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# Partner-specific induction of *Spodoptera frugiperda* immune genes in response to the entomopathogenic nematobacterial complex *Steinernema carpocapsae*-*Xenorhabdus nematophila*

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1 Partner-specific induction of *Spodoptera frugiperda* immune genes in  
2 response to the entomopathogenic nematobacterial complex *Steinernema*  
3 *carpocapsae-Xenorhabdus nematophila*

4

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## 11 **Abstract**

12 The *Steinernema carpocapsae-Xenorhabdus nematophila* association is a nematobacterial complex used  
13 in biological control of insect crop pests. The infection success of this dual pathogen strongly depends on  
14 its interactions with the host's immune system. Here, we used the lepidopteran pest *Spodoptera*  
15 *frugiperda* to analyze the respective impact of each partner in the induction of its immune responses.  
16 First, we used previously obtained RNAseq data to construct the immunome of *S. frugiperda* and analyze  
17 its induction. We then selected representative genes to study by RT-qPCR their induction kinetics and  
18 specificity after independent injections of each partner. We showed that both *X. nematophila* and *S.*  
19 *carpocapsae* participate in the induction of stable immune responses to the complex. While *X.*  
20 *nematophila* mainly induces genes classically involved in antibacterial responses, *S. carpocapsae* induces  
21 lectins and genes involved in melanization and encapsulation. We discuss putative relationships between  
22 these differential inductions and the pathogen immunosuppressive strategies.

## 23 **Keywords**

24 *Steinernema carpocapsae*, *Xenorhabdus nematophila*, *Spodoptera frugiperda*, nematobacterial complex,  
25 immunome, transcriptional response

## 26 **Abbreviations<sup>1</sup>**

### 27 **1. Introduction**

28 The *Steinernema-Xenorhabdus* nematobacterial complexes (NBCs) are natural symbiotic associations  
29 between nematodes and enterobacteria that are pathogenic for insects. The soil-living nematodes infest  
30 insects through the respiratory and/or the intestinal tract (Dowds and Peters, 2002) and reach the  
31 hemocoel, the internal body cavity, where they release their intestinal symbionts. The bacteria then grow  
32 extracellularly in the hemolymph, the insect equivalent of blood, and improve the nematodes'  
33 pathogenicity as well as their ability to reproduce in the host dead body (Forst and Clarke, 2002). Until  
34 now, about 90 species of *Steinernema* have been identified, among which several are usable as biological  
35 control agents against diverse insect crop pests (Labaude and Griffin, 2018; Lacey et al., 2015). In  
36 consequence, their interactions with insects have been extensively studied for about 50 years (Poinar and  
37 Grewal, 2012). These studies have shown that in addition to ecological and morphological parameters  
38 (Labaude and Griffin, 2018), the NBCs' interactions with the host's immune system is one of the most

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<sup>1</sup> NBC: nematobacterial complex; AMP: antimicrobial peptides; NBTA: Nutrient bromothymol blue agar; PBS: phosphate buffered saline; CFU: Colony-forming unit; GNB: Gram-negative binding protein; PGRP: Peptidoglycan recognition protein; LLP: Lysozyme-like protein; PPAAE: Prophenoloxidase-activating enzyme; IMPI: Insect metalloproteinase inhibitor protein; TEP: Thiol-ester protein

39 crucial factors influencing their ability to infest and kill a given insect (Li et al., 2007; Thurston et al.,  
40 1994; Wang et al., 1994).

41 Insects possess an elaborate immune system, which is able to respond appropriately to diverse types of  
42 pathogens and of infections. Firstly, this system involves protective external barriers such as the cuticle,  
43 or the peritrophic matrix in the midgut (Kristensen and Chauvin, 2012; Lehane, 1997). It then relies on  
44 local defenses of the surface epithelia, which repair efficiently (Ferrandon, 2013; Galko and Krasnow,  
45 2004; Rowley and Ratcliffe, 1978) and produce toxic factors such as antimicrobial peptides (AMPs)  
46 (Brey et al., 1993; Tingvall et al., 2001; Tzou et al., 2000; Wu et al., 2010) and reactive oxygen species  
47 (Ha et al., 2009). The third line of defense of insects is provided by the hemocytes, which are the  
48 circulating immune cells. They can produce diverse types of immune responses, including AMP  
49 synthesis, phagocytosis, nodulation, encapsulation, coagulation and melanization (Strand, 2008).  
50 Nodulation and encapsulation are cellular immune responses respectively consisting in the engulfment of  
51 bacterial aggregates and of large invaders via hemocytes aggregation (Strand, 2008). Together with  
52 coagulation, these responses are coupled with a melanization process consisting of series of phenolic  
53 compounds oxidations resulting in synthesis of reactive molecules and melanin that participate in  
54 pathogens trapping and killing (Jiravanichpaisal et al., 2006; Nappi and Christensen, 2005). Finally, the  
55 fat body, a functional equivalent of the mammalian liver, produces potent systemic humoral immune  
56 responses involving a massive secretion of AMP cocktails in the hemolymph. These responses can be  
57 induced by two major signaling pathways of insect immunity; the Imd pathway, which is mainly  
58 activated by Gram negative bacteria, and/or the Toll pathway, which is mainly activated by Gram positive  
59 bacteria, fungal organisms and by proteases released by pathogens (Ferrandon et al., 2007; Issa et al.,  
60 2018).

61 The *Steinernema-Xenorhabdus* NBC whose interactions with the immune system have been the most  
62 extensively studied is the *S. carpocapsae-X. nematophila* association. These interactions have firstly been  
63 studied from the NBC point of view, which allowed the identification of a multitude of immunoevasive  
64 and immunosuppressive strategies. For instance, studies in *Rhynchophorus ferrugineus* and *Galleria*  
65 *mellonella* have respectively shown that the cuticle of *S. carpocapsae* is not recognized by the host's  
66 immune system (Binda-Rossetti et al., 2016; Mastore et al., 2015) and that the nematode secretes protease  
67 inhibitors impairing the coagulation responses (Toubarro et al., 2013a; Toubarro et al., 2013b). Studies in  
68 diverse insect models have also shown that both partners produce factors impairing melanization  
69 (Balasubramanian et al., 2009; Balasubramanian et al., 2010; Crawford et al., 2012; Eom et al., 2014),  
70 hemocyte's viability (Brivio et al., 2018; Kim et al., 2005; Ribeiro et al., 1999; Ribeiro et al., 2003;  
71 Vigneux et al., 2007) and the production of cellular immune responses by several ways (Balasubramanian  
72 et al., 2009; Balasubramanian et al., 2010; Eom et al., 2014; Park and Kim, 2000; Park and Stanley, 2006;  
73 Toubarro et al., 2013b). Finally, both *X. nematophila* and *S. carpocapsae* secrete proteolytic factors

74 degrading cecropin AMPs (Caldas et al., 2002; Gotz et al., 1981) and the bacterium has also been shown  
75 to reduce more globally the hemolymph antimicrobial activity, as well as AMP transcription in  
76 lepidopteran models (Binda-Rossetti et al., 2016; Caldas et al., 2002; Duvic et al., 2012; Ji and Kim,  
77 2004).

78 On the other hand, the description of these interactions from the hosts' points of view is at its beginning.  
79 This aspect has mainly been studied in the *Drosophila melanogaster* model, with a first transcriptomic  
80 analysis of the whole larva responses to infestations by entire NBCs and by axenic nematodes (Yadav et  
81 al., 2017). This analysis has shown that several immune processes are induced by both pathogens at the  
82 transcriptional level. For instance, the authors found in each case an overexpression of genes related to  
83 the Imd and Toll pathways that was accompanied by the induction of a few AMP genes. They also found  
84 an upregulation of genes related to melanization, coagulation, or involved in the regulation of cellular  
85 immune responses (Yadav et al., 2017). Complementary gene knockout experiments in this model  
86 demonstrated an involvement of the Imd pathway in the response against *X. nematophila* (Aymeric et al.,  
87 2010) and revealed a possible involvement of the Imaginal Disc Growth Factor-2, the intestinal serine  
88 protease Jonah 66Ci (Yadav and Eleftherianos, 2019) as well as TGF- $\beta$  and JNK pathways members in  
89 the regulation of anti-nematode immunity (Yadav and Eleftherianos, 2018; Yadav et al., 2018).

90 In order to improve our understanding of the dialogue that takes place between this NBC and its host, we  
91 recently published a topologic transcriptomic analysis of the response of the lepidopteran model  
92 *Spodoptera frugiperda* to the infestation (Huot et al., 2019). This analysis was focused on the three main  
93 immunocompetent tissues that are confronted to the NBC, which are the midgut (the main entry site in  
94 the hemocoel), the hemocytes and the fat body. The RNAseq experiment showed that there was no potent  
95 or well-defined transcriptional response in the midgut. However, we observed dramatic transcriptional  
96 responses in the fat body and the hemocytes at 15 h post-infestation, which is a middle time point of the  
97 infection. In agreement with the results obtained in *D. melanogaster* whole larvae (Yadav et al., 2017),  
98 global analysis of these responses showed they are dominated by immune processes. The objective of the  
99 present study is to go further in the analysis of these induced immune responses. In order to describe them  
100 with high accuracy, we first examine the expression variations of all the immune genes that have been  
101 identified in the insect's genome. We then use tissue RT-qPCR experiments to analyze the temporal  
102 dynamics and the relative contribution of each NBC partner in the identified immune responses. Our  
103 results show that a large number of immune genes are responsive in either one or the two tissues during  
104 the infestation, with activation of antimicrobial and cellular immunities, of melanization, coagulation and  
105 of metalloprotease inhibition. These responses were found to be stable over the time post-infestation and  
106 to consist in combinations of *X. nematophila*-induced and *S. carpocapsae*-induced responses in each  
107 tissue. The *X. nematophila*-induced responses mainly correspond to genes that are classically involved in  
108 antibacterial immunity, whereas the *S. carpocapsae*-induced ones mainly include lectins and genes

109 potentially involved in melanization and encapsulation. In addition, our RT-qPCR experiments show that  
110 two previously identified candidate clusters of uncharacterized genes (Huot et al., 2019) also present  
111 partner-specific induction profiles. Our hypothesis is that they may correspond to new types of anti-  
112 nematode and antibacterial immune factors found in *Spodoptera* genus and lepidopteran species,  
113 respectively.

## 114 **2. Materials and Methods**

### 115 **2.1. Insect rearing**

116 Corn-strain *Spodoptera frugiperda* (Lepidoptera : Noctuidae) were fed on corn-based artificial diet  
117 (Poitout and Buès, 1970). They were reared at 23°C +/- 1°C with a photoperiod of 16 h/8 h (light/dark)  
118 and a relative humidity of 40 % +/- 5 %. *Galleria mellonella* (Lepidoptera : Pyralidae) were reared on  
119 honey and pollen at 28°C in dark.

### 120 **2.2. Production and storage of nematobacterial complexes**

121 *Steinernema carpocapsae-Xenorhabdus nematophila* complexes (strain SK27 isolated from Plougastel,  
122 France) were renewed by infestation of one month-old *Galleria mellonella* larvae. They were collected on  
123 White traps (White, 1927) and stored at 8°C in aerated Ringer sterile solution with 0.1 % formaldehyde.  
124 The maximal time of storage was limited to 4 weeks to avoid pathogenicity losses.

### 125 **2.3. Production of axenic nematodes**

126 Gravid *S. carpocapsae* females were extracted from *G. mellonella* dead bodies at day 4 to 6 after  
127 infestation by nematobacterial complexes (NBCs). After 5 washing steps in Ringer sterile solution, the  
128 females were surface-sterilized by 20 min incubation in 0.48 % (wt/vol) sodium hypochlorite and 3 h  
129 incubation in Ringer sterile solution supplemented with antibiotics (150 µg/mL polymyxin, 50 µg/mL  
130 colistin, 50 µg/mL nalidixic acid). The eggs were extracted by female crushing with sterile glass pestles  
131 and then washed by centrifugation (2 min, 16000 g) in Ringer sterile solution, disinfected by incubation  
132 in 0.48 % sodium hypochlorite for 5 min, and washed again twice. After microscopic observation, the  
133 intact eggs were placed on liver-agar (40 g/L Tryptycase Soja Agar [BioMérieux], 5 g/L Yeast Extract  
134 [Difco], 100 g/L porc liver) plates supplemented with antibiotics (150 µg/mL polymyxin, 50 µg/mL  
135 colistin and 50 µg/mL nalidixic acid). The plates were maintained inside a dark humid chamber for 1  
136 month to allow nematodes development. The nematodes were then suspended in Ringer sterile solution  
137 and infective juvenile stages (IJs) were sorted by pipetting under a microscope (Leica). The IJs were  
138 rinsed twice by centrifugation (2 min, 3000 g) in 1 mL Ringer sterile solution and used within minutes for  
139 experimental infection.

140 Nematodes' axenicity was verified by DNA extraction and PCR amplification. Nematodes were  
141 suspended in 200  $\mu$ L milliQ water supplemented with 200  $\mu$ L glass beads ( $\emptyset \leq 106 \mu\text{m}$ ) (Sigma). They  
142 were ground for 2 x 40 sec at 4.5 ms speed with a FastPrep homogenizer (MP Biomedicals). The debris  
143 were discarded by centrifugation (2 min, 16000 g) and 150  $\mu$ L supernatant were mixed with 200  $\mu$ L lysis  
144 buffer (Quick extract kit, Epi-centre) for a second grinding. To ensure bacterial cell lysis, the samples  
145 were incubated at room temperature for 48 h with 2  $\mu$ L Ready-Lyse Lysozyme solution at 30000 U/ $\mu$ L  
146 (Epi-centre). Protein denaturation was then performed by 10 min incubation at 90°C, and RNA was  
147 removed by 10 min incubation at 37°C with 20  $\mu$ L RNase A (20 mg/mL) (Invitrogen). DNA was  
148 extracted by successive addition of 500  $\mu$ L phenol-chloroform-isoamyl alcohol and 500  $\mu$ L chloroform,  
149 followed by centrifugations (10 min, 16000 g) and aqueous phase collections. DNA was precipitated with  
150 500  $\mu$ L 100% ethanol supplemented with 20  $\mu$ L sodium acetate and by freezing at -80°C for 2 h. After  
151 defrosting, DNA was concentrated by centrifugation (30 min, 16000 g) and the precipitates were washed  
152 twice by centrifugation (15 min, 16000 g) in 500  $\mu$ L 70% ethanol. DNA was finally suspended in 50  $\mu$ L  
153 sterile milliQ water and left at room temperature for a few hours to ensure precipitate dissolution. After  
154 DNA quantification with a Qubit fluorometer (Invitrogen), *X. nematophila* presence was assessed by PCR  
155 amplification with *Xenorhabdus*-specific primers (Xeno\_F: 5'-ATG GCG CCA ATA ACC GCA ACT A-  
156 3'; Xeno\_R: 5'-TGG TTT CCA CTT TGG TAT TGA TGC C-3'), which target a region of the  
157 XNC1\_0073 gene encoding a putative TonB-dependent heme-receptor (Cambon et al., 2020). The  
158 presence of other bacteria was assessed by 16S rRNA gene amplification with universal primers (Tailliez  
159 et al., 2006). Thirty cycles of PCR were performed using Taq polymerase (Invitrogen) in a Biorad  
160 thermocycler, with hybridization temperatures of 55°C and 50°C respectively. PCR products were then  
161 analyzed by agarose gel electrophoresis with DNAs from *X. nematophila*, *Pseudomonas protegens* and *S.*  
162 *carpocapsae-X. nematophila* complexes as controls (Supplementary Fig. 1).

#### 163 **2.4. Experimental infections**

164 Experimental infestations with NBCs were carried out on individual 2<sup>nd</sup> day 6<sup>th</sup> instar *S. frugiperda* larvae  
165 according to Huot et al. (2019). Briefly, 12-well plates were coated with pieces of filter paper (Whatman)  
166 and larvae were individually introduced in each well with a cube of artificial diet (Poitout and Buès,  
167 1970). For infestations, 150 +/- 20 NBCs in 150  $\mu$ L Ringer sterile solution were poured in each well and  
168 the plates were then maintained at 23°C. 150  $\mu$ L Ringer sterile solution were used for control  
169 experiments.

170 For intra-hemocoelic injection experiments, pathogens were injected in larvae's abdomens after local  
171 application of 70% ethanol with a paintbrush. Injections were performed using a syringe pump (Delta  
172 labo) with 1 mL syringes (Terumo) and 25G needles (Terumo). The injected larvae were then kept in 12-  
173 well plates at 23 °C with cubes of artificial diet (Poitout and Buès, 1970).

174 *X. nematophila* suspensions were prepared as described in Sicard et al. (2004). Bacterial culture was  
175 diluted in PBS and 20  $\mu$ L containing 200 +/- 50 bacterial cells were injected in the hemocoel at a rate of  
176 1.67 mL/min. 20  $\mu$ L sterile PBS were used for control larvae. The purity and number of injected *X.*  
177 *nematophila* were verified by plating 20  $\mu$ L of the bacterial suspension on NBTA (Boemare et al., 1997).

178 For NBC and axenic nematode injections, 10 +/- 3 nematodes in 20  $\mu$ L 70% Ringer and 30% glycerol  
179 solution were injected at a rate of 2.23 mL/min. Syringes were frequently renewed in order to limit  
180 nematodes concentration and sedimentation and the number of injected nematodes was verified by 10  
181 simulations of injection in Petri dishes followed by nematode counting under a stereomicroscope (Zeiss).  
182 Sterile 70% Ringer and 30% glycerol solutions were used for control larvae. To avoid accidental *per os*  
183 infections, the injected larvae were then briefly washed in sterile PBS and dried on paper towel before  
184 being placed in 12-well plates.

185 The pathogens efficacies were checked by monitoring the survival of 12 control and 12 infected larvae  
186 for 72 h after infestation or after injection.

## 187 **2.5. Production and storage of bacterial symbionts**

188 *X. nematophila* strain F1 isolated from NBCs strain SK27 was conserved at -80°C. Within 3 weeks before  
189 each experiment, they were grown for 48 h at 28°C on NBTA supplemented with erythromycin (15  
190  $\mu$ g/mL) to which the strain is resistant (Sicard et al., 2004). The colonies were then conserved at 15°C  
191 and used for overnight culture at 28°C in 5 mL Luria-Bertani broth (LB) before experiments.

## 192 **2.6. Caterpillar RNA extraction**

193 RNAs were prepared as described in Huot et al. (2019). Briefly, nine larvae per technical replicate were  
194 bled in anti-coagulant buffer (van Sambeek and Wiesner, 1999). Hemocytes were recovered by  
195 centrifugation (1 min, 800 g) at 4°C and the pellet was immediately flash-frozen with liquid nitrogen. The  
196 larvae were then dissected for fat body and midgut sampling and the tissues were flash-frozen in  
197 eppendorf tubes with liquid nitrogen. After storage at -80°C for at least 24 h, 1 mL Trizol (Life  
198 technologies) was added to the pooled tissues. The tissues were then ground by using a TissueLyzer  
199 85210 Rotator (Qiagen) with one stainless steel bead ( $\emptyset$  : 3 mm) at 30 Hz for 3 min. For optimal cell  
200 lyses, ground tissues were left at room temperature for 5 min. To extract nucleic acids, 200  $\mu$ L  
201 chloroform (Interchim) were added and the preparations were left at room temperature for 2 min with  
202 frequent vortex homogenization. After centrifugation (15 min, 15,000 g) at 4°C, the aqueous phases were  
203 transferred in new tubes and 400  $\mu$ L 70% ethanol were added. RNA purifications were immediately  
204 performed with the RNeasy mini kit (Qiagen) and contaminant DNA was removed with the Turbo DNA-  
205 free<sup>TM</sup> kit (Life Technologies).

206 RNA yield and preparation purity were analyzed by measuring the ratios  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  with a  
207 Nanodrop 2000 spectrophotometer (Thermo Scientific). RNA integrity was verified by agarose gel  
208 electrophoresis and RNA preparations were conserved at - 80 °C.

## 209 **2.7. RNAseq experiments**

210 RNAseq raw data originate from Huot et al. (2019). In brief, during 3 independent experiments, 9 larvae  
211 infested with 150 *S. carpocapsae-X. nematophila* complexes and 9 uninfected control larvae were bled  
212 and dissected at time 15 hours post-contact with NBCs or Ringer sterile solution (see Materials and  
213 Methods 2.4.). Total RNA from their hemocytes and fat bodies were extracted (see Materials and  
214 Methods 2.6.) and the corresponding libraries were prepared by MGX GenomiX (IGF, Montpellier,  
215 France) with the TruSeq Stranded mRNA sample preparation kit (Illumina). The libraries were then  
216 validated on Fragment Analyzer with a Standard Sensitivity NGS kit (Advanced Analytical Technologies,  
217 Inc) and quantified by qPCR with a Light Cycler 480 thermal cycler (Roche Molecular diagnostics).  
218 cDNAs were then multiplexed by 6 and sequenced on 50 base pairs in a HiSeq 2500 system (Illumina)  
219 with a single-end protocol. Image analysis and base calling were performed with the HiSeq Control and  
220 the RTA software (Illumina). After demultiplexing, the sequences quality and the absence of contaminant  
221 were checked with the FastQC and the FastQ Screen softwares. Data were then submitted to a Purity  
222 Filter (Illumina) to remove overlapping clusters.

223 For each sample, the reads were pseudoaligned on the *S. frugiperda* reference transcriptome version  
224 OGS2.2 (Gouin et al., 2017) using the Kallisto software (Bray et al., 2016). Differential expression  
225 between infested and control conditions were then assessed for each time point and tissue with the Sleuth  
226 software (Pimentel et al., 2017). Wald tests were used with a q-value (equivalent of the adjusted p-value)  
227 threshold of 0.01 and a beta value (biased equivalent of the log2 fold change) threshold of 1. Only  
228 transcripts with normalized counts over 5 in all three replicates of the infested and/or of the control  
229 condition were considered as reliably differentially expressed.

230 Previously annotated immune transcripts (Gouin et al., 2017) were then checked for significant  
231 expression changes and not annotated differentially expressed ones were researched with the Blast2GO  
232 software by blastx on the NCBI nr and drosophila databases (Conesa et al., 2005). To avoid mistakes  
233 related to genome fragmentation, the immune transcripts were gathered by unique gene after careful  
234 examination of their sequences and of the available genomic data (Gouin et al., 2017). The induction  
235 levels of the transcripts were then averaged by unique gene before graphical representation of the results.

## 236 **2.8. RT-qPCR experiments**

237 RT-qPCR experiments were performed with extracted total RNA (see Materials and Methods 2.6.) from  
238 hemocytes and fat bodies of 9 larvae infested or infected by injection and of 9 uninfected control larvae  
239 during 3 independent experiments.

240 For each sample, cDNAs were synthesized from 1  $\mu$ g of RNA with the SuperScript II Reverse  
241 Transcriptase (Invitrogen), according to the manufacturer's protocol.  
242 The primers (Supplementary Table 1) were designed with the Primer3Web tool (Untergasser et al., 2012).  
243 Their efficiency was estimated by using serial dilutions of pooled cDNA samples and their specificity  
244 was verified with melting curves analyses. Amplification and melting curves were analyzed with the  
245 LightCycler 480 software (Roche Molecular diagnostics).  
246 RT-qPCRs were carried out in technical triplicate, with the LightCycler 480 SYBR Green I Master kit  
247 (Roche). For each replicate and primer pair, 1.25  $\mu$ L of sample containing 50 ng/ $\mu$ L of cDNA and 1.75  
248  $\mu$ L of Master mix containing 0.85  $\mu$ M of primers were distributed in multiwell plates by an Echo 525  
249 liquid handler (Labcyte). The amplification reactions were then performed in a LightCycler 480 thermal  
250 cyclor (Roche) with an enzyme activation step of 15 min at 95°C, and 45 cycles of denaturation at 95°C  
251 for 5 sec, hybridization at 60°C for 10 sec and elongation at 72°C for 15 sec.  
252 Crossing points were determined using the Second Derivative Maximum method with the LightCycler  
253 480 software (Roche) and relative expression ratios between control and infected conditions were  
254 manually calculated according to the method of Ganger et al. (2017). The ratios were normalized to the  
255 RpL32 housekeeping gene relative levels and the EF1 gene was used as an internal control.  
256 Statistical analyses of the data were all performed with the R software (R Core Team, 2017). Differential  
257 expression significance between the control and infected conditions was assessed by paired one-tailed t-  
258 tests on  $\Delta$ Cq values. Multiple comparisons of fold changes were assessed by one-way ANOVA on  $\Delta\Delta$ Cq  
259 values followed by post hoc Tukey tests. P-values under 0.05 were considered as significant for all the  
260 above tests. The gplots package was used to draw the heatmaps and the clusters were built from a  
261 dissimilarity matrix based on Pearson correlation coefficients.

## 262 **2.9. Quantification of nematodes in the midgut lumen**

263 NBCs in the midgut lumen were quantified at several times after infestation by nematode counting in the  
264 alimentary bolus. For 3 independent experiments, 3 infested larvae were dissected and the midguts'  
265 alimentary bolus were extracted. Each alimentary bolus was then dissolved in 3 mL sterile PBS in a Petri  
266 dish ( $\emptyset$  : 35 mm) and motile nematodes were counted with a stereomicroscope (Leica).

## 267 **2.10. Quantification of *X. nematophila* in the hemolymph**

268 The concentration of *X. nematophila* strain F1 in the hemolymph was estimated by CFU counting on  
269 selective medium. For 3 independent infestation or infection by injection experiments and 3 technical  
270 replicates, hemolymph was collected by bleeding of 3 caterpillars in 200  $\mu$ L PBS supplemented with  
271 phenylthiourea (Sigma). The volumes of hemolymph were then estimated by pipetting and serial dilutions  
272 of the samples were plated on NBTA with 15  $\mu$ g/mL erythromycin. CFU were counted after 48 h

273 incubation at 28°C and the counts were reported to the estimated hemolymph volumes in order to  
274 calculate the bacterial concentrations. Hemolymph of naive larvae was also plated for control.

## 275 **2.11. Insect survival kinetics**

276 Survival kinetics were measured by 3 independent experiments on pools of 20 larvae infested with NBCs  
277 or injected with NBCs, with axenic *S. carpocapsae* nematodes or with *X. nematophila* symbionts.  
278 Survival was monitored from 0 to 72 hours after contact or injection.

## 279 **2.12. Parasitic success measurement**

280 Parasitic success was measured during 3 independent experiments on pools of 20 NBCs or axenic  
281 nematodes-injected larvae. Dead larvae were individually placed on white traps (White, 1927)  
282 approximately 2 days after their deaths. The emergence of nematodes was assessed at day 40 after  
283 injection by observation of the collection liquid with a stereomicroscope (Leica). Parasitic success was  
284 then calculated as the percentage of larvae with nematode emergence among the infected larvae.

# 285 **3. Results & Discussion**

## 286 **3.1. Hemocytes' and fat body's immune responses**

287 In order to get an accurate picture of the *S. frugiperda* transcriptional immune responses to the NBC  
288 infestation, we first used a previously published list of immune genes identified by sequence homology in  
289 the *S. frugiperda* genome (Gouin et al., 2017). We then used previously obtained RNAseq data (Huot et  
290 al., 2019) to look at their expression variations in the fat body and in the hemocytes (Supplementary  
291 Table 2A) and we completed the repertoire with additional putative immune genes that we directly  
292 identified from lists of differentially expressed genes upon NBC infestation (Supplementary Table 2B).  
293 In total, we present the annotation of 226 immune or putative immune genes of which 132 were  
294 significantly modulated at 15 h post-infestation (hpi) (Sleuth, p-value < 0.01; |Beta| > 1; all count values  
295 > 5 in at least one condition) in one or both tissues (Fig. 1). Among them, 62 were involved in  
296 antimicrobial responses (Fig. 1A), 18 were related to melanization (Fig. 1B), 23 were involved in cellular  
297 responses (Fig. 1C) and the 29 remaining genes were grouped in a category called “diverse” due to  
298 pleiotropic or poorly characterized functions (Fig. 1D).

### 299 **3.1.1. Antimicrobial responses**

300 In the antimicrobial response category, 58 genes were found to be upregulated in at least one of the two  
301 tissues (Fig. 1A). The signaling genes encoded 3 and 8 members of the Imd and Toll pathways,  
302 respectively, as well as 5 short catalytic peptidoglycan recognition proteins (PGRP-S), which are  
303 probably involved in the regulation of these pathways by peptidoglycan degradation (Myllymaki et al.,  
304 2014; Valanne et al., 2011) (Fig. 1A). Four other genes were considered as involved in recognition. They

305 encoded Gram negative binding proteins (GNBPs), which have been reported to recognize  
306 peptidoglycans or  $\beta$ -glucans and participate in the further activation of the Toll pathway (Ferrandon et al.,  
307 2007) and/or of the melanization response (Nakhleh et al., 2017) (Fig. 1A). Finally, the effector genes  
308 encoded 33 antimicrobial peptides (AMPs) belonging to all the *S. frugiperda*'s AMP families (Gouin et  
309 al., 2017) plus 4 lysozymes and lysozyme-like proteins (LLPs) (Fig. 1A). Depending on their families and  
310 on the insect species, AMPs can present varied activity spectra, ranging from antiviral or antibacterial  
311 activities to anti-fungal and anti-parasitic ones (Yi et al., 2014). Varied activity spectra have also been  
312 found for several insects' lysozymes and LLPs (Chen et al., 2018; Gandhe et al., 2007; Satyavathi et al.,  
313 2018; Sowa-Jasilek et al., 2014; Yu et al., 2002). Interestingly, all of the categories and subcategories  
314 cited above were represented in the two tissues, indicating that their antimicrobial responses are  
315 diversified and that the factors responsible for their disappearance in the hemolymph (Binda-Rossetti et  
316 al., 2016; Duvic et al., 2012) probably act at a post-transcriptional level. About a half of the genes  
317 presented similar and significant induction profiles in the hemocytes and in the fat body. This is for  
318 instance the case of the usually anti-Gram negative bacteria attacin, cecropin and gloverin AMPs (Yi et  
319 al., 2014), which were all highly induced in the two tissues (Fig. 1A), suggesting they both respond to the  
320 bacterial partner *X. nematophila*. On the other hand, all the induced GGBP, lysozyme and LLP genes  
321 were found to be either significantly induced in the hemocytes or in the fat body, and in the AMP  
322 category, tissue-specificities were observed for diapausin, defensin-like and most moricin genes (Fig.  
323 1A).

324 Only 8 antimicrobial response genes were found to be significantly downregulated (Fig. 1A).  
325 Interestingly, 4 were involved in the Imd pathway whereas the 4 remaining ones were dispersed between  
326 the AMP, GGBP and lysozyme categories (Fig. 1A). The Imd pathway downregulated factors included  
327 *sickie* and the *akirin* in the hemocytes and *SMARCC2* and *BAP60* in the fat body (Fig. 1A). In *D.*  
328 *melanogaster*, Sickie participates in the activation of Relish, the transcription factor of the Imd pathway  
329 (Foley and O'Farrell, 2004) and the akirin acts together with the Brahma chromatin-remodeling complex,  
330 containing BAP60 and SMARCC2, as cofactor of Relish to induce the expression of AMP genes (Bonnay  
331 et al., 2014). Only few data can be found in current literature on the transcriptional regulation of these  
332 factors (Bonnay et al., 2014; Cao et al. 2015; Chen et al., 2018). It is thus difficult to determine whether  
333 the downregulations we observed correspond to a normal negative feedback of the humoral immune  
334 response or to an immunosuppressive effect of the NBC. In a previous study on the *Spodoptera exigua*  
335 model, Hwang et al. (2013) have shown injection of live *X. nematophila* induces lower transcriptional  
336 inductions of several AMP genes than injection of dead bacteria (Hwang et al., 2013). It would be  
337 interesting to determine, either in the *S. exigua* or in the *S. frugiperda* model, whether reduction of AMP  
338 induction in response to live bacteria co-occurs with a downregulation of the Relish cofactors and  
339 activators we identified in the present analysis.

340 To summarize, the antimicrobial responses are potent and diversified in the two tissues, with a common  
341 induction of genes that probably respond to *X. nematophila*. Yet unexplained tissue-specific responses  
342 were observed and the results show a down-regulation of Imd pathway members that could be related to a  
343 previously described transcriptional immunosuppressive effect of the NBC. However, since at 15hpi, the  
344 full repertoire of *S. frugiperda* AMP is still expressed in response to naturally infesting NBC (Fig. 1A),  
345 this effect might not be potent enough to suppress the humoral response at this time point, suggesting that  
346 the NBC probably uses additional resistance and/or immunosuppressive strategies to survive the insect's  
347 humoral immune response.

### 348 **3.1.2. Melanization**

349 In the melanization category, 16 genes were found to be upregulated in at least one of the two tissues  
350 (Fig. 1B). These genes firstly encoded 6 serine proteases (Fig. 1B) that were considered as members of  
351 the prophenoloxidase (proPO) system. The proPO system is an extracellular proteolytic cascade ending in  
352 the maturation of the proPO zymogen into PO, which initiates the melanization process (Nakhleh et al.,  
353 2017). Among the upregulated serine proteases, PPAE2 is the only one that is known to take part in  
354 proPO processing whereas the other proteases were included in this category because of their  
355 characteristic CLIP domains and of their low homology with the serine proteases acting upstream of the  
356 Toll pathway in *D. melanogaster* (Veillard et al., 2016). The other upregulated genes in this category  
357 included 3 serpins, which are known to regulate the proPO system in several model insects (Nakhleh et  
358 al., 2017), 3 melanization enzymes, DDC, Yellow-like 1 and Punch-like (De Gregorio et al., 2001; Tang,  
359 2009) as well as 4 genes, Reeler-1 and 3 Hdd23 homologs, that are involved in melanization and nodule  
360 formation in other models (Bao et al., 2011; Qiao et al., 2014) (Fig. 1B). Despite of tissue-specific  
361 induction patterns, serine proteases and serpins were found in the two tissues (Fig. 1B), suggesting that  
362 both participate in the stimulation of the proPO system, which is consistent with results obtained in other  
363 interaction models (Yuan et al., 2017; Zou et al., 2015; Zou et al., 2010). However, with the exception of  
364 the DDC, all the melanization enzymes as well as the nodulation-related genes were specifically induced  
365 in the hemocytes (Fig. 1B), which is consistent with the very localized nature of this immune response  
366 (Tang, 2009) that is mainly mediated by hemocyte subtypes.

367 Finally, only 2 genes, PPAE1 and Yellow-like 2, were found to be significantly down-regulated in this  
368 category (Fig. 1B). Both were specifically repressed in the hemocytes, which could be due to functional  
369 interferences with their upregulated homologs (PPAE2 and Yellow-like 1) and/or to an expression in  
370 specific hemocyte subtype(s) whose proportion in the total hemocyte population would influence the  
371 observed log2 fold changes. There is also the possibility of an immunosuppressive effect of the NBC on  
372 the expression of these genes.

373 In summary, our results suggest that both the hemocytes and the fat body participate in induction and  
374 regulation of melanization in response to the NBC and no clear sign of immunosuppression at the

375 transcriptional level is detected for this response. These results are in agreement with the previous  
376 identification of diverse inhibitors of PO activity in both *S. carpocapsae* (Balasubramanian et al., 2009;  
377 Balasubramanian et al., 2010) and *X. nematophila* (Crawford et al., 2012; Eom et al., 2014) that act at the  
378 post-transcriptional level.

### 379 **3.1.3. Cellular responses**

380 In the hemocytes, 19 upregulated genes were placed in the cellular responses category (Fig. 1C). The  
381 signaling ones encoded 3 homologs of the transcription factor Krüppel (Kr) (Fig. 1C). In *D.*  
382 *melanogaster*, Kr and Kr homologs are involved in several developmental processes such as embryo  
383 patterning (Schmucker et al., 1992), organogenesis (Fichelson et al., 2012; Harbecke and Janning, 1989;  
384 Hoch and Jackle, 1998), and cell differentiation (Ivy et al., 2015). More specifically in the hemocytes, Kr  
385 has been shown to take part in hemocytes' differentiation and/or activation (Stofanko et al., 2008), a  
386 crucial step for the induction of cellular immune responses. The recognition genes encoded 3 cellular  
387 receptors of the Scavenger (SR) and Integrin families plus the hemolin, a secreted immunoglobulin-  
388 containing protein (Fig. 1C). Both Scavenger receptors and integrins are known to act as membrane  
389 receptors in phagocytosis of bacteria and apoptotic cells (Nazario-Toole and Wu, 2017). In addition,  
390 integrins are involved in diverse processes, including cell motility and adhesion, and encapsulation (Levin  
391 et al., 2005; Melcarne et al., 2019). The hemolin is known to act as an opsonin by increasing phagocytosis  
392 and nodulation of bacteria in *Manduca sexta* (Eleftherianos et al., 2007). Among the effector genes, we  
393 first identified 5 upregulated genes corresponding to conserved intracellular phagocytosis-related  
394 proteins. They included Ced-6, the Rabenosyn-5 (Rbsn-5-like), a V-ATPase subunit (ATP6V0A2-like)  
395 and 2 small GTPase Activating Proteins (Rabex-5-like, CdGAPr-like) (Nazario-Toole and Wu, 2017)  
396 (Fig. 1C). We also found genes encoding membrane proteins, such as the immunoglobulin-containing  
397 hemicentin (HMCN-like) (Barat-Houari et al., 2006) and 4 tetraspanin-like (Tsp-like) proteins (Hemler,  
398 2008) (Fig. 1C), that could participate in cell-cell adhesion and cellular immune responses. Interestingly,  
399 one of the upregulated tetraspanins (Tsp-like 3) presented 79.5% identity with the *Manduca sexta*  
400 (Lepidoptera : Noctuidae) tetraspanin D76, which takes part in hemocytes aggregation during capsule  
401 formation by trans-interacting with a specific integrin (Zhuang et al., 2007). Finally, 2 genes encoding  
402 proteins similar to the *D. melanogaster* clotting factors GP150 (Korayem et al., 2004) and a  
403 transglutaminase (Tg-like) (Lindgren et al., 2008) were also found upregulated (Fig. 1C). Only 2 genes  
404 (Ced-6-like, Rbsn-5-like) of the cellular responses category were found to be upregulated in the fat body  
405 (Fig. 1C) and both encoded intracellular proteins that are probably not related to immunity in this tissue.  
406 All the 4 down-regulated putative cellular immunity-related genes were specifically modulated in the  
407 hemocytes (Fig. 1C). They encoded 2 Rho GTPase Activating Proteins (RhoGAP-like), a scavenger  
408 receptor similar to the *D. melanogaster* Croquemort receptor (SR-B3) and a homolog of the *D.*  
409 *melanogaster* integrin  $\alpha$ -PS1. In *D. melanogaster*, Croquemort has been shown to take part in

410 phagocytosis of apoptotic cells and of the Gram positive bacterium *Staphylococcus aureus* but not of the  
411 Gram negative bacterium *Escherichia coli* (Melcarne et al., 2019; Stuart et al., 2005). Integrin  $\alpha$ -PS1 is a  
412 ligand of the extracellular matrix protein laminin (Gotwals et al., 1994). It is involved in migration and  
413 differentiation of several cell types during development (Delon and Brown, 2009; Roote and Zusman,  
414 1996; Urbano et al., 2011) but does not seem to be required for any immune process. Their down-  
415 regulations are thus probably due to their uselessness in the context of the response to the NBC.  
416 Overall, the results suggest that all types of cellular responses are transcriptionally induced at 15 hpi,  
417 including phagocytosis and nodulation, as well as encapsulation that would be adapted to the bacterial  
418 partner or the nematode, respectively. In addition, the induction of coagulation responses is particularly  
419 interesting, since many clotting factors participate in *D. melanogaster* resistance to infestation by another  
420 type of NBC, the *Heterorhabditis bacteriophora-Photorhabdus luminescens* association (Arefin et al.,  
421 2014; Hyrsl et al., 2011; Kucerova et al., 2016; Wang et al., 2010). Moreover, despite *S. carpocapsae*  
422 does not pierce the insects' cuticles as *H. bacteriophora* (Dowds and Peters, 2002), it has been shown to  
423 express at least two secreted proteases with inhibitory activities towards the formation of clot fibers and  
424 coagulation-associated pathogen trapping (Toubarro et al., 2013a; Toubarro et al., 2013b). Once again,  
425 the induction of such immune responses is consistent with the previous identification of several virulence  
426 factors of the NBC targeting cellular immunity (Balasubramanian et al., 2009; Balasubramanian et al.,  
427 2010; Brivio et al., 2018; Eom et al., 2014; Kim et al., 2005; Park and Kim, 2000; Park and Stanley,  
428 2006; Ribeiro et al., 1999; Ribeiro et al., 2003; Toubarro et al., 2013a; Vigneux et al., 2007).

#### 429 **3.1.4. Diverse immunity-related genes**

430 A total of 29 modulated genes were involved in other diverse immune processes. They included 10 up- or  
431 down-regulated signaling genes, 7 upregulated recognition genes, 8 upregulated effector genes and 5  
432 upregulated genes of unknown functions that are known to be modulated after immune challenge (Fig.  
433 1D).

434 The signaling genes firstly encoded 2 insulin-like growth factor (IGF-II-like) and 2 insulin receptor  
435 substrate homologs (IRS1-like) (Fig. 1D). Insulin signaling is known to have a deleterious impact on the  
436 induction of systemic immune responses in the fat body of *D. melanogaster* (Lee and Lee, 2018) whereas  
437 insulin increases hemocyte proliferation in the hemolymph of mosquitoes (Castillo et al., 2011) as well as  
438 in the hematopoietic organs of the lepidopteran model *Bombyx mori* (Nakahara et al., 2006). In agreement  
439 with these assertions, we found that 2 of these genes were down-regulated in the fat body, but all 4 genes  
440 were upregulated in the hemocytes (Fig. 1D). Two other signaling genes were found to be specifically  
441 overexpressed in the hemocytes. The first one is a homolog of the *Litopenaeus vannamei* (Decapoda:  
442 Penaeidae) leucine-rich repeat flightless-I-interacting protein 2 (LRRFIP2-like) (Fig. 1D), which has been  
443 shown to upregulate AMP expression in *L. vannamei* as well as in *D. melanogaster* (Zhang et al., 2013).  
444 On the other hand, 3 signaling genes were found to be strictly down-regulated (Fig. 1D). Interestingly,

445 these genes included a member of the TGF- $\beta$  pathway (BAMBI-like) in the hemocytes and a member of  
446 the JNK pathway in the fat body (Basket), two pleiotropic pathways that are currently suspected to take a  
447 part in the *D. melanogaster* immune response to nematodes after NBC infestation (Eleftherianos et al.,  
448 2016; Ozakman and Eleftherianos, 2019; Patrnogic et al., 2018; Yadav et al., 2018). The third down-  
449 regulated gene was found in the fat body and encoded MASK, an inducer of the Jak/Stat pathway (Fisher  
450 et al., 2018). In the fat body, the Jak-Stat pathway has mainly been shown to induce the expression of  
451 cytokines (Pastor-Pareja et al., 2008) and of a putative opsonin belonging to the TEP family (Lagueux et  
452 al., 2000). Remarkably, several TEP genes have been shown to participate in antibacterial immunity after  
453 NBC infestation in *D. melanogaster* (Arefin et al., 2014; Shokal and Eleftherianos, 2017; Shokal et al.,  
454 2017; Shokal et al., 2018). All of these down-regulations could thus impair the insect's immune response  
455 to the NBC. However, more detailed analyses of their functions and modulations would be required to  
456 hypothesize immunosuppressive effects of the NBCs.

457 All 7 upregulated recognition genes encoded lectins (Fig. 1D). Five of them encoded C-type lectins  
458 (CLECT), which are known to be involved in binding of diverse pathogens (Xia et al., 2018), including  
459 bacteria and nematodes (Yu and Kanost, 2004). This binding can then stimulate several immune  
460 responses, such as bacterial aggregation, melanization, phagocytosis, nodulation and encapsulation (Xia  
461 et al., 2018). The 2 others encoded galectins, which are involved in diverse aspects of mammalian  
462 immunity, including pathogens binding (Baum et al., 2014), and are considered as relevant candidate  
463 immune proteins in insects (Pace and Baum, 2002). Despite a larger set of upregulated lectins was  
464 identified in the fat body, members of these protein families were found upregulated in the two tissues.

465 In the hemocytes, the upregulated effector genes firstly encoded a homolog of the superoxide dismutase  
466 (SOD-like), a conserved detoxifying enzyme involved in responses to reactive oxygen species (Wang et  
467 al., 2018) (Fig. 1D). The 7 remaining genes encoded proteins with similarity to insect metalloproteinase  
468 inhibitors (IMPI-like) (Fig. 1D), whose functions have only been studied in the lepidopteran model  
469 *Galleria mellonella*. The only characterized IMPI encodes two proteins of which one is probably  
470 involved in the regulation of extracellular matrix remodeling and the second specifically targets  
471 metalloproteinases from pathogens (Wedde et al., 1998; Wedde et al., 2007). *S. carpocapse* and *X.*  
472 *nematophila* both express several secreted serine proteases as well as metalloproteinases during the  
473 infectious process (Caldas et al., 2002; Chang et al., 2019; Dillman et al., 2015; Hao et al., 2010; Jing et  
474 al., 2010; Lu et al., 2017; Massaoud et al., 2011). The induction of such immune responses could interfere  
475 with some of these proteinases to impair the NBC's virulence and/or survival. Interestingly, all but one of  
476 these IMPI homologs were found to be specifically upregulated in the hemocytes, a tissue-specificity that  
477 had not been highlighted in previous reports (Griesch et al., 2000; Vertyporokh and Wojda, 2017).

478 Finally, the remaining genes of unknown function encoded Spod-x-tox, a protein without antimicrobial  
479 activity which contains tandem repeats of defensin-like motifs (Destoumieux-Garzon et al., 2009), 3

480 REPAT genes, which are known to be induced in the midgut after exposure to toxins, viruses and  
481 intestinal microbiota perturbations in the close species *S. exigua* (Herrero et al., 2007; Navarro-Cerrillo et  
482 al., 2012; Navarro-Cerrillo et al., 2013), and Hdd1, which is induced in response to bacteria and  
483 peptidoglycan in the lepidopteran models *Hyphantria cunea* and *Bombyx mori* (Shin et al., 1998; Zhang  
484 et al., 2017) (Fig. 1D).

485 In summary, we found an important additional mobilization of several relevant candidate immune genes,  
486 including mainly insulin signaling factors and IMPIs in the hemocytes and lectins in the fat body. In  
487 addition, these results suggest that the candidate immune pathways TGF- $\beta$ , JNK and Jak/Stat could be  
488 down-regulated. Such down-regulations are in disagreement with the results of Yadav and colleagues  
489 (Yadav et al., 2017) in *D. melanogaster* and thus would require further investigation.

### 490 **3.2. Temporal analysis of the induced immune responses**

491 In order to put the *S. frugiperda* immune responses in relation with the infectious process, we then  
492 described their temporal dynamics in each analyzed immunocompetent tissue. To this aim, we monitored  
493 with RT-qPCR experiments the induction levels of selected representative immune genes from 5 hpi, the  
494 mean time at which nematodes release *X. nematophila* in the hemocoel, to 20 hpi, which is about 9 hours  
495 before the first insect deaths (Supplementary Fig. 2).

496 In the hemocytes, the selected genes included 15 genes of the antimicrobial response, 2 genes involved in  
497 melanization, 5 cellular response genes, 2 lectins and one IMPI-like gene. At 5 hpi, only 2 genes,  
498 encoding a leucocin antibacterial (Yi et al., 2014) AMP (Lebocin 2) and the negative regulator Pirk of the  
499 Imd pathway (Kleino et al., 2008), were found to be significantly upregulated. However, most of the  
500 selected genes that are strongly induced at later time points also presented positive log<sub>2</sub> fold changes at  
501 this time point (Fig. 2A). From 10 to 20 hpi, all selected genes but few exceptions (*cecropin D*, *Tg-like*  
502 and *Integrin  $\beta$ -like*) due to biological variability were significantly upregulated at each time point (Fig.  
503 2A). Clustering analyses based on Pearson coefficients however revealed 3 distinct clusters of  
504 covariations. The first one contained 13 genes belonging to all the categories cited above and  
505 corresponded to very stable induction patterns (Fig. 2A). The second one, which contained 8 genes  
506 involved antimicrobial and cellular responses plus the selected C-type lectin (*CLECT (ccBV)*),  
507 corresponded to slightly increasing patterns (Fig. 2A). Finally, the third one, which contained the Relish  
508 and Pelle members of the Imd and Toll pathways (Ferrandon et al., 2007), an integrin and the DDC  
509 melanization enzyme (Huang et al., 2005) genes, corresponded to slightly decreasing patterns (Fig. 2A).

510 In the fat body, the selected genes included 15 genes of the antimicrobial response, 2 genes involved in  
511 melanization, one galectin gene (*Galectin 1*) and an IMPI-like gene (*IMPI-like 3*). At 5 hpi, all 7 selected  
512 AMPs, *PGRP-S1* and *Galectin 1* were found to be upregulated (Fig. 2B). All these genes were among the  
513 most strongly overexpressed at later time points. Such as in the hemocytes, most of the selected genes  
514 were then significantly upregulated from 10 to 20 hpi (Fig. 2B). In this tissue, the genes only subdivided

515 into two main covariation clusters: a cluster of genes with stable induction patterns and a cluster of genes  
516 with increasing induction patterns. The first cluster contained 10 genes of which 8 were involved in  
517 antimicrobial responses, one encoded a melanization-related serine protease (Snake-like 2) and one  
518 encoded the Galectin 1 (Fig. 2B). The second cluster contained 9 genes, of which 7 were involved in  
519 antimicrobial responses, one encoded the DDC melanization enzyme (Huang et al., 2005) and the last one  
520 encoded the IMPI-like 3 (Fig. 2B).

521 Altogether, the results obtained for the two tissues show that most of the transcriptional immune  
522 responses induced at 15 hpi take place between 0 and 10 hpi, which is comparable to timings observed in  
523 other interaction models (Boutros et al., 2002; Erler et al., 2011; Lemaitre et al., 1997). The results also  
524 indicate that these responses are globally stable across the time post-infestation despite some distinct gene  
525 induction patterns in each category of response. Interestingly, while we were hoping to discriminate  
526 between an early response, probably activated by the nematode presence, and a later response, probably  
527 reacting to bacterial growth, we did not find any clear link between the gene inductions' dynamics and  
528 the different immune processes and pathways that were represented in our selection.

### 529 **3.3. Evaluation of each NBC partner's part in the induced immune responses**

530 In order to identify each NBC partner's relative participation in the fat body's and hemocytes' immune  
531 responses, we used RT-qPCR to compare the induction levels of the selected immune genes after  
532 independent infections by the whole NBC, the axenic nematode or the bacterial symbiont. To this aim, we  
533 decided to use a more standardized protocol of direct injection of the pathogens into the hemocoel,  
534 thereby limiting putative side effects such as early hemocoel colonization by intestinal microorganisms.  
535 Importantly, we previously compared the kinetics of *X. nematophila* growth and of *S. frugiperda* survival  
536 after injection of the entire NBC and of 200 *X. nematophila* (Supplementary Fig. 3A,B). This comparison  
537 showed that both kinetics are very similar and thus that any difference of induction level between the 2  
538 conditions would not reflect differences in bacterial load or physiological state. However, the putative  
539 impact of axenization on the nematode's physiology could not be assessed by the same way due to  
540 technical limitations and to its avirulence in absence of its bacterial symbiont (Supplementary Fig. 3B,C).  
541 In the hemocytes, 14 genes presented higher induction levels in response to *X. nematophila* than in  
542 response to the axenic nematode (Fig. 3). In the antimicrobial category, they included the negative  
543 regulator *Pirk* of the Imd pathway (Kleino et al., 2008), all the selected attacin, cecropin, gloverin,  
544 lebocin and gallerimycin AMP genes, the 2 selected PGRP-S genes, and also probably the Imd pathway  
545 transcription factor *Relish* despite non-significant statistics (Ferrandon et al., 2007) (Fig. 3A). As  
546 indicated above, the Imd pathway, as well as the attacin, cecropin and gloverin AMP families, are known  
547 to take part in anti-Gram negative bacteria immune responses (Ferrandon, 2013; Yi et al., 2014). Their  
548 induction patterns thus indicate that the antimicrobial *X. nematophila*-induced responses are well adapted  
549 to the nature of the pathogen. Moreover, these results are in agreement with the study of Aymeric et al.

(2010) showing that the Imd pathway functions in the *D. melanogaster* immune response to *X. nematophila*. In the other categories, the *X. nematophila*-induced genes encoded the DDC melanization enzyme (Huang et al., 2005), the hemolin antibacterial opsonin (Eleftherianos et al., 2007), the IMPI-like 3, and also probably the selected integrin (Integrin  $\beta$ -like) (Fig. 3B-D). Once again, all of these genes are susceptible to play a part in an immune response to a pathogenic bacterium even though most of them could act on diverse types of invaders. Surprisingly, we found that *X. nematophila* strongly over-induces the transglutaminase (Tg-like) putative clotting factor gene (Lindgren et al., 2008) (Fig. 3C). This result could suggest that the bacterium is actually the main responsible for tissue damages at this time point and/or that *Tg-like* expression is induced in response to bacteria. Importantly, this result is in agreement with the study of Yadav and colleagues (Yadav et al., 2017), who showed that the *D. melanogaster* Fondue clotting factor was induced after infestation by the NBC but not after infestation by axenic nematodes. Remarkably, most of the genes that were mostly induced by *X. nematophila* presented higher induction values in response to the bacterium alone than in response to the whole NBC. However, this observation cannot be directly interpreted as an antagonistic effect of the nematode partner since it could be due to changes in the relative proportions of each hemocyte subtype, which would not necessarily reflect absolute variations in their numbers. In addition, the nematode partner specifically induced the overexpression of the selected C-type lectin (*CLECT (ccBV)*) and was probably the main inducer of the *Galectin 1*, the tetraspanin D76 homolog (*Tsp-like 3*) and the selected diapausin AMP (*Diapausin 5*) (Fig. 3A,C,D). As mentioned before, the *M. sexta* tetraspanin D76 is known to take part in encapsulation (Zhuang et al., 2007) and some lectins can bind nematodes and participate in melanization (Yu and Kanost, 2004) as well as in all types of cellular immune responses. Once again, their induction patterns are consistent with the nature of the pathogen, since both types of molecules could be involved in classical anti-nematode immune responses, such as cellular or melanotic encapsulation (Eleftherianos et al., 2017). Finally, 5 genes, encoding the Toll pathway members Pelle and Cactus (Ferrandon et al., 2007), the selected moricin AMP (Moricin 2), the melanization-related PPAE2 and the Krüppel-like transcription factor (Kr-like factor 1), were similarly induced by each of the three pathogens (Fig. 3A-C), suggesting that these responses are induced by the 2 partners without any additive effect.

In the fat body, statistical analysis of the results firstly revealed that the induction levels of *Pirk* as well as of the selected cecropin and gloverin AMP genes were significantly lower in response to the axenic nematode than in response to the NBC and to *X. nematophila* (Fig. 4A), suggesting the bacterial partner is the main responsible for their inductions. In addition, despite non-significant statistics, the results for the selected attacin AMPs, PGRP-S6 and GNB3 genes showed similar induction patterns (Fig. 4A). As for the hemocytes, the induction patterns of *Pirk* and of the attacin, cecropin and gloverin AMP genes suggest that the fat body's antimicrobial response to *X. nematophila* is well adapted to the type of pathogen that is met. On the contrary, the induction levels of the melanization-related serine protease

585 (*Snake-like 2*) was significantly lower in response to *X. nematophila* than in response to the NBC and to  
586 the axenic nematode (Fig. 4B), suggesting that the nematode partner is the main responsible for its  
587 induction. Similar induction patterns were obtained for the Toll pathway members *Toll* and *Cactus*  
588 (Ferrandon et al., 2007) as well as for *Galectin 1* (Fig. 4A,C). As mentioned for the hemocytes, the  
589 induction of lectins and melanization-related genes in response to the nematode is consistent with the  
590 nature of the pathogen since both could participate in classical anti-nematode immune responses  
591 (Eleftherianos et al., 2017). The induction of Toll pathway members is more difficult to relate with  
592 known anti-nematode immune responses and Yadav et al. (2018) found that the inactivation of this  
593 pathway does not impact the *D. melanogaster* survival to infestation by the whole NBC or by axenic *S.*  
594 *carpocapsae*. However, the involvement of this immune pathway in anti-nematode immune responses  
595 may depend on the downstream effectors and thus be variable between insect species. Finally, the other  
596 genes did not show any clear difference of induction level after injection of the 3 pathogens, except for  
597 the gallerimycin AMP, PGRP-S1 and the DDC melanization enzyme genes, which presented a lower  
598 induction when each NBC partner was injected alone (Fig. 4A,B). These results suggest synergistic  
599 effects of the nematode and of the bacterium on the induction of these genes.

600 In summary, we found in the 2 tissues that most of the selected genes presented partner-specific induction  
601 patterns, suggesting that the immune response to the NBC corresponds to combinations of responses  
602 induced by each partner. The detailed analysis of these genes indicates that *X. nematophila* is the main  
603 inducer of most of the selected genes, and especially of the well-known antibacterial ones. On the other  
604 hand, *S. carpocapsae* is the main inducer of some melanization and encapsulation-related genes and of  
605 the selected lectins, which could all take part in classical anti-nematode immune responses. The results  
606 thus globally suggest that the hemocytes and the fat body both respond appropriately to each NBC partner  
607 despite some yet unexplained results, such as an induction of Toll pathway members in the fat body by  
608 the nematode partner.

### 609 **3.4. Expression patterns of two new clusters of candidate immune genes**

610 During our first analysis of the RNAseq data, we identified 2 new clusters of candidate immune genes  
611 (Huot et al., 2019). The first one, named the Unknown (Unk) cluster, was localized close to *Tamozhennic*,  
612 a gene encoding a nuclear porin involved in the nucleation of Dorsal, the transcription factor of the Toll  
613 pathway (Minakhina et al., 2003). It contained 5 genes predicted to encode secreted peptides and short  
614 proteins that were all highly overexpressed in the midgut, fat body and hemocytes at 15 hpi and of which  
615 4 were the unique mobilized genes at 8 hpi in the fat body. The second cluster, named the Genes with  
616 Bacterial Homology (GBH) cluster, contained 3 genes located inside a defensin-like AMP genes cluster  
617 in the *S. frugiperda* genome. The 3 genes were predicted to encode secreted proteins similar to each other  
618 and one of them was also found highly induced at 15 hpi in the 3 tissues. The particularity of these genes  
619 is that homologs are found only in lepidopteran species as well as, intriguingly, in Gram positive bacteria.

620 Here, we reexamined the expression patterns of the *Unk* and *GBH* genes and found that the 5 *Unk* genes  
621 were mainly expressed in the fat body whereas 2 of the 3 *GBH* genes were mainly expressed and induced  
622 in the hemocytes (Supplementary Table 3).

623 In order to learn more about their putative functions, we decided to analyze, as we did for the known  
624 immune genes, their induction patterns across the time post-infestation and in response to each NBC  
625 partner in the corresponding tissues. In both cases, we found that the induction dynamics of the genes  
626 were very similar to those of immune genes, with an upregulation that becomes significant at 5 or 10 hpi  
627 and with globally stable induction patterns from 10 to 20 hpi (Fig. 5A,B).

628 In the case of the GBH cluster, the results that we got for the 2 NBC-responsive genes (*GBH1* and *GBH3*)  
629 in the hemocytes indicate that they are significantly less induced after axenic nematode injection than  
630 after NBC and *X. nematophila* injections, suggesting that the bacterium is the main responsible for their  
631 up-regulation (Fig. 5C). We could hypothesize an acquisition by horizontal gene transfer from bacteria of  
632 the *GBH* genes. In this case, their putative involvement in the antibacterial immune response would be  
633 particularly interesting, since bacterial genes hijacking for immune purpose has only been reported once  
634 in metazoans, in the tick *Ixodes scapularis* (Chou et al., 2015). Such a hypothesis however requires  
635 functional confirmation.

636 In the case of the *Unk* cluster, we found that the 4 most induced genes in the fat body (*Unk2* to 5) are all  
637 strongly and similarly induced by the NBC and by the axenic nematode whereas they are not induced by  
638 *X. nematophila* (Fig. 5D). The results are very similar for the least expressed *Unk* gene (*Unk1*), for which  
639 we only found a significant induction for the injection of axenic nematodes (Fig. 5D). This partner-  
640 specific induction pattern suggests that the *Unk* genes are involved in specific aspects of the insect  
641 responses to the infestation. In addition, the putative involvement of the *Unk* genes in the response to the  
642 nematode partner seems to be in agreement with their early mobilization during the infectious process and  
643 with their overexpression in the midgut (Huot et al., 2019), which is the entry site of the nematode. In our  
644 previous study, we had hypothesized the *Unk* genes may encode new types of immune effectors (Huot et  
645 al., 2019). However, given their low levels of conservation in species as close as *S. litura* or *S. littoralis*  
646 (Supplementary Fig. 4) another hypothesis would be that they correspond to regulatory long non-coding  
647 RNAs (Johnsson et al., 2014; Qu and Adelson, 2012). In both cases, the further functional  
648 characterization of these genes could be very promising given our current lack of knowledge of the  
649 immune pathways and molecular effectors of insect anti-nematode immunity.

## 650 **4. Conclusion**

651 Here, we provide a very deep and contextualized analysis of the *S. frugiperda*'s hemocytes' and fat  
652 body's transcriptional immune responses to infestation by the *S. carpocapsae*-*X. nematophila* NBC. Our  
653 topologic analysis of these responses at 15 hpi firstly confirmed the induction of very potent and

654 diversified immune responses towards the pathogen, such as suggested by our previous analysis of the  
655 transcriptomic data (Huot et al., 2019) as well as by the study of Yadav et al. (2017) in the *D.*  
656 *melanogaster* model. The present work now shows that these responses are very stable across the post-  
657 infestation time and that they correspond to combinations of *X. nematophila*- and *S. carpocapsae*-induced  
658 responses that seem to be well adapted to the nature of each partner (Figure 6).

659 Together with results obtained in other insect models, the pieces of information collected during these  
660 analyses are of great interest for the study of the dialogue that takes place between each NBC partner and  
661 *S. frugiperda*'s immune systems. First, our results strongly suggest that the NBC immunosuppressive  
662 strategies globally have a low impact on the induction of immune responses at the transcriptional level in  
663 *S. frugiperda*. They also indicate that the nematode and/or its effects on the host are detected by the  
664 insect's immune system that in return seems to induce appropriate immune responses towards the  
665 pathogen. Such observations could help to determine the limits and/or universality of previously  
666 described immunosuppressive and immunoevasive strategies of the NBC that have been described in  
667 other models. For example, they suggest that the suppressive effect of *X. nematophila* on the expression  
668 of AMP genes (Hwang et al., 2013; Ji and Kim, 2004; Park et al., 2007) as well as the camouflage  
669 strategy of *S. carpocapsae* (Binda-Rossetti et al., 2016; Mastore et al., 2015) are probably far from  
670 sufficient to explain their success towards the *S. frugiperda*'s immune system, which could also be true  
671 for other insect species. On the other hand, we found several unexplained down-regulations of signaling  
672 genes, such as of members of the Imd, JNK, TGF- $\beta$  and Jak-Stat pathways, that had never been reported  
673 before and which could open new working trails for the study of the molecular basis of the NBC's  
674 immunosuppressive strategies. Finally, this study allowed the identification of very large panels of  
675 candidate immune genes involved in all the main components of insect immunity as well as of some yet  
676 uncharacterized genes that could encode new immune factors involved in the response to NBCs, such as  
677 the *GBH* and the *Unk* genes, which are respectively specific to lepidopterans and to some noctuid species  
678 (Huot et al., 2019).

679 Continuing this work with more functional and mechanistic approaches is now required to get an accurate  
680 picture of the molecular dialogue between the NBC and the immune system. In the longer term, using  
681 such detailed and contextualized approaches on diverse insect species could help to identify the precise  
682 causes of immune systems' failure or success towards this NBC and thus the conditions that are required  
683 for an adequate use of this pathogen against insect pests.

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### 690 **Authors' contribution**

691 L.H., N.N. and B.D. conceived this study. N.N. and B.D. directed this study. L.H. and P.-A.G. performed  
692 the infestation experiments. L.H., P.-A.G. performed dissections. L.H. and A.B. extracted and purified the  
693 RNA. J.-C.O. designed the *X. nematophila* specific primers. S.P. produced the axenic nematodes and  
694 checked their axenization. L.H. and A.B. performed the qPCRs. L.H., N.N. and B.D. analysed the data.  
695 L.H. wrote the manuscript. L.H., N.N. and B.D. revised the manuscript. All authors have read and  
696 approved the manuscript.

### 697 **Additional Information**

698 **Competing Interests:** The authors declare no competing interests.

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1103

## 1104 **Figure Legends**

1105 **Fig 1.** Expression variations of the differentially expressed immune genes after infestation by the  
1106 nematobacterial complex. Heatmaps showing the expression variations of the differentially expressed  
1107 immune genes in the hemocytes and in the fat body at a middle time point of 15 h post-infestation.  
1108 RNAseq raw data were retrieved from the study of Huot et al. (2019) in which three independent  
1109 experiments were performed with N=9 larvae exposed to 150 NBCs or to a Ringer sterile solution in each  
1110 sample. Differential expression between the infested and the control conditions was analyzed with the  
1111 Sleuth software (following pseudoalignment with the Kallisto software) using statistical thresholds of  
1112 0.01 for q-value (equivalent of adjusted p-value), -1 and +1 for Beta value (biased equivalent of log2 fold  
1113 change) and 5 for pseudocount means. The immune genes were identified by homology and classified as  
1114 (A) antimicrobial immunity-related, (B) melanization-related, (C) cellular immunity-related and (D)  
1115 diverse immune responses. Black dots indicate genes with statistically non-significant variations to the  
1116 controls in the corresponding tissue; HC : Hemocytes, FB : Fat body.

1117 **Fig 2.** Temporal dynamics of the identified immune responses after infestation by the nematobacterial  
1118 complex. Heatmaps showing the temporal evolution of the induction levels of representative immune  
1119 genes in the hemocytes (A) and in the fat body (B) after infestation by the NBC. Three independent  
1120 experiments were performed with N=9 larvae exposed to 150 NBCs and N=9 larvae exposed to Ringer  
1121 sterile solution. RT-qPCR experiments were performed in triplicate and the RpL32 housekeeping gene  
1122 was used as reference for relative quantifications. Differential expression between the infested and the  
1123 control conditions was assessed according to the method of Ganger et al. (2017) and with Student t tests  
1124 on  $\Delta Cq$ . Black dots indicate genes with statistically non-significant variations to the controls in the  
1125 corresponding tissue ( $p$ -value > 0.05). The dendrograms represent clustering analyses based on Pearson  
1126 correlation coefficients.

1127 **Fig 3.** Relative participations of *S. carpocapsae* and *X. nematophila* in the hemocytes' immune responses.  
1128 Histograms showing the induction levels (+/- SEM) of representative immune genes in the hemocytes at  
1129 13 h after independent injections of either whole NBCs, axenic nematodes or bacterial symbionts. Three  
1130 independent experiments were performed with N=9 control larvae and N=9 larvae injected with 10 NBCs  
1131 (NBC), 10 axenic *S. carpocapsae* (*S.c.*) or 200 *X. nematophila* (*X.n.*). For NBC and axenic nematode  
1132 injections control larvae were injected with 70 % Ringer - 30 % glycerol sterile solutions whereas they  
1133 were injected with sterile PBS for bacterial injections. RT-qPCR experiments were performed in triplicate  
1134 and the RpL32 housekeeping gene was used as reference for relative quantifications. Differential  
1135 expression values between the infected and the corresponding control conditions were calculated  
1136 according to the method of Ganger et al. (2017) and statistical differences between the three types of  
1137 infection were assessed by one-way ANOVA and Tukey tests on  $\Delta\Delta Cq$ . Letters indicate the statistical  
1138 groups resulting from the Tukey tests ( $p$ -value > 0.05). The genes were then gathered by type of immune

1139 response with (A) antimicrobial immunity-related, (B) melanization-related, (C) cellular immunity-  
1140 related and (D) diverse immune responses.

1141 **Fig 4.** Relative participations of *S. carpocapsae* and *X. nematophila* in the fat body's immune responses.  
1142 Histograms showing the induction levels (+/- SEM) of representative immune genes in the fat body at 13  
1143 h after independent injections of either whole NBCs, axenic nematodes or bacterial symbionts. Three  
1144 independent experiments were performed with N=9 control larvae and N=9 larvae injected with 10 NBCs  
1145 (NBC), 10 axenic *S. carpocapsae* (*S.c.*) or 200 *X. nematophila* (*X.n.*). For NBC and axenic nematode  
1146 injections control larvae were injected with 70 % Ringer - 30 % glycerol sterile solutions whereas they  
1147 were injected with sterile PBS for bacterial injections. RT-qPCR experiments were performed in triplicate  
1148 and the RpL32 housekeeping gene was used as reference for relative quantifications. Differential  
1149 expression values between the infected and the corresponding control conditions were calculated  
1150 according to the method of Ganger et al. (2017) and statistical differences between the three types of  
1151 infection were assessed by one-way ANOVA and Tukey tests on  $\Delta\Delta Cq$ . Letters indicate the statistical  
1152 groups resulting from the Tukey tests (p-value > 0.05). The genes were then gathered by type of immune  
1153 response with (A) antimicrobial immunity-related, (B) melanization-related and (C) diverse immune  
1154 responses.

1155 **Fig 5.** Transcriptional induction patterns of putative new immune genes. (A, C) Histograms showing the  
1156 induction levels (+/- SEM) of 2 GBH genes in the hemocytes (A) and of the 5 Unk genes in the fat body  
1157 (C) at several times after infestation by the NBC. Three independent experiments were performed with  
1158 N=9 larvae exposed to 150 NBCs and N=9 larvae exposed to Ringer sterile solution. RT-qPCR  
1159 experiments were performed in triplicate and the RpL32 housekeeping gene was used as reference for  
1160 relative quantifications. Differential expression between the infested and the control conditions was  
1161 assessed according to the method of Ganger et al. (2017) and with Student t tests on  $\Delta Cq$ . Black dots  
1162 indicate genes with statistically non-significant variations to the controls (p-value > 0.05). (B, D)  
1163 Histograms showing the induction levels (+/-SEM) of 2 GBH genes in the hemocytes (B) and of the 5  
1164 Unk genes in the fat body (D) at 13 h after independent injections of either whole NBCs, axenic  
1165 nematodes or bacterial symbionts. Three independent experiments were performed with N=9 control  
1166 larvae and N=9 larvae injected with 10 NBCs (NBC), 10 axenic *S. carpocapsae* (*S.c.*) or 200 *X.*  
1167 *nematophila* (*X.n.*). For NBC and axenic nematode injections control larvae were injected with 70 %  
1168 Ringer - 30 % glycerol sterile solutions whereas they were injected with sterile PBS for bacterial  
1169 injections. RT-qPCR experiments were performed in triplicate and the RpL32 housekeeping gene was  
1170 used as reference for relative quantifications. Differential expression values between the infected and the  
1171 corresponding control conditions were calculated according to the method of Ganger et al. (2017) and  
1172 statistical differences between the three types of infection were assessed by one-way ANOVA and Tukey  
1173 tests on  $\Delta\Delta Cq$ . Letters indicate the statistical groups resulting from the Tukey tests (p-value > 0.05).

1174 **Fig 6.** Hypothetical structure of the *S. frugiperda* larva's immune response to the NBC. Graphical  
1175 abstract illustrating the main hypotheses we can emit from the present RNAseq and RT-qPCR data and  
1176 from our current knowledge of *S. frugiperda* immunity. Dark green letters, lines and arrows indicate  
1177 responses that seem to be mainly induced by the nematode partner *S. carpocapsae* whereas orange ones  
1178 indicate responses that seem to be mainly induced by the bacterial symbiont *X. nematophila*. The arrows'  
1179 thicknesses and the letter sizes refer to the relative strengths of the induced transcriptional responses.  
1180

## 1181 **Supporting Information Legends**

### 1182 **Supplementary Table 1. Primers sequences and genes used in this study.**

### 1183 **Supplementary Table 2. Hemocytes and fat body RNAseq results for *S. frugiperda*'s immune genes.**

1184 RNAseq raw data were retrieved from the study of Huot et al. (2019) in which three independent  
1185 experiments were performed with N=9 larvae exposed to 150 NBCs or to a Ringer sterile solution for 15  
1186 hours. Following pseudoalignment with the Kallisto software, differential expression between the infested  
1187 and the control conditions was analyzed with the Sleuth software using statistical thresholds of 0.01 for q-  
1188 value (equivalent of adjusted p-value), -1 and +1 for Beta value (biased equivalent of log2 fold change)  
1189 and 5 for pseudocount means. The statistics corresponding to the transcripts that were considered as  
1190 significantly up- or down-regulated are highlighted in red and blue, respectively. The normalized  
1191 pseudocounts are also indicated for each individual sample, with HCn15 and FBn15 corresponding to  
1192 control larvae and HCi15 and FBi15 corresponding to infested larvae. Blast hits on the *Drosophila* and nr  
1193 NCBI databases were obtained by blastx with the Blast2GO software. (A) Immune genes previously  
1194 annotated in the *S. frugiperda*'s genome. (B) Newly identified *S. frugiperda*'s immune genes.

### 1195 **Supplementary Table 3. Hemocytes and fat body RNAseq results for the Unk and GBH putative**

1196 **new immune genes.** RNAseq raw data were retrieved from the study of Huot et al. (2019) in which three  
1197 independent experiments were performed with N=9 larvae exposed to 150 NBCs or to a Ringer sterile  
1198 solution for 15 hours. Following pseudoalignment with the Kallisto software, differential expression  
1199 between the infested and the control conditions was analyzed with the Sleuth software using statistical  
1200 thresholds of 0.01 for q-value (equivalent of adjusted p-value), -1 and +1 for Beta value (biased  
1201 equivalent of log2 fold change) and 5 for pseudocount means. The statistics corresponding to the  
1202 transcripts that were considered as significantly upregulated are highlighted in red. The normalized  
1203 pseudocounts are also indicated for each individual sample, with HCn15 and FBn15 corresponding to  
1204 control larvae and HCi15 and FBi15 corresponding to infested larvae.

### 1205 **Supplementary Fig 1. Verification of *S. carpocapsae* axenicity.**

1206 Electrophoresis gel showing the absence of bacterial contaminants in the axenized nematodes used for experimental infections. Total  
1207 DNA from ground infective stage nematodes was extracted a few hours after each experimental infection  
1208 (fresh axenic *S.c.*) and after several days of storage without antibiotics (stored axenic *S.c.*). The absence  
1209 of bacterial contaminations was assessed by agarose gel electrophoresis after PCR amplification of the  
1210 16S rRNA gene with universal primers and of the *Xenorhabdus*-specific XNC1\_0073 gene (encoding a  
1211 putative TonB-dependent heme-receptor). Whole NBCs (NBC) and a pure suspension of *X. nematophila*  
1212 (*X.n.*) were used as positive controls. A pure suspension of *Pseudomonas protegens* (*P.p.*) was used as  
1213 negative control of putative TonB-dependent heme-receptor amplification. The results indicate no  
1214 bacterial contamination is detected in the sterile axenic nematodes used for experimental infections and

1215 that even after several days of storage without antibiotics, the *X. nematophila* population did not recover  
1216 within these nematodes.

1217 **Supplementary Fig 2. Temporal monitoring of nematobacterial infestation parameters.** (A) Dotplot  
1218 showing the number of *S. carpocapsae* nematodes detected in the midgut alimentary bolus at several  
1219 times after contact with 150 NBCs. Dot colors correspond to 3 independent experiments on N=3 larvae  
1220 per time point. (B) Curve showing the temporal evolution of *X. nematophila* concentration (+/-SEM) in  
1221 the hemolymph across the time post-infestation. Three independent infestation experiments were  
1222 performed with 3 pools of 3 larvae per time point. Hemolymph was collected by bleeding and *X.*  
1223 *nematophila* was quantified by CFU counting on selective culture medium containing erythromycin. (C)  
1224 Curve showing the temporal evolution of *S. frugiperda* larvae survival percentage (+/- SEM) across the  
1225 time post-infestation. Three independent infestation experiments were performed on N=20 larvae per  
1226 experiment.

1227 **Supplementary Fig 3. Comparison of the main infection parameters after independent injections of**  
1228 **the nematobacterial complex, of axenic *S. carpocapsae* and of *X. nematophila*.** (A) Curves showing  
1229 the temporal evolution of *X. nematophila* concentration (+/-SEM) after independent injections of either  
1230 10 NBCs or 200 *X. nematophila* (*X.n.*). Three independent injection experiments were performed with 3  
1231 pools of 3 larvae per time point. Hemolymph was collected by bleeding and *X. nematophila* was  
1232 quantified by CFU counting on selective culture medium containing erythromycin. (B) Curves showing  
1233 the temporal evolution of *S. frugiperda* larvae survival percentage (+/- SEM) after independent injections  
1234 of either 10 NBCs , 10 axenic *S. carpocapsae* (*S.c.*) or 200 *X. nematophila* (*X.n.*). Three independent  
1235 injection experiments were performed on N=20 larvae per experiment. No insect death was reported for  
1236 control buffer-injected larvae. (C) Histogram showing the parasitic success (+/- SEM) (i.e.: number of  
1237 larvae with NBC emergence on total number of infested larvae) after independent injections of either 10  
1238 NBCs or 10 axenic *S. carpocapsae* (*S.c.*). Three independent injection experiments were performed on  
1239 N=20 larvae per infection type and per experiment.

1240 **Supplementary Fig 4. Alignment of deduced amino acid sequences of Unks from *S. frugiperda* with**  
1241 **those of *S. litura* and *S. littoralis*.** Nucleotide sequences were retrieved by blastn on *S. litura* and *S.*  
1242 *littoralis* genomes.

**Figure 1**

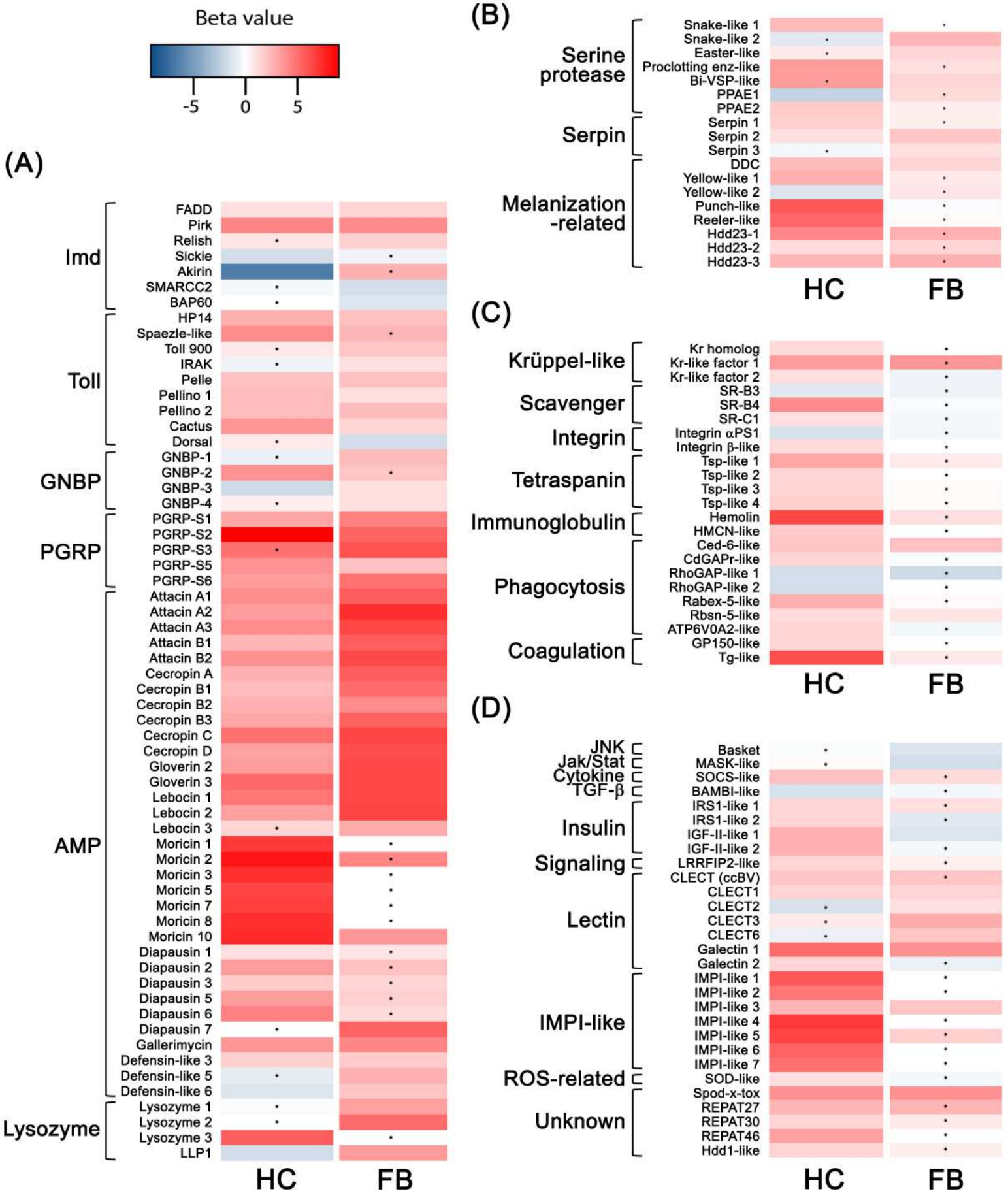


Figure 2

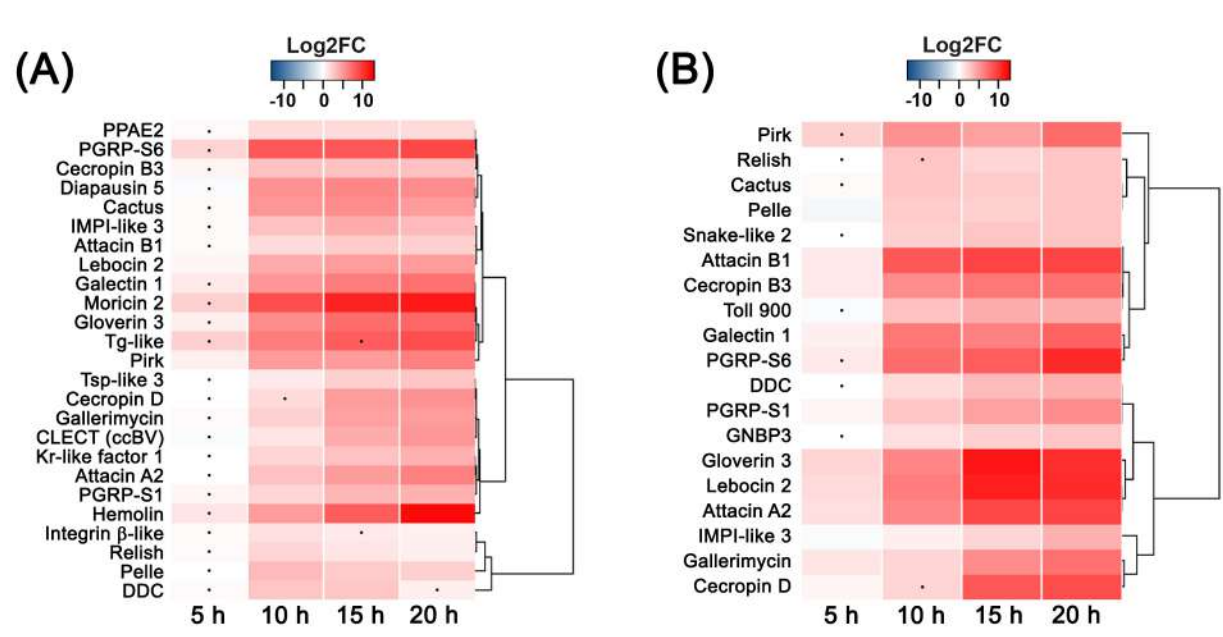


Figure 3

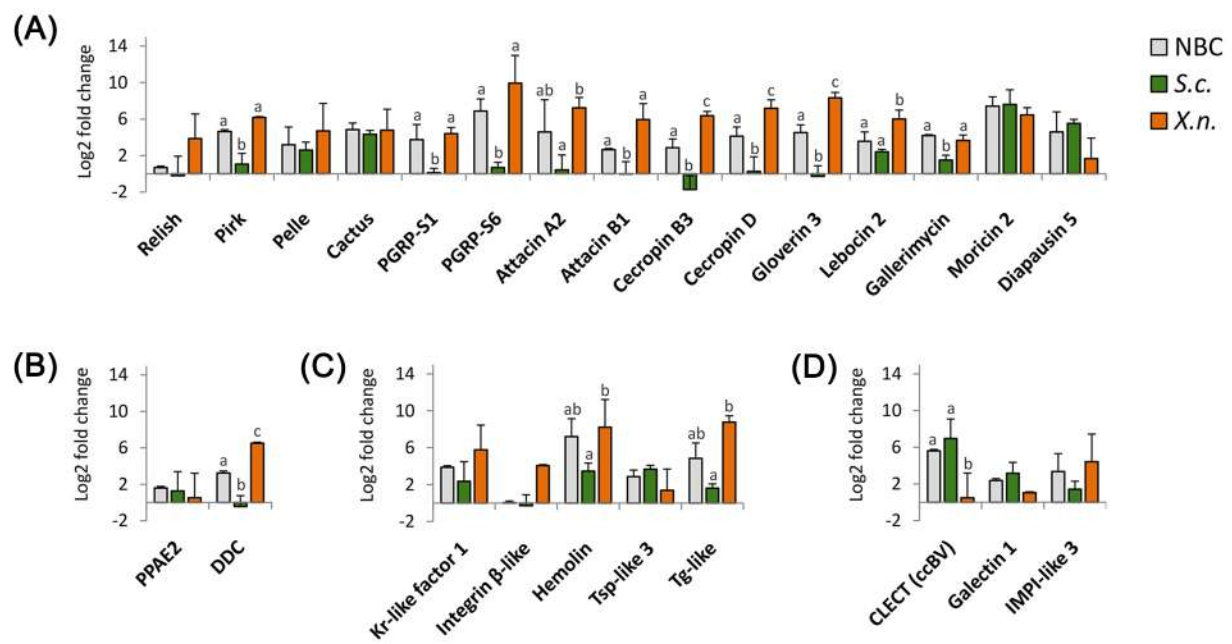


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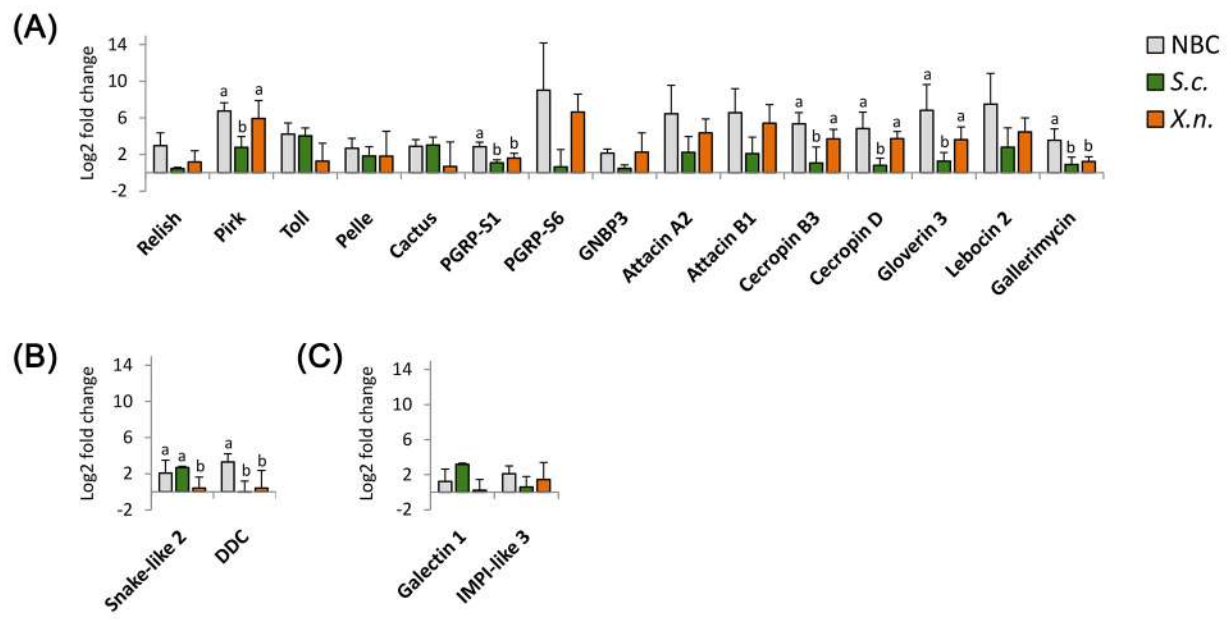


Figure 5

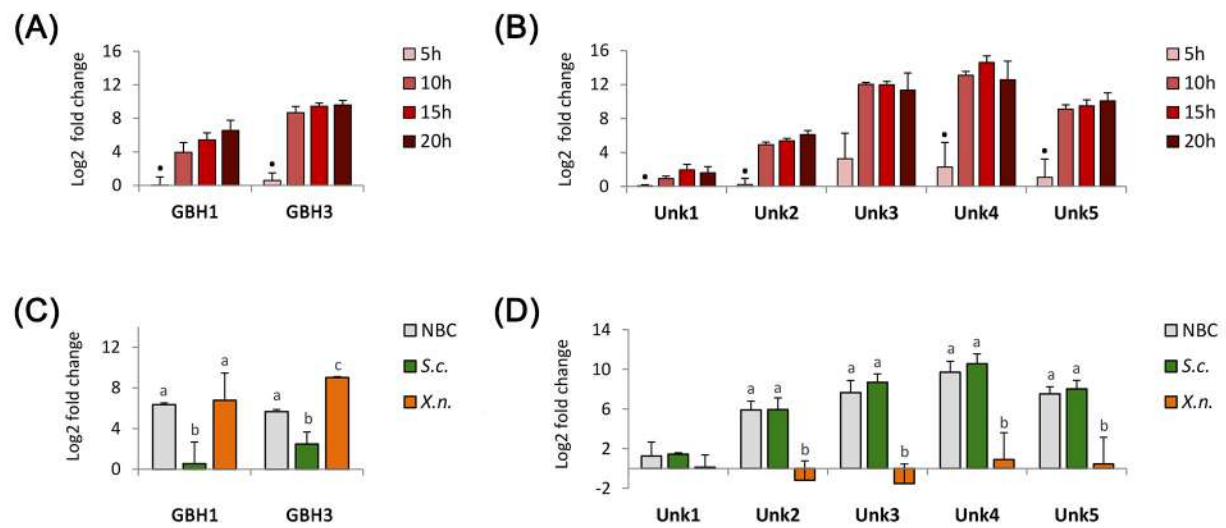


Figure 6

