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Research paper

Physiological effects of the interaction between *Nosema ceranae* and sequential and overlapping exposure to glyphosate and difenoconazole in the honey bee *Apis mellifera*

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ABSTRACT

Pathogens and pollutants, such as pesticides, are potential stressors to all living organisms, including honey bees. Herbicides and fungicides are among the most prevalent pesticides in beehive matrices, and their interaction with *Nosema ceranae* is not well understood. In this study, the interactions between *N. ceranae*, the herbicide glyphosate and the fungicide difenoconazole were studied under combined sequential and overlapping exposure to the pesticides at a concentration of 0.1 µg/L in food. In the sequential exposure experiment, newly emerged bees were exposed to the herbicide from day 3 to day 13 after emerging and to the fungicide from day 13 to day 23. In the overlapping exposure experiment, bees were exposed to the herbicide from day 3 to day 13 and to the fungicide from day 7 to day 17. Infection by *Nosema* in early adult life stages (a few hours post emergence) greatly affected the survival of honey bees and elicited much higher mortality than was induced by pesticides either alone or in combination. Overlapping exposure to both pesticides induced higher mortality than was caused by sequential or individual exposure. Overlapping, but not sequential, exposure to pesticides synergistically increased the adverse effect of *N. ceranae* on honey bee longevity. The combination of *Nosema* and pesticides had a strong impact on physiological markers of the nervous system, detoxification, antioxidant defenses and social immunity of honey bees.

1. Introduction

All living organisms are subjected to multiple stressors from anthropogenic (e.g., pollutants) and natural (e.g., pathogens) sources (Holmstrup et al., 2010). These environmental stressors can act alone or through interactions involving mixtures of pollutants, associations of pathogens, associations of pollutants and pathogens or complex combinations of pathogens and mixtures of pollutants (Feldhaar and Otti, 2020; Shahid et al., 2019). Concerning pollutants, pesticides are of particular concern for bees because they exhibit expected toxicity designed for plant protection that can elicit adverse lethal and sublethal effects (Goulson et al., 2015). Among bee species, the honey bee is considered a beneficial species and is the most economically valuable pollinator of crop monocultures worldwide (Klein et al., 2007;

McGregor, 1976; Roubik, 2002).

Honey bees can be exposed to pesticides belonging to different classes (mainly herbicides, insecticides and fungicides) either directly, during treatment for plant protection or via contact with treated plant parts, or indirectly, by the consumption of contaminated pollen, honey, guttation drops or water (Girolami et al., 2012, 2009; Krupke et al., 2012; Tapparo et al., 2011). After plant treatments, contaminated nectar and pollen can be transferred to the hive, leading to the accumulation of numerous pesticide residues in the beehive matrices, along with acaricides used for the treatment of *Varroa* infestation (Böhme et al., 2018; Chauzat et al., 2011; Kanga et al., 2019; Lambert et al., 2013; Wiest et al., 2011). In addition, farmers increasingly use tank mixing for the spraying of several pesticides together to enhance the performance of the active substances and reduce pesticide application time and cost

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(Tornisiello et al., 2013). For example, in the UK, approximately 6 different products are applied in a single spraying event in soft fruits, orchards and vegetable crops (Fryday et al., 2011). Thus, honey bees could be subjected to simultaneous, sequential or overlapping exposure to pesticides that can elicit not only additive but also synergistic adverse effects (Gill, 2012; Sgolastra, 2017; Zhu, 2017). Simultaneous exposure occurs via the ingestion of food contaminated by several pesticides, by contact with plant parts contaminated with different pesticide residues or during plant treatment with a mixture of pesticides. Sequential and overlapping exposure may be observed when consecutive treatments are applied to a crop, when bees visit different crops during foraging, when a migratory beekeeping strategy is applied, when bees consume honey or pollen successively contaminated with different pesticides or when bees are exposed to pesticides outside the hive after consuming contaminated food (Luttik et al., 2017).

Throughout their life stages, honey bees are subjected to attacks by parasitic mites, scavengers, viruses, bacteria and fungi or microsporidia such as *Nosema* spp. (Genersch et al., 2010). The interactions between pathogens and pesticides have been used to enhance the toxicity and reduce the doses of pesticides used to kill pests and minimize the ecological impacts on nontarget species and humans (Ahmed et al., 2020; Baker et al., 2020; Ericsson et al., 2007; Paula et al., 2013; Purwar and Sachan, 2006). However, a combination of pathogens and pesticides could have negative impacts on beneficial species such as the honey bee (Coulon et al., 2020; Pochini and Hoverman, 2017). Several studies have shown a significant link between virus loads and exposure to pesticides such as acaricides (Locke et al., 2012), insecticides (Coulon et al., 2020; Di Prisco et al., 2013) and fungicides (Simon-Delso et al., 2014) and between pesticides (mainly insecticides) and *Nosema* spp. (Alaux et al., 2010; Aufauvre et al., 2012; Aufauvre et al., 2014; Tesovnik et al., 2020; Vidau et al., 2011). The microsporidium *Nosema ceranae* is an obligate intracellular parasite that colonizes the epithelial cells of the honey bee gut. It is frequently associated in certain regions with colony losses (Martín-Hernández et al., 2018).

The main effects of *N. ceranae* infection are decreased honey bee survival (Aufauvre et al., 2012; Vidau et al., 2011), hormonal disruption associated with alterations in vitellogenin and juvenile hormone levels (Alaux et al., 2010; Antúnez et al., 2009; Dussaubat et al., 2010), energetic and nutritional stress (Aliferis et al., 2012; Mayack and Naug, 2010) and reduced immunocompetence (Antúnez et al., 2009; Chai-manee et al., 2012; Glavinic et al., 2017; Li et al., 2017; Sinpoo et al., 2018). On the other hand, herbicides and fungicides are among the pesticides that are frequently detected in beehive matrices (Lambert et al., 2013; Lopez et al., 2016; Mullin et al., 2010). The predominantly used herbicide is glyphosate (Benbrook, 2016). It inhibits 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), an enzyme essential for the biosynthesis of aromatic amino acids in plants and some microorganisms (Amrhein et al., 1980; Bode et al., 1984). More than 80% of honey samples of different origins have been shown to contain glyphosate at concentrations ranging between 17 and 342 µg/kg. Glyphosate is also detected in bee bread at levels around 50 µg/kg (Berg et al., 2018; El Agrebi et al., 2020; Pareja et al., 2019; Rubio et al., 2015; Thompson et al., 2019). Among fungicides, azole fungicides, including difenoconazole, exhibit broad-spectrum antifungal activity. They are applied in both preventive and curative treatments due to their systemic properties (Hof, 2001). Their mode of action is based on the inhibition of the fungal lanosterol 14 α -demethylase, which is responsible for the transformation of lanosterol into ergosterol, an essential constituent of the cytoplasmic membrane of fungi (Ji et al., 2000). Difenoconazole is present in honey, pollen, bee bread and wax at mean concentrations of 0.6, 43, 270 and 1 µg/kg, respectively (Kubik et al., 2000; Lopez et al., 2016). Thus, honey bees could be exposed to glyphosate and difenoconazole after emergence through the consumption of contaminated bee bread and honey. However, because fungicides and herbicides show low acute toxicity (LD₅₀ > 100 µg/bee), their application is allowed during the flowering period, causing particularly high exposure of honey bees

through the consumption of contaminated pollen.

While the understanding of the effect of exposure to a single pesticide has increased (Christen et al., 2019; Hesselbach and Scheiner, 2018; Nicodemo et al., 2014), there is a remaining gap in the assessment of the effects of sequential or overlapping exposure to multiple pesticides (EFSA, 2012), particularly when pesticides are associated with pathogens. Thus, the objectives of this study were to investigate the potential differences between the toxicity induced by sequential and overlapping exposure to pesticides and to determine whether the interactions between pesticides and the pathogen *N. ceranae* depend on the mode of exposure. The study was conducted on emerging bees orally exposed to pesticides to assess the sensitivity of the bees during the first stage of their adult life. The considered pesticides were the herbicide glyphosate and the fungicide difenoconazole. Attention was focused on the effect of exposure to this pesticide combination on survival, *Nosema* development, food consumption and some key physiological systems by analyzing the variations in eight life history traits that can reveal impairment in the integrity of the nervous system, immunity, defenses against oxidative stress and metabolism.

2. Materials and methods

2.1. Materials

Triton X-100, monosodium phosphate (NaH₂PO₄), sodium chloride (NaCl), sodium bicarbonate (NaHCO₃), Tris-potassium phosphate (K₃PO₄), pepstatin A, leupeptin, aprotinin, trypsin, antipain, monopotassium phosphate (KH₂PO₄), glucose, horseradish peroxidase, o-dianisidine dihydrochloride, disodium phosphate (Na₂HPO₄), disodium ethylenediaminetetraacetate dihydrate (EDTA), reduced L-glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), acetylthiocholine iodide (AcSCh), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), D-glucose-6-phosphate disodium salt hydrate (G6P), D-fructose-6-phosphate dipotassium salt (F6P), 3,4-dihydroxy-L-phenylalanine (L-DOPA), Tris base, magnesium chloride hexahydrate (MgCl₂·6H₂O), β -nicotinamide adenine dinucleotide phosphate hydrate (β -NADP⁺), 4-nitrophenyl phosphate di(Tris) salt (p-NPP), hydrogen peroxide (H₂O₂), dimethyl sulfoxide (DMSO) and a Cytochrome c oxidase Assay Kit (CYTOCOX1) were obtained from Sigma Aldrich® (Saint Quentin Fallavier, France). Difenoconazole (IUPAC name 1-[2-[2-chloro-4-(4-chloro-phenoxy)-phenyl]-4-methyl[1,3]dioxolan-2-ylmethyl]-1H-1,2,4-triazole; CAS No. 119446-68-3) and glyphosate (IUPAC name [N-(phosphonomethyl)glycine]; CAS No. 1071-83-6) (98% purity each) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). A protein solution (bee food), wax foundation sheets and pollen, certified by Ecocert, were purchased from Remuau Ltd. (Barbentane, France). Candy fondant Apifonda was purchased from Icko Apiculture (Bollène, France). The pheromone Bee Boost® (PseudoQueen) was purchased from Intko Supply Ltd (Vancouver, Canada).

2.2. Honey bees

The colonies used in this study were continuously checked to examine their health status. The experiment was performed on emerging *Apis mellifera* honey bees (≤ 24 h old) obtained from brood frames of six beehives from the experimental apiary of the *Abeilles & Environnement* Research Unit (Bee & Environment Research Unit of INRAE (Avignon, France)) in July 2018. Frames of sealed broods were collected and placed in incubators at 33 \pm 2 °C with 60 \pm 5% relative humidity. Honey bees were collected directly on the brood frames after emergence; bees from different brood frames obtained from the six beehives were randomly mixed together and randomly distributed into seven plastic cages (6 \times 8.5 \times 10 cm) in groups of 30 honey bees per dose. The cages were then placed in the dark in an incubator at 30 \pm 2 °C with 60 \pm 5% relative humidity. To mimic the hive environment, a small piece of Bee Boost® (PseudoQueen), releasing a queen mandibular pheromone, and

a small wax foundation sheet were deposited on the top of the cage. To maintain hygiene, a sheet of filter paper was placed on the bottom of each cage and replaced daily.

2.3. Infection with *N. ceranae* and treatment with pesticides

N. ceranae spore production and identification were carried out according to Roussel et al. (2015) and Paris et al. (2017) a few days before infection. Infection with *N. ceranae* was performed two hours after the emergence of honey bees. Honey bees were immobilized using sterile forceps and fed individually with 2 μ L of a 40% (w/v) sucrose solution containing 100,000 *Nosema* spores. This dose is ten times higher than that required to observe infection in 100% of honey bees (Forsgren and Fries, 2010). During the two days following emergence, honey bees were fed with water, candy and pollen ad libitum, and the few dead bees were removed and replaced with infected or uninfected honey bees according to the treatment. Each treatment consisted of seven replicates ($n = 7$ cages with 30 honey bees per cage). On the third day, chronic exposure to pesticides was initiated by replacing water, candy and pollen with a 60% (w/v) sucrose solution containing a 0.1% (v/v) final concentration of DMSO, pesticides (the herbicide glyphosate (H) and/or the fungicide difenoconazole (F)) at 0.1 μ g/L or containing no pesticides for the controls. The concentration of 0.1 μ g/L is equivalent to 0.083 μ g/kg, calculated according to a sucrose solution density of 1.23 ± 0.02 ($n = 10$). This concentration was chosen because it corresponds to the lowest concentration at which glyphosate and difenoconazole can induce a

chronic toxicity to bees through lethal and physiological effects (Almasri et al., 2020). The duration of exposure to each pesticide was ten days. The exposure was either overlapping, with a 4-day coexposure period, or sequential starting with H at day 3, followed by F at days 7 or 13 (Fig. 1). Stock solutions of pesticides were prepared by dissolving the active substance in water for glyphosate or in 100% (v/v) DMSO for difenoconazole. The stock solutions were subjected to 1/10th cascade dilutions to obtain 10X stock solutions in 1% DMSO. The stock solutions were diluted 1:10 (v/v) in sucrose syrup to obtain a final concentration of 60% sucrose, 0.1% (v/v) DMSO, 1% (v/v) Bee Food® protein solution and 0.1 μ g/L glyphosate, difenoconazole, or both for overlapping exposure. The pesticide concentrations of the stock and feeding solutions were checked according to Paradis et al. (2014) for difenoconazole and Oulkar et al. (2017) for glyphosate (Oulkar et al., 2017; Paradis et al., 2014). For each pesticide solution, the relative standard deviations (RSD) of the measured concentrations compared to the nominal concentrations were less than 10%.

The treatments in which honey bees were infected by *N. ceranae* are all given identifiers starting with "N". The groups that had received only H at day 3 were designated H and N.H, where the bees in the latter group were also infected with *Nosema*. The groups that received only F at day 7 were designated F7 and N.F7, where the bees in the latter group were also infected with *Nosema*. The groups that received only F at day 13 were designated F13 and N.F13, where the bees in the latter group were also infected with *Nosema*. The groups that received H at day 3 followed by overlapping exposure to F at day 7 were designated oH+F7 and oN.H+F7.

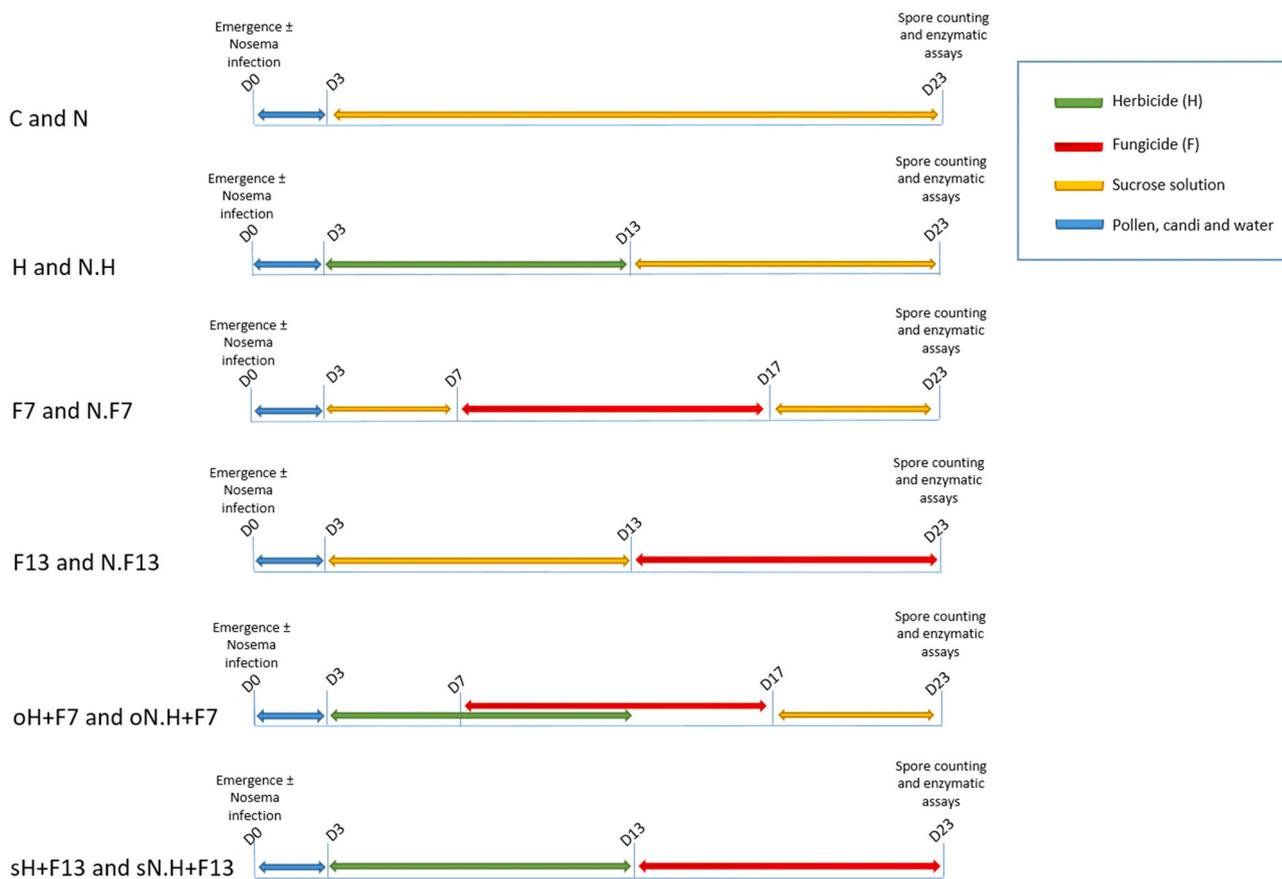


Fig. 1. Experimental design: analysis of physiological life history traits and spore loads following infection by *N. ceranae* and/or sequential or overlapping exposure to an herbicide (H) and a fungicide (F). Twelve experimental conditions were tested: six uninfected and six infected with *N. ceranae*, with seven replicates per experimental condition ($n = 7$ cages). At emergence (D0), half of the honey bees were infected with *Nosema* (N) and fed pollen, candy and water for 2 days. "o" and "s" respectively refer to overlapping (oH+F7 and oN.H+F7) and sequential (sH+F13 and sN.H+F13) glyphosate+difenoconazole treatments. On the third day (D3), the honey bees in treatments H and N.H were exposed to H for 10 days. The honey bees in the treatments F7 and N.F7 were exposed for 10 days to F from day seven (D7), while those in treatments F13 and N.F13 were exposed for 10 days to F from day 13 (D13). The honey bees in treatment oH+F7 and oN.H+F7 were exposed to H at D3 and to F at D7. The honey bees in treatment sH+F13 and sN.H+F13 were exposed to H at D3 and to F at D13.

H+F7, where the bees in the latter group were also infected with *Nosema*. The groups that received H at day 3 followed by a sequential exposure to F at day 13 were designated sH+F13 and sN.H+F13, where the bees in the latter group were also infected with *Nosema* (Fig. 1).

2.4. Survival and food consumption

Mortality and food consumption were recorded daily until the end of the experiment. The dead bees were counted at 08:00 am and discarded to preserve hygienic conditions. Individual food consumption was assessed by measuring the weight of the feeder daily and correcting the consumed food by the bees remaining alive. The feeder consisted of a 5-mL polystyrene tube with a 1.5 mm hole at the bottom. An evaporation control was included to accurately calculate the food consumed by the bees. To estimate the cumulative dose ingested by the bees, the volume of the food ingested was calculated on the basis of a sucrose density of 1.23 ± 0.02 .

2.5. Choice of physiological life history traits

The physiological effects induced by *Nosema* and pesticides were assessed by investigating the activity of eight physiological life history traits related to neural activity (acetylcholinesterase (AChE) and cytochrome c oxidase (COx)), immunity (glucose oxidase (GOx), phenoloxidase (POx) and alkaline phosphatase (ALP, also involved in metabolism)), and defense against oxidative stress and detoxification (glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PDH) and catalase (CAT)).

AChE is involved in learning and memory processing (Gauthier et al., 1992; Guez et al., 2010) through the hydrolysis of the neurotransmitter acetylcholine in cholinergic synapses (Badiou et al., 2007). COx is the terminal enzyme in the respiratory electron transport chain (Lemberg, 1969). Variations in COx activity in the head reflect changes in neuronal cell respiratory activity, which indicate perturbations in the insect nervous system potentially linked with learning deficiencies (Bennett et al., 1996; Decourtye et al., 2004). GOx is thought to confer a form of social immunity. It is secreted in the hypopharyngeal gland and is responsible for the antimicrobial properties of honey through the production of gluconic acid and hydrogen peroxide (Bucekova et al., 2014). POx contributes to the innate immune system through the activation of the melanization process to seal wound sites and encapsulate parasites and pathogens (Gonzalez-Santoyo and Cordoba-Aguilar, 2012). ALP is involved in immune function, intestinal adsorption and transport mechanisms and metabolism (Chen et al., 2011; Vlahović et al., 2009). GST transforms the peroxidation products of biological molecules formed during oxidative stress into less toxic hydroxyl derivatives and participates in the detoxification process via the conjugation of xenobiotics to reduced glutathione (GSH), making them more hydrophilic (Qin et al., 2013; Yan et al., 2013). G6PDH regenerates NADPH through the pentose phosphate pathway to indirectly promote the formation of GSH (Efferth et al., 2006). CAT transforms hydrogen peroxide (H_2O_2 , a toxic reactive oxygen species (ROS)) into water and oxygen, directly leading to the reduction of oxidative stress (Felton and Summers, 1995).

The physiological markers were assessed in tissues in which they are relevant and where their biological activity is particularly high. AChE, GOx and COx were assessed in the head (Alaux et al., 2010; Armengaud et al., 2000; Belzunces et al., 1988); CAT and ALP in the midgut (Badiou-Bénéteau et al., 2012; Carvalho et al., 2013); POx and G6PDH in the abdomen (Alaux et al., 2010; Renzi et al., 2016); and GST in the head, abdomen and midgut (Almasri et al., 2020) (Table 1).

2.6. Tissue homogenization and analysis of physiological life history traits

The changes in physiological life history traits were analyzed in surviving honey bees 23 days after emergence (equivalent to a maximum 20-day exposure period). To avoid animal suffering when

Table 1

Distribution of common and specific markers in honey bee tissues.

	Head	Abdomen	Midgut
Common marker	GST	GST	GST
Specific markers	GOx	POx	ALP
	AChE	G6PDH	CAT
	COx		

Distribution of the physiological markers between the different honey bee compartments. GOx, AChE and COx were measured in the head, POx and G6PDH in the abdomen and ALP and CAT in the midgut. GST was analyzed in all three compartments.

sampling the tissues, the honey bees were first anesthetized with CO_2 , the head was immediately separated from the thorax, and the midgut was pulled out from the abdomen. Then, the heads, abdomens (devoid of the intestinal tract) and midguts were placed in 2 mL tubes, weighed and stored at $-80^\circ C$ until analyses. For each treatment, seven repetitions ($n = 7$ samples) of pooled tissues from three bees per sample were analyzed, and each sample was assayed in triplicate during the measurement of enzymatic activity. The tissues were homogenized using a high-speed Qiagen TissueLyzer II at 30 Hz in five periods of 30 s at 30 s intervals after the addition of the extraction medium [10 mM sodium chloride, 1% (w/v) Triton X-100, 40 mM sodium phosphate, pH 7.4 and protease inhibitors (2 $\mu g/mL$ of pepstatin A, leupeptin and aprotinin, 0.1 mg/mL soybean trypsin inhibitor and 25 units/mL antipain)] to produce 10% (w/v) tissue extracts. After homogenization, the extracts were centrifuged at $4^\circ C$ for 20 min at $15,000 \times g_{av}$, and the supernatants were kept on ice for further enzyme assays. The heads used for the measurement of COx were subjected to similar tissue grinding and centrifugation procedures, but the tissues were homogenized in buffer containing 2 mM EDTA, 5 mM DTT, 1 mM F6P, 3.5 mM G6P, 0.5% (v/v) Triton X-100 and 25 mM Tris-potassium phosphate, pH 7.8 (Suarez et al., 2005).

GOx was measured by following the formation of oxidized o-dianisidine at 430 nm in medium containing the head extract, 100 mM glucose, 2.5 units peroxidase, 0.3 mM o-dianisidine and 125 mM monopotassium phosphate, pH 7.0 (Kairo et al., 2017). AChE was measured at 412 nm in medium containing the head extract, 1.5 mM DTNB, 0.3 mM AcSch and 100 mM sodium phosphate, pH 7.0 (Belzunces et al., 1988). COx was measured in the head extract by following the decrease in absorbance at 550 nm resulting from the conversion of ferrocytochrome c into ferricytochrome c. The Cytochrome c Oxidase Assay Kit (CYTOCOX1®) from Sigma Aldrich was used to measure cytochrome c oxidase activity. Briefly, cytochrome c was previously reduced into ferrocytochrome c at $25^\circ C$ for 20 min in the presence of 0.5 mM DTT. The degree of reduction was considered suitable for the enzyme assay when the A_{550}/A_{565} ratio was between 10 and 20. The reaction medium contained 10 μM ferrocytochrome c, 5 mM KCl, 25 μM DTT and 50 mM Tris-HCl, pH 7.8. POx was measured by following the transformation of L-DOPA into melanin at 490 nm in medium containing the abdominal extract, 0.4 mg/mL L-DOPA, 20 mM NaCl and 10 mM monosodium phosphate, pH 7.2 (Kairo et al., 2017). G6PDH was measured by following the formation of NADPH at 340 nm in medium containing the abdominal extracts, 1 mM G6P, 0.5 mM $NADP^+$, 10 mM $MgCl_2$ and 100 mM Tris-HCl, pH 7.4 (Renzi et al., 2016). ALP was measured at 410 nm by following the formation of p-nitrophenol in medium containing the midgut extract, 20 μM $MgCl_2$, 2 mM p-NPP and 100 mM Tris-HCl, pH 8.5 (Badiou-Bénéteau et al., 2012). CAT was measured by following the decomposition of H_2O_2 in medium containing the midgut extract, 10 mM H_2O_2 and 100 mM sodium phosphate, pH 7.0 (Beers and Sizer, 1952). GST was measured at 340 nm in the head, abdomen and midgut in medium containing the extract, 1 mM EDTA, 2.5 mM GSH, 1 mM CDNB and 100 mM sodium phosphate, pH 7.4 (Badiou-Bénéteau et al., 2012).

2.7. *Nosema ceranae* spore count

To determine the effect of different types of pesticide combinations on the infection success of *Nosema*, 24 honey bees per treatment modality were anesthetized, and their whole intestinal tracts were dissected after head removal to avoid animal suffering. Three extracts of 8 guts were produced to quantify the number of *Nosema* spores. The guts were supplemented with 4 mL of distilled water and placed in Bioreba extraction bags. The guts were homogenized using a Bioreba ball-bearing head. The spore concentration in the homogenates was determined by counting the number of spores in a hemacytometer chamber (Fries et al., 2006).

2.8. Statistical analysis

Statistical analyses were performed using RStudio version 1.1.463 statistical software. Survival analyses were performed using the packages *survival* and *survminer* (Kassambara and Kosinski, 2018; Therneau, 2015), and the Kaplan-Meier method was used, followed by a post hoc test for the comparison of survival between treatments. The Kruskal-Wallis test, followed by pairwise comparisons using the Wilcoxon rank test (with Benjamini-Hochberg correction), was used to compare the cumulative individual food consumption between treatments. The effects of the treatments on enzymatic activities were determined by ANOVA followed by Tukey's HSD test when the data followed a normal distribution or by a Kruskal-Wallis test followed by a post hoc Dunn's test (with Benjamini-Hochberg correction using the *agricolae* package (de Mendiburu, 2013)) when the data followed a nonnormal distribution.

3. Results

3.1. Effects of *Nosema* and the sequence of exposure to pesticides on honey bee survival

The effects of the sequence of exposure to pesticides on honey bee survival were assessed in both bees infected with *Nosema* and uninfected bees (Fig. 2 and Table S1). The analysis of survival revealed that the mortality rates of uninfected honey bees exposed to individual pesticides (H, F7, and F13) were not significantly different from the mortality rates of control honey bees (C). No effect of sequential exposure to the herbicide followed by the fungicide was observed (sH+F13 versus H, F13 and C). The day on which the bees started to be exposed to the fungicide did not significantly influence the toxicity of difenoconazole (F13 versus F7). Differences in mortality rates were observed only in H (17.7%) and sH+F13 (15.2%), which induced mortality rates significantly higher than that induced by F7 (7.6%).

The mortality rates of all *Nosema*-infected groups were significantly higher than those of their uninfected counterparts (C versus N; H versus N.H; F7 versus N.F7; F13 versus N.F13; oH+F7 versus oN.H+F7 and sH+F13 versus sN.H+F13), revealing a strong adverse effect of *Nosema* on honey bee survival. Based on the observed mortality rates, the toxicity of the *Nosema*-pesticide treatments could be ranked as follows: sN.H+F13 (43.8%), N (45.2%), N.H (52.4%), N.F7 (52.9%), N.F13 (54.3%), oN.H+F7 (62.9%). However, upon *Nosema* infection, overlapping exposure to the herbicide and the fungicide (oN.H+F7) not only induced significantly higher toxicity than that observed in the controls (N or C) but also induced higher toxicity than that induced by sequential exposure (sN.H+F13) (Fig. 2 and Table S1).

3.2. Effects of *Nosema* and the sequence of exposure to pesticides on food consumption

The effect of sequential and overlapping exposure to pesticides on potential energetic stress was assessed through the daily monitoring of food consumption in bees infected with *Nosema* and uninfected bees

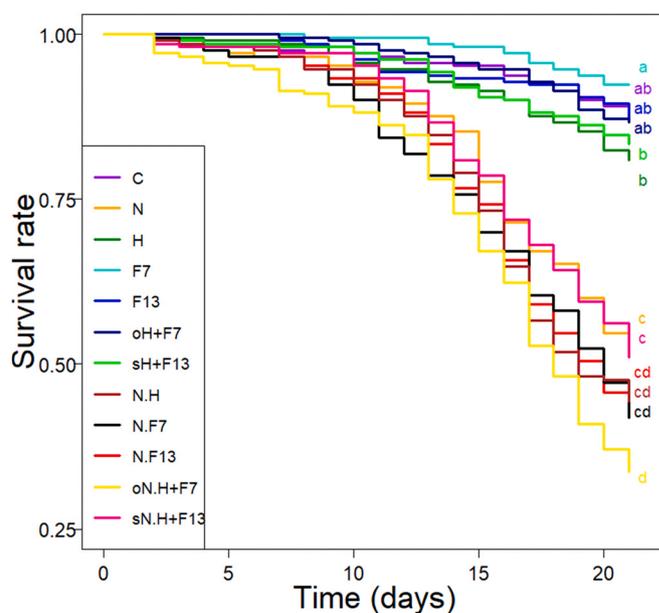


Fig. 2. Effects of *N. ceranae*-pesticide interactions on honey bee survival. Emerging honey bees were infected with *N. ceranae* (N) and then exposed to glyphosate (H) and/or difenoconazole (F) at 0.1 $\mu\text{g/L}$. H corresponds to honey bees exposed to H for 10 days starting at day 3, F7 corresponds to honey bees exposed to F for 10 days starting at day 7, and F13 corresponds to honey bees exposed to F for 10 days starting at day 13. C corresponds to control honey bees that were neither infected nor exposed to pesticides. "o" and "s" respectively refer to overlapping (oH+F7 and oN.H+F7) and sequential (sH+F13 and sN.H+F13) glyphosate+difenoconazole treatments. The data represent the mean proportion of surviving honey bees during 23 days after emergence. The mortalities recorded from 7 replicates of 30 bees per treatment were analyzed using the Kaplan-Meier method followed by a post hoc test for the comparison of survival between treatments. Data with different letters are significantly different ($p < 0.05$).

(Fig. 3 and Table S2). In general, honey bees from the *Nosema*-infected and uninfected groups consumed similar amounts of food. In addition, no significant difference in food consumption was observed between the honey bees exposed to glyphosate and difenoconazole either individually or in combination. The only difference in food consumption found among uninfected bees was between F13 and oH+F7, while the only differences among infected bees were found between N.F13 or sN.H+F13 and N.H and between N and N.H. On the basis of a food density of 1.23 ± 0.02 ($n = 10$) and a pesticide concentration of 0.1 $\mu\text{g/L}$, honey bees ingested a cumulative dose of glyphosate over 10 days ranging from 18.8 to 26.7 ng/bee, which corresponded to $1/3.8 \times 10^6$ to $1/5.3 \times 10^6$ of the glyphosate LD_{50} , while the cumulative dose of difenoconazole ranged from 18.3 to 26.7 ng/bee, which corresponded to $1/3.8 \times 10^6$ to $1/5.5 \times 10^6$ of the difenoconazole LD_{50} (LD_{50} of both pesticides ≥ 100 $\mu\text{g/bee}$) (National Center for Biotechnology Information, 2019).

3.3. Effects of the sequence of exposure to pesticides on the *Nosema* spore load

The number of spores present in the honey bee gut reflects the infection success of *N. ceranae*. *Nosema* spores were not detected in uninfected honey bees. However, in the groups infected with *N. ceranae*, the spore count showed a tendency to be higher in bees exposed to both pesticides either alone or in combination than in unexposed honey bees, although this difference was not significant (18.5×10^6 for N, 19.9×10^6 for N.F7, 21.4×10^6 for sN.H+F13, 21.7×10^6 for N.F13, 22.8×10^6 for N.H and 24.6×10^6 for oN.H+F7) (Table S3).

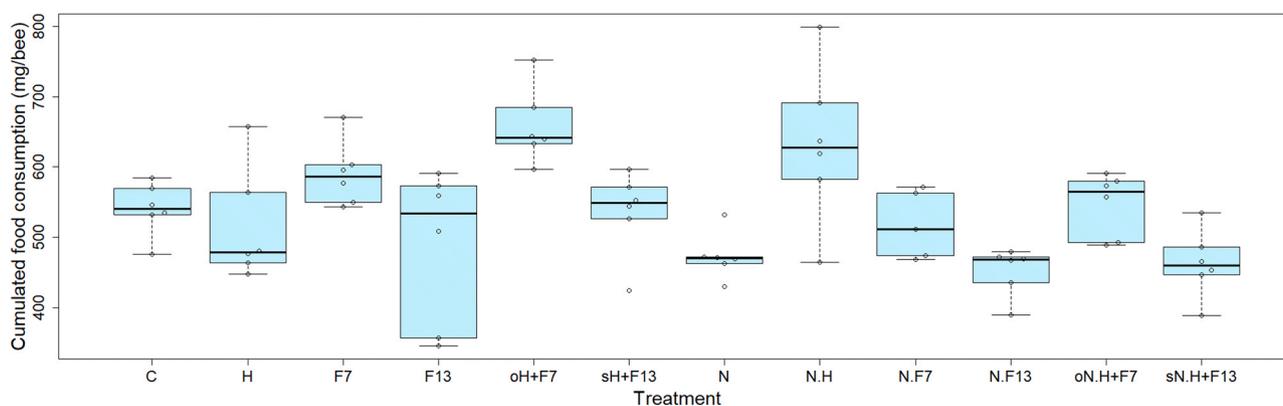


Fig. 3. Effects of *N. ceranae*-pesticide interactions on honey bee food consumption. Emerging honey bees were infected with *N. ceranae* (N) and then exposed to glyphosate (H) and/or difenoconazole (F) at 0.1 $\mu\text{g/L}$. H corresponds to honey bees exposed to H for 10 days starting at day 3, F7 corresponds to honey bees exposed to F for 10 days starting at day 7, and F13 corresponds to honey bees exposed to F for 10 days starting at day 13. C corresponds to control honey bees that were neither infected nor exposed to pesticides. “o” and “s” respectively refer to overlapping (oH+F7 and oN.H+F7) and sequential (sH+F13 and sN.H+F13) glyphosate+difenoconazole treatments. Food consumption was evaluated daily for 23 days. Box plots represent the cumulative individual consumption (mg/bee) at day 23 as determined from 7 cages of 30 bees per treatment. Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with Benjamini-Hochberg correction.

3.4. Physiological effects of exposure to *Nosema* and pesticides

To detect the physiological effects induced by *Nosema*, the sequence of exposure to pesticides and *Nosema*-pesticide interactions, eight physiological markers were analyzed in the honey bee head, abdomen and midgut. To compare the activity levels of the markers obtained at day 23 for each of the 11 treatment modalities, enzymatic activities were expressed as percentages of the control values (Fig. 4 and Table S4).

Head AChE increased to 134% of the control (C) activity ($114.6 \pm 5.0 \text{ mAU}\cdot\text{min}^{-1}\cdot\text{mg of tissue}^{-1}$) for sH+F13. There was no significant difference in head AChE activity between the control and *Nosema*-infected bees (N). However, the activity increased in all infected bees exposed to pesticides, regardless of the modality of exposure (152% of control activity for N.H, 141% for N.F7, 130% for N.F13, 135% for oN.H+F7 and 128% for sN.H+F13).

Head GOx increased to 336% and 301% of the activity in the control (C) ($1.2 \pm 0.5 \text{ mAU}\cdot\text{min}^{-1}\cdot\text{mg of tissue}^{-1}$) in oH+F7 and sH+F13, respectively. However, the activity decreased to 31% of control activity in N.F13.

Head COx increased to 301% of the activity in the control (C) ($46.5 \pm 8.8 \text{ mAU}\cdot\text{min}^{-1}\cdot\text{mg of tissue}^{-1}$) and to 560% of the activity in *Nosema*-infected bees (N) ($25.0 \pm 12.9 \text{ mAU}\cdot\text{min}^{-1}\cdot\text{mg of tissue}^{-1}$) only in N.F13. All other treatments did not induce a significant alteration of COx activity.

Head GST did not undergo significant alteration. Abdomen GST increased to 311% of control activity ($25.7 \pm 33.3 \text{ mAU}\cdot\text{min}^{-1}\cdot\text{mg of tissue}^{-1}$) for F7 and 237% for oN.H+F7. Midgut GST decreased after overlapping (oH+F7 and oN.H+F7) and sequential (sH+F13 and sN.H+F13) exposure to glyphosate and difenoconazole in both infected and uninfected honey bees. Expressed as a percentage of control (C) activity ($131.0 \pm 12.2 \text{ mAU}\cdot\text{min}^{-1}\cdot\text{mg of tissue}^{-1}$), GST activity was 69% for oH+F7, 79% for sH+F13, 66% for N.H, 75% for oN.H+F7 and 71% for sN.H+F13. Under exposure to a single pesticide, a decrease was observed only for N.H, in which GST activity represented 66% of the control activity.

Abdomen POx activity was altered only under N.F13, showing a decrease to 43% of that in the control ($7.7 \pm 2.1 \text{ mAU}\cdot\text{min}^{-1}\cdot\text{mg of tissue}^{-1}$). For abdomen G6PDH, no significant change was observed.

Midgut ALP was not altered, regardless of the treatment modality. The activity of midgut CAT decreased in the bees of all groups infected with *Nosema*. Expressed as a percentage of control activity ($1.0 \pm 0.2 \text{ mAU}\cdot\text{min}^{-1}\cdot\text{mg of tissue}^{-1}$), CAT activity was 64% in N and 51% in N.H, 67% in N.F7, 79% in N.F13, 71% in oN.H+F7 and 59% in sN.H+F13.

The analysis of the effects of infection by *Nosema* on the modulation of physiological markers under exposure to pesticides revealed different types of modulation. Infection with *Nosema* elicited increases in the activity of (i) AChE in H, F7 and oH+F7; (ii) head COx in F13; and (iii) abdomen GST in oH+F7. Infection with *Nosema* elicited decreases in the activity of (i) head GOx in F7, F13, oH+F7 and sH+F13; (ii) head GST in F13 and oH+F7; (iii) abdomen POx in F13; (vi) abdomen GST in F7; (v) midgut GST in H; and (vi) midgut CAT in H, F7, oH+F7 and sH+F13.

In general, overlapping and sequential exposure to glyphosate and difenoconazole induced similar changes in the physiological markers. However, in uninfected bees, sequential exposure induced a greater change in AChE activity than did overlapping exposure (sH+F13 > oH+F7). In infected bees, overlapping exposure induced a greater change in abdomen GST activity than did sequential exposure (oN.H+F7 > sN.H+F13).

4. Discussion

In this study, infection with *N. ceranae* is the main factor influencing honey bee survival. A significant decrease in survival was observed in all *Nosema*-infected groups, confirming that this pathogen is able to reduce honey bee survival if infection occurs in the early stages of the life of adult bees (Aufauvre et al., 2012, 2014; Dussaubat et al., 2012). The exposure of infected honey bees to glyphosate or difenoconazole individually did not increase the adverse effect of *Nosema*. This result is in accordance with those of previous studies showing no significant effect of glyphosate on *Nosema* infection (Blot et al., 2019). However, pronounced changes in gene expression were reported in honey bees exposed to prochloraz (imidazole fungicide) at larval stage and then infected with *Nosema ceranae* after emergence (Glavinic et al., 2019). Concerning the effect of sequential exposure, exposure to glyphosate first and then difenoconazole (sN.H+F13) did not modulate the effect of *Nosema* on longevity. In contrast, there was a synergistic adverse interaction effect on longevity of overlapping exposure to the pesticides (oN.H+F7) and *Nosema*. This could be due to the effect of the detoxification system of infected honey bees on the sequence of exposure. Infection by *Nosema* induces the overexpression of genes encoding cytochrome P450 (CYP450) monooxygenases (Dussaubat et al., 2012), which are enzymes involved in the metabolism of xenobiotics (Mao et al., 2009; Mao et al., 2011). Hence, since the metabolism of glyphosate leads to nontoxic metabolites (Blot et al., 2019), the overexpression of CYP450 may increase the metabolism and decrease the toxicity of glyphosate. Azole fungicides, including difenoconazole, are known to be

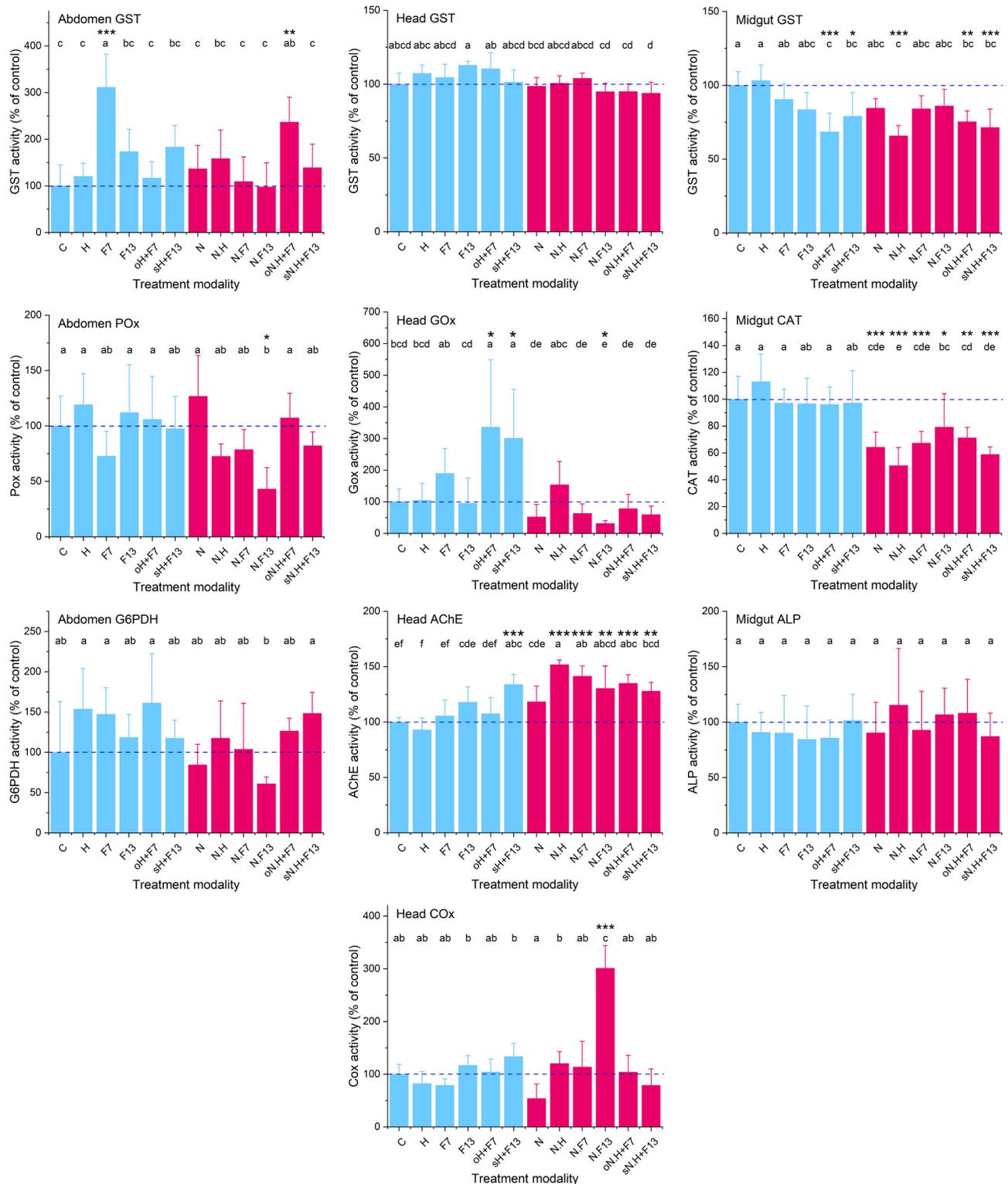


Fig. 4. Effects of *N. ceranae*-pesticide interactions on physiological markers of honey bees. Emerging honey bees were infected with *N. ceranae* (N) and then exposed to glyphosate (H) and/or difenoconazole (F) at 0.1 µg/L. H corresponds to honey bees exposed to H for 10 days starting at day 3, F7 corresponds to honey bees exposed to F for 10 days starting at day 7, and F13 corresponds to honey bees exposed to F for 10 days starting at day 13. C corresponds to control honey bees that were neither infected nor exposed to pesticides. “o” and “s” respectively refer to overlapping (oH+F7 and oN.H+F7) and sequential (sH+F13 and sN.H+F13) glyphosate+difenconazole treatments. A multiple marker approach was applied at day 23 after emergence, which corresponded to the maximum exposure period, to study the effects of the *N. ceranae*-pesticide interaction on the nervous system (AChE and COx), immune system (GOx and POx), oxidative stress and detoxification system (GST, CAT and G6PDH) and metabolism (ALP). GST was measured in the head, midgut and abdomen. AChE, GOx and COx were measured in the head. CAT and ALP were measured in the midgut. G6PDH and POx were measured in the abdomen. To make the data comparable, the enzymatic activities were expressed as percentages of the control values. The exposure modalities above and below the dashed horizontal line indicate increases and decreases in enzymatic activity, respectively, compared to that in the control (C). Data with different letters are significantly different ($p < 0.05$). Asterisks indicate significant differences from the control group (C): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

strong inhibitors of CYP450s involved in pesticide metabolism and are able to induce synergistic effects in association with insecticides (Colin and Belzunces, 1992; Johnson et al., 2013; Thompson and Wilkins, 2003). Thus, under overlapping exposure, difenoconazole may enhance the toxicity of glyphosate by inhibiting its metabolism, which is less likely under sequential exposure.

The cumulative ingested doses of glyphosate and difenoconazole were more than one million times less than their respective LD₅₀ values. In all cases of exposure, honey bees consumed similar amounts of food, confirming that neither glyphosate nor difenoconazole exhibited an attractive or a repellent effect, at least at the concentration of 0.1 µg/L. These results do not support the hypothesis that an increase in pesticide toxicity in bees infected by *N. ceranae* could result from an increase in pesticide intake (Alaux et al., 2010). The absence of an effect of glyphosate on food consumption was previously reported in summer honey bees exposed to this herbicide at concentrations of 0.21 and 1.08 g/kg (Blot et al., 2019). In addition, infection by *N. ceranae* did not result in an increase in food consumption, which is in accordance with the results of a previous study (Aufauvre et al., 2012). This confirms that the increase in food consumption is not a pertinent key symptom of infection by *N. ceranae* despite the dependency of this parasite on the energetic resources of the host (Liu, 1984) and the increase in energetic stress following infection (Martín-Hernández et al., 2011; Mayack and Naug, 2009).

Glyphosate and difenoconazole, either alone or in combination, did not have an effect on *Nosema* proliferation success in gut epithelial cells, even in the sequential oN.H+F7 treatment, which elicited the highest mortality. The absence of an effect of the sequential oN.H+F7 treatment on the *Nosema* count confirms that the strongest adverse effects induced by the interaction between *Nosema* and pesticides are not necessarily due to enhanced *Nosema* proliferation, as was previously shown for *Nosema*-fipronil and *Nosema*-thiacloprid interactions (Vidau et al., 2011). Thus, these results confirm that the *Nosema* spore count is not a suitable indicator of the mechanism involved in adverse *Nosema*-pesticide interactions (Collison et al., 2016).

The *Nosema*-pesticide interaction induced an impairment of the nervous system of honey bees. This was revealed by (i) an increase in AChE activity in all *Nosema* pesticide treatments. This enzyme is involved in learning and memory processes in insects (Gauthier et al., 1992), and the perturbation of its activity by pesticides alters the motor functions and behavior of honey bees (Williamson et al., 2013). In addition, the increase in AChE activity under all *Nosema*-pesticide treatments clearly shows that the influence of *Nosema* on honey bee physiology extends beyond the site of action of the parasite. (ii) An increase in COx activity under the N.F13 treatment, reflecting an increase in neuronal cell respiratory activity that could be linked to learning deficiencies (Bennett et al., 1996; Decourtye et al., 2004). Therefore, the interactions of *Nosema* with pesticides could contribute over time to colony collapse due to alterations in the behavior, foraging performance and homing flights of honey bees.

Infection by *Nosema* did not significantly alter GOx activity, which is in accordance with the results of two other studies (Alaux et al., 2010; Kairo et al., 2017). However, when *Nosema* was associated with difenoconazole whose exposure was initiated 10 days after infection (modality N.F13), a decrease in GOx activity below the physiological level was observed. In addition, infection by *Nosema* abolished the increase in GOx activity induced by overlapping (oH+F7) and sequential (sH+F13) exposure to glyphosate and difenoconazole. Thus, these results suggest that infection by *N. ceranae* tends to suppress the protective immune effect generated by the induction of GOx, possibly as a strategy of the parasite to protect itself from the deleterious impact of H₂O₂ produced by this enzyme.

Globally, *Nosema* infection does not affect the melanization process. This conclusion was supported by the absence of changes in phenoloxidase activity in all *Nosema*-infected groups in our study except for N.F13 and by two other studies (Alaux et al., 2010; Kairo et al., 2017; Vázquez

et al., 2020). The absence of an effect of *Nosema* on phenoloxidase activity is not correlated with the downregulation of the serine protease SP22 and SP40 genes in *Nosema*-infected honey bees (Aufauvre et al., 2014). These two genes are involved in the activation of prophenoloxidase pathways (Kanost and Clarke, 2005), and a decrease in their expression should result in a decrease in POx activity, which was not observed. Therefore, the alteration of the expression of POx-regulating genes does not always reflect changes at the phenotypic level, probably because of possible posttranslational modifications and regulation. However, honey bees do not rely solely on the melanization process to combat pathogens. A humoral immune response can also be achieved through antimicrobial peptides (AMPs). *N. ceranae* infection was previously reported to downregulate several AMP-coding genes (Antúnez et al., 2009; Aufauvre et al., 2014; Chaimanee et al., 2012). Thus, as in the case of POx, the downregulation of AMP genes might not reflect a decrease in AMP levels in honey bee hemolymph, making it difficult to predict the effect of *Nosema* on the humoral immune response.

One way in which pesticides could increase the susceptibility of honey bees to pathogenic infection (Aufauvre et al., 2012) is by impairing immunocompetence (i.e., the ability to mount a functional immune response) (Collison et al., 2016; Wilson-Rich et al., 2009). In our study, neither glyphosate nor difenoconazole, either individually or under sequential or overlapping exposure, decreased honey bee immunocompetence, as they did not impact POx activity or, hence, melanization. Such an absence of an effect of glyphosate on the humoral response was previously reported in honey bee larvae, in which an absence of changes in prophenoloxidase-activating enzyme (PPOact) and several AMP genes was found (Gregorc et al., 2012). However, the large decrease in POx activity elicited by the interaction between *Nosema* and difenoconazole exposure starting at day 13 (N.F13) showed that difenoconazole can weaken the immune defenses of bees infected by *Nosema*. Thus, it appears that pesticides can accentuate the physiological weakening elicited by infectious agents by impairing the immune system. In addition, the fact that N.F13, but not N.F7 ($p = 0.967$), decreased POx activity reveals that the period of exposure may be critical for the impairment of immune defenses.

The effects of *Nosema*, glyphosate and difenoconazole on antioxidant defenses strongly depend on the type of stressor and appear to mainly be tissue specific. Neither glyphosate nor difenoconazole affected the activity of antioxidant enzymes. However, glyphosate has been reported to induce oxidative stress in adult honey bees at higher concentrations under identical exposure durations (Helmer et al., 2015; Jumarie et al., 2017). This suggests that glyphosate and difenoconazole may cause oxidative stress after a quantity of pesticides exceeding a certain threshold is ingested, which depends on the level and duration of exposure. Combined exposure to glyphosate and difenoconazole (oH+F7 and sH+F13) had a stronger impact on oxidative stress than either pesticide applied individually. This was reflected in the decrease in GST activity in the midguts of honey bees exposed to both pesticides, independent of the exposure sequence. The decrease in GST activity could lead to an increase in oxidative damage and the toxicity of xenobiotics, as GST transforms lipid peroxidation products into less toxic hydroxyl derivatives and participates in the detoxification of xenobiotics (du Rand et al., 2015). Therefore, the detoxification system could become overwhelmed, and the oxidative balance could be impaired by combined exposure to several pesticides, which may lead to an increase in the pathogenicity of *Nosema* (Goblirsch, 2018). In the midgut, *N. ceranae* did not affect GST activity, which contrasts with the increase in activity observed in infected honey bees 7 and 10 days after infection (Dussaubat et al., 2012; Vidau et al., 2011). Moreover, midgut CAT activity decreased 23 days after infection under the six exposure modalities involving infection compared with the results of their uninfected counterparts (Control versus N; H versus N.H; F7 versus N.F7; oH+F7 versus oN.H+F7; sH+F13 versus sN.H+F13). This result contrasts with the increase in midgut CAT gene expression observed 7 days after infection (Dussaubat et al., 2012). The differences in the changes in

midgut CAT and GST observed between this study and previously published studies (Dussaubat et al., 2012; Vidau et al., 2011) might be linked to the changes in gene expression and enzymatic activities that occur during honey bee aging (Aufauvre et al., 2014). This could be explained by an increase in the production of reactive oxygen species (ROS) upon infection by *Nosema* and coexposure to glyphosate and difenoconazole. The increase in ROS triggers an increase in antioxidant defenses to protect the host from the potential harmful effects of ROS. A battery of enzymatic and nonenzymatic antioxidants are activated, which could explain the upregulation of the expression and the increases in the activities of CAT and GST in the midguts of infected honey bees observed during the first few days after infection (Corona and Robinson, 2006; Ha et al., 2005; Sies, 1993). The activation of these enzymes reduces the levels of ROS markers (soluble peroxides and protein carbonylation) (Paris et al., 2017). A decrease in the level of ROS, which serve as substrates for antioxidant enzymes, induces decreases antioxidant enzymes via a retro-control mechanism, which is in accordance with the decrease in CAT in the midguts 23 days after infection and the return to the normal physiological levels of CAT in the head, G6PDH in the abdomen and GST in the midgut.

5. Conclusion

The present study demonstrates that overlapping, but not sequential, exposure to an herbicide (glyphosate) and a fungicide (difenoconazole) at environmental concentrations synergistically increases the adverse effect of *Nosema* on honey bee longevity. Either alone or under overlapping and sequential exposure, glyphosate and difenoconazole induce disruptions in the nervous system, immunity, detoxification system and antioxidant defenses, particularly when they interact with *N. ceranae*. These findings reveal that the physio-pathological state of the honey bee should be considered a key variable in the assessment of pesticide toxicity in the registration procedure of phytopharmaceuticals.

CRedit authorship contribution statement

Hanine Almasri: Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Maryline Pioz, Maryam Alamil:** Formal analysis, Data curation. **Dai-ana Antonia Tavares, Déborah Sené:** Investigation. **Sylvie Tchamitchian, Marie Diogon:** Investigation, Resources. **Marianne Cousin:** Writing - review & editing. **Jean-Luc Brunet:** Writing - review & editing, Supervision. **Luc P. Belzunces:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2021.112258](https://doi.org/10.1016/j.ecoenv.2021.112258).

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