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Imidacloprid increases the prevalence of the intestinal parasite *Lotmaria passim* in honey bee workers

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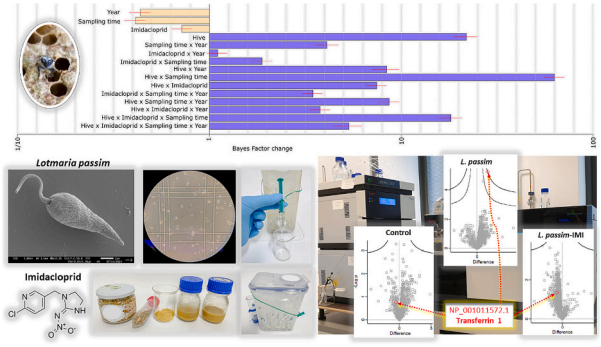
HIGHLIGHTS

- Sublethal exposure to imidacloprid at 2.5 μg/L boosts infection by *L. passim*.
- A single xenobiotic compound can elicit harmful effects on *L. passim*.
- Novel data support the harmful effects of imidacloprid on natural systems.
- Imidacloprid neutralized the increased transferrin 1 expression by *L. passim*.
- A methodology for risk assessment of pesticide–*L. passim* interaction was optimized.

GRAPHICAL ABSTRACT



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ABSTRACT

A challenge in bee protection is to assess the risks of pesticide-pathogen interactions. *Lotmaria passim*, a ubiquitous unicellular parasite in honey bees, is considered harmful under specific conditions. Imidacloprid causes unpredictable side effects. Research indicates that both *L. passim* and imidacloprid may affect the physiology, behavior, immunity, microbiome and lifespan of honey bees. We designed cage experiments to test whether the infection of *L. passim* is affected by a sublethal dose of imidacloprid. Workers collected at the time of emergence were exposed to *L. passim* and 2.5 μg/L imidacloprid in the coexposure treatment group. First, samples of bees were taken from cages since they were 5 days old and 3 days postinfection, i.e., after finishing an artificial 24 h *L. passim* infection. Additional bees were collected every two additional days. In addition, bees frozen at the time of emergence and collected from the unexposed group were analyzed. Abdomens were analyzed using qPCR to determine parasite load, while corresponding selected heads were subjected to a label-free proteomic analysis. Our results show that bees are free of *L. passim* at the time of emergence. Furthermore, imidacloprid considerably increased the prevalence as well as parasite loads in individual bees. This means that imidacloprid facilitates infection, enabling faster parasite spread in a colony and potentially to surrounding colonies. The proteomic

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analysis of bee heads showed that imidacloprid neutralized the increased transferrin 1 expression by *L. passim*. Importantly, this promising marker has been previously observed to be upregulated by infections, including gut parasites. This study contributes to understanding the side effects of imidacloprid and demonstrates that a single xenobiotic/pesticide compound can interact with the gut parasite. Our methodology can be used to assess the effects of different compounds on *L. passim*.

1. Introduction

Approximately 80 % of flowering plants are dependent on animal-mediated pollination. The weakening of pollination services has direct economic consequences and endangers sustainable food production (Klein et al., 2007; Potts et al., 2010; Ollerton et al., 2011). Apart from so many species of wild pollinators, managed honey bees are the key pollinators of both agricultural crops and native plants. Therefore, honey bees critically contribute to food production and maintaining plant diversity (Klein et al., 2007; Gallai et al., 2009; Calderone, 2012). In addition, beekeeping is an important cultural heritage (Tautz, 2008). The actual number of managed colonies is affected by popularity and interest in beekeeping, and this hobby has been in boom in recent years. Although the current number of beehives is not in decline and tends to uptrend on a global scale (Phiri et al., 2022), high annual losses of honey bee colonies continue despite the extensive worldwide effort by the scientific community to reveal the causes. In recent years, overwintering losses of honey bee colonies have increased, and in some countries and regions, losses commonly reach over 30 % but have been reported to be up to >50 % (Bruckner et al., 2020; Ferland et al., 2022; Gray et al., 2023). The causes of colony deaths have been accurately identified only in some cases and are usually linked to official governmental investigations on the diseases listed in the WOAH Terrestrial Animal Health Code – mainly varroosis, American foulbrood and European foulbrood (WOAH, 2022) – and officially confirmed pesticide poisoning incidents (Barnett et al., 2007; Kadlikova et al., 2021). However, most honey bee colony losses remain unexplained, and the general causes are obscured by the complexity of the different biotic and abiotic factors at play (vanEngelsdorp et al., 2009; Goulson et al., 2015; Motta et al., 2018; Insolia et al., 2022; Gray et al., 2023; Pervez and Manzoor, 2023). Research has indicated that bees can be negatively affected by pesticides through the effect of the latter on microorganisms associated with honey bees. Exposure of honey bees to pesticides can affect the beneficial gut bacterial microbiome, which may lead to dysbiosis (Kakumanu et al., 2016; Motta et al., 2018; Rouze et al., 2019; El Khoury et al., 2021). However, pesticides can also interact with honey bee pathogens, and coexposure can be the main driver of dysbiosis (O'Neal et al., 2018; Paris et al., 2020). Currently, one of the key challenges in bee protection is to assess the risks of pesticide–pathogen interactions by investigating sublethal effects and realistic exposure levels (Harwood and Dolezal, 2020; Straub et al., 2022). Thus, there is a need to perform controlled experiments to assess the impact of these interactions. Moreover, the experiments should be adjusted to pathogens and the tested pesticides since the biological processes that these factors affect alone and together with potential synergistic effects can be different.

Various pathogens and pests belonging to diverse groups, including viruses, bacteria, fungi, protists, mites and insects, can harm honey bees (vanEngelsdorp et al., 2009; Genersch et al., 2010; Insolia et al., 2022). Many of the diverse honey bee pathogens are traditionally recognized because the disease signs are obvious. However, numerous other pathogens cannot be recognized by beekeepers and veterinarians and are difficult to identify by researchers. One such case is that of bee-gut-inhabiting trypanosomatids, which can appear similar to other factors affecting honey bee physiology, behavior, immunity, microbiome and lifespan (Runckel et al., 2011; Ravoet et al., 2013; Schwarz and Evans, 2013; Strobl et al., 2019; Arismendi et al., 2020). Importantly, the recognition of the main honey bee trypanosomatid was inaccurate until later, when the traditionally recognized *Crithidia mellificae* was distinguished from a newly described and globally more dominant trypanosomatid, *Lotmaria passim*, by Schwarz et al. (2015). Although *L. passim* is the predominant trypanosomatid in the honey bee *Apis mellifera* worldwide, its pathogenic effect on the host is not clear (Arismendi et al., 2020; Gomez-Moracho et al., 2020; Nanetti et al., 2021). Research has shown that the prevalence of *L. passim* can vary in honey bee colonies and apiaries, and coinfections with other gut parasites occur (Hubert et al., 2017; Tritschler et al., 2017; Michalczyk et al., 2020, 2022a; Williams et al., 2021; Aguado-Lopez et al., 2023). In addition to year and season affecting the prevalence of *L. passim* (Stevanovic et al., 2016; Vejnovic et al., 2018), substantial variations in *L. passim* incidence have been observed in workers of different ages and developmental stages (larva, pupa, adults) (Arismendi et al., 2022; Michalczyk et al., 2022b). However, the absence of the parasite in eggs indicated that transovarial transmission does not occur (Arismendi et al., 2022). Currently, it is not clear whether *L. passim* is present in bees at the time of emergence because, to our knowledge, such samples were not analyzed since groups of newly emerged bees were collected from brood frames (Schwarz and Evans, 2013; Gomez-Moracho et al., 2020; Arismendi et al., 2022; Buendia-Abad et al., 2022).

Pesticides are among the important factors that are necessary to consider in relation to honey bee colony losses. Although diagnosing acute poisoning due to pesticide exposure is important, sublethal pesticide exposures can have higher effects if the entire colony is further affected. The threat of sublethal effects of pesticide exposure is that they may gradually impact colonies unnoticed by beekeepers, i.e., these effects include mainly disordered development, longevity, immunity, reproduction and behavior (Wu et al., 2011; Sanchez-Bayo and Goka, 2014; Goulson et al., 2015; Kadlikova et al., 2021; Douglas et al., 2022). Realistic field exposure to neonicotinoids poses sublethal risks for honey bees and other pollinators (Easton and Goulson, 2013; Stanley et al., 2016; Tsvetkov et al., 2017; Siviter et al., 2021) and has become one of the main focuses of scientific and government interest in relation to potential harmful effects on bees, beneficial invertebrates and biodiversity in general (Frank and Tooker, 2020; Klingelhofer et al., 2022). These insecticides are systemic; however, this mode of action, which is highly beneficial in pest control, also poses a high risk to pollinators, which deliver the substances to colonies in nectar and pollen. Although the doses are low, the risk of sublethal field-realistic doses to pollinators is high (Blacquiere et al., 2012; EFSA, 2012; Goulson, 2013). Due to these risks, the use of three highly toxic neonicotinoids (imidacloprid, clothianidin and thiamethoxam) commonly used in seed dressings has been severely restricted in the European Union (EU) since 2013 (EC, 2013). The ban was later confirmed in 2018 (EC, 2018), and the approval of their use in plant protection products (PPPs) has expired in the EU (EC, 2023). Additionally, outside the EU, re-evaluations of the neonicotioids are intensively re-evaluated, and the authorities' finalized reviews and others are ongoing. In general, outside the EU, there is not such a strict attitude to the three neonicotinoids, and instead, they were not, until present, evaluated as dangerous for bees and other pollinators, although earlier serious concerns indicated banning. Thus, according to re-evaluation decisions by the Pest Management Regulatory Agency (PMRA), the three are currently approved for use in Canada (PMRA, 2019a, 2019b, 2021). Notably, the United States Environmental Protection Agency (EPA) plans to complete an extensive review in 2024 (EPA, 2023). Furthermore, for instance, they are also approved in Brazil (Friedrich et al., 2021; de Assis et al., 2022).

Imidacloprid (IMI) poses the highest risk to nontarget organisms,

especially pollinators, as indicated by many studies, but the other neonicotinoids have similar risks to some extent. The adverse sublethal effects of neonicotinoids on honey bees (and other bee species) can be summarized as impaired neural function, navigation, learning, memory, longevity, reproduction, and immunity (Bortolotti et al., 2003; Medrzycki et al., 2003; Decourtye et al., 2004a, 2004b; Feltham et al., 2014; Tan et al., 2014; Ciereszko et al., 2017; Walderdorff et al., 2018). However, IMI causes insidious side effects, with delayed and time-cumulative toxicity (Rondeau et al., 2014; Dively et al., 2015; Sanchez-Bayo and Tennekes, 2020). These effects of IMI can be explained by its metabolism, since it is transformed to the more toxic metabolite IMI-olefin in bees (Suchail et al., 2001; Erban et al., 2019b) and plants (Seifrtova et al., 2017). In bumblebees, IMI-olefin showed cumulative properties, and together with the parent compound, sublethal exposure impaired the mevalonate pathway and fatty acid synthesis. In addition, IMI and IMI-olefin were shown to interact with sterol regulatory element-binding protein cleavage-activating protein (SCAP) (Erban et al., 2019b). These results are consistent with the suggested endocrine disruptive effect of IMI in animals (Baines et al., 2017; Mikolic and Brcic Karaconji, 2018). Importantly, the risks of IMI must be evaluated further because the side effects have not been explained completely in all contexts. In addition, IMI is still present in the environment because it can be used in PPPs in different countries outside the exemplary EU ban. In addition, IMI is currently authorized for use in biocides (e.g., against ants) in the EU (EP, 2021; ECHA, 2023). Note that in the EU, PPPs and biocides are differently regulated. For instance, IMI was recently detected in the larval provisions of solitary bees in croplands and community gardens (allotments) in Czechia, an EU member in central Europe (Slachta et al., 2023).

The high importance of IMI side effects and the fact that *L. passim* and IMI have been identified to cause similar effects on honey bee colonies in some respects (effects on bee physiology, behavior, immunity, microbiome and lifespan) makes it possible that their simultaneous presence in bees may result in synergistic effects on bees. Incidentally, IMI exposure has been identified to increase the abundance of the gut microsporidian parasites *Nosema* spp. in honey bees (Pettis et al., 2012). Note that *Nosema ceranae* and *Nosema apis* were reclassified as *Vairimorpha ceranae* and *Vairimorpha apis*, respectively (Tokarev et al., 2020).

In this study, we sought to determine whether low sublethal exposure to IMI affects the abundance of another intestinal parasite, *L. passim*. We optimized the experimental procedure to test the interactions between the gut parasite and pesticides, while the additional objective was to determine whether bees at the time of emergence contained *L. passim*. Additionally, we analyzed the heads of honey bees using label-free proteomics to identify the possible effect of *L. passim* and IMI coexposure on bees.

2. Materials and methods

2.1. Experimental honey bees

The honey bees used in this study originated from an apiary at the Crop Research Institute (CRI), Ruzyne, Prague. The queens of colonies were from the same genetic line, and in the particular year of analysis, the queens were sisters. Only colonies with no obvious disease symptoms were used for the analyses and manipulative experiments. This means that the colonies rapidly built up, and damaged cappings or varroosis symptoms were not observed.

Several types of analyses were performed in this study. Some of the analyses were used for verification of *L. passim* in sample types, although the main analysis focused on *L. passim*—IMI interactions. The main bee samples were emerging bees since they were used in manipulative experiments, but numerous emerging bees were directly frozen and used to verify the absence of the parasite *L. passim*. Prior to the collection of emerging bees, frames were taken from the colony for a short period of approximately a few minutes. It was repeated until enough bees were collected. Note that the frames were not placed in a thermostat to allow the bees to emerge. Instead, the bees were individually collected at the time of emergence, just after chewing through the cell caps. All the collected emerging bees were verified to be unparasitized by *Varroa destructor* mites in the cells, were without any obvious defects, were not fed by other bees and did not feed on nutrients from the comb. **i)** Some of the emerging bees were placed in Eppendorf tubes, and immediately after collection, they were frozen on dry ice and later stored in a deep freezer until use. These samples were used to verify using qPCR the absence/presence of *L. passim* in the bees at the time of emergence. **ii)** Most of the emerging bees were used in a manipulative experiment (Section 2.4) to investigate the effect of time and IMI on the abundance of *L. passim*. **iii)** To estimate the prevalence of *L. passim* infection in the apiary, we shook down bees to a plastic bag from a brood comb from a selected colony, and the bag was immediately placed in a box with dry ice. The bees were subsequently stored in a deep freezer until use. Furthermore, 90 randomly selected bees from each of the three colonies were analyzed using qPCR.

2.2. Lotmaria passim axenic clonal culture

For exposure to the parasite, axenic cell cultures of *L. passim* were prepared. The strain was isolated in May/June 2020 from the hindgut of honey bees collected at the Bee Research Institute in Dol (Maslovice, Czechia). Bees were dissected within 6 h after capture, and the removed digestive tract of infected individuals was processed for cultivation, axenization, and cloning following a previously described protocol (Votypka et al., 2020). Parasites were maintained at 23 °C in brain heart infusion (BHI) medium (Bacto™) supplemented with hemin (10 mg/mL in 0.1 M NaOH), 10 % heat-inactivated fetal bovine calf serum (FBS; Gibco), and 250 μg/mL amikacin (Amikin, Bristol-Myers Squibb). The cells were then placed in cell culture flasks (Nunc) in a horizontal position and incubated at 23 °C, and every 2 weeks, they were transferred to new flasks. Before experimental infection, the isolated clone of *L. passim* was multiplied in a thermostat, washed by centrifugation (1,500 ×*g* for 5 min), resuspended in saline solution and counted using a hemocytometer (Burker counting chamber) under a microscope. The parasite *L. passim* was provided to bees in 20 % sugar beet solution from a syringe (Fig. 1).

2.3. Experimental cage, feed, IMI

For manipulative experiments, 1,800 mL (15.1 × 10.8 × 18.5 cm) angular LOCKnLOCK plastic food storage containers/jars with a pour spout with a flap cover on the lid were used. Each box was adjusted for the experiments (Fig. 1). The round lid of the pour spout was punctured to allow air flow, but to prevent contamination, the lid was covered with a filter paper disc. Two holes were made on the sides: i) one for the feeder to provide nutrition during the experiment and ii) one for the syringe to perform an oral parasite infection. The inside of the container was smeared with virgin beeswax obtained from a young bee colony without any previous veterinary/pesticide treatments. At the bottom, a sand mat for birds (Wivral) was placed to maintain cleanliness in the cage. In addition, hydrogel (Floria) in a bag made of white nonwoven fabric was used to prevent wetting of the cage.

For preparation of feed for the bees during the experiment, we used Apisyrup 71/73 (E D & F Man Ingredients). In 2021, the stock syrup had the following exact parameters: i) Brix 72.8; ii) pH 4.2; iii) hydroxymethylfurfural (HMF) 29 ppm; iv) sugars: fructose/glucose/saccharose of 42/32/26 ratio; and other sugars were under the limit of quantification (LOQ), which was 0.5 % based on the HPLC method. In 2022, the exact parameters of the stock syrup were i) Brix 72.4; ii) pH 3.8; iii) HMF 24 ppm; and iv) sugars: fructose/glucose/saccharose of 40/28/32 ratio. In addition, we enriched the syrup with proteins because sugars alone are not an appropriate diet for long-term experiments. Note that pollen

Fig. 1. Experimental cage.

and other plant-derived/nectar proteins are commonly present in honey, and hive bees use bee bread prepared from pollen and nectar/pollen to feed the brood. The source of proteins was bee pollen from a local beekeeper (Naturalis) at a site far from agricultural pesticide use in the White Carpathian Mountains in South Moravia (Czechia). Note that we used the same batch of pollen in all experiments, while the glass packages were stored unsealed at 5 °C and in the dark until processing. The final content of pollen was calculated to be 5 g in 1 kg of the syrup-derived feed. To remove residual water, the pollen was lyophilized overnight in tubes covered with filters in a PowerDry LL3000 (Thermo). Then, the pollen was pulverized in a coffee mill (Bosch). Furthermore, the pollen was dissolved at a ratio of 1 g per 20 g in boiled water. In addition, the pollen in water solution was mixed using a submersible ultrasonic homogenizer (Bandelin) at 10-s pulses for 2 min. A series of 20-mL aliquots of the homogenized pollen-water stock solution were autoclaved in 100-mL glass bottles (Simax). Thus, any microbial contamination from pollen as well as spoiling were prevented. The bottles were stored at 5 °C in the dark until use. The procedure of preparation of these pollen stocks in bottles is illustrated in Fig. S1. For feeding the bees during experiments, pollen-water feed was freshly prepared, while syrup was dissolved to a final 50 % concentration.

A water stock solution of IMI was prepared from a powdered PESTANAL® analytical standard (Supelco, Sigma–Aldrich). With respect to solubility in water, IMI was solubilized in boiled water in the dark overnight by continuous stirring using a magnetic stirrer. The obtained stock solution was divided into aliquots that were stored in a freezer at −28 °C until use. Note that we did not use any additional dissolving (organic) agent other than water. The required content of IMI was added during the process of dissolving the Apisyrup to the 50 % experimental concentration. The final IMI concentration in the syrup-pollen feed was 2.5 μg/L.

2.4. Manipulative experiment Lotmaria-IMI

A schematic of the experimental procedure with the timing of feed provision, *L. passim* exposure, IMI and sample collection is shown in Fig. 2. During the experiments, bees were kept at an optimum brood rearing temperature of 35 °C (Medrzycki et al., 2010; Cho et al., 2022) in precise (accuracy ±0.2 °C) microprocessor-controlled stainless thermostats with forced air circulation (POL-EKO-Aparatura). First, 50 bees that were collected at the time of emergence were placed per box. The bees were collected in a short period of time and the time of the beginning of the feeding in each box was recorded. All bees were adapted for the first 24 h and were fed syrup-pollen feed. Then the feed was removed, and 24 h of infection with *L. passim* in a sugar solution was performed using a syringe. Next, bees were fed syrup-pollen feed, which in the IMI exposure group was enriched with IMI to a final concentration of 2.5 μg per L of feed. Feed was repeatedly replaced with fresh. Briefly, 6 workers were collected from every experimental box every other day, with the first collection performed 72 h (3 days) after ending *L. passim* infection. The experimental exposure of *L. passim* per 10 μL was calculated to be ~1.5×10^4 cells. The minimal concentration for infection is considered 5,000–25,000 cells (Brown et al., 2003; Logan et al., 2005). Low passages were used in the experiment. In 2021, cell culture passage 8 was used, while in 2022, passage 11 was used.

2.5. qPCR analysis

For qPCR analysis, entire abdomens of bees that were cut from deep-frozen bees were used. The abdomens were surface-sterilized using 96 %

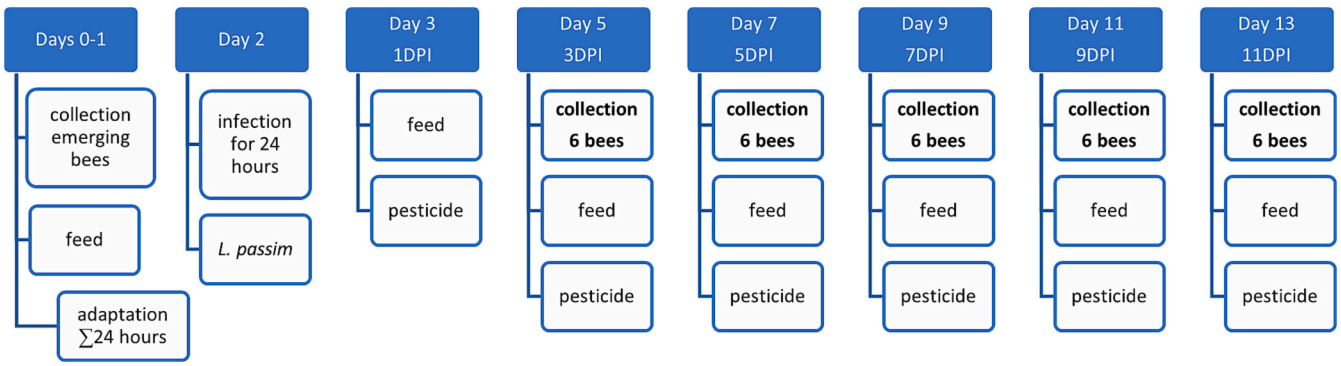


Fig. 2. Schematic of the experimental procedure with timing of feed provision, *L. passim* exposure, IMI exposure and sample collection. DPI – days postinfection (days after termination of 24 h of *L. passim* exposure).

ethanol and cleaned using a sterile physiological solution. DNA was extracted using a High Pure PCR Template Preparation Kit (Roche Life Science) according to the recommended instructions. Primers targeting the *L. passim* cytochrome *b* and Hymenoptera 18S rRNA genes and TaqMan probes (HEX and Cy5, respectively) were used according to Xu et al. (2018), and each sample was measured in technical triplicates. The entire preparation process for qPCR was performed in a flowbox on ice. The reaction mixture contained 5 μL of Maxima Probe/ROX Master mix (Thermo), 1 μL of primers (reverse + forward) and probe (at final concentration of 100 nM), and 2 μL of nuclease-free water. Eight microliters of this mixture was pipetted into a 96-well PCR plate, and 2 μl of DNA or negative control (water) was added to each well. To calculate the amount of DNA in the sample, standards of known concentration were prepared. Cells were counted in a Burker chamber under a microscope and subsequently diluted. Between each subsequent dilution, the previous suspension was mixed and vortexed. The volume of the standards was chosen to be the same as the volume of the elution solution for DNA isolation from bee abdomens (200 μl) to better calculate the DNA concentration in a bee sample. This dilution series (covering a range of 3.2×10^2–5×10^6 of cells) was also added to the plate, sealed with a coverslip, and centrifuged for 30 s at 3000 ×*g*. The analyses were performed on a LightCycler® 480 System (Roche), with the cycle starting with 10 min of initial denaturation (95 °C), followed by 40 cycles of denaturation (95 °C for 15 s), annealing (60 °C for 30 s), and extension (72 °C for 30 s).

2.6. Data analysis

The data were analyzed in R version 4.2.2 (R Core Team, 2022). The data were evaluated using multifactor *analysis of variance* (*ANOVA*). In addition, we comprehensively compared impact factors such as replicate, time of sampling (age of bees/days post infection), year of experiment, exposure (*L. passim*, IMI), hive (experimental cage) and interaction between factors with Bayesian statistics (Morey et al., 2015; Puga et al., 2015) using the BayesFactor package in R v0.9.12–4.4 (Etz, 2015).

2.7. Proteomic analysis of honey bee heads

Based on the results of qPCR analysis, honey bee heads that remained after the abdomen was cut were selected. Overall, five heads of bees were selected from each of the experimental exposures performed in 2021: i) control without *L. passim* and IMI exposure and with confirmed no *L. passim* detected; ii) bees positive for *L. passim* from the experimental exposure of *L. passim*; and iii) bees positive for *L. passim* from the experimental exposure of *L. passim* combined with 2.5 μL IMI coexposure. The heads were homogenized, processed and analyzed analogously to as described earlier (Erban et al., 2019b), but carbamidomethyl was the fixed modification. Briefly, the tryptic digests were analyzed by Dionex Ultimate 3000 nanoliquid chromatography (nanoLC) coupled with an Orbitrap Fusion Tribrid mass spectrometer (Thermo). The data were evaluated using label-free quantification (LFQ) algorithms in MaxQuant version 2.4.1.0 (Cox et al., 2014). The search database of 23,520 RefSeq sequences was downloaded from NCBI on 13 May 2023. Furthermore, the data were evaluated in Perseus version 2.0.7.0 (Tyanova et al., 2016). Briefly, contaminants and low abundance data were removed, the LFQ intensities were log2 transformed, and missing values were replaced by values from normal distribution. Each of the three trials was compared against a complement using Spearman rank correlation. Furthermore, we inspected the original LFQ data before imputation of the missing values to verify the positivity of the results in each of the replicates (honey bee head).

3. Results

3.1. Presence and prevalence of L. passim in the apiary

The qPCR analysis of bees from the brood comb showed that all three tested colonies from the CRI apiary were infected with *L. passim* (Table S1). The estimated natural occurrence of bees positive for *L. passim* in the colonies was 63 % (57 positive per 90 bees), 44 % (40 positive per 90 bees) and 17 % (15 positive per 90 bees). There were large differences in the parasite load in individual *L. passim*-positive bees. In the colony with the highest prevalence, approximately $1.6 \times 10^6 \pm 2.6 \times 10^6$ cells of *L. passim* were quantified in the positive samples. Among the 57 positive bees, 17 bees had very high ($>10^6$) levels of *L. passim* cells, and 19 bees had levels of $>10^5$ cells. In the second colony, 4 bees reached levels $>10^6$ cells, and 4 bees reached levels $>10^5$ cells. In the third colony with the lowest prevalence, all positive bees had $<10^5$ *L. passim* cells. Among all the samples, the top five abundances of *L. passim* in one bee were found to be 9.5×10^6, 9.32×10^6, 8.09×10^6, 8.08×10^6 and 7.70×10^6 cells, while the highest value was found in the colony with the middle prevalence. The other listed values were found in the colony with the highest prevalence.

3.2. Absence of L. passim in bees at the time of emergence

The qPCR analysis of bees that were collected at the time of emergence and consequently frozen on dry ice showed that none of the 60 emerging bees were positive for *L. passim*. *Lotmaria passim* was not detected in 20, 20 and 20 bees analyzed from three different colonies in which the prevalences of 63, 44 and 17 % were recorded, respectively (see Section 3.1). This enabled us to consider that at the time of emergence, the bees were free of the parasite.

Note that except for this analysis, we performed previous analyses of emerging bees on the presence of *L. passim* using nested PCR (unpublished results), and the results of the analysis of 3×20 bees showed that no emerging bees were positive for *L. passim* (unpublished results).

3.3. Absence of L. passim in a control experiment

All 30 bees from a blank experiment (control box) that was performed in the same age-dependent design as the *L. passim* and *L. passim*-IMI exposures in 2021 were negative for the parasite. The results showed that in this trial, no bees between 5 and 13 days of age developed *L. passim* infection. This result supported the finding that the emerging bees (Section 3.2) are free of *L. passim*.

3.4. Exposure to L. passim and L. passim-IMI

Fig. 3 shows details of the positive results and number of *L. passim* cells in the subsequently performed exposures to *L. passim* (Fig. 3A) and *L. passim*-IMI (Fig. 3B) in 2021 and 2022. Infection was successful in all 12 experimental boxes/hives. From each box, 30 bees out of a total of 50 initial bees were successfully collected until the 13th day of age. Thus, from each box, representing a replicate group of bees in a time series, a total of 30 (5×6) bees were collected and analyzed. The lowest infection rate and level of *L. passim* cells were found in the first sampling, i.e., in 5-day-old bees that were three days postinfection (DPI). Later, the infection rate increased, and there was an apparent trend toward increased abundance of *L. passim* cells over time since exposure. Comparison of the individual results of positive cases and absolute abundances of *L. passim* cells per bee showed higher values in the IMI coexposures. The highest numbers of *L. passim* cells per bee in *L. passim*-IMI exposures reached the highest levels of 7.3×10^6, 5.9×10^6 and 5.3×10^6 cells. These cell counts are similar to those observed in naturally infected bees in the CRI apiary (Section 3.1, Table S1). The levels of *L. passim* cells without IMI coexposure were lower, with the top three abundances found in individual bees being 3.7×10^6, 1.9×10^6 and 1.1

$\times 10^6$ cells.

Statistical analysis using multifactor ANOVA (Table 1) as well as Bayesian statistics (Fig. 4) confirmed that IMI increased the prevalence of *L. passim* infection. Additionally, the sampling time or age of the bees had a significant and even higher impact on the results. Furthermore, the statistical analysis showed that there was a difference in the prevalence of *L. passim* infection between 2021 and 2022 when replicates were performed. Importantly, IMI increased the number of positive cases/ prevalence and abundance of *L. passim* cells in both years.

3.5. Proteomic analysis of honey bee heads

Overall, 3217 protein hits of *A. mellifera* were identified using label-free nanoLC–MS/MS analysis. Filtering the dataset of hits that had fewer than 70 % valid values in a group of exposures resulted in 2563 proteins that were processed for detail. Statistical analysis (Fig. 5A) revealed that

A — ***L. passim***

	3DPI	5DPI	7DPI	9DPI	11DPI
	5-day	7-day	9-day	11-day	13-day
A - 2021	-	11,400	33,400	4,500	51,200
	-	-	-	17,400	104,400
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
B - 2021	-	52,200	58,600	-	601,000
	-	24,000	23,000	-	508,000
	-	-	-	-	638,000
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
C - 2021	-	16,960	91,400	-	-
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
A - 2022	91,400	406,000	26,400	133,400	3,660,000
	14,520	99,600	1,888,000	76,600	1,098,000
	4,060	744,000	1,090,000	532,000	-
	165,600	48,200	-	-	-
	-	996,000	-	-	-
	-	-	-	-	-
B - 2022	-	-	-	7,100	-
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
C - 2022	12,340	38,000	139,400	63,800	238,000
	8,720	126,000	-	5,340	13,620
	7,040	18,160	-	-	28,600
	10,120	4,080	-	-	83,200
	-	-	-	-	-
	-	-	-	-	-
P	8	13	8	8	11
%P	22	36	22	22	31
AV	39,225	198,815	418,775	105,018	638,547
SD	58,647	320,177	695,211	178,458	1,059,266

B — ***L. passim*-IMI**

	3DPI	5DPI	7DPI	9DPI	11DPI
	5-day	7-day	9-day	11-day	13-day
D - 2021	-	32,000	-	56,000	1,260
	-	-	-	-	110,200
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
E - 2021	-	39,600	74,000	224,000	145,000
	-	21,800	81,400	2,600,000	142,600
	-	13,120	-	14,980	962,000
	-	-	-	9,200	-
	-	-	-	12,620	-
	-	-	-	6,080	-
F - 2021	22,000	186,200	86,600	270,000	336,000
	358,000	26,600	326,000	16,780	202,000
	39,000	16,500	19,780	182,200	140,800
	-	344,000	18,980	430,000	1,106,000
	-	28,000	13,280	81,800	54,400
	-	-	380,000	-	964,000
D - 2022	14,560	7,860	1,014,000	2,400,000	4,700,000
	3,420	672,000	93,000	5,300,000	8,200
	51,800	198,400	21,800	266,000	638,000
	4,440	67,000	89,600	250,000	314,000
	9,220	224,000	562,000	2,560,000	330,000
	12,780	60,000	398,000	-	-
E - 2022	5,960	118,600	508,000	3,200,000	5,920,000
	81,600	115,600	1,144,000	1,082,000	142,400
	113,600	254,000	2,300,000	428,000	7,260,000
	53,600	444,000	256,000	2,520	-
	13,740	-	-	-	-
	-	-	-	-	-
F - 2022	5,260	296,000	57,400	206,000	1,294,000
	8,020	486,000	64,800	73,600	3,120
	8,800	103,000	17,840	1,490,000	23,400
	-	230,000	2,980,000	2,380,000	1,566,000
	-	316,000	6,540	4,320	2,920
	-	-	35,800	1,156	-
P	17	24	24	27	24
%P	47	67	67	75	67
AV	47,400	179,178	439,534	872,121	1,098,596
SD	85,870	176,071	750,829	1,339,329	1,966,550

Fig. 3. Overview of *L. passim*-infected bees after infection A) without and B) with exposure to 2.5 μL IMI in feed. The experiment was performed in 2021 and 2022. In each year, three replicates of *L. passim* exposure alone and three replicates of *L. passim* exposure combined with 2.5 μL IMI coexposure were performed. Sampling was performed 5 times and consisted of the collection of 6 individual bees.

Legend: DPI – days postinfection; **x-day** – age of bees since emergence; **P** – number of positive bees in a total of 36 bees per age/DPI; **%P** – percentage of positive bees per age/DPI; **AV** – average number of *L. passim* cells in positive bees; **SD** – standard deviation of the number of *L. passim* cells in positive bees.

Table 1
Multifactor *analysis of variance* (*ANOVA*) of the manipulative experiment of *L. passim*-IMI coexposure. The analysis showed that *L. passim* prevalence was significantly affected by the year of the experiment, sampling date (i.e., age of bees) and imidacloprid exposure.

Observed factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Signif.
Year	1	7.51×10^{12}	7.51×10^{12}	16.578	5.87×10^{-5}	***
Sampling_time	5	1.12×10^{13}	2.25×10^{12}	4.956	0.000218	***
Imidacloprid	1	7.36×10^{12}	7.36×10^{12}	16.228	6.99×10^{-5}	***
Hive	6	1.87×10^{12}	3.12×10^{11}	0.688	0.659376	
Year:Sampling_time	5	4.78×10^{12}	9.56×10^{11}	2.109	0.064093	
Year:Imidacloprid	1	3.51×10^{12}	3.51×10^{12}	7.74	0.005714	**
Sampling_time:Imidacloprid	4	5.77×10^{12}	1.44×10^{12}	3.181	0.013874	*
Year:Hive	6	3.24×10^{12}	5.41×10^{11}	1.193	0.309482	
Sampling_time:Hive	20	5.62×10^{12}	2.81×10^{11}	0.62	0.897445	
Year:Sampling_time:Imidacloprid	4	3.09×10^{12}	7.72×10^{11}	1.702	0.149126	
Year:Sampling_time:Hive	20	9.32×10^{12}	4.66×10^{11}	1.028	0.427805	
Residuals	326	1.48×10^{14}	4.53×10^{11}			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05.

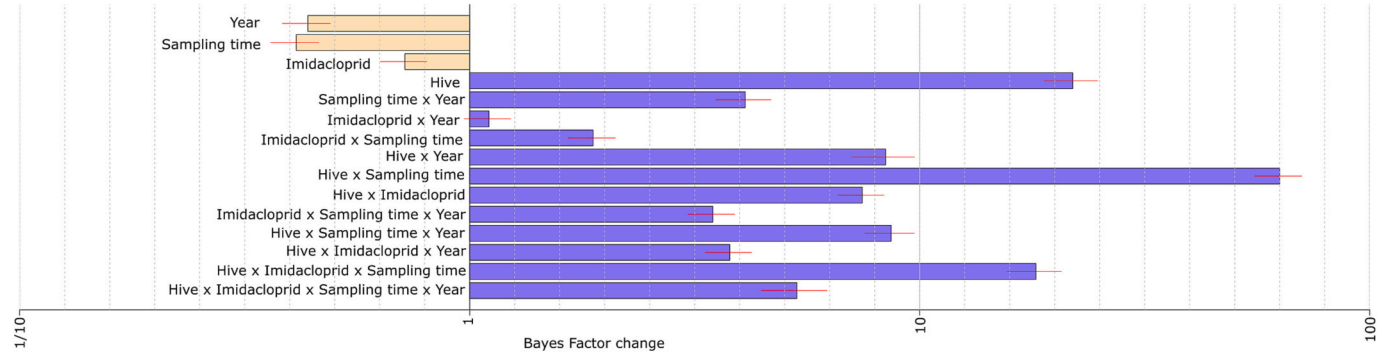


Fig. 4. Output of Bayesian statistics of the manipulative experiment of *L. passim*-IMI coexposure. The analysis showed that *L. passim* prevalence was significantly affected by the year of the experiment, sampling date (i.e., age of bees) and imidacloprid exposure. However, the experimental cage (i.e., hive) did not affect *L. passim* prevalence.

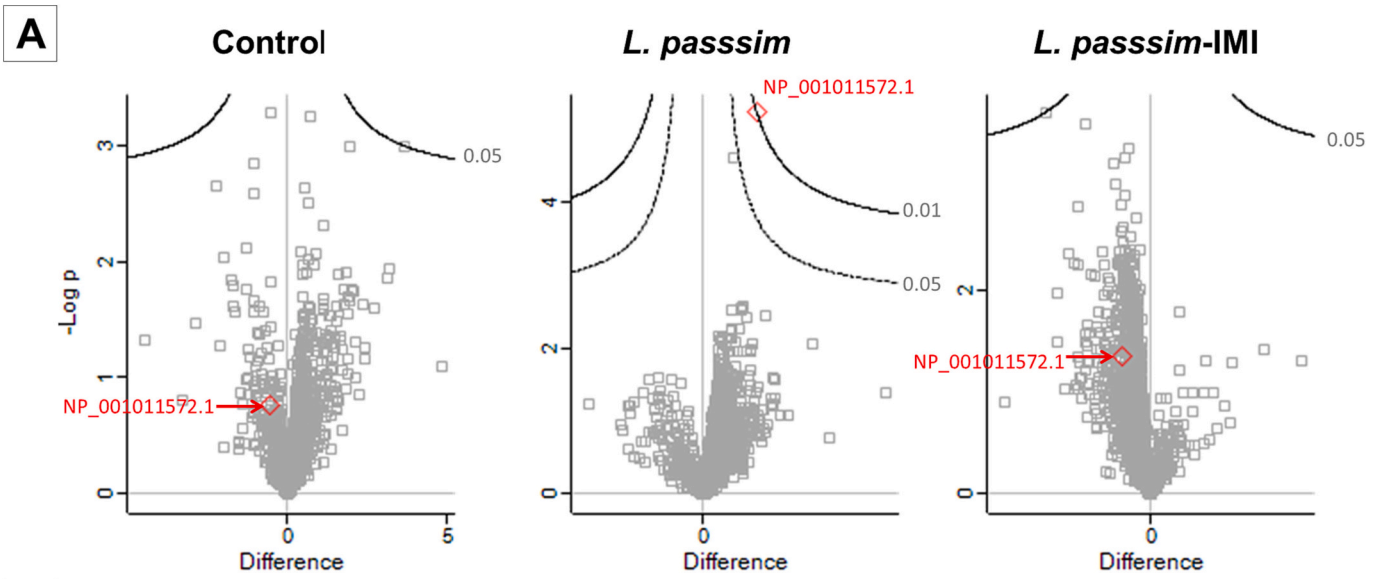
only transferrin 1 precursor (GenBank: NP_001011572.1) significantly differed. We found that this protein was highly abundant in the analysis since a total of 2529 MS/MS counts were retrieved. In addition, this protein was reliably identified in all of the honey bee head samples that were analyzed. Comparison of the average Log2 LFQ intensities (Fig. 5B) showed that the levels of transferrin 1 were similar in the control (without *L. passim* or IMI exposure) and *Lotmaria*-IMI (coexposure), while transferrin 1 abundance was higher in *L. passim* exposure, and the difference in log2-fold change was 1.30 and 1.46, respectively. Interestingly, there was no difference between the control and *Lotmaria*-IMI despite the loads of *L. passim* being higher in the coexposure.

4. Discussion

In this study, our main objective was to reveal whether low field-realistic IMI exposure of 2.5 μg/L (Schmuck et al., 2001; Bonmatin et al., 2003; Blacquiere et al., 2012) can affect the prevalence of *L. passim* parasites in honey bees. Importantly, our results showed that exposure of bees to *L. passim* with IMI coexposure resulted in a higher prevalence than in the case of *L. passim* exposure without coexposure to the pesticide. The increased infestation numbers presented for *L. passim* are a consequence of the parasite loads in individual bees being substantially increased. This means that IMI has the potential to facilitate the spread of *L. passim* in the colony and surrounding colonies, which increases the potential for adverse effects on honey bees that can occur under certain conditions (Arismendi et al., 2020; Gomez-Moracho et al., 2020; Nanetti et al., 2021). The potential excessive occurrence of *L. passim* and possible adverse effects provide additional information relevant to the risk assessment of IMI on bees. To some extent, our findings resemble the previous observation that IMI exposure increased the abundance of *Vairimorpha* (*Nosema*) in honey bees (Pettis et al., 2012). We suggest that there may be synergistic effects between IMI and gut parasites that may increase their abundance. Overall, the increased abundance of a parasite/pathogen is a prerequisite condition to cause harmful effects on the host. However, the infestation numbers of *L. passim* differ by year and even by season (Stevanovic et al., 2016; Vejnovic et al., 2018). According to our results from the manipulative experiment, it is likely that IMI or exposures with similar effects can be the factor that increases *L. passim* abundance apart from the natural course.

We confirmed the natural presence of *L. passim* in the experimental CRI apiary, which was a source of emerging bees for manipulative experiments. Our observed maximal parasite loads reaching 10^6–10^7 *L. passim* per bee in naturally infected colonies were similar to those found in other studies (Xu et al., 2018; Arismendi et al., 2022). However, in the manipulative experiments, the maximal numbers of parasites were similar to the highest levels in natural infections only under IMI coexposure. Overall, we observed that the high levels of parasite load were linked to increased prevalence in the experiment (IMI coexposure) similar to the naturally infected colonies.

In the manipulative experiments, the bees were collected at different ages and DPIs. Similar to previous studies, the parasite loads increased with the age of honey bees (Strobl et al., 2019; Michalczyk et al., 2022b), and survival decreased with age, although the speed of death was observed to vary in studies (Gomez-Moracho et al., 2020; Liu et al., 2020). However, taken together with our study, approximately 10 days of age appears to be a critical period at which a high increase in *L. passim* abundance occurs in individual bees, and later, the bees can rapidly decline (Gomez-Moracho et al., 2020). Consistent with this, in our manipulative experiment, we successfully collected 30 bees in all cages out of 50 initials when the bees were 13 days old and 11 DPI; however, the bees at 15 days of age were not collected due to low numbers in some replicates. The abundance of parasites in individual bees as well as the



B

			Control			
Sample id	A12	B2	B3	B4	B5	**Average**
Cell counts	0	0	0	0	0	0
Log2 LFQ intensity	28.1	27.7	28.0	27.7	27.3	27.77
			L. passim			
Sample id	G10	G11	G3	G7	G9	**Average**
Cell counts	508,000	51,200	104,400	610,000	638,000	382,320
Log2 LFQ intensity	28.8	29.7	29.2	29.2	28.4	29.07
			L. passim-IMI			
Sample id	A11	A5	A6	A7	A9	**Average**
Cell counts	964,000	962,000	336,000	202,000	1,106,000	714,000
Log2 LFQ intensity	27.8	27.5	27.4	27.5	27.9	27.62

Fig. 5. Analysis of the proteome differences in honey bee heads. A) Hawaii plot revealed that only one protein was found to be significantly different and upregulated by the presence of *L. passim*. B) Results of the individual inspection of the significant protein transferrin 1 precursor. Notably, there is a minimal variation in the Log2 LFQ intensities in five biological replicates within each of three exposures, while there is a high variation in cell counts of the parasite.

prevalence rate can be strongly affected by various environmental factors. Incidentally, Gomez-Moracho et al. (2020) observed that the growth of *L. passim* as well as *C. mellificae* has been observed to be affected by culture media; therefore, it can be suspected that the growth of *L. passim* in bees can be affected by differences in bee nutrition and other environmental conditions that also vary during season. Colonies that can collect many different substances in the surrounding environment, including pesticides, can circulate them and affect bees (Ardalani et al., 2021; Seshadri and Bernklau, 2021). The results of our experiment show that coexposure to a single substance (IMI) can increase *L. passim* to high abundances that are similar to the highest observed in the outside environment. We believe that this is indicative of a synergistic effect between a pesticide and parasite/pathogen.

Furthermore, our results provided strong support that the bees at the time of emergence do not carry *L. passim*. This is supported by the fact that we did not detect any bees positive for *L. passim* at the time of emergence and by the fact that emerging bees without exposure did not develop the infection. Additionally, our results confirmed that only some bees developed the infection after exposure to *L. passim*. Thus, the distribution of the parasite in worker bees is interrupted, similar to that in eggs (Arismendi et al., 2022). Although *L. passim* has been found to be present in bee broods and detected in pupae (Taric et al., 2020; Arismendi et al., 2022), our results indicate that bees at the time of emergence that underwent complete metamorphosis in the capped cell could also be free of *L. passim*.

The finding that emerging bees lack *L. passim* infection is important in relation to the experimental design used. In manipulative experiments, we used only bees that were collected at the time of emergence. In other studies, newly emerged bees for experiments with *L. passim*/Trypanosomatidae were collected from brood frames after they emerged in a thermostat (Schwarz and Evans, 2013; Gomez-Moracho et al., 2020; Arismendi et al., 2022; Buendia-Abad et al., 2022). Notably, the recommended OECD toxicity tests on honey bees allow newly emerged bees of similar age (OECD, 1998, 2017). In a 10-day feeding chronic oral toxicity test to honey bees, young bees with a maximum age of 2 days should be used (OECD, 2017), but this means that the individuals may differ up to 48 h of age. This increment in bee individuals allows testing exposures on bees with different physiology in developmental age, i.e., 0,1-day and 2-day bees will differ substantially due to different times of feeding prior to the experiment. Instead, in our experiment, during this 2-day period range, we performed a 24 h adaptation and next 24 h infection with *L. passim* on the emerging bees collected in a short time. Our method is supposed to be more accurate for comparisons, but performing such an experiment is more difficult, and we can be limited by

fewer bees for the experiment. Thus, it is necessary to decide which question is key to answer. In our case, it was the difference between *L. passim* and *L. passim*-IMI exposures. Therefore, we used only one control cage without any exposure in 2021. Additional controls were not necessary because the control was used to verify that *L. passim* did not develop infection from the emerging bees. Moreover, compared to the OECD tests, mainly 10-day feeding (average mortality across replicates ≤15 % (OECD, 2017)), we did not consider mortality in controls since the experiment was to determine the effect of *L. passim* and IMI coexposure, and the bees were used in this comparison. Finally, for analyses we collected the bees gradually with a 2-day increment since they were 5 days old/3 days DPI, and until they were 13 days old/11 DPI, we successively collected a total of 30 bees from each experimental cage. Later, the bees were not collected because they can rapidly decline due to *L. passim* infection (Gomez-Moracho et al., 2020). Overall, we suggest that bees collected at the time of emergence that had no previous contact with colony members or could not be infected by food stores are appropriate for host–pathogen studies using the trypanosomatid *L. passim*. Using emerging bees in experiments ensures different routes of infection by *L. passim* and excludes contact with the ubiquitous parasite *V. destructor*. In addition, the age of the bees at the time of emergence was accurate regarding the time of development, and the related benefits for physiology and host–pathogen studies have been previously specified (Erban et al., 2016, 2019a).

Notably, in our study, we also observed substantially different infection success between the years when the experiment was performed, while IMI increased the prevalence of *L. passim* in both years/repeats. In particular, in each year, all replicates exposed to *L. passim* and coexposed to IMI were performed on the same occasion. Interestingly, the factor "hive" (experimental box) did not significantly affect the results. Thus, the entire experiment significantly differed only between the experimental years. Another possible factor affecting infection success is the strain and different passages (Buendia-Abad et al., 2021). However, we used low passages of the same axenic clonal culture in both independent replicate years. In particular, in natural infections of *L. passim*, there can be variations in *L. passim* strains (Buendia-Abad et al., 2021). Thus, an open question is whether different strains of *L. passim* can be differentially affected by IMI exposure. Finally, in our experiment, we used the same pollen in both years of the experiment. According to the exact parameters of the stock syrup used to prepare the syrup-pollen feed, there were slightly different pH values and fructose/glucose/saccharose ratios, which are in the accepted range in production. We do not expect that these variations in stock syrup could cause such an effect on the success of *L. passim* infection, although culture media affect the growth of the parasite (Gomez-Moracho et al., 2020).

Finally, we sought to identify whether proteomes of the heads of 13-day-old honey bees were affected by *L. passim* and coexposure to *L. passim* and IMI. Although we performed a comprehensive label-free proteomic analysis, we found only one significantly different protein. The explanation is that the proteomes in honey bee heads were relatively highly variable, and therefore, the one significant marker, transferrin 1, appears to be the key affected marker. We stress that transferrin 1 was a high-abundance protein in all samples of our analysis, as indicated by MS/MS counts and intensities, which makes this marker highly reliable. Interestingly, transferrin 1 was found to be upregulated by *L. passim* occurrence only, while levels of this protein marker were found to be similar in the control and *L. passim*-IMI coexposure. This means that *L. passim* caused the upregulation of transferrin 1 and that IMI coexposure neutralized the effect on this marker. Similar levels of transferrin 1 despite variation in *L. passim* loads in samples of each of the exposure variants support that transferrin 1 is a steady marker. The abundance of this marker was minimally affected by different loads of the parasite, but it appears that the effect of IMI in the coexposure is strong because the effect of IMI on the expression of transferrin 1 exceeds the higher average loads of *L. passim* in the coexposure.

A question is the mechanism that can explain the upregulation of transferrin 1 by *L. passim* and cancelling the abundance by IMI. Transferrin 1 is an iron binding and transporting protein that is induced by infection and can be part of a host defense (Yoshiga et al., 1999; Iatsenko et al., 2020; Najera et al., 2021). Importantly, Rodriguez-Garcia et al. (2021) found it to be upregulated (at the mRNA level) due to *V.* (*N.*) *ceranae* infection, in agreement with transferrin 1 playing a key role in the battle for iron. Moreover, they found that RNAi achieved suppression of transferrin 1 and led to reduced *V.* (*N.*) *ceranae* transcription activity, alleviated iron loss and enhanced immunity linked to better survival of the bees (Rodriguez-Garcia et al., 2021). Moreover, bloodstream trypanosomes obtain iron from transferrin, which is a host iron carrier protein (Taylor et al., 2013). Taken together, our observation that *L. passim* infection was linked to an increased level of transferrin 1 is in agreement with the role and responses of the protein to the infection. However, the resulting neutralization of transferrin 1 linked to increased infection success of *L. passim* by IMI remains unknown. It appears that IMI is a compound that similarly increases the abundance of gut parasites in honey bees such as *L. passim* (our study) and *Vairimorpha* (*Nosema*) (Pettis et al., 2012). A possible explanation is that the unexpected effect of IMI is through modulation of a mevalonate pathway, as observed in bumblebees (Erban et al., 2019b). It is possible that IMI affects ferroptosis-based cell death of the parasite (Zou and Schreiber, 2020; Li et al., 2022), which is linked to the mevalonate pathway (Hao et al., 2018; Zheng and Conrad, 2020).

5. Conclusions

In this study, we show that the occurrence of *L. passim* in honey bee colonies can be affected by IMI. Our results contribute to understanding the adverse effects of IMI, which is a substance that has been found to be harmful to honey bees and other pollinators. However, it is not clear how *L. passim* can adversely affect honey bee colonies. If a substance such as IMI increases the prevalence and abundance of gut parasites such as *L. passim* in individuals, it is likely that adverse effects will arise. Importantly, the increased prevalence linked to higher loads in individuals of any parasite/pathogen should lead to facilitated spread in colonies and between colonies. Notably, the increases in *L. passim* due to IMI have a synergistic nature, and for both stressors, similar adverse effects have been described. The proteomic analysis of honey bee heads showed that IMI can neutralize the increase in the expression of the immune marker transferrin 1 caused by parasite infection. It is possible that different synthetic or natural substances can affect the incidence of *L. passim* similarly or in the opposite way as IMI. Incidentally, we indicate that our findings regarding the interaction between *L. passim* and IMI are similar to those of studies related to *Vairimorpha* spp. (*Nosema* spp.), the effect on prevalence and transferrin 1. Finally, we also developed a methodology to test the interactions between xenobiotics and trypanosomatid parasites in bees. Finally, our results indicate that bees at the time of emergence are free of *L. passim*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166973.

CRediT authorship contribution statement

Tomas Erban conceptualized the study, wrote the main manuscript and supervised the work. Kamila Parizkova performed the experiment, processed the samples and analyzed using qPCR. Bruno Sopko performed statistical analysis of the data. Pavel Talacko performed the proteomic nanoLC-MS/MS analysis and evaluated the data, while Tomas Erban evaluated the data to detail. Jana Jarosova contributed to qPCR analysis. Jan Votypka isolated the *L. passim* and contributed to the parasite experimental exposure. Martin Markovic verified the references, edited the draft version and checked formality. All authors participated in preparation of the manuscript and improved the final work.

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

The accession number for the raw nanoLC–MS/MS runs reported in this paper is MassIVE MSV000092818 (doi: 10.25345/C5RN30J5M) and be assigned ProteomeXchange PXD045086.

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