Nosema and Imidacloprid Synergy affects Immune-strength-related enzyme activity In the Honey bee, *Apis mellifera*

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SUMMARY

The dramatic depopulation of honey bee colonies has not yet been associated to a single culprit, although many potential contributing factors have been identified. In an attempt to address the impact of an association of two factors on the honey bee, *Apis mellifera*, we studied the effects of a joint exposure to the microsporidian *Nosema* and the systemic insecticide imidacloprid, in the context of parasitisation by the mite *Varroa destructor*. Young adult bees were exposed to doses found in the field, in a lab rearing experiment. Survival, feeding behaviour, and specific activity of two immunity-related enzymes were investigated. Parasitisation by *Nosema* combined with sublethal intoxication to imidacloprid triggered energetic stress and mortality. The association of these two factors did not affect phenoloxidase specific activity, but decreased the activity of glucose oxidase. These results reveal a weakening of the jointly treated bees, suggesting that the depopulation phenomenon is likely to be triggered by multifactorial causes and that the two factors studied were amongst.

INTRODUCTION

The honey bee, *Apis mellifera*, is an essential component to modern agriculture. It has been estimated that one third of the food consumed in the world is due, directly or indirectly, to honey bee pollination (Free, 1993) and the commercial value of pollination due to insects, primarily honey bees, reached 153 billion euro in 2005 (Gallai et al., 2009).

However, honey production and agricultural pollination around the world have been threatened over the last several years since heavy colony losses have been experienced by beekeepers over the winter period (Forster et al., 2005). A recent significant decrease in bee populations, described as the Colony Collapse Disorder (CCD), has been observed in the United States and in Europe and constitute another sign of population decline (for review, see Anderson et al., 2008). Several factors have been proposed to explain such high bee mortality, including widespread development of bacterial, viral and fungal pathogens (Cox-Foster et al., 2007), landscape changes due to modification in agriculture practises and intensity of pesticide use (Haubruge et al., 2006).

However, monofactorial analyses have failed to explain these heavy bee losses so far. They are currently being replaced by multifactorial approaches, to account for synergy hypothesis. In particular, since pathogens are not able to explain losses on their own, it is likely honey bees are suffering from compromised immune systems which pathogens are able to take advantage of or are responsible for (for review, see Oldroyd, 2007). Therefore, variations in immune activities can be regarded as a good sign of weakness, in the context of physiological disorders which could lead to bee population decrease.

Insects have an immune system closely related to the mammalian innate

immune system, which includes cellular and humoral responses involving the participation of critical enzymes (Hoffmann, 2003). Among them, phenoloxidase (PO) is implicated in the immune reaction of melanisation, which is the common response to parasite entry in invertebrates (Decker and Jaenicke, 2004; Söderhäll and Cerenius, 1998). The PO pathway plays a fundamental role in encapsulation of non phagocytised foreign elements through melanin synthesis (Lourenço et al., 2005), and therefore constitutes a good reporter of immune challenge at the individual level.

In addition to PO, glucose oxidase (GOX) is also an important enzyme to honey bee immunity. GOX belongs to carbohydrate-metabolizing enzyme family and catalyses the oxidation of β-D-glucose to D-gluconic acid and hydrogen peroxide (Bak, 1967), which confers antiseptic properties to the food (Musser et al., 2005). Expressed in the hypopharyngeal glands (Ohashi et al, 1999), this enzyme is secreted into larval food by nurse bees (Santos et al., 2007; Sano et al., 2004) and into honey by forager bees (White et al., 1967), which contributes to food sterilization and therefore to prevention of diseases. Thus, GOX appears to provide immunological protection at the colony level.

Pathogens and pesticides are the main factors that have been described to have a potential role in the heavy colony losses observed worldwide. Here, we present one of the first attempts to investigate the causes of these population decreases based on multifactorial hypothesis. Young adult bees were used as sample population in order to study factors internal to the colony. To this end, joined and/or disjoined infection by the parasite *Nosema* sp. and sublethal intoxication by the neonicotinoid pesticide imidacloprid were explored. Treatment doses were determined in accordance to those found in field conditions.

Nosema disease is one of the most prevalent adult honey bee diseases (Bailey, 1991; Matheson, 1996) and is caused by two described species of microsporidia, *Nosema apis* and *Nosema ceranae*. *Nosema Apis* was described more than a hundred years ago and infects the gut of adult bees, causing dysentery (Zander, 1909). *Nosema ceranae*, which also develops in the ventricular epithelial cells, has been very recently reported (Higes et al., 2006; Tsai et al., 2005). If none of the two species could be directly linked to colony losses, *Nosema ceranae* seems to be responsible for signs of colony weakness (Higes et al., 2008; Martin-Hernandez et al., 2007). Moreover, this later microsporidia species has been widely infecting *Apis mellifera* colonies in the United States for at least a decade, timing which is concomitant to the apparition of the bee depopulation phenomenon (Chen et al., 2008).

Systemic insecticides, and more particularly neonicotinoids such as imidacloprid, have been suspected to contribute to the colony losses (Frazier et al., 2008) although a great controversy remains amongst the scientific community concerning the toxicity of these products, especially at the sublethal level (Nguyen et al., 2009). However, it has been shown this class of pesticides not only penetrates the leaves they are spread on, but also enter the pollen and nectar of the plant (Chauzat el al., 2006). Consistently with this observation, several studies have shown behavioural changes on memory processes, following experimental exposure to imidacloprid (Yang et al., 2008; Decourtye et al., 2004).

Moreover, one of the main sources of stress for honey bees is the varroa mite, *Varroa destructor*. This parasite weakens colonies directly by feeding on the haemolymph and indirectly by serving as a vector of bee viruses (Chen et al., 2004). As this mite is currently present in bee hives all over the world, varroa parasitisation was used in this study as a third parameter, in addition to *Nosema* infection and imidacloprid intoxication.

The work presented in this report evaluates the immune response of honey bees jointly and/or disjointly infected by *Nosema* and intoxicated by imidacloprid, in presence or absence of varroa. To this end, an estimation of mortality rates, feeding behaviour, as well as measurements of the enzymatic activities of PO and GOX using biochemical assays, were performed. This study on the honey bee immunity is part of a broader project measuring other physiological parameters in order to detect the potential synergy between *Nosema* and imidacloprid, in context of varroa infestation.

RESULTS

Mortality rate of bees jointly and/or disjointly infected by *Nosema* and intoxicated by imidacloprid

Bee mortality was assessed on adult bees aged 1 to 10 days. Preliminary experiments showed that an imidacloprid concentration of 7 µg/L of feeding syrup corresponds to a sublethal dose in an acute intoxication assay (LD_{50}= 150 ng/bee at 48 h). Here we investigate the potential effect on mortality of such a dose, when chronically delivered to young bees. This mortality assay also investigated the hypothesis of a synergy between *Nosema* and imidacloprid. Four groups of bees were included, depending on the treatment they received: control, *Nosema* alone, imidacloprid alone, both *Nosema* and imidacloprid (n=9 cages for each group, n=30 bees per cage). Within one treatment group, bees from three different colonies were analysed, with three cages replicates for each hive. Two hives were uninfected by the parasitic mite *Varroa destructor* ("varroa free" hives – V^-) and one hive was highly parasitised by varroa ("varroa parasitised" hives – V^+).

The cumulated mortality rate increased with time for all treatment conditions, but remained lower than 10% in the control group (Figure 1.). GLM performed on all data revealed a significant time effect (t=13.51, $p<10^{-15}$). This same analysis indicated the absence of colony effect (t=1.18, NS). In addition, an important treatment effect was detected. Indeed, all three treatment groups exhibited significantly higher mortality rates than the control group (Imidacloprid, t=7.32, $p<10^{-10}$; *Nosema*, t=6.12, $p<10^{-7}$, *Nosema*/Imidacloprid, t=6.40, $p<10^{-8}$). Interestingly, on the last two days of rearing, the mortality rate of the jointly treated group was higher than the rate of the disjointly treated groups. In particular, on day 9 and 10 mortality rates of the *Nosema*/Imidacloprid group equalled the sum of the mortality rates of the *Nosema* and imidacloprid groups (Figure 1.).

Based on these observations, two sampling dates were chosen for enzymatic activity measurements (see next sections), according to the presence or absence of significant mortality rate detected in the cages. The first one, corresponding to the 5th day after the start of the experiment, was selected for the low mortality rate observed in the four treatment groups. The second sampling was performed 10 days after the beginning of the experiment, as bees exposed to *Nosema* and Imidacloprid displayed a significantly higher mortality than

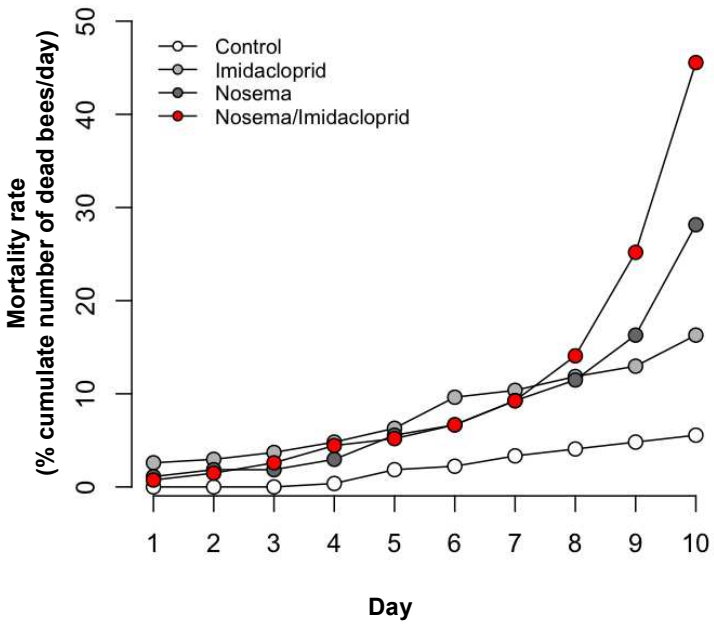

Figure 1. Bees jointly and/or disjointly exposed to *Nosema* and imidacloprid show increased mortality rates in lab rearing conditions.

Mortality is expressed as percentage of the cumulated number of dead bees per cage and per day (n=270). GLM analysis revealed a significant time effect ($p<10^{-15}$), as well as significant differences between treated and control groups (Imidacloprid, $p<10^{-10}$; *Nosema*, $p<10^{-7}$; *Nosema*/imidacloprid, $p<10^{-8}$). Four treatment groups (Control, Imidacloprid, *Nosema*, *Nosema*/Imidacloprid) were included (n=9 cages per group), and reared for 10 days. 3 colonies were analysed, with 3 cage replicates for each colony. 30 bees were placed in each cage at day 1. Asterisks indicate dates for which the mortality rate of *Nosema*/imidacloprid treated bees is higher than in *Nosema* or imidacloprid treated bees.

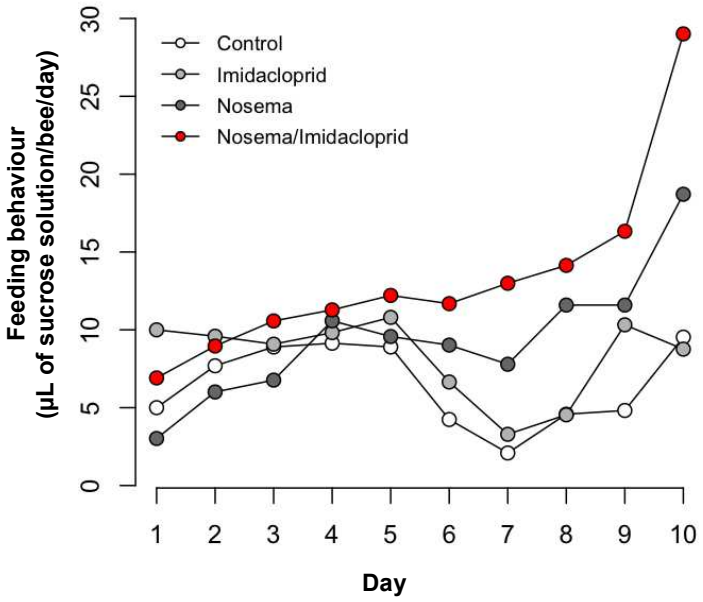

Figure 2. Infection by *Nosema* and joint exposure to *Nosema* and imidacloprid induce energetic stress in lab reared bees.

Feeding behaviour is expressed as the amount of sucrose solution (50% w/v, *ad libitum* delivery) consumed per day and per bee (n=30 bees per cage).

Two-way repeated measures ANOVA revealed that bees jointly exposed to *Nosema* and imidacloprid absorbed more sucrose than any other treatment group (p=0.003). Four treatment groups (Control, Imidacloprid, *Nosema*, *Nosema*/Imidacloprid) were included (n=9 cages per group), and reared for 10 days. 3 colonies were analysed, with 3 cage replicates for each colony. 30 bees were placed in each cage at day 1.

bees exposed to other treatments. On the 10^{th} day, 46% (± 7%) of the *Nosema*/Imidacloprid treated bees were dead. The number of living bees left in these cages being just high enough to allow sampling for subsequent measures, the rearing process was stopped at this date. 10 days also corresponds to a limit time for rearing after which mortality effects due to artificial rearing conditions can appear (personal data).

Feeding behaviour of bees jointly and/or disjointly infected by *Nosema* and intoxicated by imidacloprid

Comparison of the feeding behaviour between the four treatment groups was performed by estimating the amount of sucrose absorbed per day and per bee, as shown in Figure 2. These measures were performed on the same cages as those used for the mortality assay (n=9 cages per treatment).

Two-way repeated measures ANOVA revealed that the amount of sucrose absorbed significantly increased with time ($F_{9.288}$=17.17, $p<10^{-3}$). The treatment effect ($F_{3.288}$=16.84, $p<10^{-3}$) and the interaction between the factors treatment and time ($F_{3.288}$=5.69, $p<10^{-3}$) were also significant. Indeed, post-hoc analysis using Bonferroni unpaired t-tests indicated that bees treated with *Nosema* or with *Nosema* and imidacloprid absorbed more sucrose than bees from the control group (*Nosema*, t=2.95, p=0.01; *Nosema*/imidacloprid, t=6.85, $p<10^{-3}$). In addition, sucrose consumption was higher in the *Nosema*/imidacloprid group than in the *Nosema* group (t=3.90, p=0.003). However, the daily sucrose consumption did not differ between control bees and bees exposed to imidacloprid (t=1.78, NS).

Determination of the compartment for enzymatic activity measurements

The first preliminary enzymatic assay has been performed in order to determine the optimum compartment to be used for the measurement of phenoloxidase (PO) and glucose oxidase (GOX) activities.

In the case of PO, measurements were carried out into the haemolymph, the thorax, the abdomen and the abdomen devoid of the digestive tract (Figure 3.A.i.). 32 bees were analysed for each compartment.

A mean specific activity of 695 $muA.min^{-1}.mg^{-1}$ of proteins was recorded in the haemolymph, which corresponds to the highest PO enzymatic activity measured (Haem. - Figure 3.A.i.). However, measurements in haemolymph were abandoned since variability of PO activity

was there particularly high (SEM, ± 30%; box and whiskers). Although the abdomen and the thorax are two compartments containing haemolymph, specific activities in these two body parts did not exceed 23 and 18 muA.min^{-1}.mg^{-1} of proteins, respectively (TotAbd., Thorax – Figure 3.A.i.).

Interestingly, PO specific activity in abdomens devoid of the digestive tract reached 126 muA.min^{-1}.mg^{-1} of proteins, which is 5.1 times higher than the activity detected in total abdomens (VoidAbd. – Figure 3.A.i.). Moreover, variability in voided abdomens was about half of that measured in the haemolymph (SEM, ± 17%).

Further measurements were performed to compare the specific activities of PO between nurse and forager bees, as it has been shown that PO exhibits age related differences in activities (Wilson-Rich et al.,

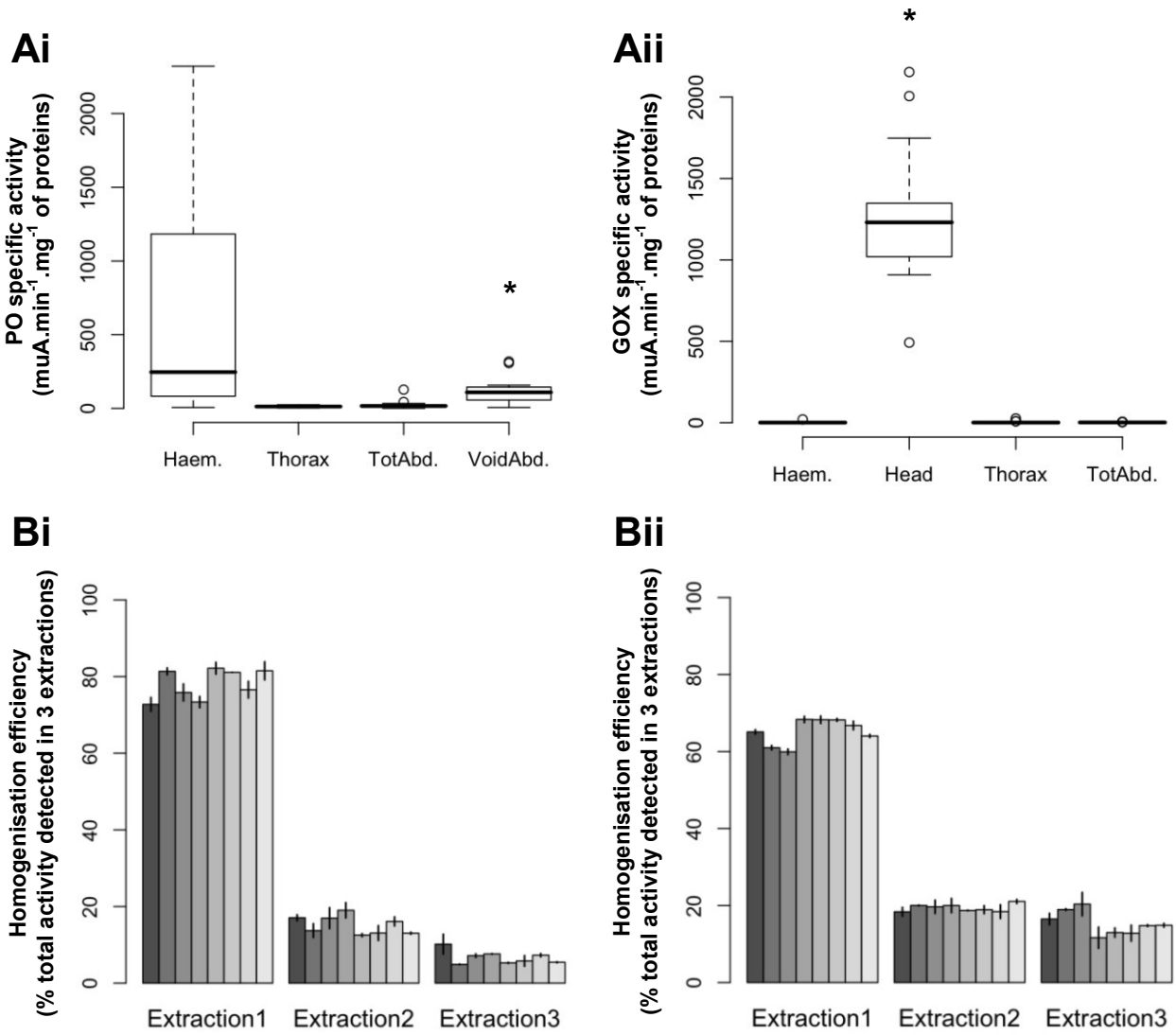

Figure 3. Voided abdomens and heads were determined as reference compartments for the measure of phenoloxidase (PO) and glucose oxidase (GOX) activities, respectively.

Ai-Bi. Characterisation of PO enzymatic activity.

Aii-Bii. Characterisation of GOX enzymatic activity.

(A) Determination of the optimum compartment to perform enzymatic measurements (n=32 bees for each compartment). Boxes show 50% of the individuals with line denoting medians. Whiskers encompass 90% of the individuals, beyond which outliers are represented by circles. Asterisks indicate the compartment chosen as reference for subsequent enzymatic measurements.

(B) Determination of the homogenisation rate efficiency in the reference compartments (n=8 bees). Measures are expressed as percentage of the total activity detected in the 3 extractions. Error bars indicate the SEM from the triplicate measurements of absorbance for each sample.

Heam., Heamolymph; Abd., Abdomen; VoidAbd, Abdomen devoid of rectum and intestine.

2008). The same activity ratio between the two age groups was found in both the haemolymph and voided abdomens (data not shown), confirming the reliability of the later compartment to detect changes in PO activity between different groups of bees. Therefore, all subsequent measurements of PO enzymatic activities were performed on voided abdomens.

GOX activities were investigated in the haemolymph, the abdomen, the thorax and the head (Figure 3.A.ii.). 16 bees were analysed for each compartment.

Enzymatic activity was very low in the thorax, abdomen and haemolymph, and did not exceed 5 muA.min^{-1}.mg^{-1} of proteins (Thorax, Abd., Haem. – Figure 3.A.ii.). On the contrary, GOX specific activity reached 1258 muA.min^{-1}.mg^{-1} of protein in the head (Head – Figure 3.A.ii.). Moreover, variability between samples was very low (SEM, ± 7%) in this same body segment. The head was therefore chosen as the reference compartment for all subsequent measurements of GOX enzymatic activities.

The second preliminary assay investigated the efficiency of the homogenisation process in each of the two compartments chosen to estimate PO and GOX activities. To this end, three successive homogenisations were performed on each sample, and the enzymatic activity was measured in the three supernatants obtained (n=16 bees for each enzyme). For each homogenisation process, activities were expressed as percentage of the total enzymatic activity detected in the 3 extractions.

Results obtained for PO are presented on Figure 3.B.i. The first homogenisation extract contained on average 78% (SEM, ± 1.4%) of the total enzymatic activity, the second 15.2% (SEM, ± 0.9%), and the third 6.7% (SEM, ± 0.6%).

In the case of GOX (Figure 3.B.ii.), the first homogenisation extract contained in average 65.2% (SEM, ± 1.2%) of the total enzymatic activity, the second 19.4% (SEM, ± 0.3%), and the third 15.3% (SEM, ± 1.1%).

Since first extraction rates were higher than 2/3 for both enzymes and highly reproducible, subsequent enzymatic measures were performed on the supernatant resulting from the first homogenisation process only.

Effect of joined and/or disjoined infection by *Nosema* and sublethal intoxication by imidacloprid on specific activity of phenoloxidase

Specific activity of phenoloxidase (PO) was assessed on voided abdomens dissected on bees belonging to four groups, according to the treatment they received (Control, *Nosema*, Imidacloprid, *Nosema*/Imidacloprid), on day 4 (D4) and 9 (D9) of the assay (Figure 4.A.). 6 cages were reared for each of the four treatment conditions and 8 bees sampled for PO activity measurements (n=48 bees for each group). Similarly to the mortality assay, newly emerged bees coming from 3 different colonies were caged, with 2 replicates per hive. The 3 colonies included 2 “varroa free” hives and 1 “varroa parasitised” hive.

Two-way ANOVA performed on all data revealed that replicates of each colony behaved similarly regarding the specific activity of the immunity-related enzyme PO (($F_{1.352}$=1.33, NS).

Similarly, PO activity was not affected by the treatments ($F_{3.352}$=1.57, NS). In particular, the activity of this enzyme was not significantly different between control bees and bees jointly treated by *Nosema* and imidacloprid.

However, PO specific activities significantly differed between colonies ($F_{2.352}$=17.00, $p<10^{-7}$). Further analysis showed that the variation detected between colonies was specific to day 10. Figure 4.B.i. presents specific activities for the three colonies at day 5 and Figure 4.B.ii. presents results at day 10. Two-way ANOVA performed on day 5 data showed no colony effect at this date ($F_{2.174}$=1.49, NS). On the contrary, colonies sampled at day 10 displayed markedly different responses in PO activity ($F_{2.173}$=18.61, $p<10^{-7}$). No treatment effect was revealed at day 5 ($F_{3.174}$=1.3, NS) or day 10 ($F_{3.173}$=1.28, NS).

As showed on Figure 4.A., further analysis of the two-way ANOVA performed on all data indicated that PO activity significantly increased between day 5 and day 10 ($F_{1.352}$=10.90, $p<10^{-4}$). Figure 4.C. presents the results obtained at day 5 versus day 10, for the two “varroa free” colonies (Figure 4.C.i and 4.C.ii.) and the “varroa parasitised” colony (Figure 4.C.iii.). Two-way ANOVAs performed on each hive separately revealed that this date difference in PO

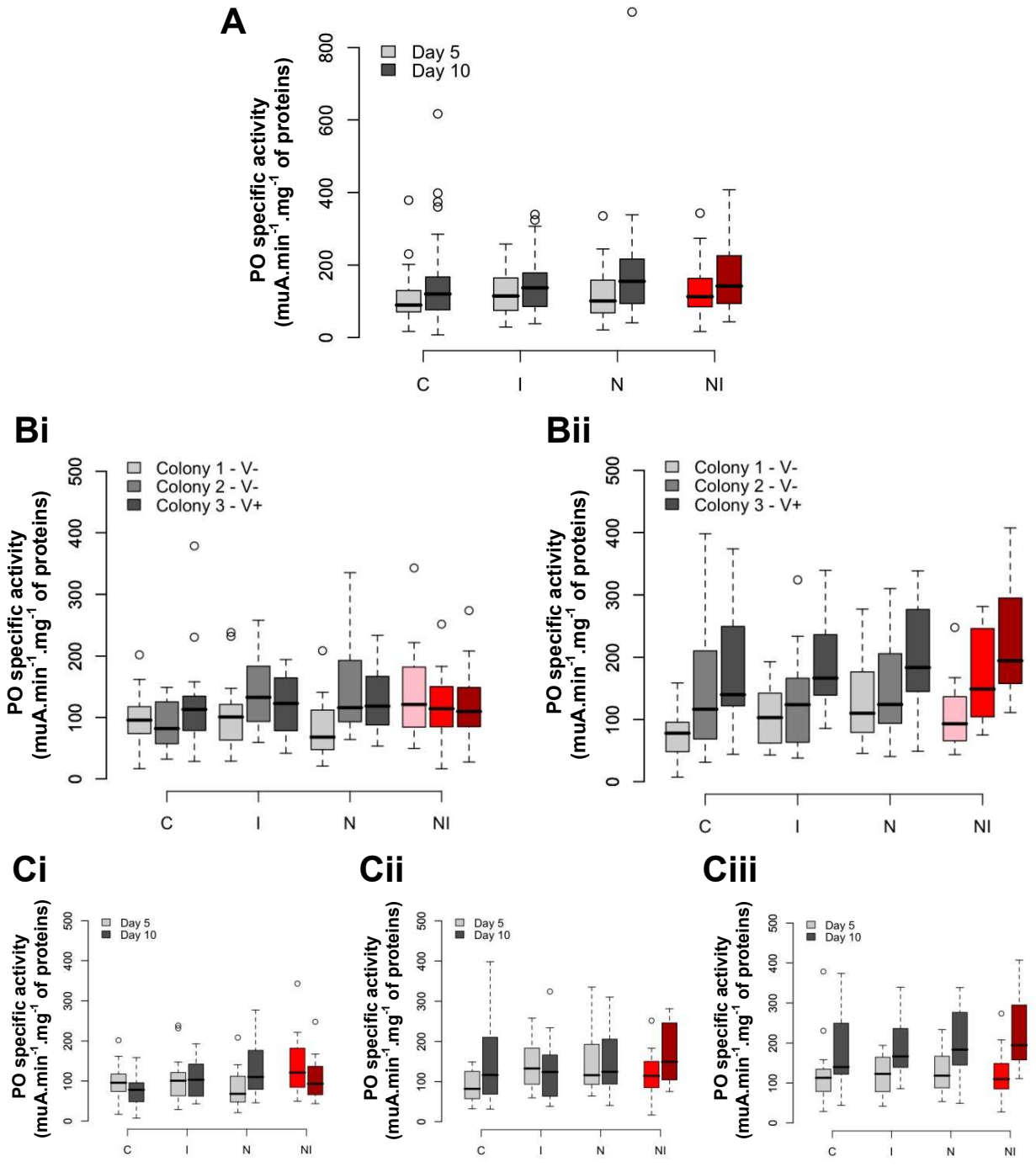

Figure 4. Joint and/or disjoint exposure to *Nosema* and imidacloprid does not affect phenoloxidase (PO) specific activity but varroa parasitism increases PO activity

(A) Global representation of PO specific activity at day 5 and day 10 for the four treatment groups (n=48). Two-way ANOVA revealed a significant date effect ($p<10^{-4}$), and the absence of treatment effect (p=0.2).

(B) Comparison of PO specific activity between colonies, at day 5 (i) and day 10 (ii), (n=16). Two-way ANOVA highlighted a colony effect at day 10 ($p<10^{-7}$), but not at day 5 (p=0.2).

(C) Comparison of PO specific activity between day 5 and day 10, for the 3 colonies (n=16). Two-way ANOVA revealed the absence of date effect for the 2 "varroa free" colonies (i, $p_1=0.9$; ii, $p_2=0.04$). The date effect was highly significant in the "varroa parasitized" colony (iii, $p<10^{-5}$).

3 colonies were analysed, with 2 cage replicates for each colony. 8 bees were sampled in each cage and at each date (day 5 and day 10) for PO activity measurements. Boxes show 50% of the individuals with line denoting medians. Whiskers encompass 90% of the individuals, beyond which outliers are represented by circles. Data obtained from bees jointly exposed to *Nosema* and imidacloprid are indicated in red. C, control; I, imidacloprid; N, *Nosema*; NI, *Nosema*/imidacloprid; V-, "varroa free" colony; V+, "varroa parasitized" colony.

activity was significant only in the "varroa parasitised" hive ($F_{1.118}$=22.78, $p<10^{-5}$) and not in any of the two "varroa free" hives ($F_{1.112}$=0.0077, NS; $F_{1.116}$=4.34, NS).

Effect of joined and/or disjoined infection by *Nosema* and sublethal intoxication by imidacloprid on specific activity of glucose oxidase

Specific activity of glucose oxidase (GOX) was assessed on heads dissected from bees which had been exposed jointly or disjointly to *Nosema* and Imidacloprid for 9 days (Figure 5.). As for PO activity measurements, 3 colonies were sampled, with 2 replicates per colony. Therefore, each treatment was repeated on 6 cages, in which 8 bees were sampled for GOX activity measurements (n=48 bees for each group).

As showed on Figure 5.A., two-way ANOVA performed on all data revealed a significant effect of the treatments on the specific activity of GOX ($F_{3.182}$=4.62, $p<4.10^{-3}$). Unpaired t-tests indicated that the *Nosema*/Imidacloprid group exhibited a significantly lower GOX specific activity than the control group (t=2.54, $p<10^{-2}$). Although the two other treatment groups showed slightly higher activities than the control group within one given colony (Figure 5.B.), this difference was not significant when considered globally (*Nosema*, t=1.38, NS; Imidacloprid, t=0.34, NS).

Further analysis highlighted that the response of GOX activity was reproducible (Figure 5.B.). Indeed, two-way ANOVA did not reveal any significant difference within hive replicates ($F_{1,182}$=0.92, NS) or within colonies ($F_{2,182}$=1.91, NS).

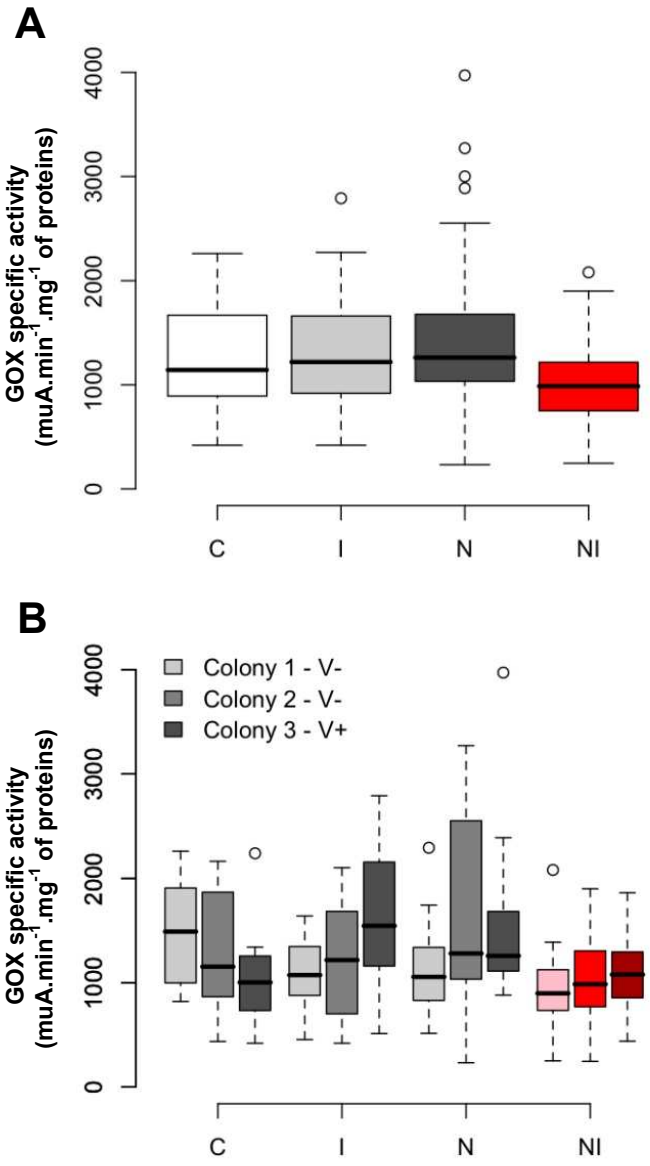

Figure 5. Joint exposure to *Nosema* and imidacloprid decreases glucose oxidase (GOX) specific activity

(A) Global representation of GOX specific activity at day 10 for the four treatment groups (n=48). Two-way ANOVA followed by unpaired t-test revealed that joint exposure to Nosema and imidacloprid decreases GOX activity ($p<10^{-2}$).

(B) Comparison of GOX specific activity between colonies, at day 10 (n=16). Two-way ANOVA highlighted the absence of colony effect (p=0.2).

3 colonies were analysed, with 2 cage replicates for each colony. 8 bees were sampled in each cage for GOX activity measurements. Boxes show 50% of the individuals with line denoting medians. Whiskers encompass 90% of the individuals, beyond which outliers are represented by circles. Data obtained from bees jointly exposed to *Nosema* and imidacloprid are indicated in red.

C, control; I, imidacloprid; N, *Nosema*; NI, *Nosema*/imidacloprid.

DISCUSSION

In this study, we performed mortality, feeding behaviour and enzymatic assays to assess the effect of joint and /or disjoint infection by the microsporidian *Nosema* and sublethal intoxication to the neonicotinoid insecticide imidacloprid on the activity of two immunity-related enzymes, in artificial rearing conditions and in the context of parasitisation by *Varroa destructor*. The two factors *Nosema* and imidacloprid displayed synergistic effects on feeding behaviour, mortality and on the specific activity of the glucose oxidase (GOX) enzyme. This study also revealed that the activity of phenoloxidase (PO) was not affected by *Nosema* and/or imidacloprid. Parasitisation by varroa may lead to an activity increase of this later enzyme. These observations bring interesting perspectives concerning the immune and energetic responses displayed by the honey bee, *Apis mellifera*, exposed to a combination of environmental stresses.

Enzymatic measurement techniques are reliable for detecting specific activity variations in different groups of bees.

Biochemical assays were developed to measure the specific activities of two immunity-related enzymes: phenoloxidase (PO) and glucose oxidase (GOX).

GOX activity was maximal in the head. This segment corresponds to the compartment of principal accumulation of this enzyme, since it is known to be synthesised in the bee hypopharyngeal glands (Ohashi et al., 1999) and is incorporated to the food in the salivary glands (Merkx-Jacques and Bede, 2005).

PO activity was particularly high in the haemolymph, which is in accordance with the immune functions displayed by this body fluid in insects (Gillespie and Kanost, 1997). Haemolymph plays a key role in the cellular immune response, which includes as main component the PO pathway. Although the haemolymph displayed the maximal PO activity detected in this study, measures were not performed on this fluid because of the important variability between individuals. This may be due to the variation in volume of haemolymph and to the difficulty of estimating the total amount of haemolymph circulating in one bee. Measurements were performed on abdomens devoid of the digestive tract, a compartment containing haemolymph and the fat bodies which are also involved in the cellular immune response (Lemaitre et al., 2006). Interestingly, the PO activity detected in this compartment was higher than in the one measured in total abdomens. This difference can be interpreted by the presence of inhibitors or inhibitory enzymes in the digestive tract, as it has already been described for P450 enzymes (Krieger and Wilkinson, 1971). A reduction in PO activity may also be the result of the action of enzymes that degrade melanin, which is the product of the reaction catalysed by PO.

PO activity do not display any variation in response to joint and/or disjoint exposure to *Nosema* and imidacloprid

PO activity measurements were used as a tool to assess the immune response of bees reared with *Nosema* and/or imidacloprid. No variation due to these two factors in the specific activity of this enzyme was detected, either on 5-day old or 10-day old bees. This suggests that the PO pathway, which is involved in the mechanism of elimination of non phagocytised foreign elements, is not mobilised in such conditions of rearing.

This absence of treatment effect on such a key immunity-related enzyme may indicate the immune system is not activated in response to the two factors *Nosema* and/or imidacloprid. Possibly, imidacloprid does not affect the immune system. Indeed, most of the sublethal effects of this pesticide have been revealed on neurological processes, such as learning and memory (Guez et al, 2001), that could affect orientation abilities. However, it has been shown that *Nosema* affects the bee immune system (Antunez et al., 2009). *Nosema ceranae* suppresses the immune reaction, whereas *Nosema apis* activates defence mechanisms, mainly through the production of peptides and enzymes with antimicrobial actions. Therefore, when both *Nosema ceranae* and *apis* are parasitising a colony, which is the case in our study, the two species could have antagonist effects on the immune system, in particular on the PO pathway. As a result, no variation in PO activity may be observed although this enzyme might be recruited in response to *Nosema* and/or imidacloprid

exposure. Such antagonist effects, could also happen when the colony is exposed to different stimuli, such as pathogens and pesticides.

Alternatively, the PO pathway could be implicated consequently to the infection by *Nosema* and the intoxication to imidacloprid, but the response may not be immediate. Indeed, this study investigated the consequences of an exposure to these two factors for 10 days only. Therefore, if the treatment effect on PO is delayed, or if this enzyme is activated in a pathogen dose dependant manner, it would have been necessary to perform measurements later in the bee life in order to detect any activity variation.

In any case, in order to have a more complete estimation of the mobilisation of the immune system, other parameters should be estimated. It would be interesting to measure the activity of other immunity related enzymes, such as glucose dehydrogenase or lysozyme. However, our attempts to develop a spectrophotometry dosage technique for lysozyme in the honey bee have not been successful so far. In addition to the activity, mRNAs levels of the same enzymes or of immune response genes, such as defensin, could be estimated. Finally, interest could be given to behavioural studies since it has been shown defence against pathogens and parasite can involve behaviour modification within an infected colony (Hart, 1988).

PO activity variation could be triggered by varroa parasitisation

Enzymatic assays performed on PO failed to reveal any effect of the two factors *Nosema* and imidacloprid. However, a highly significant colony effect was detected. This result suggests that other factors may be implicated in the PO response, such as the genetic background, or the presence of pathogens. Consistently with this view, one single colony, which was infected by the parasitic mite *Varroa destructor,* was responsible for this colony effect. Indeed, 10-day old bees coming from this hive exhibited higher PO activities than other bees, independently from the treatment they received during the rearing period. This suggests that varroa may activate the PO pathway, and this could be due to the multiplication of viral infections in “varroa parasitised” bees (Sammataro et al., 2000). This possible incidence of varroa parasitisation confirms that a combination of many factors may trigger weakening of a honey bee colony.

PO and GOX may not participate in the same immunity and defence mechanisms

Measurements performed on GOX activity also highlight the complexity of the responses developed consequently to an environmental stress. Indeed, the activity of this enzyme was very similar between hives in response to joint and/or disjoint exposure to *Nosema* and imidacloprid. Thus, varroa may not affect this part of the carbohydrate-metabolising pathway.

Moreover, the intensity of the GOX specific activity, contrarily to PO activity, was treatment dependant. GOX activity was lowered on bees jointly exposed to *Nosema* and imidacloprid compared to the control bees, whereas no difference was detected on bees which received single treatment by *Nosema* or imidacloprid. This result is consistent with the multifactorial hypothesis proposed to explain the colony weakening phenomenon.

The differential response displayed by PO and GOX consequently to *Nosema* and/or imidacloprid exposure suggests that these two enzymes may not have the same immune functions. Indeed, PO appears to be a reliable tool to estimate the immune state of a single bee at the time of sampling, since variations in activity reveals an activation of the process of elimination of non phagocytised foreign elements (Lourenço et al., 2005). On the contrary, variations in GOX activity may not have major consequences for the individual which is host for this reaction. More likely, such variations would give indications on the future immune state of the colony. Indeed, a decrease in GOX activity reduces the potential of sterilisation of the food subsequently delivered to larvae. These developing bees could therefore be more subject to pathogen attacks. Thus, the significant decrease in GOX specific activity measured on bees jointly exposed to *Nosema* and imidacloprid may presage a weakening of the colony, due to the development of opportunistic pathogens.

Nosema and Imidacloprid seem to have synergistic effects on the bee physiology

In addition to GOX specific activity, two other physiological parameters measured in this study seem to rely on synergistic effects of *Nosema* and imidacloprid.

The mortality rate of 10 day-old bees jointly treated with *Nosema* and imidacloprid equalled the sum of the mortality rates obtained with single exposures, revealing that *Nosema* and imidacloprid have additive effects on mortality for these doses of imidacloprid and *Nosema*. Moreover, bees treated with both *Nosema* and imidacloprid consumed more sucrose than bees exposed to *Nosema* alone.

Likely, the feeding data obtained for the *Nosema* and *Nosema*/imidacloprid groups are the result of an energetic stress in these bees. This phenomenon is commonly observed when bees are reared in the presence of an environmental stress (Thompson and Redak, 2008, personal observations). In particular, *Nosema ceranae* has been shown to increase the hunger level of infected bees (Mayack et al., 2008). This energetic demand can be due to the mounting of an immune response, which is an energetically expensive process (Schid-Hempel, 2005). In the case of parasitism, the pathogen energetic dependence may be by-passed through the induction of such an energetic stress (Agnew and Koella, 1997).

In addition, the decrease in GOX activity detected in this study when bees are jointly exposed to *Nosema* and imidacloprid could accentuate this situation of energetic stress. Indeed, bees have a diet particularly rich in carbohydrates, but their diversity is limited. Three specific enzymes, alpha-glucosidase, amylase and GOX, are involved in the synthesis of the sugar forms which are not available in the food (for review, see Kunieda et al., 2006). Thus, a decrease in GOX enzymatic activity may trigger an increase in energetic demand in order to overcome the lack of particular carbohydrates.

Moreover, since imidacloprid was delivered in the food (to reproduce consumption of contaminated honey in field conditions), the increase in the amount of sucrose absorbed by bees exposed to both *Nosema* and imidacloprid is likely to lead to a higher exposure to the insecticide. Therefore, some imidacloprid effects on physiology could be revealed in such bees, especially if they are dose dependant. As previously described, parasites are often responsible for energetic stress in their host. Considering the multiplicity of pathogens currently infecting honey bee colonies, this higher energetic demand could increase bee exposure to chemical residues present in honey.

Consistently with this view, mortality rates were higher in the jointly than disjointly treated groups in the last days of rearing. In particular, a significant mortality effect was detected on the imidacloprid group. This observation is in accordance with previous intoxication studies (Suchail et al., 2001), but in contradiction with recent results obtained in a field survey on the impact of maize seeds treated with this insecticide (Nguyen et al., 2009). The significant mortality rate observed for *Nosema* treated bees can be attributed to the presence of the species *Nosema ceranae* in our spore solution. Indeed, this species has been recently described as pathogenic for bees (Higes et al., 2008). Moreover, the mortality rate in the 3 treated groups was significant only after 6 days of treatment. Interestingly, young bees appear to be more resistant to intoxications than older bees. In fact the LD50 values obtained for a 48 hour exposure to imidacloprid was 2.5 times higher on newly emerged bees than on nurse and forager bees (Suchail et al., 2001; preliminary studies). Possibly, the mortality effect of the treatments can be delayed. In this case, in-hive exposure to a combination of insecticide and parasite may affect the next generation of forager bee population. Thus, the colony collapse disorder (CCD) could be partly due to factors internal to the hive, although this phenomenon is primarily characterised by the death of “outside” bees (Cox-Foster and vanEngelsdorp, 2009). It could therefore be useful to repeat our study in field conditions, in order to increase the duration of the assays. This could allow assessing the effect of joint and/or disjoint exposure to *Nosema* and imidacloprid on the lifespan, behaviour and immune state of bees, from their emergence until they become foragers. Such an experiment would be an opportunity to investigate the effects of multifactorial exposures on the queen physiology. More particularly, long term

survival and laying ability measurements could be of use since beekeepers have noticed a reduction in their queen laying longevity.

Mortality effects and GOX activity decrease suggest a colony weakening

The effect of joint exposure to *Nosema* and imidacloprid on mortality and GOX activity may be the sign of a weakening of the treated bees. In addition, bees treated with both factors often had a "wet" aspect in cages, revealing a ventilation defect. This physiological weakening may have deleterious effects on the ability of bees to develop an effective immune response in the case of pathogen attacks. Such weakened bees could therefore be more vulnerable to viruses and other opportunistic infections, a phenomenon which is commonly observed on colonies suffering from CCD (Cox-Foster et al., 2007).

Furthermore, the mortality curves obtained, especially in the case of exposure to *Nosema* or *Nosema* and imidacloprid, exhibited a high exponential factor. Indeed, from day 8 to day 10, the mortality rate doubled each day in the jointly treated group. Such sudden mortality events may reflect the events of losses that are observed for CCD (Stokstad, 2007).

In this study, we investigated the effects of two factors, one parasite and one insecticide, on the honey bee immunity, feeding behaviour and survival. Joint exposure to *Nosema* and imidacloprid lead to weakened bee populations, both at the individual and the group level. These effects were not observed at the same degree when the exposures were disjoint. Therefore, honey bee colonies suffering from the depopulation phenomenon are likely to be in a complex condition that can be triggered by large combinations of primary causes. Bees exposed to these environmental factors may be in turn a target for opportunistic infections which can be responsible for the physiological disorders. Thus, remedies to the bee population decrease may require long-term changes to both beekeeping and agricultural practices.

EXPERIMENTAL PROCEDURES

Chemicals

All chemicals were obtained from Sigma Aldrich, except for imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine] which was provided by Cluzeau (Dr. Ehrenstorfer) and for the protein assay dye reagent which was obtained from Bio-Rad.

Honey bee colonies and artificial rearing

Colonies were maintained at the Institut National de la Recherche Agronomique Laboratory of Bees and Environment. Experiments were performed during spring 2009 (February to May). Bees were taken from hives located on the same study site in Avignon, and were a mixture of European subspecies (*Apis mellifera*) typically used for beekeeping in southeast of France, primarily *Mellifera*.

Varroa mite (*Varroa destructor*) levels were assessed in colonies using the sugar shake method (Macedo and Ellis, 2002); two "varroa free" hives and one infected hive were chosen for the study, in order to respect the degree of infestation of the study site (data given by the beekeepers in charge of the apiary).

To determine the compartment for measuring the enzymatic activities, nurse bees were caught inside one single colony uninfected by varroa and sampled as bees visiting unsealed brood cells. For further characterization of the phenoloxidase activity, forager bees carrying pollen baskets were caught at the entrance of the colony.

Pesticide exposure and parasite infection were performed on young adult bees of known age, reared in the laboratory after emergence. To provide newly emerged bees, honey combs containing late stage pupae were placed in a 34°C incubator. Newly emerged bees were transferred in cages (10.5 x 7.5 x 11.5 cm) and kept in an incubator (28°C, non-controlled humidity) for 9 days. Bees were fed *ad libitum* a freshly prepared 50% (w/v) sucrose/water solution 10 hours a day (containing or not imidacloprid). The rest of the time, they were fed bee candy (70% honey, 30% icing sugar) and water. The feeders were replaced each day at the same time of the day and the daily sucrose consumption was measured for each cage. The amount of sucrose absorbed was expressed per day (10 hour period) and per bee, by dividing the amount consumed in a cage by the number of bees in this cage. Bees were also given pollen for the first three days to provide a source of proteins (Grogan et al., 1979), and treated with 3 queen equivalent of queen mandibular pheromone (QMP) (Pherotech Canada) during all the experiment. This amount should help simulate natural rearing conditions (for review, see Slessor et al., 2005). 30 bees were placed in each cage for the mortality assay

(first experiment), 120 for the physiological trait assay (the number of bees used in this second experiment was determined to provide enough bees for sampling, considering the mortality rate observed in the first experiment). Dead bees were removed every morning from the cages, to limit stress and evaluate the mortality rate. The experiment was repeated on three hives, with three replicates for the first experiment and two for the second. Each replicate included four groups, according to the treatment they received: control, *Nosema* alone, imidacloprid alone, both *Nosema* and imidacloprid.

Experimental infection with *Nosema* and parasite determination

On the first day after emergence, bees were starved for 4 h and individually fed with 2 µL of a freshly prepared 50% sucrose solution containing 200,000 spores of *Nosema sp*. This dose corresponds to an average rate of infection, according to Higes et al., 2007 and our preliminary studies. Newly emerged bees were held with their mouthparts touching a droplet of the spore solution at the tip of a micropipette until it had consumed the entire droplet (Malone et al., 1999).

Spores were isolated from two source colonies of the apiary that had been revealed to be highly infected by *Nosema sp.*, according to the protocol developed by OIE (World Organisation for Animal Health, 2008). Intestines of forager bees were dissected and individually homogenised in distilled water (20 % w/v) with a pellet pestle homogenizer connected to an IKA RW14 laboratory stirrer, 3 times 10 s with a 20 s interval. The spore concentration of the suspension was determined using a haemocytometer chamber.

Identification of the *Nosema* species was performed after induction of the spore germination. After DNA extraction, specific fragments of the two species *Nosema apis* and *N. ceranae* were amplified by PCR (Martin-Hernandez et al., 2007). Electrophoresis of the PCR products revealed that our spore solution contained both species of *Nosema*, *N. apis* and *N. ceranae*.

Imidacloprid treatment

Previous studies have showed that imidacloprid can be found in hives in significant amounts (Aubert et al., 2008): imidacloprid was present in concentration reaching 5 µg/L of honey. This represents a concentration of 7 µg/L of sugar syrup, which is the dose chosen for this study in order to respect field conditions of exposure. Preliminary results obtained on young bees confirmed that this dose corresponds to a sublethal exposition (data not shown).

Bees were chronically exposed to imidacloprid by ingesting imidacloprid-containing sugar syrup (50% sucrose solution, w/v) 10 hours per day. This method allowed for chronic treatment with minimal animal disturbance.

Sample collection for enzymatic activity assays

Bees collected either in the hive or in cages were slightly CO_2 anesthetized. The different compartments were dissected, immediately placed in ice cooled tubes and frozen at -20°C until homogeneisation. In order to determine the best compartment for measuring the immune enzyme activities, five compartments were tested: head, thorax, abdomen, abdomen devoid of its digestive tract, haemolymph. The insect body segments were simply isolated by disrupting the junction head-thorax and thorax-abdomen. Haemolymph was extracted from the dorsal part of the abdomen, using a microcapillary tube introduced between the second and third tergite. Intestine and rectum were removed from the abdomen by pulling on the sting.

The different compartments were homogenised at 4°C, using a mixer-mill disruptor (TissueLyser - Qiagen), in sodium 10% (w/v) phosphate buffer (80 mM NaH_2PO_4 – 20 mM NaCl – 1% (w/v) Triton X-100 – pH 7.4) containing a mixture of 2 mg/ml of antipain, leupeptin and pepstatin A, 25 units/ml of aprotinin, 0.1 mg/ml of trypsin inhibitor. The homogenates were then centrifuged at 15000 g for 20 min at 4°C. The supernatant was used for further analysis of enzymatic activities and protein contents. In the case of successive extractions, the supernatant was removed, the pellet was rinsed once with one volume of extraction medium, and the homogenisation step was repeated identically.

Phenoloxidase activity assay

PO activity assays are based on the conversion of L-Dopa (3,4-Dihydroxy-L-phenylalanine) to melanin, which apparition can be measured by spectrophotometry at 490 nm.

Haemolymph, thorax, abdomen and abdomen devoid of rectum and intestine extracts were measured.

Freshly extracted samples were combined with extraction buffer (80 mM NaH_2PO_4 – 20 mM NaCl – 1% (w/v) Triton X-100) prior to enzymatic measurement. Tissue homogenates were diluted using a 2:3 extract ratio, and haemolymph with a 1:50 ratio.

The reaction mixture consisted in 90 µL of distilled water and 20 µL of sodium phosphate buffer (100 mM NaH_2PO_4 – 200 mM NaCl – pH 7.2), to which 50 µL of freshly extracted sample was added. The microplate was incubated 5 min at 37°C before adding 40 µL of the enzymatic substrate L-Dopa (2 mg/mL) to each well. PO activity was quantified by recording the change in

sample absorbance at 490 nm every 16 s for 10 min. Analysis was repeated three times for each sample. Absorbance data were obtained using a Bioteck Synergy HT100 plate reader.

Glucose oxidase activity assay

Glucose oxidase (GOX) catalyses the conversion of β-D-glucose and O_2 to D-gluconolactone (D-gluconic acid) and H_2O_2 (Bentley, 1963). H_2O_2, in the presence of o-dianisidine, can in turn be converted by peroxidase into oxydised O-dianisidine, a spectrophometically active compound.

Haemolymph, abdomen, thorax and head extracts were measured.

GOX activity was assayed after adapting the method of White et al (1963) to honey bee compartments. The reaction mixture consisted in 100 µL of distilled water and 50 µL of potassium phosphate buffer (500 mM KH2PO4 – pH 7.0), containing 20 µL of glucose (100 mM) and 10 µL of horseradish peroxidase (2.5 U). Glucose solution was prepared 1 h in advance to allow β-mutarotation of the glucose and the reaction mixture was incubated for 10 min at 37°C prior to absorbance measurement. 10 µL (25 µL for thorax and abdomen extracts) of freshly homogenised sample was incubated 10 min at 37°C in the microplate. Extracts were combined with the reaction mixture to which 20 µL of o-dianisidine (3 mM) was extemporaneously added. GOX activity was quantified by recording the change in sample absorbance at 430 nm every min for 1h30. Analysis was repeated three times for each sample.

Protein determination

The protein concentration of each sample was determined by a standard Bradford protein quantification assay (Bradford, 1976), adapted to microplate. Bovine serum albumine (BSA) dilutions were used as standards (concentrations ranging from 0 to 0.375 mg/mL). Samples were diluted in distilled water (1:20 to 1:50 ratios according to the abundance of protein in each compartment) and 10 µL of this solution was mixed with 150 µL of distilled water and 40 µL of protein assay dye reagent concentrate. After thorough homogenisation, the microplate was incubated for 30 min in obscurity and absorbance was measured at 595 nm. Protein concentrations were determined by using the equation of the linear regression obtained from the standard curve, which was repeated for each plate.

Computer modelling of the kinetic curves of PO and GOX enzymes

Enzymatic activities were calculated by modelling the kinetic curves obtained from the measures of absorbance change and determining the slope of the steady-rate phase in the curves obtained. This data was then normalised by the protein concentration of the sample to estimate specific activities for each enzyme. They were expressed as variations of milli-unit of absorbance per mg of protein (muA.min-1.mg-1 of protein).

For PO, a classical enzymatic model adapted to first order reactions was used (Cornish-Bowden, 1995; Rault et al., 1991). Kinetic absorbance curves were adjusted according to a time function as follows:

$$A = C_0 + (1 - e^{-k.t}) + v_s.t,$$

where C_0 (absorbance at t=0), k (the time constant of the activation process) and v_s (the steady-state rate of the activation process) are the constants to estimate.

For GOX, the model was adapted from logistic equations (Murray, 2008; Verhulst, 1845). This logistic function is suitable to fit the coupled reaction, as it accounts for the delay related to the first reaction step. Kinetic absorbance curves were adjusted according to a time function as follows:

$$A = \frac{A_c.Co.e^{k.t}}{A_c + Co(e^{k.t} - 1)},$$

where C_0 (absorbance at t=0), A_c (maximum absorbance) and k (the time constant of the activation process) are the constants to estimate.

Statistical analyses

Statistical analyses were performed using the software R (version 2.7) and the level of significance of 1% was accepted for all tests. For enzymatic and feeding behaviour measurements, the effect of the rearing process was estimated using analysis of the variance (two-way ANOVA and repeated measures two-way ANOVA, respectively). Further investigation of the effect of one particular rearing condition (joint or disjoint exposure to *Nosema* and imidacloprid, colonies, replicates, dates) was performed using unpaired t-tests. In the mortality assay, daily counts of the number of dead bees of corresponding replicates were added together, then cumulated with time and finally log linearised. Analysis was performed using a generalised linear model function (GLM) (McCullagh and Nelder, 1989).

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