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Original article

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Abstract – *Apis mellifera* often encounters the fungicide boscalid in agricultural landscape. In this study, we assessed the impacts of ingesting boscalid and Pictor Pro® (a commercial formulation of boscalid) at environmentally relevant doses during the larval development phase. Following chronic exposure, we measured survival, development time, and gene expression in both larvae and in 10-day-old workers. The genes analyzed were involved in immunity, detoxification, development, and mitochondrial activity pathways. We found no significant impact of boscalid in larval survival and emergence. However, genes related to cytochrome b, succinate dehydrogenase, and catalase were downregulated in larvae and adult workers exposed to boscalid, though the differences were not significant when compared to the solvent control. Late variation was observed in 10 days adult bees' overexpression of the vitellogenin gene, which is linked to development. The effects observed with boscalid molecule were not seen with the commercially formulated product, Pictor Pro®.

***Apis mellifera* / In vitro / fungicide / SDHI / field concentration / vitellogenin**

1. INTRODUCTION

The process of pollination, facilitated by insects, plays an essential role in the successful reproduction of flowering plants and is vital to the functioning of terrestrial ecosystems, providing vital ecosystem services that are essential to human production (Garibaldi et al. 2014; Ollerton et al. 2011). Their role in plant reproduction is crucial, contributing to the production of fruit, vegetables, and seeds in many species. Approximately 75% of crop species and 90% of all flowering plants (Tepedino 1979) require pollinators to reproduce (Klein et al. 2007; Tepedino 1979). Overall, pollinators provide a global service worth $153 billion to food production (Gallai et al. 2009).

Insect populations, including honey bees, are declining worldwide and which raises significant concerns and has prompted extensive inquiries into the contributing factors (Hallmann et al. 2017). This decline is the result of multiple factors acting separately or in combination (Goulson et al. 2015). Key factors include habitat modification and loss (Kuchling et al. 2018), parasites and diseases (Fünfhaus et al. 2018), and

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pesticides (Sanchez-Bayo & Goka 2014) which are major factors contributing to the decline of bees. Many chemicals can have harmful effects on bees, including immediate mortality at high doses and sublethal effects, such as alterations in development, behavior, and overall capacity at lower doses (Henry et al. 2012).

Pesticides, including insecticides, fungicides, and herbicides, are widely used in agriculture to safeguard crops against pests and diseases (Mitchell et al. 2017). The total number of 454 approved active substances in the EU in May 2023, which translates into a vast number of commercial formulations incorporating one or more active substances with adjuvants (EU Pesticides Database—Active substances 05/2023). Bees can be exposed to pesticides through various pathways, including as direct exposure while foraging on treated crops, spray drift, contamination of food sources, and the presence of pesticides in hive components (Sanchez-Bayo & Goka 2014). Although the concentrations of many pesticides found in beehives are relatively low compared to the oral and contact LD50, recent meta-analyses suggest that the effects of fungicides on bees are insufficiently studied and potentially underestimated, particularly in real-world conditions (Di Noi et al. 2021). For bee larvae, one primary route of pesticide exposure is through the consumption of pollen contaminated with pesticides from treated plants. Studies have shown a correlation between pesticide quantities and the presence of pollen grains in worker jelly, indicating the risk of larval exposure to pesticides (Böhme et al. 2019; Drummond et al. 2018). These findings highlight the necessity for further investigation into the impacts of pesticide exposure on bees, including a more thorough understanding of sublethal effects on larval development and health status.

Fungicides are not specifically designed to target bees and other pollinating insects, so knowledge of their potential risks is limited. However, previous studies suggest that fungicides can affect navigation, learning, and larval development in bees (Liao et al. 2019). Among the various fungicides used in conventional agricultural, boscalid is widely used due to its broad spectrum of action and effectiveness against fungal diseases (Li et al. 2021). In France, this active molecule is found alone or in mixtures in 47 commercial products covered by French regulation (ANSES 2023). Pictor Pro® is a commonly used commercial formulation of boscalid, applied to crops such as peas, beans, and oilseed rape after the first flower emerges (BASF France SAS 2022).

Notably boscalid, a commonly used, is frequently detected in hive matrices such as honey, beeswax, pollen, and worker jelly (Böhme et al. 2018; Friedle et al. 2021). Boscalid belongs to the carboxamide family and acts by inhibiting the activity of succinate dehydrogenase, an enzyme essential for the mitochondrial respiratory chain in fungal organisms (Stammler et al. 2008). Additionally, recent studies have shown that exposure to boscalid can have harmful effects on bees, particularly by reduction in the longevity of worker bees (Fisher II et al. 2022; Simon-Delso et al. 2018). Exposure can also result in sublethal effects, such as reduced capacities and behavioral changes of individuals, including decreased pollen consumption, impaired learning, earlier foraging, and a reduced wing beat frequency (DesJardins et al. 2021; Fisher et al. 2021a, b; Fisher II et al. 2022; Liao et al. 2019). Although the effects of several pesticides (chlorpyrifos, imidacloprid, amitraz, fluvalinate, coumaphos, myclobutanil, chlorothalonil, etc.) on gene expression have been studied in larvae, no research has specifically examined the effects of boscalid (Gregorc et al. 2012; Shi et al. 2020; Tesovnik et al. 2017).

Boscalid has been shown to alter feeding behavior by disrupting the nutritional intake necessary for the overall health and survival of the colony, particularly during the winter (Fisher et al. 2021a, b). In the *Apis cerana*, the genes involved in detoxification and immunity were also strongly upregulated following exposure to a pesticide cocktail containing boscalid (Dong et al. 2023). Moreover, exposure to boscalid caused physiological changes in queens, notably in the expression of the vitellogenin gene, and negatively affected colonies in particular in brood production (Pineaux et al. 2023).

This study aims to better understand the effects of a fungicide, boscalid, on honeybees' gene expression. We chronically exposed larvae to boscalid at field concentration. We used a parsimonious approach by only considering the exposure of the larvae to an equivalent amount of boscalid that they could ingest through contaminated pollen in the environment. The effects of boscalid on gene expression were measured few days after chronic exposure of the larvae. Additionally, gene expression in adult workers was measured 10 days after emergence. The target genes were linked to metabolic pathways of immunity, detoxification, development, and mitochondrial activity. The results of this study will contribute to a better assessment of the risks associated with the use of this fungicide in the field and help to minimize adverse effects on pollinators.

2. MATERIALS AND METHODS

Experiments were conducted in the entomological experimental unit of INRAE, Le Magneraud (46° 8′ 59,104″N, 0° 41′ 28,609″W), France between April and August 2021. In this study, we recorded larval and pupal mortality and adult emergence rates.

We compared all variables between the untreated larvae (control), treated with 0.1% acetone (solvent control), and treated with pesticides. The two treatments were as follows: exposure to 3.05 ng pure same dose of active molecule to a commercial solution (Pictor Pro®: contains 50% boscalid).

2.1. Larval rearing

One-day-old larvae were collected from three colonies of *A. mellifera* and reared in vitro until emergence. For artificial rearing, we followed the method adapted by Aupinel et al. (2007) and Papach et al. (2017).

Comb with first instar larvae was obtained by enclosing the queen with a frame of built-up brood to an isolated Italian cage for 30 h. Then, the queen was released, and the freshly laid frame was left in the hive for 1 more day to obtain first cycle larvae (L1) for grafting.

All grafts were performed in the laboratory at room temperature. The transplants were performed by the same experimenters. The first larval rearing was carried out, until the eighth day of rearing in the laboratory. The larvae (L1) were transferred into disinfected plastic grafting dishes. The cups containing with the L1 were kept in an incubator at 34.5 ± 5 °C from day 1 to day 8 with a relative humidity (RH) of $95 \pm 5\%$ (K_2SO_4 solution). Cups were filled with a mixture of royal jelly, aqueous sugar, and yeast extract with or without pesticide treatment (see below for details) on days 1, 3, 4, 5, and 6. Larvae were then collected on day 8 for molecular biology analyses.

A second round of grafting was performed with the same protocol to obtain adult workers. The first part of the protocol was like the first grafting round. Then the larvae were transferred to a new clean plate when they had finished feeding (days 7 and 8). The plates containing the pupae were transferred to a sealed incubator at 34.5 ± 5 °C, with $80 \pm 5\%$ RH (saturated NACL solution). Before emergence (day 15), pupal plates were placed in crystal polypropylene emergence boxes ($11 \times 15 \times 12$ cm) at 34.5 ± 5 °C and $50\% \pm 5\%$ RH with 50% agarose-sugar solution ad libitum. A 5-mm piece of Be-Boost® (PseudoQueen) was attached to a piece of wax (approximately 5×5 cm) in each emergence box. After emergence, the bees were transferred to a new polypropylene box, similarly equipped but with the addition of pollen powder. They were maintained under laboratory conditions for ten days at 34.5 ± 5 °C and $50\% \pm 5\%$ RH before being collected for molecular biology analysis.

For the first series, larvae were collected at day 8, and for the second series, worker bees were collected at 10 days of age. The individuals were collected alive and immediately stored at − 80 °C until gene expression analyses (the experimental design is graphically summarized in Appendix 1).

2.2. Pesticide exposure

Firstly, we estimated boscalid contamination in the natural environment based on data in the literature. The concentration of boscalid found in pollen brought back by bees to the hives varies significantly worldwide. For example, the maximum dose reported in California is 6060 ng/g (Fisher et al. 2021a, b), in Canada is 2200 ng/g (Tsvetkov et al. 2017), and at 1496.4 ng/g in Germany (Böhme et al. 2018). In our study, we base our concentrations on the exposure levels found in Germany, because the agricultural landscape is comparable to that of France and reflects environmental conditions relevant to the European context. We use the quantity of 1496.4 ng/g, and considering that a bee larva feeds on around 0.002 g of pollen in 4 days (Babendreier et al. 2004), we estimated that under natural conditions, the larvae would consume 3.05 ng of boscalid.

Stock solutions of boscalid (99% purity, Cluzeau Info Labo, France) were prepared in acetone and added to the larval diet. Pictor Pro® is a commercial solution and is composed of 50% boscalid. Thus, the concentration of boscalid, and commercial solution were 19.5 ng/g and 38.9 ng/g in diet B and C, respectively (Appendix 1). The stock solutions of the pesticides were prepared at the start of the beekeeping season and stored at − 20 °C; they were only thawed when used. Each larva was chronically exposed to water (control), 0.1% of acetone only (solvent control), or 3.05 ng of boscalid solution or 6.1 ng of Pictor Pro® for 4 days in their diet (day 3 to day 6).

2.3. Laboratory analysis

We measured the expression of seven genes (Appendix 1) at both larvae and adult stage (10-day-old workers). The analyses were performed at the laboratory Ecology and Biology of Interactions (Vienne-France) in 2021, on frozen individuals. The individuals analyzed came from two colonies used for grafting. In larvae and bees, gene expression was analyzed in 13–16 individuals per modality.

2.4. Extraction of total RNA

Each larva was homogenized in 900 µl of QIAzol (Qiagen) with three beads of 2-mm diameter beads, using a FastPrep-24 (MP) (one time 30 s at 6.5 m/s). For each bee, the abdomen was homogenized in 900 µl of QIAzol (Qiagen) with three beads of 2-mm diameter beads, using a FastPrep-24 (MP) (two times 30 s at 6.5 m/s with 15 min in ice for adult bees). The homogenate was incubated for 5 min at room temperature. Total RNA was isolated from each larva using an RNeasy Plus Universal Mini kit (QIAGEN), with optional DNA digestion step by adding 50 µl of DNAse. The final volume of the suspension was 50 µl. RNA was quantified using a NanoDrop 1000 Spectrophotometer (ThermoFisher).

2.5. Quantification of gene expression

Reverse transcription was performed with SuperScript® First-Stand cDNA Synthesis Reaction Kit (Invitrogen) using 500 ng RNA in 20 µl reaction volumes containing random hexamer primer. The RNase H step was performed to digest the RNA residue, by adding one µl of RNase H and incubating at 37 °C for 20 min, according to the producer's protocol. The expression levels of two reference genes and five target genes were quantified by qPCR.

The epMOTION automated system (Eppendorf) was used to prepare the qPCR plates. For the RT-qPCR assays, LighCycler 480 SYBER-GREEN I Master (Roche) qPCR was used. Each reaction of ten consisted of 1.5 µl sample cDNA, 0.5 µM forward and 0.5 µM reverse primers (Integrated DNA Technologies), and 7.5 µl master mix, using the LighCycler 480 (Roche) detection system. Amplification was performed with the following program: 10 min at 95 °C and 35 cycles, denaturation cycles of 10 s at 95 °C, hybridation cycles of 10 s at 60 °C, and elongation cycle of 20 s at 72 °C. At the end of this program, a melting curve is generated by measuring the fluorescence over a range of 65 to 97 °C to verify the presence of the desired amplicon. All reactions were performed in triplicate, and the variation

between the triples was less than 1 Ct. Controls, containing water, were included in each assay. The average cycle threshold values of our different target genes were normalized to the geometric mean of the reference genes. The ribosomal protein S5 and β-actin were found to have stable expression levels. The ΔCt value of each group was subtracted from the ΔCt value of the solvent control to obtain ΔΔ Ct. For gene expression profiling was conducted by collecting 14–16 larvae of each modality from two different colonies, witch were immediately frozen on 8 days. Additionaly, and 16 bees from two differents colonies were frozen 10 days after emergence for blind analysis.

2.6. Statistical analyses

Statistical analyses were conducted using statistical software R 4.0.3 (R Core Team 2020) and add to package survival 3.3–1 (Therneau 2023), emmeans 1.8.5 (Lenth et al. 2023), and Lme4 1.1–32 (Bates et al. 2023).

The survival of individuals was analyzed with a Cox proportional hazard model (Therneau 2023). We checked the proportionality of the hazard ratios using a score test in the "cox.zph" function of the *survival* package. Selection of the best-fitted model was based on analyses of variance and the Akaike information criterion (AIC) (Guthery et al. 2003). The modality, the experimenter transferring the larvae at D8, and the hive of origin of the larvae were considered fixed effect variables in the model.

The variation in emergence days of bees was analyzed using a linear random effects model built in the *lme4* package. Model selection was performed by analysis of variance and AIC (Guthery et al. 2003). Variations in emergence days between rearing plates were accounted in a random effect. The exposure modalities and the hive of origin of the larvae were also included in the model as fixed effect variables. Pairwise comparisons were conducted using R package *emmeans* (Tukey's HSD) to identify differences among treatment modalities and the impact of the hive of origin of the larvae.

We assessed whether the effects of the different modalities altered gene expression using linear models included in the r software. For each of the genes, we also considered the effects of the hive of origin of the individuals reared in vitro. To identify differences between treatment modalities and the impact of the hive of origin of the larvae, pairwise comparisons were performed using the R *emmeans* package (Tukey's HSD).

3. RESULTS

3.1. Larva survival

Larval survival during in vitro rearing was not significantly impacted by the different treatments added to their diet. Larvae in both control and solvent control modalities had the same chance of survival (Tukey test $= -0.301$; $p = 0.480$). There was no significant difference in survival rates between larvae exposed to boscalid or Pictor Pro® and those exposed to solvent control (All comparison: Tukey test < 0.24; $p > 0.668$) (Figure 1).

Larvae survival was not significantly different between the colonies R3 and R6 (Tukey test $= -0.01$; $p = 0.997$) but mortality was significantly higher mortality for the hive R4 (Tukey test $= -0.54$; $p = 0.013$). In addition, larvae manipulation at 8 days (D8) was performed by two experimenters, and we observed a significant difference of larvae survival between these two groups (Tukey test $= 0.920$; $p < 0.005$; Appendix 2).

3.2. Development time

The duration of larval development until emergence was not affected by exposure to boscalid or Pictor Pro®, with an average of 19.2 days (all comparison: Tukey test, $p < 0.24$; $p > 0.6770$ (Figure 2)). Additionally, comparisons among the colonies of origin of the larvae showed no effect on the time required for the larvae to emerge (Tukey test < 0.302; $p > 0.2653$; Appendix 2).

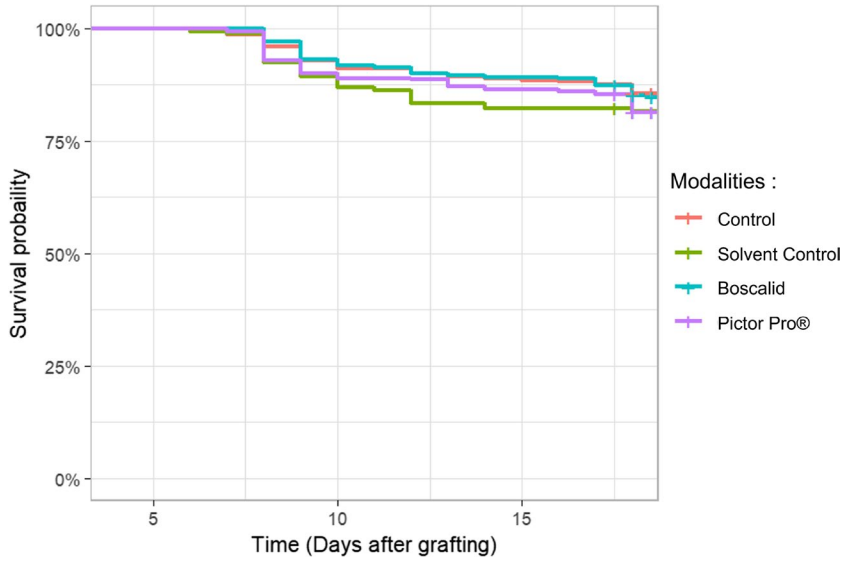

Figure 1. Larvae survival when unexposed (control), exposed to solvent (solvent control), to boscalid, and to Pictor Pro®. Larval survival did not differ significantly between the experimental groups (log rank test; $\chi.^2 = 3.2$; $p = 0.4$).

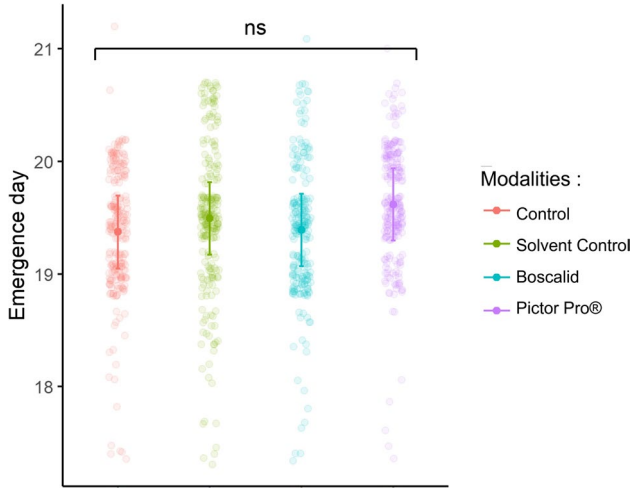

Figure 2. Days of workers' emergence unexposed (control), exposed to solvent (solvent control), to boscalid, and to Pictor Pro® (from left to right). Larger points represent mean ± SE and transparent points represent raw data. Pairwise comparison (Tukey's HSD): ns when $p > 0.05$.

3.3. Larvae's gene expression

Larvae exposed to solvent control showed significantly lower expression levels of cytochrome b, which is associated with mitochondrial activity, compared with control larvae (Tukey test = −0.25; $p = 0.006$, Figure 3C). Larvae exposed to boscalid had significantly lower expression levels of catalase (detoxification) and cytochrome b (mitochondrial activity), compared to solvent control larvae (respectively Tukey test = −0.25 (Figure 3A); $p = 0.006$ and

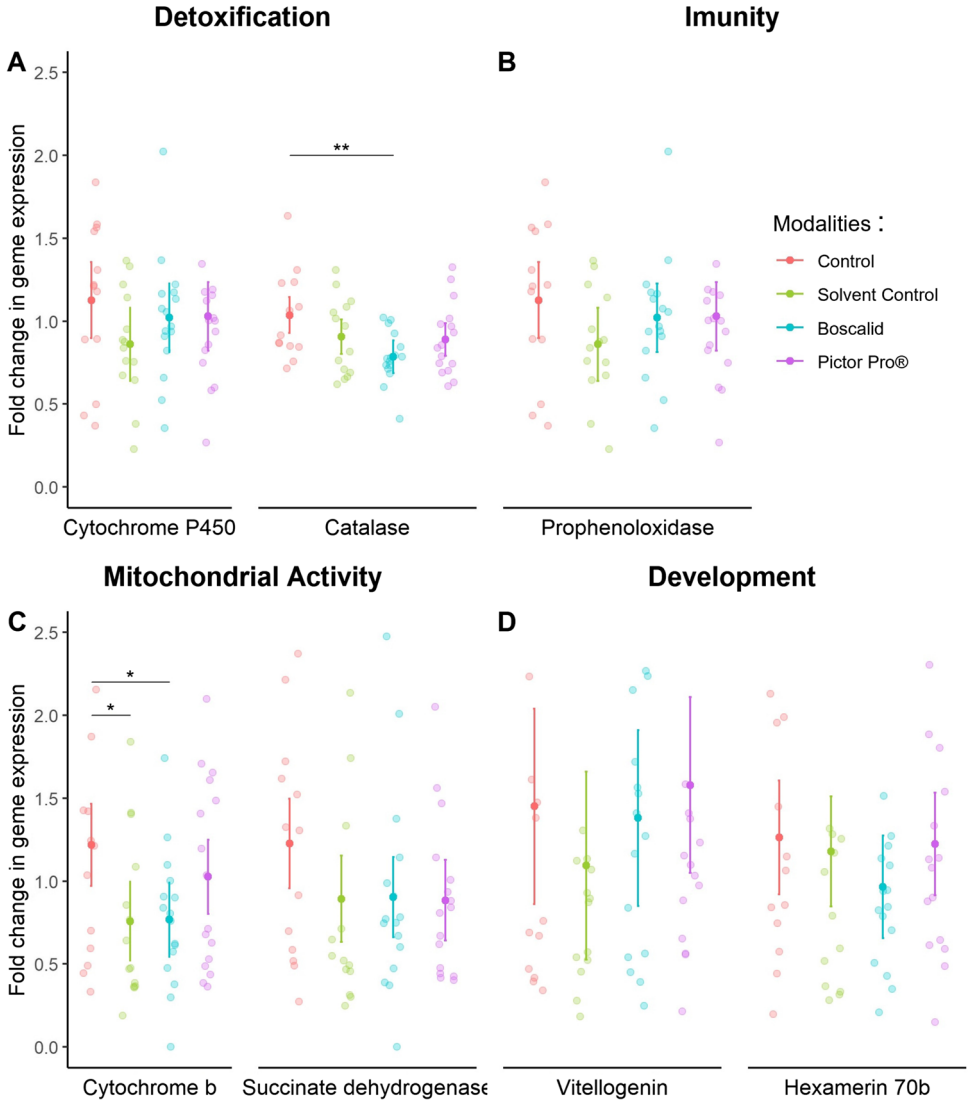

Figure 3. Relative expression of seven genes: cytochrome P450, catalase, prophenoloxidase, cytochrome b, succinate dehydrogenase, vitellogenin, and hexamerin, in treated larvae with control, solvent control, boscalid, and Pictor Pro® (from left to right). Genes are grouped by function: **A** detoxification; **B** immunity: **C** mitochondrial activity; **D** development. Relative gene expression was calculated using the 2-ΔΔCt (Livak, 2001). Larger points represent mean ± SE and transparent points represent raw data. Pairwise comparison (Tukey's HSD): * when $p < 0.05$; ** when $p < 0.01$ (Appendix 3).

Tukey test = −0.45; $p = 0.042$ (Figure 3C)). Gene expression of larvae exposed to Pictor Pro® did not significantly differ for any of the genes compared to larvae exposed to solvent control (all comparisons: Tukey test < 0.147, $p > 0.195$). For genes prophenoloxidase, succinate dehydrogenase, and hexamerin, no significant differences were observed between treatments (all comparison: Tukey test < 0.147, $p > 0.195$ (Figure 3, Appendix 3)).

The colonies of origin from which the larvae had been collected influenced the expression

levels of several genes. Larvae from the colony R3 had significantly higher expression levels of cytochrome 450 (Tukey test = −8.36; $p < 0.001$) and lower expression levels for catalase (Tukey test = −0.191; $p < 0.001$), for prophenoloxidase (Tukey test = −0.248; $p = 0.025$), for cytochrome b (Tukey test = −0.63; $p < 0.001$), for succinate dehydrogenase (Tukey test = −0.792; $p < 0.001$), for vitellogenin (Tukey test = −1395; $p < 0.001$), and for hexamerin (Tukey test = −1015; $p < 0.001$), compared to larvae from colony R4 (Appendix 3).

3.4. Worker's gene expression

Ten-day-old workers exposed to control solvent showed significantly lower expression levels of succinate dehydrogenase, associated with mitochondrial activity, than workers exposed to control (Tukey test = −544; $p = 0.599$ (Figure 4C)). The activity of succinate dehydrogenase was altered in 10-day-old workers after exposure to boscalid (Figure 4C). Workers exposed to boscalid tended to under-express the succinate dehydrogenase gene (Tukey test = −0.514; $p = 0.075$) and significantly under-expressed the cytochrome b gene (Tukey test = −0.463; $p = 0.036$) compared with workers exposed to solvent control. In addition, workers exposed to boscalid had higher levels of vitellogenin gene expression, associated with development, than workers in the solvent control group (Tukey test = 0.835; $p = 0.045$). Gene expression of workers exposed to Pictor Pro® did not significantly differ for any genes compared those exposed to solvent control (Tukey test < 0.214; $p > 0.564$). For the other five genes, associated with detoxification (cytochrome P450, catalase), immunity (prophenoloxidase), and development (hexamerin), no significant differences were observed between bees from the different modalities (all comparison: Tukey test < 0.238; $p > 0.133$ (Figure 4)).

We have a strong effect of the grafting colony. Workers from the colony R3 had significantly lower gene expression levels for succinate dehydrogenase and vitellogenin (Tukey test = −1.879; $p < 0.001$ and Tukey test = −1.000; $p < 0.001$, respectively), higher expression levels for catalase and prophenoloxidase (Tukey test = 0.495; $p < 0.001$ and Tukey test = 0.246; $p = 0.010$, respectively) compared to workers from the colony R4 (Appendix 4). There was no significant difference in the expression of cytochrome P450, cytochrome b and hexamerin genes between workers from the colonies R3 and R4 (all comparison: Tukey test ≤ 0.538; $p \geq 0.343$).

4. DISCUSSION

4.1. Choice of intoxication dose

The aim of our study was to examine the effects of exposure to boscalid at doses present in the environment, and the resulting gene expression changes in bees during both the larval and adult stages. A previous study estimated that the LD50 values on day 8 and day 15 were 86.786 µg/larva and 78.782 µg/larva, respectively (Simon-Delso et al. 2017), Here, larval individuals were chronically exposed to a sublethal does at a concentration of 3.05 ng/larvae. Our approach was parsimonious, particularly regarding larval exposure, which was assessed based on boscalid contamination in the pollen that feeds the larvae (Böhme et al. 2018). Limited information is available on the pathway of contamination, accumulation, or degradation of boscalid in different bee matrices and their effects on larval physiology (Wueppenhorst et al. 2022). Boscalid metabolites have been quantified in bee samples collected from various colonies in France, but their harmful effects on beehives remain unclear (Jabot et al. 2016). However, the ingestion of contaminated food and pollen from pesticide-treated plants is one of the primary pathways of larval exposure (Babendreier et al. 2004; Böhme et al. 2018). Additionally, bee larvae come direct into contact with wax, which may contain pesticide residues from various sources, such as antiparasitic treatments used in hives, pesticide applications on crops, or environmental contamination (Friedle et al. 2021; Xiao et al. 2022). These different

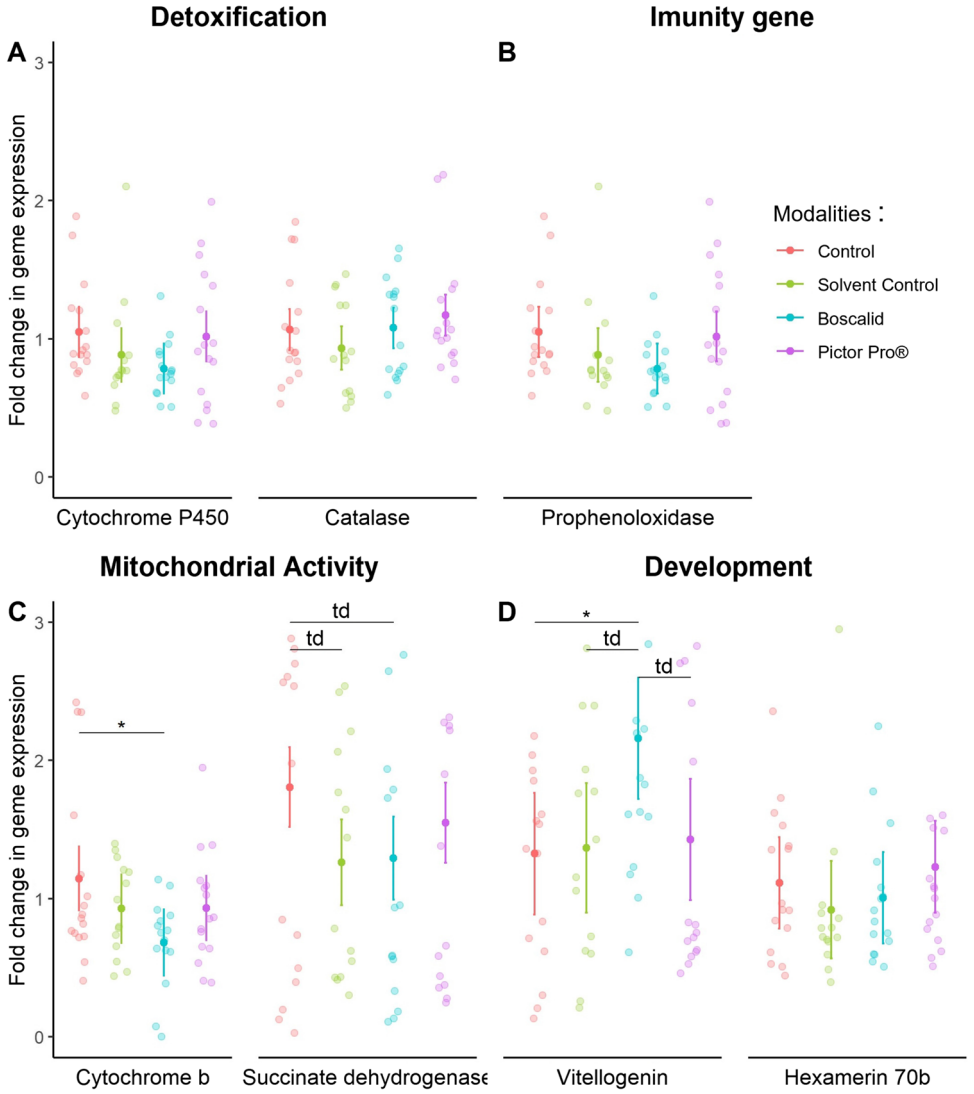

Figure 4. Relative expression of seven genes: cytochrome P450, catalase, prophenoloxidase, cytochrome b, succinate dehydrogenase, vitellogenin, and hexamerin, in treated worker bees of 10 days with control, solvent control, boscalid, and Pictor Pro® (from left to right). Genes are grouped by function: **A** detoxification, **B** immunity, **C** mitochondrial activity, **D** development. Relative gene expression was calculated using the 2-ΔΔ Ct (Livak, 2001). Larger points represent mean ± SE, and transparent points represent raw data. Pairwise comparison (Tukey's HSD): when $p < 0.1$; * when $p < 0.05$ (Appendix 4).

exposure routes contribute to the accumulation of pesticides in bee larvae, potentially leading to lethal or sublethal consequences. We have likely underestimated the pesticide doses to which bee larvae may be exposed in the field by considering only one route and a limited exposure period.

4.2. Impact on workers' larvae

As expected, we did not observe a significant impact on larval survival following exposure to boscalid. Previous studies using doses 10 to 10,000 times higher than those used in this

study did not report on larval mortality or development time until bee emergence (Simon-Delso et al. 2018). However, other studies have shown an effect of boscalid on larval mass during the prenymphal stage (Glass et al. 2021).

We assessed the consequences of this exposure on gene expression to better understand the physiological impact of this molecule on bees. Our results show the expression of specific genes was significantly impacted by the different larval treatments with boscalid. We observed the downregulation of the cytochrome b transcription gene. This gene is associated with cellular respiration as it is responsible for transmembrane electron transfer, by which redox energy is converted into a proton motive force (Esposti et al. 1993). However, the commercial product Pictor® had no significant effect on gene expression. Other fungicides have been shown to have a significant impact on gene expression in larvae. For instance, chlorothalonil caused overexpression of genes related to immunity (defensin or toll) and detoxification-related genes (cytochrome P450) (He et al. 2022; Lu et al. 2023). The measured effects appear to be dose-dependent and typically occurring at high doses, at doses even below the LD50 (He et al. 2022; Lu et al. 2023). In addition, larval exposure to the fungicide carbendazim has been found to reduce the expression of royal jelly proteins in the heads of bees (Wang et al. 2021).

4.3. Delayed impact on adult workers

We assess the long-term effects by examining the gene expression in 10-day-old workers that were exposed during the larval phase. The worker bees showed slight modifications in gene expression. We observed a clear effect of boscalid on downregulation of the cytochrome b gene and a less pronounced effect on the succinate dehydrogenase gene, which is associated with mitochondrial activity. A previous study, conducted on *Apis cerana*, found that mixture of fungicides containing boscalid significantly upregulated gene transcripts associated with immune response and coding for enzymes involved in oxidative phosphorylation and metabolism (Dong et al. 2023). The observed decrease in transcripts related to cellular respiration, notably cytochrome b, could potentially reduce the bees' flight capacity (Chance & Sacktor 1958). It has been shown that high doses of boscalid can reduce thorax size (Glass et al. 2021), which may impact foraging by decreasing wingbeat frequency (Liao et al. 2019). Furthermore, under field conditions, boscalid has been found to reduce the survival rate of worker bees (Fisher II et al. 2022).

We also observed an upregulation of the vitellogenin gene associated with development. A study conducted on queens exposed to the same doses of pesticides had a significant impact on vitellogenin gene expression in queens (Pineaux et al. 2023). Long-term upregulation of the vitellogenin gene could lead to earlier foraging behavior (Fisher et al. 2021a, b). These findings highlight the potential effects of boscalid exposure on long-term capacity of bees. Young bees exposed during the larval phase exhibited changes in motion behavior (Tadei et al. 2019).

4.4. Individual sensitivity in pesticide

In our study, we observed different sensitivities to boscalid in workers from different colonies. The queens used were sisters, but the workers could be genetically distinct, which may have influenced their sensitivity to pesticides. For example, some colonies may possess natural pesticide resistance genes, making them less likely to be affected by certain substances (Rinkevich et al. 2015).

4.5. Commercial solutions and pesticide synergy

Literature reviews reveal a significant lack of information on the sublethal and combined toxicity of pesticides to bees, particularly in regulatory toxicological bioassays (Barascou et al. 2021; Tosi et al. 2022). This is especially considering given the substantial variety of

different pesticide residues found in all hive matrices (Friedle et al. 2021; Sanchez-Bayo and Goka 2014; Tosi et al. 2018). Our study focused on one commercial formulation, Pictor Pro®, and we did not observe specific effects of this formulation at sublethal doses of 6.1 ng/larvae). However, there is some evidence in the literature of adverse effects caused by adjuvant ingredients present in these formulations, such as increased nuptial flight mortality after exposure of queen bees (Pineaux et al. 2023). This highlights the need to assess the risk of inactive ingredients and commercial pesticide formulations to bees (Wernecke et al. 2022). Other commercial products, in particular Pristin®, a mixture of boscalid, and pyraclostrobin, have been shown to affect bees' individual capacities, in particular, on the size of their thorax and their microbiota, their pollen consumption behavior, and reductions in colony population (Fisher et al. 2021a, b; Fisher II et al. 2022; Glass et al. 2021).

4.6. Limits of laboratory tests

While laboratory sublethality studies are useful for addressing compound-specific concerns, it is crucial to complement them with field and semi-field trials which may reveal different effects (Thompson et al. 2007). Laboratory studies can underestimate the true effects of fungicides by failing to accurately reflect the complex interactions and real-world conditions that bees encounter in their natural environment (Henry et al. 2012). The sensitivity of bees to boscalid may vary according to different factors, such as the caste and age of the bee, the general health of the colony, and the interaction between other pesticides and pathogens, as has been shown with other pesticides (Coulon et al. 2020; Wood et al. 2020). This has also been demonstrated with many pesticide combinations (David et al. 2016; Wood et al. 2020). Therefore, it is important to conduct in-depth studies to assess the specific effects of boscalid on different bee species and to consider the complex interactions between this pesticide and other environmental stressors that bees face. Despite its usefulness in crop protection, it is crucial to better understand the potential effects of boscalid on non-target organisms, particularly bees, which play an essential role in crop pollination and biodiversity preservation. Recent studies have emphasized the need to assess the risks associated with boscalid use and to adopt more sustainable agricultural practices to minimize impacts on bees and other pollinator populations (Perrot et al. 2022; Wintermantel et al. 2019).

5. CONCLUSION

The aim of this study was to assess the effects of exposure to boscalid at field concentration on the survival, development time, and gene expression of honeybee larvae. The results indicate that boscalid exposure had no significant effect on the survival and emergence of the larvae compared with the control groups. However, we observed effects on gene expression, suggesting that boscalid impact on certain physiological processes in honeybee larvae. Larvae exposed to boscalid showed significantly lower levels of the expression of certain genes associated with detoxification and mitochondrial activity (catalase and cytochrome b). Additionally, analyses revealed late impacts on adult bees exposed during the larval phase, with effects on genes linked to mitochondria and development (cytochrome b, succinate dehydrogenase, vitellogenin). Notably, the effects observed with the boscalid molecule were not observed with the commercial formulation Pictor Pro®. Differences in gene expression between larvae and adult bees from different colonies indicate the influence of origin of the expression levels of the genes studied. This highlights the importance of considering individual and environmental variations when interpreting the results. Overall, this study contributes to our understanding of the potential effects of boscalid exposure on larvae. The results suggest that boscalid can modulate the gene expression of certain

enzymes and mitochondrial activities. Although the functional consequences were not explored in this study, the results obtained help to explain the physiological and behavioral impacts on bees described in the literature.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

VDP: Methodology and analysis. SG, CMV, DR, and TL: Resources editing. VDP, FJR, and PA: Writing. VDP, FJR, and PA: Conceptualization and investigation. FJR and PA: Funding acquisition; supervision; and project administration.

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DATA AVAILABILITY

All the relevant experimental data are included in the manuscript.

DECLARATIONS

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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