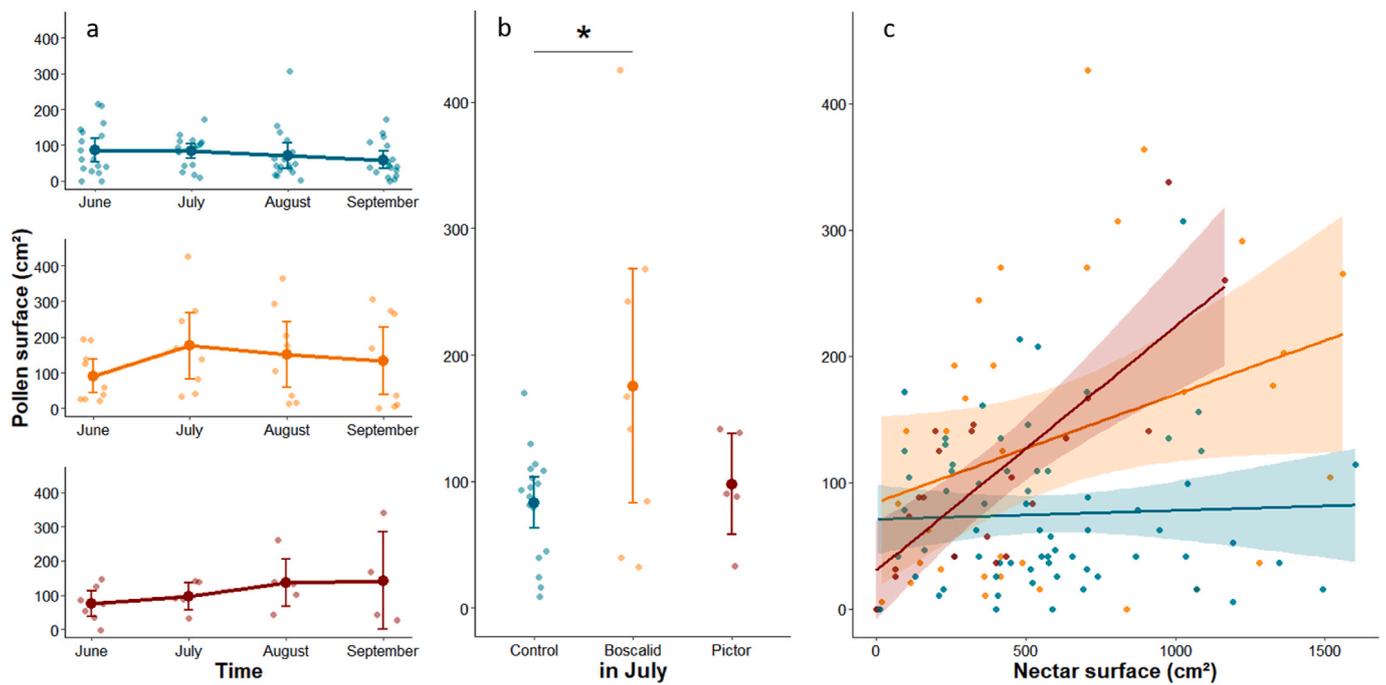


Fig. 1. Queen survival over four months according to treatment. Colored areas represent 95% confidence intervals.

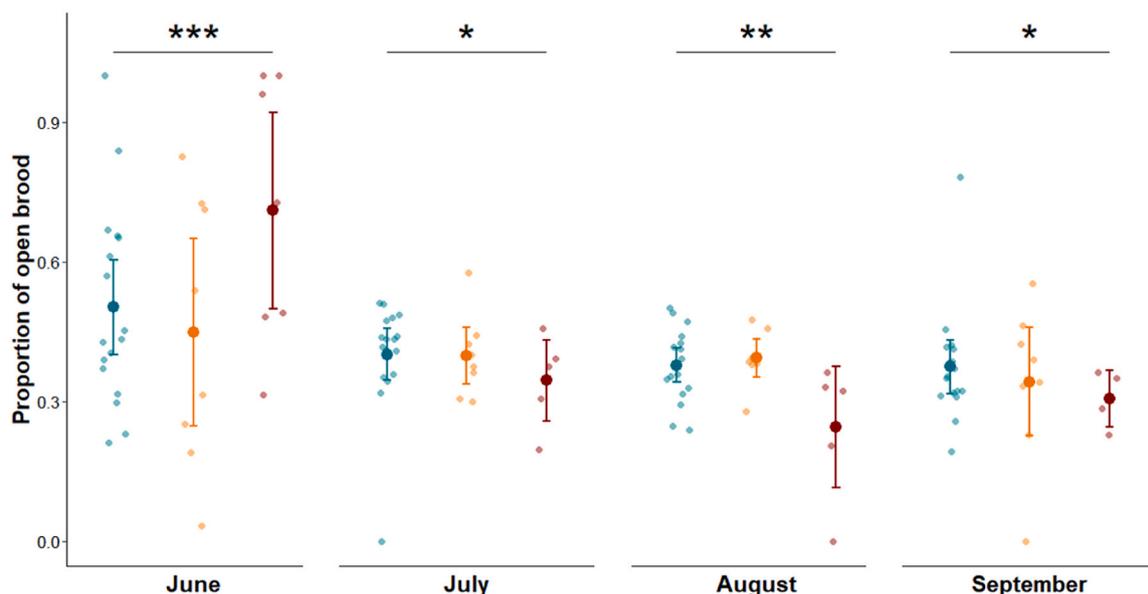


**Fig. 2.** Frame surface occupied with pollen (cm<sup>2</sup>) (a) over time and according to treatment (interaction,  $P = 0.047$ ), (b) according to treatment in July (\* when  $P = 0.038$ ) and (c) according to treatment and frame surface occupied with nectar (cm<sup>2</sup>) (interaction,  $P = 0.008$ ). Colors correspond to treatment: control in blue, boscalid in orange and Pictor Pro® in red. In (a) and (b), the bigger dots represent mean  $\pm$  SE. In (c), shaded areas denote 95% confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

exposed to boscalid ( $175.5 \pm 133.8$  cm<sup>2</sup>;  $t = -2.54$ ,  $P = 0.038$ ) but not to Pictor Pro® ( $97.8 \pm 45.4$  cm<sup>2</sup>;  $t = -0.25$ ,  $P = 0.81$ ; Fig. 2b). The amount of pollen stored in colonies headed by queens exposed to boscalid remained higher than in control colonies afterwards, although the difference was not significant after correction for multiple comparisons (August:  $t = -2.18$ ,  $P = 0.095$ ; September:  $t = -2.04$ ,  $P = 0.11$ ). To investigate the consequences of increased pollen storage on nectar

surface in the model. Pollen surface was significantly associated with this interaction ( $X^2_1 = 9.62$ ,  $P = 0.008$ ; Fig. 2c). In colonies headed by queens exposed to boscalid, pollen surface tended to increase with nectar surface whereas these two parameters were independent in control colonies ( $t = -2.35$ ,  $P = 0.06$ ).

Furthermore, the proportion of open brood surface relative to total brood surface was significantly different between treatments over time (interaction:  $X^2_1 = 71.90$ ,  $P < 0.001$ ). More precisely, in June, one month



**Fig. 3.** Proportion of frame surface occupied with open brood relative to total brood surface over time and according to treatment (interaction,  $P < 0.001$ ). Colors correspond to treatment: control in blue, boscalid in orange and Pictor Pro® in red. The bigger dots represent mean  $\pm$  SE. \*, \*\* and \*\*\* represent significant differences between colonies headed by queens exposed to Pictor Pro® and the two other treatments (control or boscalid) when  $P < 0.05$ , 0.01 and 0.001, respectively. Control colonies and colonies headed by queens exposed to boscalid were never significantly different ( $P > 0.35$ ). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

after queen introduction, the proportion of open brood was lower in control colonies ( $50.2 \pm 21.5\%$ ) than in colonies headed by queens exposed to Pictor Pro® ( $71.0 \pm 28.5\%$ ;  $z = -5.80$ ,  $P < 0.001$ ) but not to boscalid ( $44.8 \pm 29.1\%$ ;  $z = 0.94$ ,  $P = 0.34$ ; Fig. 3). This trend reversed afterwards, with the proportion of open brood remaining constant through the season in control colonies whereas it dropped in colonies headed by queens exposed to Pictor Pro® (July:  $z = 2.88$ ,  $P = 0.012$ ; August:  $z = 2.79$ ,  $P = 0.008$ ; September:  $z = 2.24$ ,  $P = 0.037$ ; Fig. 3).

Evaluation of varroa intensity at the end of the season revealed significant differences between treatments ( $X^2_1 = 7.91$ ,  $P = 0.019$ ). Colonies headed by queens exposed to boscalid had more varroa mites (mean  $\pm$  SD:  $8.00 \pm 5.71$ ) than control colonies ( $4.65 \pm 3.74$ ;  $z = 2.16$ ,  $P = 0.046$ ) and colonies headed by queens exposed to Pictor Pro® ( $2.40 \pm 1.14$ ;  $z = 2.62$ ,  $P = 0.026$ ; Fig. 4). Queen treatment was not significantly associated with population size ( $X^2_1 = 2.57$ ,  $P = 0.28$ ; Fig. S1), surface occupied with nectar ( $X^2_1 = 2.35$ ,  $P = 0.31$ ; Fig. S2), open brood ( $X^2_1 = 4.35$ ,  $P = 0.11$ ; Fig. S3) or capped brood ( $X^2_1 = 3.40$ ,  $P = 0.18$ ; Fig. S3), and foraging activity ( $X^2_1 = 2.99$ ,  $P = 0.29$ ; Fig. S4).

### 3.3. Paternity frequency

Among queens that successfully produced brood ( $n = 31$ ), we observed a 23.4% decrease in effective paternity frequency in queens exposed to boscalid ( $6.2 \pm 2.7$  males;  $n = 8$  queens) compared to control queens ( $8.1 \pm 3.7$  males;  $n = 17$ ; Fig. 5a), although the difference was not significant ( $X^2_1 = 0.98$ ,  $P = 0.39$ ). Similar result was found regarding mating number ( $X^2_1 = 0.51$ ,  $P = 0.76$ ).

### 3.4. Queen physiology

The number of spermatozoa stored in the spermatheca was tightly correlated with mating number (Pearson's correlation  $r = 0.65$ ,  $P < 0.001$ ; Fig. 5b). Queens exposed to boscalid had significantly fewer spermatozoa stored in their spermatheca ( $3.65 \pm 1.17$  million;  $n = 7$ ) than control queens ( $5.14 \pm 1.57$  million;  $n = 15$ ;  $t = -2.48$ ,  $P = 0.025$ ;

Fig. 5c). The three queens exposed to Pictor Pro® also had a reduced number of spermatozoa stored in their spermatheca ( $4.09 \pm 0.46$  million). There was no significant difference in ovary weight between treatments ( $t = -0.95$ ,  $P = 0.37$ ).

Queens exposed to boscalid exhibited significantly lower expression levels of *vitellogenin* in ovaries ( $t = -2.80$ ,  $P = 0.013$ ) and higher expression levels of *hexamerin 70b* in fat bodies ( $t = 2.58$ ,  $P = 0.032$ ) compared to control queens (Fig. 6). Regarding the other nine genes, no significant difference was observed between queens exposed to boscalid and control queens ( $P > 0.15$ ; Figs. 6 and 7, Table S3).

## 4. Discussion

Many studies investigated how pesticides impact pollinators but only a few concerned reproductive individuals. This lack is especially true for a pesticide family which safety for pollinators has been recently questioned, namely SDHI fungicides (Bénit et al., 2019). In this study, we found that the SDHI fungicide boscalid decreased honeybee queen quality. In particular, exposure to the commercial solution Pictor Pro® (50% boscalid) dramatically increased queen mortality, notably during or shortly after the nuptial period. Indeed, among queens exposed to Pictor Pro®, over half (57%) disappeared during the first month while 26% of unexposed queens were not found in the colony after this period (the risk of not returning to the hive after a flight was estimated to be 15–30%; Schluns et al., 2005). There was no evidence of laying in the colonies headed by queens that disappeared during the first month. Mean laying age of introduced queens is up to 19 days old in bad weather conditions (Pérez-Sato and Ratniek, 2006), which is what we experienced in May 2021. This suggests that those queens might have failed their nuptial flights. However, we cannot discriminate whether they died when performing nuptial flights or if they were killed by workers for failing to do so. Boscalid may reduce queen ability to perform nuptial flights by decreasing flight performance or by disrupting flight-associated cognitive abilities such as memory, learning or orientation, as previously found in workers and solitary bees (Artz and Pitts-Singer, 2015; Liao et al., 2019; DesJardins et al., 2021). We measured mRNA levels for two memory-related genes (PKAr1 and PKAc1) that were previously shown to be down-regulated in honey bees exposed to neonicotinoids (Christen et al., 2016) and miticides (Chai-manee and Pettis, 2019) but we found no such change in the brain of honeybee queens exposed to boscalid. Because we performed this analysis on established queens (i.e. on queens that stopped flying three months ago), the cessation of flight might have led to a downregulation of genes encoding for activities related to nuptial flights (e.g. memory, orientation, flight; Lockett et al., 2016).

Although some honeybee queens exposed to boscalid successfully established a colony, they had a decreased reproductive quality. The more polyandrous a queen is, the more genetically diverse its colony will be and the better it will develop (Mattila and Seeley, 2007). Queens exposed to boscalid reproduced with 23% less males than unexposed ones, although the difference was not significant. We might underestimate the impact of boscalid on polyandry because we performed physiological analyses on surviving queens only and dead queens might have reduced mating rate. Boscalid also led to a 29% decrease in the number of spermatozoa found in the spermatheca of four-months old queens. Among queens exposed to boscalid, 38% were considered as poorly mated while only 6% of unexposed queens were so (i.e. less than 3 million spermatozoa stored in the spermatheca, as defined by 46). Previous studies found a similar decrease following queen exposure to other pesticides during development (Milone and Tarpy, 2021; Williams et al., 2015; Rangel and Tarpy, 2015). A decrease in sperm quantity should not directly result from reduced mating number because the ejaculate of a single male contains ~10 million spermatozoa (Kairo et al., 2016; Woyke, 1960). Although an important amount of sperm is ejected before reaching the spermatheca, a queen mating with a few number of males should have enough sperm to lay eggs throughout their life (Koeniger

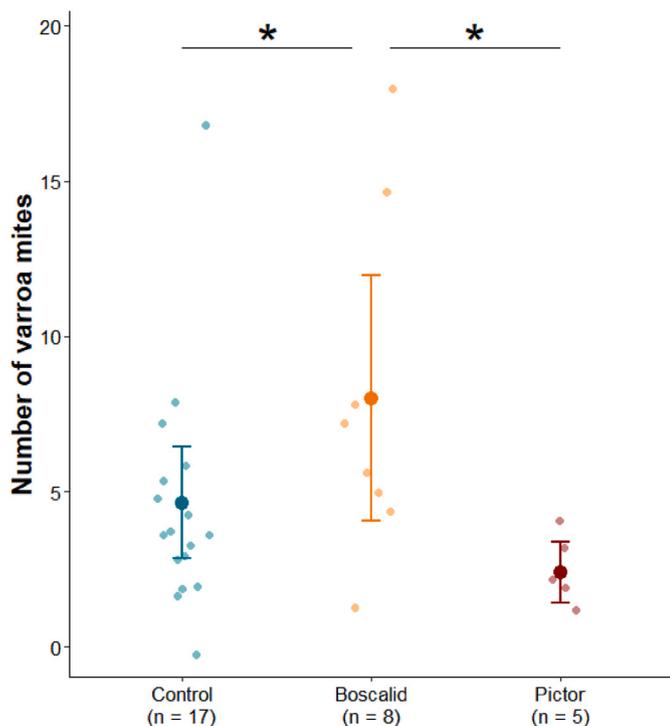
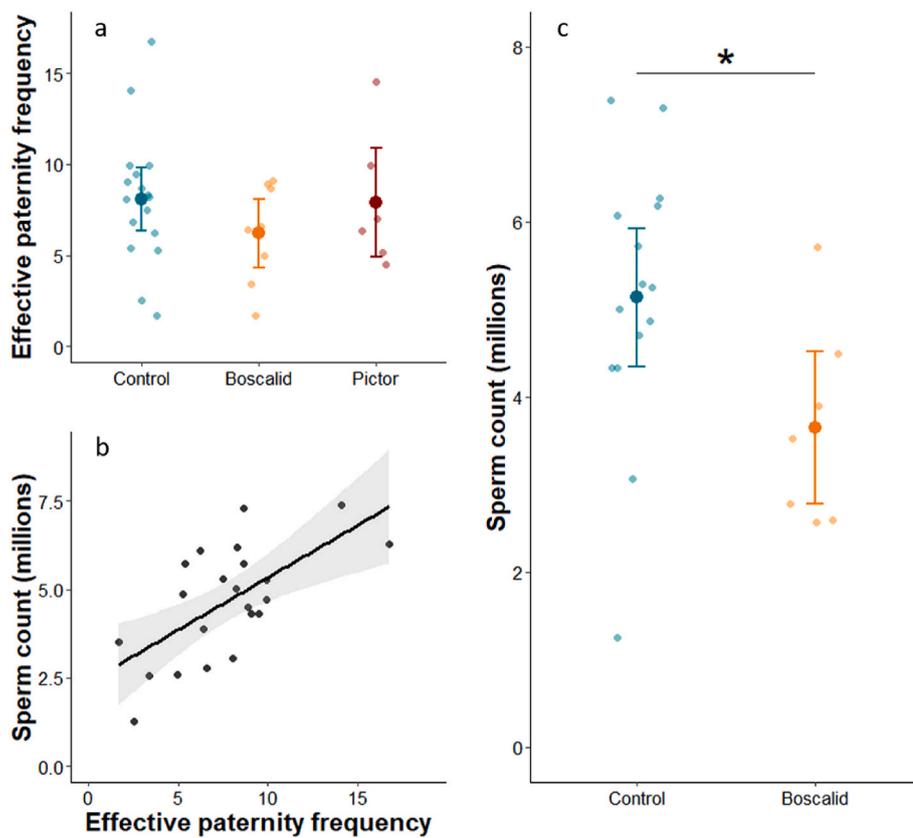
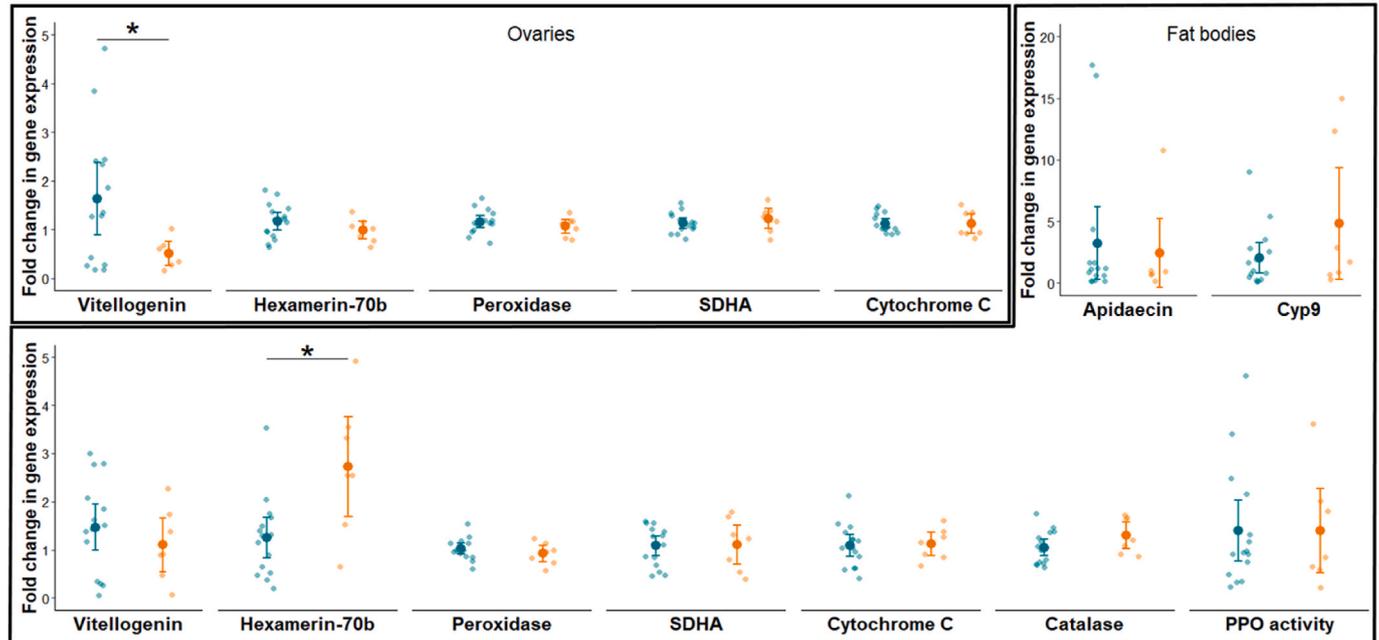


Fig. 4. *Varroa destructor* intensity within colonies in late August according to treatment ( $P = 0.019$ ). The bigger dots represent mean  $\pm$  SE. \* when  $P < 0.05$ .



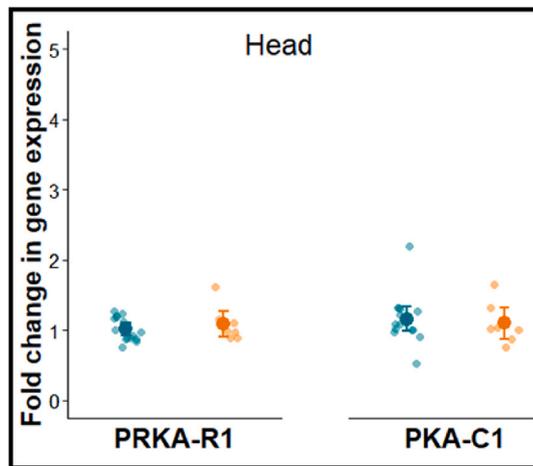
**Fig. 5.** Queen reproductive quality: (a) effective paternity frequency according to treatment, (b) number of spermatozoa stored in the spermatheca according to effective paternity frequency (Pearson's correlation  $r = 0.65$ ,  $P < 0.001$ ) and (c) according to treatment. Colors correspond to treatment: control in blue, boscalid in orange and Pictor Pro® in red. Only three Pictor Pro® queens were alive at the end of season and they were thus excluded from sperm count analyses. In (a) and (c), the bigger dots represent mean  $\pm SE$ . \* when  $P < 0.05$ . In (b), shaded area denotes 95% confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 6.** Relative gene expression in ovaries and fat bodies according to treatment: control ( $n = 15$ , in blue) and boscalid ( $n = 7$ , in orange). Only three Pictor Pro® queens were alive at the end of season and they were thus excluded from gene expression analyses. The bigger dots represent mean  $\pm SE$ . \* when  $P < 0.05$ . The relative gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method (Livak, 2001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and Koeniger, 2000). We hypothesize that boscalid might have disrupted post-copulatory sperm storage mechanisms, for instance by limiting migration of spermatozoa from oviducts to the spermatheca (Gessner and Ruttner, 1977) or by accelerating the depletion of the

spermatheca. Sperm depletion may result from an increase in the number of sperm used by the queen to fertilize an egg (Baer et al., 2016) and from an increased death rate of spermatozoa (Chaimanee et al., 2016). Sperm are preserved in the spermatheca for years partly because



**Fig. 7.** Relative gene expression in the head according to treatment: control ( $n = 15$ , in blue) and boscalid ( $n = 7$ , in orange). Only three Pictor Pro® queens were alive at the end of season and they were thus excluded from gene expression analyses. The bigger dots represent mean  $\pm$  SE. The relative gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method (Livak, 2001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the spermathecal fluid contains proteins involved in a large range of protective functions, including energy metabolism and antioxidant defenses (Baer et al., 2009; Paynter et al., 2017). Previous studies found differential expression of genes coding for such proteins in the spermatheca of queens exposed to pesticides after mating (Chaimanee and Pettis, 2019; Chaimanee et al., 2016; McAfee et al., 2020). However, it remains unknown whether queen exposure to pesticides before mating would lead to similar change in the expression of protective genes in the spermathecal fluid. Future work should focus on the short-term consequences of boscalid intoxication on queen physiology to identify the underlying mechanisms of decreased queen reproductive quality.

In addition to decrease queen reproductive quality, queen exposure to boscalid also had detrimental consequences on the colonies they later established. One month after queen introduction, colonies headed by queens exposed to the commercial solution Pictor Pro® had a higher proportion of open brood than colonies headed by unexposed queens. At this period, Pictor Pro® colonies tended to have fewer capped brood than control ones (although not statistically significant) while the amount of open brood was similar between treatments (Fig. S3). One explanation may be that Pictor Pro® queens started laying later than control ones, resulting in most brood cells being not capped yet when colonies were checked for the first time. This trend then reversed, with Pictor Pro® colonies having a lower proportion of open brood than control colonies during the rest of the summer. This could result from a decreased laying rate in queens exposed to Pictor Pro®, as previously found in colonies fed with neonicotinoid-treated pollen (Wu-Smart and Spivak, 2016) and when queens were raised in miticide-laden wax (Walsh et al., 2020). A decreased proportion of open brood may also partly result from increased open brood loss (via reduced egg hatchability or increased larval mortality), as found in colonies fed with insecticide- or fungicide-treated food (Fine, 2020; Traynor et al., 2021). Although we could not directly measure queen laying rate or brood loss, we found that two reproduction-related genes, namely *vitellogenin* and *hexamerin 70b*, were differentially expressed in the ovaries and the fat bodies, respectively, of queens exposed to boscalid. Vitellogenin is a protein involved in egg-yolk formation and is thus strongly associated with queen sexual maturity (Kocher et al., 2008; Zhang et al., 2011). Decreased *vitellogenin* expression in the ovaries of queens exposed to boscalid may thus have led to the laying of eggs with suboptimal energy reserves, which in turn may have decreased their hatchability or

increased the mortality of developing larvae (Diss et al., 1996). Vitellogenin is also involved in immune functions, including the transfer of immune-priming signals from the queen to its offspring (Salmela et al., 2015). Thus, queens exposed to boscalid may have produced workers with increased susceptibility to pathogens. Accordingly, we found an increased varroa intensity in colonies headed by queens exposed to boscalid. In addition to decrease *vitellogenin* expression in ovaries, queen exposure to boscalid was also associated with increased expression of *hexamerin 70b* in fat bodies. Hexamerins are storage proteins mostly involved in larvae development but also possibly in reproduction (Martins et al., 2010; Martins et al., 2008). The source of amino acid used by queens for egg formation does not usually come from hexamerins but rather directly from the dietary-proteins contained in royal jelly and directed to vitellogenesis (Martins et al., 2008). We could speculate that the royal jelly consumed by queens exposed to boscalid may have been altered, as a result of the abnormal increase in pollen storage we observed in their colonies (see below). Because an increased expression of hexamerins has been associated with increased consumption of pollen in young workers (Martins et al., 2010), queens exposed to boscalid may have used hexamerins as a source of energy reserves for eggs to compensate for the decreased production of vitellogenin.

The brood anomalies found in colonies headed by exposed queens could result from low queen reproductive quality but also from decreased worker performance. Indeed, nurse bees affect larvae survival by performing hygienic behaviors throughout larvae development. In particular, nurses actively participate to limit varroa outbreak by detecting and removing mites from capped cells (Harbo and Harris, 2005). The increased number of varroa mites found in our colonies headed by queens exposed to boscalid may indicate failure to detect mites or poor hygienic behaviors (Mondet et al., 2015). In contrast, there was a decreased number of varroas in colonies headed by queens exposed to Pictor Pro®. This difference between pesticide treatments may be explained by the adjuvants contained in the commercial solution Pictor Pro®, i.e. ammonium sulfate and sodium sulfate, although their toxicity for bees is unknown. Larvae also depends on forager bees through food supplies. In case of shortage in pollen supply, nurse bees neglect young larvae, thereby increasing their risk of being cannibalized. Moreover, old larvae correspond to a development stage with the highest pollen demand and to face the lack of pollen, nurse bees cap larvae earlier than expected (Schmickl and Crailsheim, 2001; Schmickl and Crailsheim, 2002). This scenario results in a decreased proportion of open brood relative to capped brood, and could thus explain our findings in colonies headed by queens exposed to Pictor Pro®. However, regarding pollen storage, colonies headed by queens exposed to boscalid actually stored more pollen than control colonies after sunflower started flowering. A similar increase in pollen collection was found in colonies fed with pollen treated with Pristine®, a commercial pesticide containing 25% boscalid (Fisher et al., 2021a). Workers exposed to Pristine® forage earlier and have an increased tendency to forage for pollen (Fisher et al., 2021a; Fisher et al., 2021b). This is likely a response to perceived nutritional stress because workers fed with Pristine®-treated pollen have increased protein concentration in their feces, suggesting disruption of protein absorption (Degrandi-Hoffman et al., 2015). Thus, nurses of colonies headed by exposed queens may have responded to protein deficits by giving less food to younger larvae and by capping older larvae earlier, thereby resulting in a decreased proportion of open brood relative to capped brood. In other words, protein absorption disorders caused by boscalid may have led workers to act like if there was a shortage in pollen supply. Protein malnutrition may further explain why queens exposed to boscalid had decreased expression of *vitellogenin* because queens use dietary-proteins to produce this egg-yolk precursor (Zhang et al., 2011).

Interestingly, we found similar effects on pollen storage than in studies that directly exposed workers or the whole colony (Fisher et al., 2021a; Fisher et al., 2021b). One possible explanation may be that

changes in gene expression induced by exposure to boscalid in queens were transferred to the next generation. Such epigenetic inheritance can occur through the transfer of DNA methylation profiles from parents to offspring (Skvortsova et al., 2018). Contrary to mammals in which most DNA methylation marks are erased during embryogenesis, there is almost no remodeling of DNA methylation during development in honey bees (Xu et al., 2019; Harris et al., 2019). A limitation of epigenetic reprogramming would make possible the inheritance of DNA methylation induced by environmental factors, including exposure to contaminants (Head, 2014). Recently, another SDHI fungicide, flutolanil, has been proposed to have transgenerational effects in zebrafish through alterations of DNA methylation profiles (Teng et al., 2020). It remains to be investigated if boscalid induces similar changes in methylation profiles or other epigenetic marks in bees and whether these changes are passed through generations. Alternatively, queen exposure to boscalid may have changed pollen storage behavior in workers via reduced vitellogenin provisioning to the eggs. Nutritional stress in larvae has consequences on adult worker physiology and behavior, including precocious foraging onset, which should enhance food collection at the colony level (Wang et al., 2016). Reduced levels of vitellogenin have been also associated with faster transition from nursing to foraging (Amdam et al., 2011). To further explore the consequences of queen exposure to boscalid (or other foraging distorter pesticides), future research could measure foraging behavior and expression levels of genes related to foraging and digestion in the progeny.

## 5. Conclusions

Overall, our results indicate that sublethal, field-realistic doses of boscalid negatively impacts honeybee queens during the mandatory nuptial flight period by causing their precocious death and by decreasing their reproductive quality. These findings underline the need to perform toxicity assessments of fungicides on pollinators by evaluating sublethal effects, including reproductive disorders, in natural conditions and at field relevant concentrations (Barascou et al., 2021; Cullen et al., 2019). In addition, queen exposure to boscalid before mating had long-lasting effects, as shown by colony-level anomalies in brood production and pollen storage. These adverse effects of boscalid on the queen's progeny suggest that even if an intoxicated queen successfully produces substitute queens, they might be of reduced quality, thereby limiting colony recovery. Future works should investigate whether the detrimental effects of boscalid and other SDHI fungicides on pollinators are transferred from generation to generation through epigenetic alterations, as shown in fish (Teng et al., 2020), and how such changes would affect population dynamic.

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## Credit author statement

Maxime Pineaux: Conceptualization; Data curation; Methodology; Formal analysis; Investigation; Visualization; Writing – original draft. Stéphane Grateau: Resources; Writing – review & editing. Tiffany Lirand: Resources; Writing – review & editing. Pierrick Aupinel: Conceptualization; Funding acquisition; Supervision; Writing – review & editing. Freddie-Jeanne Richard: Conceptualization; Funding

acquisition; Investigation; Supervision; Project administration; Writing – original draft.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data are available on Open Science Framework: Pineaux, M., et al. (2022). Data - *Apis mellifera* queens exposure to boscalid. DOI 10.17605/OSF.IO/PKG7U

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2023.121131>.

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