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Fipronil alters the *circadian clock-controlled protein* gene and the action of juvenile hormone in bees, *Apis mellifera* L.

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Abstract

Apis mellifera bees are considered efficient pollinators of wild plants and crops; however, during the food search, foraging bees can become contaminated with persistent insecticides in the environment, which can harm bees' health lethally or gradually over time. Gene expression analysis is a way of investigating the changes caused in the body by pesticide contamination. Using the transcriptome, the present study investigated the effect of fipronil on the gene expression of bees in the forage phase contaminated by ingestion for 4 hours. The insecticide fipronil, at an environmentally relevant dose (2.5 ppb), downregulated the transcription of the *circadian clock-controlled protein* gene, a gene encoding transport proteins controlled by the circadian rhythm, suggesting that exposure to fipronil can affect the circulation of juvenile hormone in the body and, consequently, harm the development of the colony.

Introduction

The biological or circadian clock regulates numerous cellular, molecular, and physiological processes, including breathing, sleep cycles, locomotion, body temperature, food metabolism, and immune system functioning (Patke et al., 2020). In bees, this mechanism also influences mating, oviposition, larval development, pupation, and hatching of the adult individual (Saunders, 2020). In foragers, the circadian clock interferes with the cognitive system, navigation, solar compass, and communication capacity for recruitment in the search for food resources (Tomioka & Matsumoto, 2015).

In *Apis mellifera*, the circadian clock also regulates the juvenile hormone binding proteins (JHBP), responsible for transporting and protecting juvenile hormone (JH) from degradation by the enzyme juvenile hormone esterase (JHE) found in the hemolymph (Marchler-Bauer et al., 2017). During the journey between the *corpora allata* gland and the target tissue (Figure 1), JHBP proteins regulate their availability and activity in target tissues, acting as a reservoir that gradually releases them when necessary to regulate development and bee behavior (Suzuki et al., 2011).

Foragers are the most susceptible to environmental risks, such as pollutants and pesticide exposure. These factors

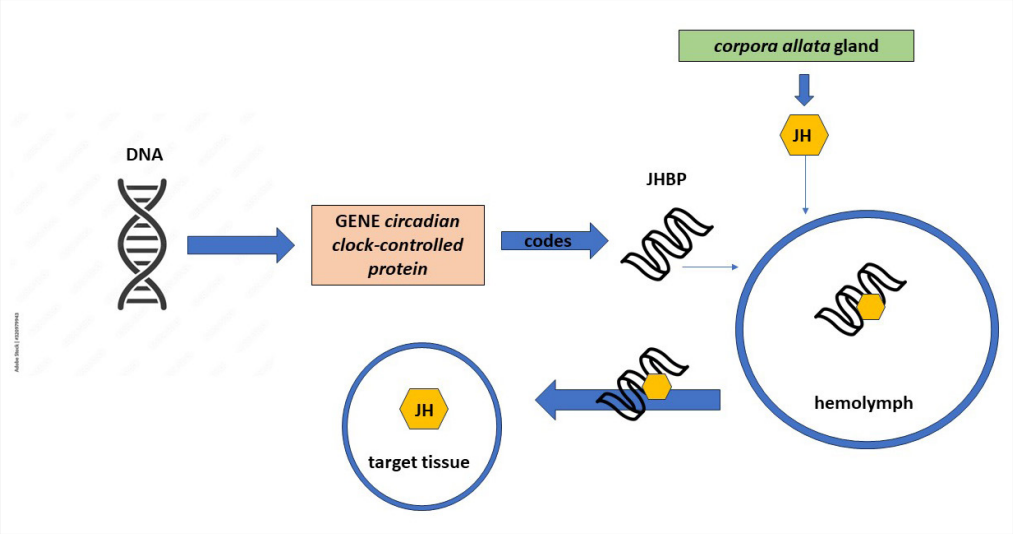

Fig 1. Process from encoding the juvenile hormone binding protein (JHBP) that binds to free juvenile hormone in the hemolymph, transporting it to the target tissue.

can weaken their health, even in sublethal doses, which do not kill bees immediately, but cause morphological, physiological, and behavioral disorders (Traynor et al., 2021). Phenylpyrazole insecticides, such as fipronil, are examples of harmful pesticides for bees as they act in the synaptic neural region, blocking the action of the gamma-aminobutyric acid (GABA) receptor, and preventing the entry of chlorine ions into the postsynaptic neuron. The result is a flow of uninterrupted nerve impulses, leading to exhaustion of the central nervous system until the insect dies (Simon-Delso et al., 2015).

Given the above, the present study aimed to identify, through transcriptome analysis, changes in the gene expression related to the juvenile hormone of *Apis mellifera* caused by the insecticide fipronil.

Material and methods

The experiment used Africanized *A. mellifera* colonies maintained in a standard Langstroth hive, which standardized the number of brood frames and food, containing naturally mated queens. Two frames containing closed broods were wrapped in perforated tissue from each colony and later returned to the hives. After emergence, around 250 bees were marked on the thorax with a non-toxic pen (Uni posca, Mitsubishi Pencil, Tokyo, Japan) and reintroduced to the original colonies (De Barros et al., 2021; Astolfi et al, 2022).

After twenty-one days, 120 marked bees were collected and distributed into six Petri dishes (three replicates per treatment), fasting for three hours to empty the crop. The control group received a mixture of honey and water at a concentration of 1:1, and the fipronil group was challenged with the same food plus the insecticide (Regent 800WG - BASF Agri-Production SAS), at its environmentally relevant dose of 2.5 ppb (Zaluski et al., 2020). Feeders were weighed before and after consumption. The bees were exposed for four hours, and after this period, 15 bees from each group were anesthetized at a temperature of approximately 5°C, placed in a Falcon tube, and sent for transcriptome analysis (Astolfi et al., 2022).

TRIzol ® method protocol, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, USA), and each sample passed through the QubitTM Fluorometer 2.0 (Thermo Fisher Scientific, Waltham, USA) to quantify extracted concentrations.

cDNA libraries using the SureSelect Specific RNA Library Preparation Kit Yarn (Agilent Technologies, Santa Clara, CA, USA) as per the manufacturer's instructions, and library products were sequenced using the Illumina NextSeq Platform (Illumina, San Diego, CA, USA).

To examine adapter content and evaluate the quality of raw reads, the FASTQC program was used, and data alignment was performed using the Burrows-Wheeler aligner (BWA) v0.7.12, employing the NCBI reference Amel_HAv3.1 (RefSeq with access appropriate: GCF_003254395.2).

The feature count matrix was generated using HTSeq v0.11.2 and the GTF annotation file from Amel_HAv3.1; For data analysis, visualization and plotting, RStudio was used in the R language and ggplot2 v3.3.2 package and performed the differential expression analysis, the edgeR v3.30.3 package was used, available in the Bioconductor software project for R. Genes with low expression were filtered, remaining genes that presented a count per million greater than one in at least two samples; the normalization of counts was performed with TMM normalization and the generalized log-linear negative binomial was applied in the differential expression analysis, with the Benjamini and Hochberg procedure (FDR) for test corrections. Genes with adjusted p values < 0.05 were considered significantly misregulated.

Results

Regarding food consumption, there was no significant difference between the control and treatment groups (47.0 ± 7.7 µL/bee and 51.4 ± 30.2 µL/bee, respectively).

Forager bees contaminated with a sublethal dose of fipronil showed a 42% downregulation in *circadian clock-controlled protein* gene expression compared to bees in the control group. This gene encodes the GB13060-PA protein, a member of the JHBP family.

Table 1. Differentially expressed gene.

Number of NCBI access	Gene locus	Gene name	General gene function	log2FoldChange	Direction of regulation
XM_001122696.5	LOC726981	*circadian clock-controlled protein* (protein regulated by the circadian clock)	Youth hormone transporter protein	-1.252075785	Downregulated (42%)

Discussion

The work demonstrated that the insecticide Fipronil, even at environmentally relevant doses, promoted a reduction in *circadian clock-controlled protein* gene expression, whose function is the transcription of juvenile hormone (JH) transporter protein.

This hormone helps regulate the bee's life cycle, from the larval stage to the adult stage, acting on embryogenesis, regulating the transition between the different stages of development, such as the transformation of larva into pupa and pupa into adult bee; acting as a signal for the start of these transitions, ensuring that they occur at the right time (Robinson; Vargo, 1997). During the larval stage of the future queen of *Apis mellifera*, JH is produced in high quantities, causing polyphenism, a phenomenon of caste differentiation (queen and worker bees) that present different morphological, physiological, and behavioral characteristics, which can be influenced by age, function in the colony, and environmental conditions. This mechanism is an adaptive strategy that promotes the reproductive and functional maintenance of the colony, in addition to maintaining behavioral control of adult worker bees through neurogenesis (Hartfelder et al., 2012).

Previous studies indicate that morphological changes were caused by fipronil contamination, resulting in a 46% reduction in the height of the epithelial secretory cells of the mandibular gland and a decrease in the number and area of acini in the hypopharyngeal glands of nursing bees exposed to this insecticide compared to honey bees. From the control group, modifications that affect the production of protein components of royal jelly, compromising their quality and quantity, and consequently, impairing the nutrition of young larvae and queens (Zaluski et al., 2017; Zaluski et al., 2020).

Poor quality royal jelly can result in the birth of a deficient queen, since in the larval development of the *Apis mellifera* queen bee, royal jelly enhances the activation of biochemical signaling pathways, insulin/IGF systems (insulin/ Insulin-like Growth Factor) and TOR (Target of Rapamycin), increasing the synthesis of juvenile hormone in the *corpora allata* (Cameron et al., 2013), causing body enlargement, especially abdominal, differentiation of ovarian cells and inhibition of specific structures of worker bees, such as corbiculae in the last pair of paws and well-developed hypopharyngeal glands (Li et al., 2021).

Decreased *circadian clock-controlled protein* gene expression can promote the reduction of JH in target tissues, which can affect the metamorphosis process, altering cell differentiation and normal development of tissues and organs that form the new individual (Xu et al., 2022). Colonies contaminated with fipronil exhibit physiological changes in the larvae, preventing their complete growth until reaching the last stage; apparently, the most serious anomalies occur in the head and thorax of pupae, sites of synthesis for juvenile and ecdysteroid hormones that act in larval development, pupation, and emergence of adult bees (Robinson & Vargo, 1997).

Changes in JH levels they can also affect the distribution mechanism of functions within the colony; this situation was observed in the study that tested the treatment of young adult bees with synthetic JH, causing early foraging stimulation (Lippi et al., 2024), as well as the reduction of JH, after removal of the *corpora allata* gland, caused the delay in bee promotion to foraging activity (Sullivan et al., 2000).

Conclusion

The results of the present work suggest that honey bees exposed to fipronil exhibit a reduction in the expression of the *circadian gene clock-controlled protein*, resulting in a decrease in the coding for JHBP proteins and consequently affecting the distribution of juvenile hormone in the bee's body.

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Authors' Contributions

All authors contributed similarly to the subject conception, writing, and manuscript review.

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