

1 Partner-specific induction of *Spodoptera frugiperda* immune genes in  
2 response to the entomopathogenic nematobacterial complex *Steinernema*  
3 *carpocapsae-Xenorhabdus nematophila*  
4 Running Title: Specific response of *Spodoptera* immune genes to *Steinernema* nematode or  
5 its bacterial symbiont

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## 12 **Abstract**

13 The *Steinernema carpocapsae*-*Xenorhabdus nematophila* association is a nematobacterial complex (NBC)  
14 used in biological control of insect crop pests. The ability of this dual pathogen to infest and kill an insect  
15 strongly depends on the dialogue between the host's immune system and each partner of the complex. Even  
16 though this dialogue has been extensively studied from the two partners' points of view in several insect  
17 models, still little is known about the structure and the molecular aspects of the insects' immune response  
18 to the dual infection. Here, we used the lepidopteran pest *Spodoptera frugiperda* as a model to analyze the  
19 respective impact of each NBC partner in the spatiotemporal immune responses that are induced after  
20 infestation. To this aim, we first analyzed the expression variations of the insect's immune genes in the fat  
21 bodies and hemocytes of infested larvae by using previously obtained RNAseq data. We then selected  
22 representative immune genes for RT-qPCR investigations of the temporal variations of their expressions  
23 after infestation and of their induction levels after independent injections of each partner. We found that  
24 the fat body and the hemocytes both produce potent and stable immune responses to the infestation by the  
25 NBC, which correspond to combinations of bacterium- and nematode-induced ones. Consistent with the  
26 nature of each pathogen, we showed that *X. nematophila* mainly induces genes classically involved in  
27 antibacterial responses, whereas *S. carpocapsae* is responsible for the induction of lectins and of genes  
28 expected to be involved in melanization and cellular encapsulation. In addition, we found that two clusters  
29 of unknown genes dramatically induced by the NBC also present partner-specific induction profiles, which  
30 paves the way for their functional characterization. Finally, we discuss putative relationships between the  
31 variations of the expression of some immune genes and the NBC's immunosuppressive strategies.

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## 33 **Author summary**

34 Entomopathogenic nematodes (EPNs) are living in the soil and prey upon insect larvae. They enter the  
35 insect by the natural orifices, and reach the hemocoel through the intestinal epithelium. There, they release  
36 their symbiotic bacteria that will develop within the insect and eventually kill it. Nematodes can then feed  
37 and reproduce on the insect cadaver. By using transcriptomic approaches, we previously showed that  
38 Lepidoptera larvae (caterpillars of the fall armyworm *Spodoptera frugiperda*) produce a strong immune  
39 response in reaction to infestation by EPNs. However, we do not know if this immune reaction is triggered  
40 by the nematode itself -*Steinernema carpocapsae* - or its symbiotic bacteria - *Xenorhabdus nematophila*.  
41 To answer this question, we present in this work a careful annotation of immunity genes in *S. frugiperda*  
42 and surveyed their activation by quantitative PCR in reaction to an injection of the bacteria alone, the axenic  
43 nematode or the associated complex. We found that the immune genes are selectively activated by either  
44 the bacteria or the nematode and we discuss the implication of which pathway are involved in the defense  
45 against various pathogens. We also show that a cluster of newly discovered genes, present only in  
46 Lepidoptera, is activated by the nematode only and could represent nematicide genes.

## 47 Introduction

48 The *Steinernema-Xenorhabdus* nematobacterial complexes (NBCs) are natural symbiotic associations  
49 between nematodes and enterobacteria that are pathogenic for insects. The soil-living nematodes infest  
50 insects through the respiratory and/or the intestinal tract (1) and reach the hemocoel, the internal body  
51 cavity, where they release their intestinal symbionts. The bacteria then grow extracellularly in the  
52 hemolymph, the insect equivalent of blood, and improve the nematodes' pathogenicity as well as their  
53 ability to reproduce in the host dead body (2). Until now, about 90 species of *Steinernema* have been  
54 identified, among which several are usable as biological control agents against diverse insect crop pests (3,  
55 4). In consequence, their interactions with insects have been extensively studied for about 50 years (5).  
56 These studies have shown that in addition to ecological and morphological parameters (3), the NBCs'  
57 interactions with the host's immune system is one of the most crucial factors influencing their ability to  
58 infest and kill a given insect (6-8).

59 Insects possess an elaborate immune system, which is able to respond by adapted ways to diverse types of  
60 pathogens and of infections. This system firstly relies on protective external barriers such as the cuticle, or  
61 the peritrophic matrix in the midgut (9, 10). It then relies on local defenses of the surface epitheliums,  
62 which repair efficiently (11-13) and produce toxic factors such as antimicrobial peptides (AMPs) (14-17)  
63 and reactive oxygen species (18). The third line of defense of insects is provided by the hemocytes, which  
64 are the circulating immune cells. They can produce diverse types of immune responses, including AMP  
65 synthesis, phagocytosis, nodulation, encapsulation, coagulation and melanization (19). Nodulation and  
66 encapsulation are cellular immune responses respectively consisting in the engulfment of bacterial  
67 aggregates and of large invaders via hemocytes aggregation (19). Together with coagulation, these  
68 responses are coupled with a melanization process consisting in series of phenolic compounds oxidations  
69 resulting in synthesis of reactive molecules and melanin that participate of pathogens trapping and killing  
70 (20, 21). Finally, the fat body, a functional equivalent of the mammalian liver, produces potent systemic  
71 humoral immune responses involving a massive secretion of AMP cocktails in the hemolymph. These  
72 responses can be induced by two major signaling pathways of insect immunity; the Imd pathway, which is  
73 mainly activated by Gram negative bacteria, and/or the Toll pathway, which is mainly activated by Gram  
74 positive bacteria, fungal organisms and by proteases released by pathogens (22, 23).

75 The *Steinernema-Xenorhabdus* NBC whose interactions with the immune system have been the most  
76 extensively studied is the *S. carpocapsae-X. nematophila* association. These interactions have firstly been  
77 studied from the NBC point of view, which allowed the identification of a multitude of immunoevasive  
78 and immunosuppressive strategies. For instance, studies in *Rhynchophorus ferrugineus* and *Galleria*  
79 *mellonella* have respectively shown that the cuticle of *S. carpocapsae* is not recognized by the host's  
80 immune system (24, 25) and that the nematode secretes protease inhibitors impairing the coagulation  
81 responses (26, 27). Studies in diverse insect models have also shown that both partners produce factors

82 impairing melanization (28-31), hemocyte's viability (32-36) and the production of cellular immune  
83 responses by several ways (27-29, 31, 37, 38). Finally, both *X. nematophila* and *S. carpocapsae* secrete  
84 proteolytic factors degrading cecropin AMPs (39, 40) and the bacterium has also been shown to reduce  
85 more globally the hemolymph antimicrobial activity, as well as AMP transcription in lepidopteran models  
86 (24, 39, 41, 42).

87 On the other hand, the description of these interactions from the hosts' points of view is at its beginning.  
88 This aspect has mainly been studied in the *Drosophila melanogaster* model, with a first transcriptomic  
89 analysis of the whole larva responses to infestations by entire NBCs and by axenic nematodes (43). This  
90 analysis has shown that several immune processes are induced by both pathogens at the transcriptional  
91 level. For instance, the authors found in each case an overexpression of genes related to the Imd and Toll  
92 pathways that was accompanied by the induction of a few AMP genes. They also found an upregulation of  
93 genes related to melanization, coagulation, or involved in the regulation of cellular immune responses (43).  
94 Complementary gene knockout experiments in this model demonstrated an involvement of the Imd  
95 pathway in the response against *X. nematophila* (44) and revealed a possible involvement of the Imaginal  
96 Disc Growth Factor-2, the intestinal serine protease Jonah 66Ci (45) as well as TGF- $\beta$  and JNK pathways  
97 members in the regulation of anti-nematode immunity (46, 47).

98 In order to improve our understanding of the dialogue that takes place between this NBC and its host, we  
99 recently published a topologic transcriptomic analysis of the response of the lepidopteran model  
100 *Spodoptera frugiperda* to the infestation (48). This analysis was focused on the three main  
101 immunocompetent tissues that are confronted to the NBC, which are the midgut (the main entry site in the  
102 hemocoel), the hemocytes and the fat body. The RNAseq experiment showed that there was no potent or  
103 well-defined transcriptional response in the midgut. However, we observed dramatic transcriptional  
104 responses in the fat body and the hemocytes at 15 h post-infestation, which is a middle time point of the  
105 infection. In agreement with the results obtained in *D. melanogaster* whole larvae (43), global analysis of  
106 these responses showed they are dominated by immune processes. The objective of the present study is to  
107 go further in the analysis of these induced immune responses. In order to describe them with high accuracy,  
108 we first examine the expression variations of all the immune genes that have been identified in the insect's  
109 genome. We then use tissue RT-qPCR experiments to analyze the temporal dynamics and the relative  
110 contribution of each NBC partner in the identified immune responses. Our results show that a large number  
111 of immune genes are responsive in either one or the two tissues during the infestation, with activation of  
112 antimicrobial and cellular immunities, of melanization, coagulation and of metalloprotease inhibition.  
113 These responses were found to be stable over the time post-infestation and to consist in combinations of *X.*  
114 *nematophila*-induced and *S. carpocapsae*-induced responses in each tissue. The *X. nematophila*-induced  
115 responses mainly correspond to genes that are classically involved in antibacterial immunity, whereas the  
116 *S. carpocapsae*-induced ones mainly include lectins and genes potentially involved in melanization and  
117 encapsulation. In addition, our RT-qPCR experiments show that two previously identified candidate

118 clusters of uncharacterized genes (48) also present partner-specific induction profiles. Our hypothesis is  
119 that they may correspond to new types of anti-nematode and antibacterial immune factors found in  
120 *Spodoptera* genus and lepidopteran species, respectively.

## 121 **Results & Discussion**

### 122 **Hemocytes' and fat body's immune responses**

123 In order to get an accurate picture of the *S. frugiperda* transcriptional immune responses to the NBC  
124 infestation, we first used a previously published list of immune genes identified by sequence homology in  
125 the *S. frugiperda* genome (49). We then looked at their expression variations in the fat body and in the  
126 hemocytes (S1A Table) and we completed the repertoire with additional putative immune genes that we  
127 directly identified from our RNAseq data (S1B Table). In total, we present the annotation of 226 immune  
128 or putative immune genes of which 132 were significantly modulated at 15 h post-infestation (hpi) (Sleuth,  
129 p-value < 0.01; |Beta| > 1; all count values > 5 in at least one condition) in one or both tissues (Fig 1).  
130 Among them, 62 were involved in antimicrobial responses (Fig 1A), 18 were related to melanization (Fig  
131 1B), 23 were involved in cellular responses (Fig 1C) and the 29 remaining genes were grouped in a category  
132 called “diverse” due to pleiotropic or poorly characterized functions (Fig 1D).

133 **Antimicrobial responses.** In the antimicrobial response category, 58 genes were found to be  
134 upregulated in at least one of the two tissues (Fig 1A). The signaling genes encoded 3 and 8 members of  
135 the Imd and Toll pathways, respectively, as well as 5 short catalytic peptidoglycan recognition proteins  
136 (PGRP-S), which are probably involved in the regulation of these pathways by peptidoglycan degradation  
137 (50, 51) (Fig 1A). Four other genes were considered as involved in recognition. They encoded Gram  
138 negative binding proteins (GNBPs), which have been reported to recognize peptidoglycans or  $\beta$ -glucans  
139 and participate in the further activation of the Toll pathway (22) (Fig 1A). Finally, the effector genes  
140 encoded 33 antimicrobial peptides (AMPs) belonging to all the *S. frugiperda*'s AMP families (49) plus 4  
141 lysozymes and lysozyme-like proteins (LLPs) (Fig 1A). Depending on their families and on the insect  
142 species, AMPs can present varied activity spectra, ranging from antiviral or antibacterial activities to anti-  
143 fungal and anti-parasitic ones (52). Varied activity spectra have also been found for several insects'  
144 lysozymes and LLPs (53-57). Interestingly, all of the categories and subcategories cited above were  
145 represented in the two tissues, indicating that their antimicrobial responses are diversified and that the  
146 factors responsible for their disappearance in the hemolymph (24, 41) probably act at a post-transcriptional  
147 level. About a half of the genes presented similar and significant induction profiles in the hemocytes and  
148 in the fat body. This is for instance the case of the usually anti-Gram negative bacteria attacin, cecropin and  
149 gloverin AMPs (52), which were all highly induced in the two tissues (Fig 1A), suggesting they both  
150 respond to the bacterial partner *X. nematophila*. On the other hand, all the induced GGBP, lysozyme and  
151 LLP genes were found to be either significantly induced in the hemocytes or in the fat body, and in the

152 AMP category, tissue-specificities were observed for diapausin, defensin-like and most moricin genes (Fig  
153 1A).

154 Only 8 antimicrobial response genes were found to be significantly downregulated (Fig 1A). Interestingly,  
155 4 were involved in the Imd pathway whereas the 4 remaining ones were dispersed between the AMP, GGBP  
156 and lysozyme categories (Fig 1A). The Imd pathway downregulated factors included *sickie* and the *akirin*  
157 in the hemocytes and *SMARCC2* and *BAP60* in the fat body (Fig 1A). In *D. melanogaster*, Sickie  
158 participates in the activation of Relish, the transcription factor of the Imd pathway (58) and the akirin acts  
159 together with the Brahma chromatin-remodeling complex, containing BAP60 and SMARCC2, as cofactor  
160 of Relish to induce the expression of AMP genes (59). Given the potent induction of anti-Gram negative  
161 bacteria immune responses in the two tissues, the down-regulation of these genes could be attributed to  
162 immune regulations. However, it has been shown that in the close species *S. exigua*, live *X. nematophila*  
163 reduces the expression of several AMP genes, including attacin, cecropin and gloverin (42, 60, 61). It would  
164 thus be of particular interest to determine whether the observed down-regulations are related to this  
165 immunosuppressive effect.

166 To summarize, the antimicrobial responses are potent and diversified in the two tissues, with a common  
167 induction of genes that probably respond to *X. nematophila*. Yet unexplained tissue-specific responses were  
168 observed and the results show a down-regulation of Imd pathway members that could be related to a  
169 previously described transcriptional immunosuppressive effect of the NBC. However, this effect would not  
170 be potent enough to suppress the humoral responses at this time point, suggesting that the NBC probably  
171 uses other immunosuppressive strategies in this model.

172 **Melanization.** In the melanization category, 16 genes were found to be upregulated in at least one of the  
173 two tissues (Fig 1B). These genes firstly encoded 6 serine proteases (Fig 1B) that were considered as  
174 members of the prophenoloxidase (proPO) system. The proPO system is an extracellular proteolytic  
175 cascade ending in the maturation of the proPO zymogen into PO, which initiates the melanization process  
176 (62). Among the upregulated serine proteases, PPAE2 is the only one that is known to take part in proPO  
177 processing whereas the other proteases were included in this category because of their characteristic CLIP  
178 domains and of their low homology with the serine proteases acting upstream of the Toll pathway in *D.*  
179 *melanogaster* (63). The other upregulated genes in this category included 3 serpins, which are known to  
180 regulate the proPO system in several model insects (62), 3 melanization enzymes, DDC, Yellow-like 1 and  
181 Punch-like (64, 65) as well as 4 genes, Reeler-1 and 3 Hdd23 homologs, that are involved in melanization  
182 and nodule formation in other models (66, 67) (Fig 1B). Despite of tissue-specific induction patterns, serine  
183 proteases and serpins were found in the two tissues (Fig 1B), suggesting that both participate in the  
184 stimulation of the proPO system, which is consistent with results obtained in other interaction models (68-  
185 70). However, with the exception of the DDC, all the melanization enzymes as well as the nodulation-

186 related genes were specifically induced in the hemocytes (Fig 1B), which is consistent with the very  
187 localized nature of this immune response (65) that is mainly mediated by hemocyte subtypes.  
188 Finally, only 2 genes, PPAE1 and Yellow-like 2, were found to be significantly down-regulated in this  
189 category (Fig 1B). Both were specifically repressed in the hemocytes, which could be due to functional  
190 interferences with their upregulated homologs (PPAE2 and Yellow-like 1).  
191 In summary, our results suggest that both the hemocytes and the fat body participate in induction and  
192 regulation of melanization in response to the NBC and no sign of transcriptional immunosuppression is  
193 detected for this response. These results are in agreement with the previous identification of diverse PO  
194 inhibitors in both *S. carpocapsae* (28, 29) and *X. nematophila* (30, 31).

195 **Cellular responses.** In the hemocytes, 19 upregulated genes were placed in the cellular responses  
196 category (Fig 1C). The signaling ones encoded 3 homologs of the transcription factor Krüppel (Kr) (Fig  
197 1C). In *D. melanogaster*, Kr and Kr homologs are involved in several developmental processes such as  
198 embryo patterning (71), organogenesis (72-74), and cell differentiation (75). More specifically in the  
199 hemocytes, Kr has been shown to take part in hemocytes' differentiation and/or activation (76), a crucial  
200 step for the induction of cellular immune responses. The recognition genes encoded 3 cellular receptors of  
201 the Scavenger (SR) and Integrin families plus the hemolin, a secreted immunoglobulin-containing protein  
202 (Fig 1C). Both Scavenger receptors and integrins are known to act as membrane receptors in phagocytosis  
203 of bacteria and apoptotic cells (77). In addition, integrins are involved in diverse processes, including cell  
204 motility and adhesion, and encapsulation (78, 79). The hemolin is known to act as an opsonin by increasing  
205 phagocytosis and nodulation of bacteria in *Manduca sexta* (80). Among the effector genes, we first  
206 identified 5 upregulated genes corresponding to conserved intracellular phagocytosis-related proteins. They  
207 included Ced-6, the Rabenosyn-5 (Rbsn-5-like), a V-ATPase subunit (ATP6V0A2-like) and 2 small  
208 GTPase Activating Proteins (Rabex-5-like, CdGAPr-like) (77) (Fig 1C). We also found genes encoding  
209 membrane proteins, such as the immunoglobulin-containing hemicentin (HMCN-like) (81) and 4  
210 tetraspanin-like (Tsp-like) proteins (82) (Fig 1C), that could participate in cell-cell adhesion and cellular  
211 immune responses. Interestingly, one of the upregulated tetraspanins (Tsp-like 3) presented 79.5% identity  
212 with the *Manduca sexta* (Lepidoptera : Noctuidae) tetraspanin D76, which takes part in hemocytes  
213 aggregation during capsule formation by trans-interacting with a specific integrin (83). Finally, 2 genes  
214 encoding proteins similar to the *D. melanogaster* clotting factors GP150 (84) and a transglutaminase (Tg-  
215 like) (85) were also found upregulated (Fig 1C). Only 2 genes (Ced-6-like, Rbsn-5-like) of the cellular  
216 responses category were found to be upregulated in the fat body (Fig 1C) and both encoded intracellular  
217 proteins that are probably not related to immunity in this tissue.

218 All the 4 down-regulated putative cellular immunity-related genes were specifically modulated in the  
219 hemocytes (Fig 1C). They encoded 2 Rho GTPase Activating Proteins (RhoGAP-like), a scavenger  
220 receptor similar to the *D. melanogaster* Croquemort receptor (SR-B3) and a homolog of the *D.*



221 *melanogaster* integrin  $\alpha$ -PS1. In *D. melanogaster*, Croquemort has been shown to take part in phagocytosis  
222 of apoptotic cells and of the Gram positive bacterium *Staphylococcus aureus* but not of the Gram negative  
223 bacterium *Escherichia coli* (79, 86). Integrin  $\alpha$ -PS1 is a ligand of the extracellular matrix protein laminin  
224 (87). It is involved in migration and differentiation of several cell types during development (88-90) but  
225 does not seem to be required for any immune process. Their down-regulations are thus probably due to  
226 their uselessness in the context of the response to the NBC.

227 Overall, the results suggest that all types of cellular responses are transcriptionally induced at 15 hpi,  
228 including phagocytosis and nodulation, as well as encapsulation that would be adapted to the bacterial  
229 partner or the nematode, respectively. In addition, the induction of coagulation responses is particularly  
230 interesting, since many clotting factors participate in *D. melanogaster* resistance to infestation by another  
231 type of NBC, the *Heterorhabditis bacteriophora*-*Photorhabdus luminescens* association (91-94).  
232 Moreover, despite *S. carpocapsae* does not pierce the insects' cuticles as *H. bacteriophora* (1), it has been  
233 shown to express at least two secreted proteases with inhibitory activities towards the formation of clot  
234 fibers and coagulation-associated pathogen trapping (26, 27). Once again, the induction of such immune  
235 responses is consistent with the previous identification of several virulence factors of the NBC targeting  
236 cellular immunity (26, 28, 29, 31-38).

237 **Diverse immunity-related genes.** A total of 29 modulated genes were involved in other diverse  
238 immune processes. They included 10 up- or down-regulated signaling genes, 7 upregulated recognition  
239 genes, 8 upregulated effector genes and 5 upregulated genes of unknown functions that are known to be  
240 modulated after immune challenge (Fig 1D).

241 The signaling genes firstly encoded 2 insulin-like growth factor (IGF-II-like) and 2 insulin receptor  
242 substrate homologs (IRS1-like) (Fig 1D). Insulin signaling is known to have a deleterious impact on the  
243 induction of systemic immune responses in the fat body of *D. melanogaster* (95) whereas insulin increases  
244 hemocyte proliferation in the hemolymph of mosquitoes (96) as well as in the hematopoietic organs of the  
245 lepidopteran model *Bombyx mori* (97). In agreement with these assertions, we found that 2 of these genes  
246 were down-regulated in the fat body, but all 4 genes were upregulated in the hemocytes (Fig 1D). Two  
247 other signaling genes were found to be specifically overexpressed in the hemocytes. The first one is a  
248 homolog of the *Litopenaeus vannamei* (Decapoda: Penaeidae) leucine-rich repeat flightless-I-interacting  
249 protein 2 (LRRFIP2-like) (Fig 1D), which has been shown to upregulate AMP expression in *L. vannamei*  
250 as well as in *D. melanogaster* (98). On the other hand, 3 signaling genes were found to be strictly down-  
251 regulated (Fig 1D). Interestingly, these genes included a member of the TGF- $\beta$  pathway (BAMBI-like) in  
252 the hemocytes and a member of the JNK pathway in the fat body (Basket), two pleiotropic pathways that  
253 are currently suspected to take a part in the *D. melanogaster* immune response to nematodes after NBC  
254 infestation (47, 99-101). The third down-regulated gene was found in the fat body and encoded MASK, an  
255 inducer of the Jak/Stat pathway (102). In the fat body, the Jak-Stat pathway has mainly been shown to

256 induce the expression of cytokines (103) and of a putative opsonin belonging to the TEP family (104).  
257 Remarkably, several Tep genes have been shown to participate in antibacterial immunity after NBC  
258 infestation in *D. melanogaster* (91, 105-107). All of these down-regulations could thus impair the insect's  
259 immune response to the NBC. However, more detailed analyses of their functions and modulations would  
260 be required to hypothesize immunosuppressive effects of the NBCs.

261 All 7 upregulated recognition genes encoded lectins (Fig 1D). Five of them encoded C-type lectins  
262 (CLECT), which are known to be involved in binding of diverse pathogens (108), including bacteria and  
263 nematodes (109). This binding can then stimulate several immune responses, such as bacterial aggregation,  
264 melanization, phagocytosis, nodulation and encapsulation (108). The 2 others encoded galectins, which are  
265 involved in diverse aspects of mammalian immunity, including pathogens binding (110), and are  
266 considered as relevant candidate immune proteins in insects (111). Despite a larger set of upregulated  
267 lectins was identified in the fat body, members of these protein families were found upregulated in the two  
268 tissues.

269 In the hemocytes, the upregulated effector genes firstly encoded a homolog of the superoxide dismutase  
270 (SOD-like), a conserved detoxifying enzyme involved in responses to reactive oxygen species (112) (Fig  
271 1D). The 7 remaining genes encoded proteins with similarity to insect metalloproteinase inhibitors (IMPI-  
272 like) (Fig 1D), whose functions have only been studied in the lepidopteran model *Galleria mellonella*. The  
273 only characterized IMPI encodes two proteins of which one is probably involved in the regulation of  
274 extracellular matrix remodeling and the second specifically targets metalloproteinases from pathogens  
275 (113, 114). *S. carpocasape* and *X. nematophila* both express several secreted serine proteases as well as  
276 metalloproteinases during the infectious process (39, 115-120). The induction of such immune responses  
277 could interfere with some of these proteinases to impair the NBC's virulence and/or survival. Interestingly,  
278 all but one of these IMPI homologs were found to be specifically upregulated in the hemocytes, a tissue-  
279 specificity that had not been highlighted in previous reports (121, 122).

280 Finally, the remaining genes of unknown function encoded Spod-x-tox, a protein without antimicrobial  
281 activity which contains tandem repeats of defensin-like motifs (123), 3 REPAT genes, which are known to  
282 be induced in the midgut after exposure to toxins, viruses and intestinal microbiota perturbations in the  
283 close species *S. exigua* (124-126), and Hdd1, which is induced in response to bacteria and peptidoglycan  
284 in the lepidopteran models *Hyphantria cunea* and *Bombyx mori* (127, 128) (Fig 1D).

285 In summary, we found an important additional mobilization of several relevant candidate immune genes,  
286 including mainly insulin signaling factors and IMPIs in the hemocytes and lectins in the fat body. In  
287 addition, these results suggest that the candidate immune pathways TGF- $\beta$ , JNK and Jak/Stat could be  
288 down-regulated. Such down-regulations are in disagreement with the results of Yadav and colleagues (43)  
289 in *D. melanogaster* and thus would require further investigation.

## 290 **Temporal analysis of the induced immune responses**

291 In order to put the *S. frugiperda* immune responses in relation with the infectious process, we then described  
292 their temporal dynamics in each analyzed immunocompetent tissue. To this aim, we monitored with RT-  
293 qPCR experiments the induction levels of selected representative immune genes from 5 hpi, the mean time  
294 at which nematodes release *X. nematophila* in the hemocoel, to 20 hpi, which is about 9 hours before the  
295 first insect deaths (S1 Fig).

296 In the hemocytes, the selected genes included 15 genes of the antimicrobial response, 2 genes involved in  
297 melanization, 5 cellular response genes, 2 lectins and one IMPI-like gene. At 5 hpi, only 2 genes, encoding  
298 a lebocin antibacterial (52) AMP (Lebocin 2) and the negative regulator Pirk of the Imd pathway (129),  
299 were found to be significantly upregulated. However, most of the selected genes that are strongly induced  
300 at later time points also presented positive log<sub>2</sub> fold changes at this time point (Fig 2A). From 10 to 20 hpi,  
301 all selected genes but few exceptions (cecropin D, Tg-like and Integrin  $\beta$ -like) due to biological variability  
302 were significantly upregulated at each time point (Fig 2A). Clustering analyses based on Pearson  
303 coefficients however revealed 3 distinct clusters of covariations. The first one contained 13 genes belonging  
304 to all the categories cited above and corresponded to very stable induction patterns (Fig 2A). The second  
305 one, which contained 8 genes involved antimicrobial and cellular responses plus the selected C-type lectin  
306 (CLECT (ccBV)), corresponded to slightly increasing patterns (Fig 2A). Finally, the third one, which  
307 contained the Relish and Pelle members of the Imd and Toll pathways (22), an integrin and the DDC  
308 melanization enzyme (130) genes, corresponded to slightly decreasing patterns (Fig 2A).

309 In the fat body, the selected genes included 15 genes of the antimicrobial response, 2 genes involved in  
310 melanization, one galectin gene (Galectin 1) and an IMPI-like gene (IMPI-like 3). At 5 hpi, all 7 selected  
311 AMPs, PGRP-S1 and Galectin 1 were found to be upregulated (Fig 2B). All these genes were among the  
312 most strongly overexpressed at later time points. Such as in the hemocytes, most of the selected genes were  
313 then significantly upregulated from 10 to 20 hpi (Fig 2B). In this tissue, the genes only subdivided into two  
314 main covariation clusters: a cluster of genes with stable induction patterns and a cluster of genes with  
315 increasing induction patterns. The first cluster contained 10 genes of which 8 were involved in antimicrobial  
316 responses, one encoded a melanization-related serine protease (Snake-like 2) and one encoded the Galectin  
317 1 (Fig 2B). The second cluster contained 9 genes, of which 7 were involved in antimicrobial responses, one  
318 encoded the DDC melanization enzyme (130) and the last one encoded the IMPI-like 3 (Fig 2B).

319 Altogether, the results obtained for the two tissues show that most of the transcriptional immune responses  
320 induced at 15 hpi take place between 0 and 10 hpi, which is comparable to timings observed in other  
321 interaction models (131-133). The results also indicate that these responses are globally stable across the  
322 time post-infestation despite some distinct gene induction patterns in each category of response.  
323 Interestingly, while we were hoping to discriminate between an early response, probably activated by the  
324 nematode presence, and a later response, probably reacting to bacterial growth, we did not find any clear

325 link between the gene inductions' dynamics and the different immune processes and pathways that were  
326 represented in our selection.

### 327 **Evaluation of each NBC partner's part in the induced immune responses**

328 In order to identify each NBC partner's relative participation in the fat body's and hemocytes' immune  
329 responses, we used RT-qPCR to compare the induction levels of the selected immune genes after  
330 independent infections by the whole NBC, the axenic nematode or the bacterial symbiont. To this aim, we  
331 decided to use a more standardized protocol of direct injection of the pathogens into the hemocoel, thereby  
332 limiting putative side effects such as early hemocoel colonization by intestinal microorganisms.  
333 Importantly, we previously compared the kinetics of *X. nematophila* growth and of *S. frugiperda* survival  
334 after injection of the entire NBC and of 200 *X. nematophila* (S2A and S2B Fig). This comparison showed  
335 that both kinetics are very similar and thus that any difference of induction level between the 2 conditions  
336 would not reflect differences in bacterial load or physiological state. However, the putative impact of  
337 axenization on the nematode's physiology could not be assessed by the same way due to technical  
338 limitations and to its avirulence in absence of its bacterial symbiont (S2B and S2C Fig).

339 In the hemocytes, 14 genes presented higher induction levels in response to *X. nematophila* than in response  
340 to the axenic nematode (Fig 3). In the antimicrobial category, they included the negative regulator Pirk of  
341 the Imd pathway (129), all the selected attacin, cecropin, gloverin, lebocin and gallerimycin AMPs, the 2  
342 selected PGRP-S, and also probably the Imd pathway transcription factor Relish (22) (Fig 3A). As indicated  
343 above, the Imd pathway, as well as the attacin, cecropin and gloverin AMP families, are known to take part  
344 in anti-Gram negative bacteria immune responses (11, 52). Their induction patterns thus indicate that the  
345 antimicrobial *X. nematophila*-induced responses are well adapted to the nature of the pathogen. Moreover,  
346 these results are in agreement with the study of Aymeric and colleagues (44) showing that the Imd pathway  
347 functions in the *D. melanogaster* immune response to *X. nematophila*. In the other categories, the *X.*  
348 *nematophila*-induced genes encoded the DDC melanization enzyme (130), the hemolin antibacterial  
349 opsonin (80), the IMPI-like 3, and also probably the selected integrin (Integrin  $\beta$ -like) (Fig 3B, 3C and 3D).  
350 Once again, all of these genes are susceptible to play a part in an immune response to a pathogenic  
351 bacterium even though most of them could act on diverse types of invaders. Surprisingly, we found that *X.*  
352 *nematophila* strongly over-induces the transglutaminase (Tg-like) putative clotting factor (85) (Fig 3C).  
353 This result could suggest that the bacterium is actually the main responsible for tissue damages at this time  
354 point and/or that Tg-like expression is induced in response to bacteria. Importantly, this result is in  
355 agreement with the study of Yadav and colleagues (43), who showed that the *D. melanogaster* Fondue  
356 clotting factor was induced after infestation by the NBC but not after infestation by axenic nematodes.  
357 Remarkably, most of the genes that were mostly induced by *X. nematophila* presented higher induction  
358 values in response to the bacterium alone than in response to the whole NBC. However, this observation  
359 cannot be directly interpreted as an antagonistic effect of the nematode partner since it could be due to

360 changes in the relative proportions of each hemocyte subtype, which would not necessarily reflect absolute  
361 variations in their numbers. In addition, the nematode partner specifically induced the overexpression of  
362 the selected C-type lectin (CLECT (ccBV)) and was probably the main inducer of the Galectin 1, the  
363 tetraspanin D76 homolog (Tsp-like 3) and the selected diapausin AMP (Diapausin 5) (Fig 3A, 3C and 3D).  
364 As mentioned before, the *M. sexta* tetraspanin D76 is known to take part in encapsulation (83) and some  
365 lectins can bind nematodes and participate in melanization (109) as well as in all types of cellular immune  
366 responses. Once again, their induction patterns are consistent with the nature of the pathogen, since both  
367 types of molecules could be involved in classical anti-nematode immune responses, such as cellular or  
368 melanotic encapsulation (134). Finally, 5 genes, encoding the Toll pathway members Pelle and Cactus (22),  
369 the selected moricin AMP (Moricin 2), the melanization-related PPAE2 and the Krüppel-like transcription  
370 factor (Kr-like factor 1), were similarly induced by each of the three pathogens (Fig 3A, 3B and 3C),  
371 suggesting that these responses are induced by the 2 partners without any additive effect.

372 In the fat body, statistical analysis of the results firstly revealed that the induction levels of Pirk as well as  
373 of the selected cecropin and gloverin AMPs were significantly lower in response to the axenic nematode  
374 than in response to the NBC and to *X. nematophila* (Fig 4A), suggesting the bacterial partner is the main  
375 responsible for their inductions. In addition, despite non-significant statistics, the results for the selected  
376 attacin AMP, PGRP-S6 and GGBP3 showed similar induction patterns (Fig 4A). As for the hemocytes, the  
377 induction patterns of Pirk and of the attacin, cecropin and gloverin AMPs suggest that the fat body's  
378 antimicrobial response to *X. nematophila* is well adapted to the type of pathogen that is met. On the  
379 contrary, the induction levels of the melanization-related serine protease (Snake-like 2) was significantly  
380 lower in response to *X. nematophila* than in response to the NBC and to the axenic nematode (Fig 4B),  
381 suggesting that the nematode partner is the main responsible for its induction. Similar induction patterns  
382 were obtained for the Toll pathway members Toll and Cactus (22) as well as for Galectin 1 (Fig 4A and  
383 4C). As mentioned for the hemocytes, the induction of lectins and melanization-related genes in response  
384 to the nematode is consistent with the nature of the pathogen since both could participate in classical anti-  
385 nematode immune responses (134). The induction of Toll pathway members is more difficult to relate with  
386 known anti-nematode immune responses and Yadav and colleagues (47) found that the inactivation of this  
387 pathway does not impact the *D. melanogaster* survival to infestation by the whole NBC or by axenic *S.*  
388 *carpocapsae*. Therefore, the involvement of this immune pathway in anti-nematode immune responses may  
389 depend on the downstream effectors and thus be variable between insect species. Finally, the other genes  
390 did not show any clear difference of induction level after injection of the 3 pathogens, except for the  
391 gallerimycin AMP, PGRP-S1 and the DDC melanization enzyme, which presented a lesser induction when  
392 each NBC partner was injected alone (Fig 4A and 4B). These results suggest synergistic effects of the  
393 nematode and of the bacterium on the induction of these genes.

394 In summary, we found in the 2 tissues that most of the selected genes presented partner-specific induction  
395 patterns, suggesting that the immune response to the NBC corresponds to combinations of responses

396 induced by each partner. The detailed analysis of these genes indicates that *X. nematophila* is the main  
397 inducer of most of the selected genes, and especially of the well-known antibacterial ones. On the other  
398 hand, *S. carpocapsae* is the main inducer of some melanization and encapsulation-related genes and of the  
399 selected lectins, which could all take part in classical anti-nematode immune responses. The results thus  
400 globally suggest that the hemocytes and the fat body both respond by adapted ways to each NBC partner  
401 despite some yet unexplained results, such as an induction of Toll pathway members in the fat body by the  
402 nematode partner.

### 403 **Expression patterns of two new clusters of candidate immune genes**

404 During our first analysis of the RNAseq data, we identified 2 new clusters of candidate immune genes (48).  
405 The first one, named the Unknown (Unk) cluster, was localized close to Tamozhennic, a gene encoding a  
406 nuclear porin involved in the nucleation of Dorsal, the transcription factor of the Toll pathway (135). It  
407 contained 5 genes predicted to encode secreted peptides and short proteins that were all highly  
408 overexpressed in the midgut, fat body and hemocytes at 15 hpi and of which 4 were the unique mobilized  
409 genes at 8 hpi in the fat body. The second cluster, named the Genes with Bacterial Homology (GBH)  
410 cluster, contained 3 genes located inside a defensin-like AMP cluster in the *S. frugiperda* genome. The 3  
411 genes were predicted to encode secreted proteins similar to each other and one of them was also found  
412 highly induced at 15 hpi in the 3 tissues. The particularity of these genes is that homologs are found only  
413 in lepidopteran species as well as, intriguingly, in Gram positive bacteria. Here, we reexamined the  
414 expression patterns of the Unk and GBH genes and found that the 5 Unk genes were mainly expressed in  
415 the fat body whereas 2 of the 3 GBH genes were mainly expressed and induced in the hemocytes (S2 Table).  
416 In order to learn more about their putative functions, we decided to analyse, as we did for the known  
417 immune genes, their induction patterns across the time post-infestation and in response to each NBC partner  
418 in the corresponding tissues. In both cases, we found that the induction dynamics of the genes were very  
419 similar to those of immune genes, with an upregulation that becomes significant at 5 or 10 hpi and with  
420 globally stable induction patterns from 10 to 20 hpi (Fig 5A and 5B).  
421 In the case of the GBH cluster, the results that we got for the 2 NBC-responsive genes (GBH1 and GBH3)  
422 in the hemocytes indicate that they are significantly less induced after axenic nematode injection than after  
423 NBC and *X. nematophila* injections, suggesting that the bacterium is the main responsible for their up-  
424 regulation (Fig 5C). We could hypothesize an acquisition by horizontal gene transfer from bacteria of the  
425 GBH genes. In this case, their putative involvement in the antibacterial immune response would be  
426 particularly interesting, since bacterial genes hijacking for immune purpose has only been reported once in  
427 metazoans, in the tick *Ixodes scapularis* (136). Such a hypothesis however requires functional confirmation.  
428 In the case of the Unk cluster, we found that the 4 most induced genes in the fat body (Unk2 to 5) are all  
429 strongly and similarly induced by the NBC and by the axenic nematode whereas they are not induced by  
430 *X. nematophila* (Fig 5D). The results are very similar for the least expressed Unk gene (Unk1), for which

431 we only found a significant induction for the injection of axenic nematodes (Fig 5D). This partner-specific  
432 induction pattern suggests the Unk genes are involved in specific aspects of the insect responses to the  
433 infestation. In addition, the putative involvement of the Unk genes in the response towards the nematode  
434 partner seems to be in agreement with their early mobilization during the infectious process and with their  
435 overexpression in the midgut, which is the entry site of the nematode. In our previous study, we had  
436 hypothesized the Unk may encode new types of immune effectors (48). However, given their low levels of  
437 conservation in species as close as *S. littura* or *S. littoralis* (S4 Fig) another hypothesis would be that they  
438 correspond to regulatory long non-coding RNAs (137, 138). In both cases, the further functional  
439 characterization of these genes could be very promising given our current lack of knowledge of the immune  
440 pathways and molecular effectors of insect anti-nematode immunity.

## 441 **Conclusion**

442 Here, we provide a very deep and contextualized analysis of the *S. frugiperda*'s hemocytes' and fat body's  
443 transcriptional immune responses to infestation by the *S. carpocapsae-X. nematophila* NBC. Our topologic  
444 analysis of these responses at 15 hpi firstly confirmed the induction of very potent and diversified immune  
445 responses towards the pathogen, such as suggested by our previous analysis of the transcriptomic data (48)  
446 as well as by the study of Yadav and colleagues (43) in the *D. melanogaster* model. The present work  
447 establishes that these responses are very stable across the post-infestation time and that they correspond to  
448 combinations of *X. nematophila*- and *S. carpocapsae*-induced responses that seem to be well adapted to the  
449 nature of each partner (Fig 6).

450 The pieces of information collected during these analyses are of great interest for the study of the dialogue  
451 that takes place between each NBC partner and their hosts' immune systems. First, our results strongly  
452 suggest that the NBC immunosuppressive strategies globally have a low impact on the induction of immune  
453 responses at the transcriptional level. They also indicate that the nematode and/or its effects on the host are  
454 detected by the insect's immune system that in return seems to induce adapted immune responses towards  
455 the pathogen. Such observations could help to identify the limits of previously described  
456 immunosuppressive and immunoevasive strategies of the NBC. For example, they suggest that the  
457 suppressive effect of *X. nematophila* on the expression of AMP genes (42, 60, 61) as well as the camouflage  
458 strategy of *S. carpocapsae* (24, 25) are probably far from sufficient to explain their success towards the  
459 immune system in the case of *S. frugiperda*. Nevertheless, we found several unexplained down-regulations  
460 of signaling genes, such as of members of the Imd, JNK, TGF- $\beta$  and Jak-Stat pathways, that represent  
461 interesting working trails for the study of the molecular basis of the NBC's immunosuppressive strategies.  
462 Finally, this study allowed the identification of very large panels of candidate immune genes involved in  
463 all the main components of insect immunity as well as of some yet uncharacterized genes that could encode  
464 new immune factors involved in the response to the complex.

465 Continuing this work with more functional and mechanistic approaches is now required to get an accurate  
466 picture of the molecular dialogue between the NBC and the immune system. In the longer term, such  
467 approaches could help to identify the precise causes of the immune system's failure against this NBC and  
468 thus the conditions that are required for an adequate use of this NBC against insect pests.

## 469 **Materials and Methods**

### 470 **Insect rearing**

471 Corn variant *Spodoptera frugiperda* (Lepidoptera : Noctuidae) were fed on corn-based artificial diet (139).  
472 They were reared at 23°C +/- 1°C with a photoperiod of 16 h/8 h (light / dark) and a relative humidity of  
473 40 % +/- 5 %. *Galleria mellonella* (Lepidoptera : Pyralidae) were reared on honey and pollen at 28°C in  
474 dark.

### 475 **Production and storage of nematobacterial complexes**

476 *Steinernema carpocapsae-Xenorhabdus nematophila* complexes (strain SK27 isolated from Plougastel,  
477 France) were renewed by infestation of one month-old *Galleria mellonella* larvae. They were collected on  
478 White traps (140) and stored at 8°C in aerated Ringer sterile solution with 0.1 % formaldehyde. The  
479 maximal time of storage was limited to 4 weeks to avoid pathogenicity losses.

### 480 **Production of axenic nematodes**

481 Gravid *S. carpocapsae* females were extracted from *G. mellonella* dead bodies at day 4 to 6 after infestation  
482 by nematobacterial complexes. After 5 washing steps in Ringer sterile solution, the females were surface-  
483 sterilized by 20 min incubation in 0.48% (wt/vol) sodium hypochlorite and 3 h incubation in Ringer sterile  
484 solution supplemented with antibiotics (150 µg/mL polymyxin, 50 µg/mL colistin, 50 µg/mL nalidixic  
485 acid). The eggs were extracted by female crushing with sterile glass pestles and then washed by  
486 centrifugation (2 min, 16000 g) in Ringer sterile solution, disinfected by incubation in 0.48% sodium  
487 hypochlorite for 5 min, and washed again twice. After microscopic observation, the intact eggs were placed  
488 on liver-agar (40 g/L Tryptycase Soja Agar [BioMérieux], 5 g/L Yeast Extract [Difco], 100 g/L porc liver)  
489 plates supplemented with antibiotics (150 µg/mL polymyxin, 50 µg/mL colistin and 50 µg/mL nalidixic  
490 acid). The plates were maintained inside a dark humid chamber for 1 month to allow nematodes  
491 development. The nematodes were then suspended in Ringer sterile solution and infective juvenile stages  
492 (IJs) were sorted by pipetting under a microscope (Leica). The IJs were rinsed twice by centrifugation (2  
493 min, 3000 g) in 1 mL Ringer sterile solution and used within minutes for experimental infection.  
494 Nematodes' axenicity was verified *a posteriori* by DNA extraction and PCR amplification. Nematodes  
495 were suspended in 200 µL milliQ water supplemented with 200 µL glass beads ( $\emptyset \leq 106 \mu\text{m}$ ) (Sigma).  
496 They were grinded for 2 x 40 sec at 4.5 ms speed with a FastPrep homogenizer (MP Biomedicals). The  
497 debris were discarded by centrifugation (2 min, 16000 g) and 150 µL supernatant were mixed with 200 µL



498 lysis buffer (Quick extract kit, Epi-centre) for a second grinding. To ensure bacterial cell lysis, the samples  
499 were incubated at room temperature for 48 h with 2  $\mu$ L Ready-Lyse Lysozyme solution at 30000 U/ $\mu$ L  
500 (Epi-centre). Protein denaturation was then performed by 10 min incubation at 90°C, and RNA was  
501 removed by 10 min incubation at 37°C with 20  $\mu$ L RNase A (20 mg/mL) (Invitrogen). DNA was extracted  
502 by successive addition of 500  $\mu$ L phenol-chloroform-isoamyl alcohol and 500  $\mu$ L chloroform, followed by  
503 centrifugations (10 min, 16000 g) and aqueous phase collections. DNA was precipitated with 500  $\mu$ L 100%  
504 ethanol supplemented with 20  $\mu$ L sodium acetate and by freezing at -80°C for 2 h. After defrosting, DNA  
505 was concentrated by centrifugation (30 min, 16000 g) and the precipitates were washed twice by  
506 centrifugation (15 min, 16000 g) in 500  $\mu$ L 70% ethanol. DNA was finally suspended in 50  $\mu$ L sterile  
507 milliQ water and left at room temperature for a few hours to ensure precipitate dissolution. After DNA  
508 quantification with a Qubit fluorometer (Invitrogen), *X. nematophila* presence was assessed by PCR  
509 amplification with *Xenorhabdus*-specific primers (Xeno\_F: 5'-ATG GCG CCA ATA ACC GCA ACT A-  
510 3'; Xeno\_R: 5'-TGG TTT CCA CTT TGG TAT TGA TGC C-3'), which target a region of the XNC1\_0073  
511 gene encoding a putative TonB-dependent heme-receptor. The presence of other bacteria was assessed by  
512 16S rRNA gene amplification with universal primers (141). Thirty cycles of PCR were performed using  
513 Taq polymerase (Invitrogen) in a Biorad thermocycler (Biorad), with hybridization temperatures of 55°C  
514 and 50°C respectively. PCR products were then analyzed by agarose gel electrophoresis.

## 515 **Experimental infections**

516 Experimental infestations with nematobacterial complex were carried out on individual 2<sup>nd</sup> day 6<sup>th</sup> instar *S.*  
517 *frugiperda* larvae according to (48). Larvae were kept at 23°C in 12-well plates with an artificial diet (139).  
518 Briefly, each well was coated with a piece of filter paper (Whatman) and 150 +/- 20 NBCs in 150  $\mu$ L Ringer  
519 solution were poured in each larva-containing well. 150  $\mu$ L Ringer sterile solution were used for control  
520 larvae.

521 For intra-hemocoelic injection experiments, pathogens were injected in larvae's abdomens after local  
522 application of 70% ethanol with a paintbrush. Injections were performed using a syringe pump (Delta labo)  
523 with 1 mL syringes (Terumo) and 25G needles (Terumo). *X. nematophila* suspensions were prepared as  
524 described in Sicard et al (2004)(142). Bacterial culture was diluted in PBS and 20  $\mu$ L containing 200 +/-  
525 50 bacterial cells were injected in the hemocoel at a rate of 1.67 mL/min. 20  $\mu$ L sterile PBS was used for  
526 control larvae. The purity and number of injected *X. nematophila* were verified by plating 20  $\mu$ L of the  
527 bacterial suspension on NBTA (143). For NBC and axenic nematode injections, 10 +/- 3 nematodes in 20  
528  $\mu$ L solution at 70% Ringer and 30% glycerol were injected at a rate of 2.23 mL/min. Syringes were  
529 frequently renewed in order to limit nematodes' concentration and sedimentation and the number of  
530 injected nematodes was verified by 10 simulations of injection in Petri dishes followed by nematode  
531 counting under a microscope (Zeiss). Sterile solutions at 70% Ringer and 30% glycerol were used for  
532 control larvae. To avoid accidental *per os* infections, the injected larvae were then briefly washed in sterile

533 PBS and dried on paper towel before being placed in 12-well plates. The pathogens efficacies were checked  
534 by monitoring 12 control and 12 infected larvae's survival for 72 h after infestation or after injection.

### 535 **Production and storage of bacterial symbionts**

536 *X. nematophila* strain F1 isolated from nematobacterial complexes strain SK27 was conserved at -80°C.  
537 Within 3 weeks before each experiment, they were grown for 48 h at 28°C on NBTA with erythromycin  
538 (15 µg/mL). The colonies were then conserved at 15°C and used for overnight culture at 28°C in 5 mL  
539 Luria-Bertani broth (LB) before experiments.

### 540 **RNA extraction**

541 RNAs were prepared as described in Huot et al (2019) (48). Briefly, nine larvae per technical replicate were  
542 bled in anti-coagulant buffer (144). Hemocytes were recovered by centrifugation (1 min, 800 g) at 4°C and  
543 the pellet was immediately flash-frozen with liquid nitrogen. The larvae were then dissected for fat body  
544 and midgut sampling and the tissues were flash-frozen in eppendorf tubes with liquid nitrogen. After  
545 storage at -80°C for at least 24 h, 1 mL Trizol (Life technologies) was added to the pooled tissues. The  
546 tissues were then grounded by using a TissueLyzer 85210 Rotator (Qiagen) with one stainless steel bead  
547 (Ø : 3 mm) at 30 Hz for 3 min. For optimal cell lyses, grounded tissues were left at room temperature for 5  
548 min. To extract nucleic acids, 200 µL chloroform (Interchim) were added and the preparations were left at  
549 room temperature for 2 min with frequent vortex homogenization. After centrifugation (15 min, 15,000 g)  
550 at 4°C, the aqueous phases were transferred in new tubes and 400 µL 70% ethanol were added. RNA  
551 purifications were immediately performed with the RNeasy mini kit (Qiagen) and contaminant DNA was  
552 removed with the Turbo DNA-free™ kit (Life Technologies).

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

### 556 **RNAseq experiments**

557 RNAseq raw data originate from Huot et al (2019) (48). In brief, libraries were prepared by MGX GenomiX  
558 (IGF, Montpellier, France) with the TruSeq Stranded mRNA Sample preparation kit (Illumina). The  
559 libraries were then validated on Fragment Analyzer with a Standard Sensitivity NGS kit (Advanced  
560 Analytical Technologies, Inc) and quantified by qPCR with a Light Cycler 480 thermal cycler (Roche  
561 Molecular diagnostics). cDNAs were then multiplexed by 6 and sequenced on 50 base pairs in a HiSeq  
562 2500 system (Illumina) with a single-end protocol. Image analysis and base calling were performed with  
563 the HiSeq Control and the RTA softwares (Illumina). After demultiplexing, the sequences quality and the  
564 absence of contaminant were checked with the FastQC and the FastQ Screen softwares. Data were then  
565 submitted to a Purity Filter (Illumina) to remove overlapping clusters.

566 For each sample, the reads were pseudoaligned on the *S. frugiperda* reference transcriptome version  
567 OGS2.2 (49) using the Kallisto software (145). Differential expression between infested and control  
568 conditions were then assessed for each time point and tissue with the Sleuth software (146). Wald tests  
569 were used with a q-value (equivalent of the adjusted p-value) threshold of 0.01 and a beta value (biased  
570 equivalent of the log<sub>2</sub> fold change) threshold of 1. Only transcripts with normalized counts over 5 in all  
571 three replicates of the infested and/or of the control condition were considered as reliably differentially  
572 expressed.

573 Previously annotated immune transcripts (49) were then checked for significant expression changes and  
574 not annotated differentially expressed ones were researched with the Blast2GO software by blastx on the  
575 NCBI nr and drosophila databases (147). To avoid mistakes related to genome fragmentation, the immune  
576 transcripts were gathered by unique gene after careful examination of their sequences and of the available  
577 genomic data (49). The induction levels of the transcripts were then averaged by unique gene before  
578 graphical representation of the results.

### 579 **RT-qPCR experiments**

580 cDNAs were synthesized from 1 µg of RNA with the SuperScript II Reverse Transcriptase (Invitrogen),  
581 according to the manufacturer's protocol.

582 The primers (S3 Table) were designed with the Primer3Web tool (148). Their efficiency was estimated by  
583 using serial dilutions of pooled cDNA samples and their specificity was verified with melting curves  
584 analyses. Amplification and melting curves were analyzed with the LightCycler 480 software (Roche  
585 Molecular diagnostics).

586 RT-qPCR were carried out in triplicate for each biological sample, with the LightCycler 480 SYBR Green  
587 I Master kit (Roche). For each sample and primer pair, 1.25 µL of sample containing 50 ng/µL of cDNA  
588 and 1.75 µL of Master mix containing 0.85 µM of primers were distributed in multiwell plates by an Echo  
589 525 liquid handler (Labcyte). The amplification reactions were then performed in a LightCycler 480  
590 thermal cycler (Roche) with an enzyme activation step of 15 min at 95°C, and 45 cycles of denaturation at  
591 95°C for 5 sec, hybridization at 60°C for 10 sec and elongation at 72°C for 15 sec.

592 Crossing points were determined using the Second Derivative Maximum method with the LightCycler 480  
593 software (Roche) and relative expression ratios between control and infected conditions were manually  
594 calculated according to the method of Ganger et al (2017)(149). The ratios were normalized to RpL32  
595 housekeeping gene relative levels and the EF1 gene was used as an internal control.

596 Statistical analyses of the data were all performed with the R software (150). Differential expression  
597 significance between the control and infected conditions was assessed by paired one-tailed t-tests on  $\Delta Cq$   
598 values. Multiple comparisons of fold changes were assessed by one-way ANOVA on  $\Delta\Delta Cq$  values followed  
599 by post hoc Tukey tests. P-values under 0.05 were considered as significant for all the above tests. The

600 gplots package was used to draw the heatmaps and the clusters were built from a dissimilarity matrix based  
601 on Pearson correlation coefficients.

### 602 **Quantification of nematodes in the midgut lumen**

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

### 607 **Quantification of *X. nematophila* in the hemolymph**

608 The concentration of *X. nematophila* in the hemolymph was estimated by CFU counting. For 3 independent  
609 infection experiments and 3 technical replicates, hemolymph was collected by bleeding of 3 caterpillars in  
610 200  $\mu$ L PBS supplemented with phenylthiourea (Sigma). The volumes of hemolymph were then estimated  
611 by pipetting and serial dilutions of the samples were plated on NBTA with 15  $\mu$ g/mL erythromycin. CFU  
612 were counted after 48 h incubation at 28°C and the counts were reported to the estimated hemolymph  
613 volumes in order to calculate the bacterial concentrations. Hemolymph of naive larvae was also plated for  
614 control.

### 615 **Insect survival kinetics**

616 Survival kinetics were performed in triplicate on pools of 20 infested or injected larvae. Survival was  
617 monitored from 0 to 72 hours after contact or injection. Naïve larvae were used as control for infestations  
618 whereas larvae injected with PBS were used for controls of *X. nematophila* injections and larvae injected  
619 with 70% Ringer - 30% glycerol solutions were used for controls of nematobacterial complexes and  
620 nematodes injections.

### 621 **Parasitic success measurement**

622 Parasitic success was measured in triplicate on pools of 20 nematobacterial complexes or axenic  
623 nematodes-injected larvae. Dead larvae were individually placed on white traps (140) approximately 2 days  
624 after their deaths. The emergence of nematodes was assessed at day 40 after injection by observation of the  
625 collection liquid with a microscope (Leica). Parasitic success was then calculated as the percentage of  
626 larvae with nematode emergence among the infected larvae.

627

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## 633 **Authors' contribution**

634 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  
635 the infestation experiments. L.H., P.-A.G. performed dissections. L.H. and A.B. extracted and purified the  
636 RNA. J.-C.O. designed the *X. nematophila* specific primers. S.P. produced the axenic nematodes and  
637 checked their axenization. L.H. and A.B. performed the qPCRs. L.H., N.N. and B.D. analysed the data.  
638 L.H. wrote the manuscript. L.H., N.N. and B.D. revised the manuscript. All authors have read and approved  
639 the manuscript.

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## 1004 **Figure Legends**

1005 **Figure 1.** Expression variations of the differentially expressed immune genes after infestation by the  
1006 nematobacterial complex. Heatmaps showing the expression variations of the differentially expressed  
1007 immune genes in the hemocytes and in the fat body at a middle time point of 15 h post-infestation.  
1008 Infestation experiments were performed in triplicate with N=9 larvae per sample. RNAseq data were  
1009 analyzed with the Kallisto/Sleuth softwares using statistical thresholds of 0.01 for p-values, -1 and +1 for  
1010 Beta value (biased equivalent of log<sub>2</sub> fold change) and 5 for pseudocount means. The immune genes were  
1011 identified by homology and classified as (A) antimicrobial immunity-related, (B) melanization-related, (C)  
1012 cellular immunity-related and (D) diverse immune responses. Black dots indicate genes with statistically  
1013 non-significant variations to the controls in the corresponding tissue; HC : Hemocytes, FB : Fat body.

1014 **Figure 2.** Temporal dynamics of the identified immune responses after infestation by the nematobacterial  
1015 complex. Heatmaps showing the temporal evolution of the induction levels of representative immune genes  
1016 in the hemocytes (A) and in the fat body (B) after infestation by the nematobacterial complex. RT-qPCR  
1017 relative quantifications were performed on triplicate samples of N=9 larvae per sample with the RpL32  
1018 housekeeping gene as reference. Differential expression was assessed with Student t tests on  $\Delta Cq$  (149)  
1019 and black dots indicate genes with statistically non-significant variations to the controls in the  
1020 corresponding tissue (p-value > 0.05). The dendrograms represent clustering analyses based on Pearson  
1021 correlation coefficients.

1022 **Figure 3.** Relative participations of *S. carpocapsae* and *X. nematophila* in the hemocytes' immune  
1023 responses. Histograms showing the induction levels (+/- SEM) of representative immune genes in the  
1024 hemocytes at 13 h after independent injections of either 10 nematobacterial complexes (NBC), 10 axenic  
1025 *S. carpocapsae* (*S.c.*) or 200 *X. nematophila* symbionts (*X.n.*). RT-qPCR relative quantifications were  
1026 performed on triplicate samples of N=9 larvae per sample with the RpL32 housekeeping gene as reference  
1027 and buffer-injected control larvae. Letters show statistical differences between treatments from one-way  
1028 ANOVA and Tukey tests on  $\Delta\Delta Cq$  (149). The genes were gathered by type of immune response with (A)  
1029 antimicrobial immunity-related, (B) melanization-related, (C) cellular immunity-related and (D) diverse  
1030 immune responses.

1031 **Figure 4.** Relative participations of *S. carpocapsae* and *X. nematophila* in the fat body's immune responses.  
1032 Histograms showing the induction levels (+/- SEM) of representative immune genes in the fat body at 13  
1033 h after independent injections of either 10 nematobacterial complexes (NBC), 10 axenic *S. carpocapsae*  
1034 (*S.c.*) or 200 *X. nematophila* symbionts (*X.n.*). RT-qPCR relative quantifications were performed on  
1035 triplicate samples of N=9 larvae per sample with the RpL32 housekeeping gene as reference and buffer-  
1036 injected control larvae. Letters show statistical differences between treatments from one-way ANOVA and

1037 Tukey tests on  $\Delta\Delta Cq$  (149). The genes were gathered by type of immune response with (A) antimicrobial  
1038 immunity-related, (B) melanization-related and (C) diverse immune responses.

1039 **Figure 5.** Transcriptional induction patterns of putative new immune genes. (A, C) Histograms showing  
1040 the induction levels ( $\pm$  SEM) of 2 GBH genes in the hemocytes (A) and of the 5 Unk genes in the fat body  
1041 (C) at several times after infestation by the nematobacterial complex. RT-qPCR relative quantifications  
1042 were performed on triplicate samples of N=9 larvae per sample with the RpL32 housekeeping gene as  
1043 reference. Differential expression was assessed with Student t tests on  $\Delta Cq$  (149) and black dots indicate  
1044 genes with statistically non-significant variations to the controls ( $p$ -value  $> 0.05$ ). (B, D) Histograms  
1045 showing the induction levels ( $\pm$ -SEM) of 2 GBO genes in the hemocytes (B) and of the 5 Unk genes in the  
1046 fat body (D) at 13 h after independent injections either 10 nematobacterial complexes (NBC), 10 axenic *S.*  
1047 *carpocapsae* (*S.c.*) or 200 *X. nematophila* (*X.n.*). RT-qPCR relative quantifications were performed on  
1048 triplicate samples of N=9 larvae per sample with the RpL32 housekeeping gene as reference and buffer-  
1049 injected control larvae. Letters show statistical differences between treatments from one-way ANOVA and  
1050 Tukey tests on  $\Delta\Delta Cq$  (149).

1051 **Figure 6.** Hypothetical structure of the *S. frugiperda* larva's immune response to the nematobacterial  
1052 complex. Graphical abstract illustrating the main hypotheses we can emit from the present RNAseq and  
1053 RT-qPCR data and from our current knowledge of *S. frugiperda* immunity. Dark green letters, lines and  
1054 arrows indicate responses that seem to be mainly induced by the nematode partner *S. carpocapsae* whereas  
1055 orange ones indicate responses that seem to be mainly induced by the bacterial symbiont *X. nematophila*.  
1056 The arrows' thicknesses and the letter sizes refer to the relative strengths of the induced transcriptional  
1057 responses. AMP: AntiMicrobial Peptides, IMPI: Induced MetalloProteinase Inhibitors.

1058

## 1059 **Supporting Information Legends**

1060 **S1 Table. Hemocytes and fat body RNAseq results for *S. frugiperda*'s immune genes.** (A) Results for  
1061 the previously annotated *S. frugiperda*'s immune genes, (B) Results for the newly identified *S. frugiperda*'s  
1062 immune genes. The statistics of the transcripts that were considered as significantly (Sleuth :  $|\text{Beta}| > 1$ ;  $qval$   
1063  $< 0.01$ ; pseudocounts  $> 5$  in all the samples of at least one condition) up- or down-regulated are highlighted  
1064 in red and blue, respectively. The Beta value gives a biased estimate of the log2 fold change. The  $qval$   
1065 ( $qval$ ) is an equivalent of the adjusted  $p$ -value. The following columns give the normalized pseudocounts  
1066 (Kallisto) for each individual sample, with HCn15 and FBn15 corresponding to control larvae and HCi15  
1067 and FBi15 corresponding to infested larvae. Blast hits on the *Drosophila* and nr NCBI databases were  
1068 obtained by blastx with the Blast2GO software.



1069 **S2 Table. Hemocytes and fat body RNAseq results for the Unk and GBH putative new immune genes.**

1070 The statistics of the transcripts that were considered as significantly upregulated (Sleuth : Beta>1; qval <  
1071 0.01; pseudocounts > 5 in all the samples of at least one condition) are highlighted in red. The Beta value  
1072 gives a biased estimate of the log2 fold change. The qvalue (qval) is an equivalent of the adjusted p-value.  
1073 The following columns give the normalized pseudocounts (Kallisto) for each individual sample, with  
1074 HCn15 and FBn15 corresponding to control larvae and HCi15 and FBi15 corresponding to infested larvae.

1075 **S3 Table. Primers sequences and genes used in this study.**

1076 **S1 Fig. Temporal monitoring of nematobacterial infestation parameters.** (A) Dotplot showing the  
1077 number of *S. carpocapsae* detected in the midgut alimentary bolus at several times after infestation by the  
1078 nematobacterial complex. Infestations were performed by putting in contact individual larvae with 150  
1079 nematobacterial complexes (at time 0) in cell culture plates. Dot colors correspond to 3 independent  
1080 experiments on N=3 larvae per time point. (B) Curve showing the temporal evolution of *X. nematophila*  
1081 concentration (+/-SEM) in the hemolymph across the time post-infestation. Infestation experiments were  
1082 performed in triplicate with 3 pools of 3 larvae per time point. *X. nematophila* were quantified by CFU  
1083 counting on selective culture medium. (C) Curve showing the temporal evolution of *S. frugiperda* larvae's  
1084 survival percentage (+/- SEM) across the time post-infestation. Infestation experiments were performed in  
1085 triplicate on N=20 larvae per experiment.

1086 **S2 Fig. Comparison of the main infection parameters after independent injections of the**  
1087 **nematobacterial complex, of axenic *S. carpocapsae* and of *X. nematophila*.** (A) Curves showing the  
1088 temporal evolution of *X. nematophila* concentration (+/-SEM) after independent injections of either 10  
1089 nematobacterial complexes (NBC) or 200 *X. nematophila* (*X.n.*). Injection experiments were performed in  
1090 triplicate with 3 pools of 3 larvae per time point and *X. nematophila* were quantified by CFU counting on  
1091 selective culture medium. (B) Curves showing the temporal evolution of *S. frugiperda* larvae's survival  
1092 percentage (+/- SEM) after independent injections of either 10 nematobacterial complexes (NBC), 10  
1093 axenic *S. carpocapsae* (*S.c.*) or 200 *X. nematophila* (*X.n.*). Injection experiments were performed in  
1094 triplicate on N=20 larvae per experiment. No insect death was reported for control buffer-injected larvae.

1095 (C) Histogram showing the parasitic success (+/- SEM) (i.e.: number of larvae with nematobacterial  
1096 complex emergence on total number of infested larvae) after independent injections of either 10  
1097 nematobacterial complexes (NBC) or 10 axenic *S. carpocapsae* (*S.c.*). Injection experiments were  
1098 performed in triplicate on N=20 larvae per experiment.

1099 **S3 Fig. Verification of *S. carpocapsae* axenicity.** Electrophoresis gel showing the absence of bacterial  
1100 contaminants in the axenized nematodes. Total DNAs from grinded infective stage nematodes (axenic *S.c.*)  
1101 were extracted and the absence of bacterial contaminants was verified by PCR amplification of the 16S  
1102 rRNA gene with universal primers and of the *Xenorhabdus*-specific gene (see Materials and Methods).  
1103 Whole nematobacterial complexes (NBC) and a pure suspension of *X. nematophila* (*X.n.*) were used as  
1104 positive controls. A pure suspension of *P. protegens* (*P.p.*) was used as negative control for putative TonB-  
1105 dependent heme-receptor amplification.

1106 **S4 Fig. Alignment of deduced amino acid sequences of Unks from *S. frugiperda* with those of *S. litura***  
1107 **and *S. littoralis*.** Nucleotide sequences were retrieved by blastn on *S. litura* and *S. littoralis* genomes.

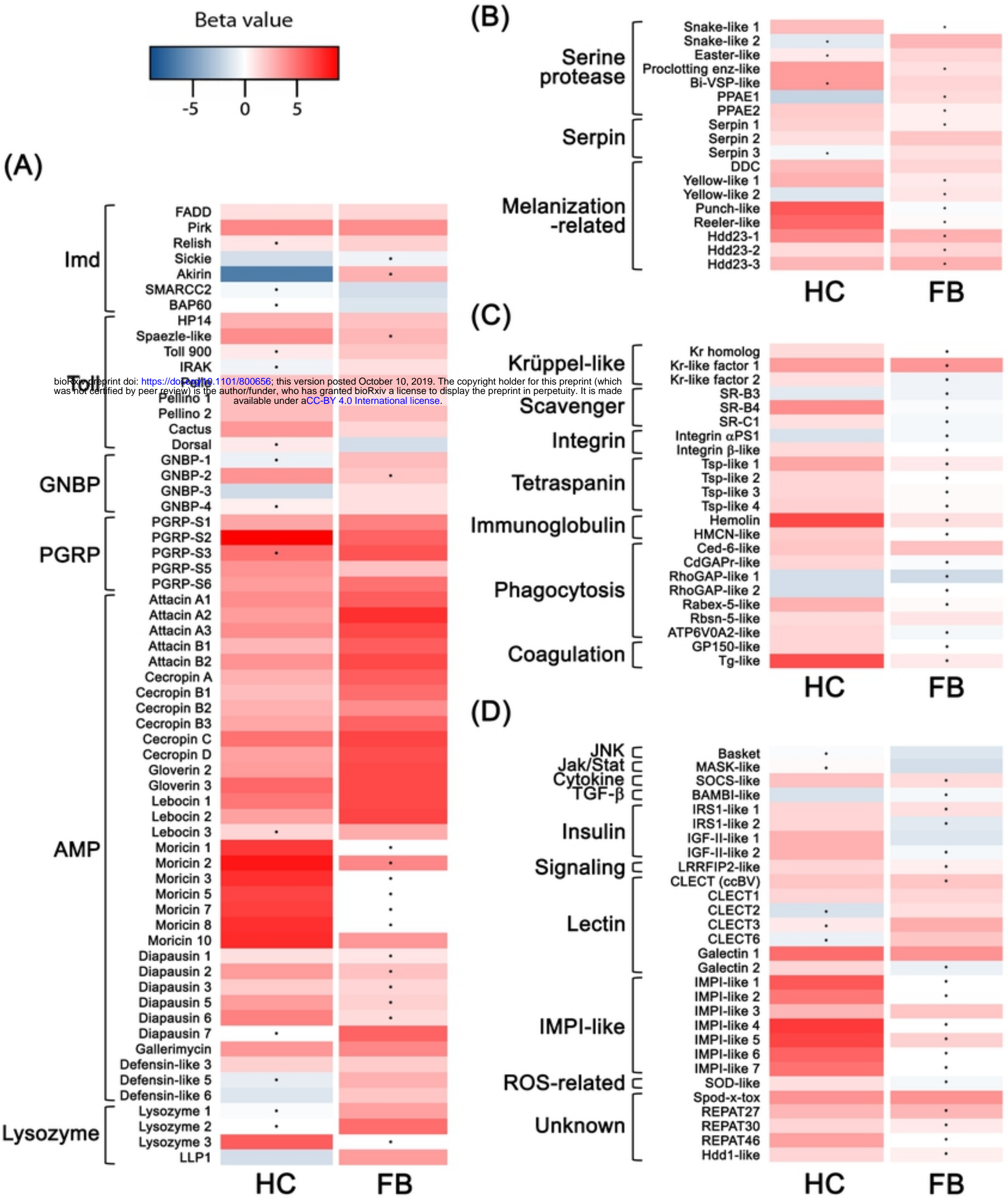
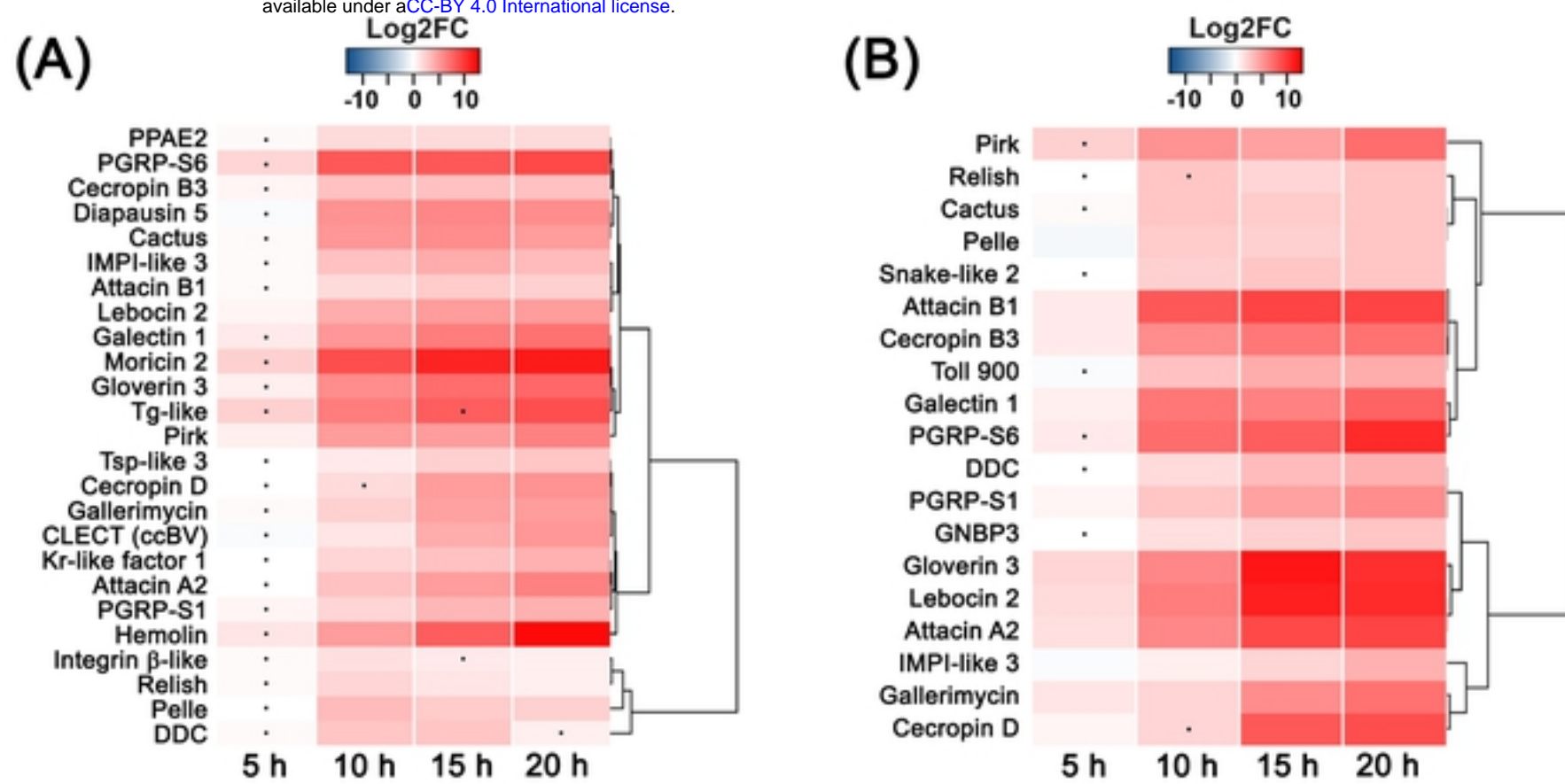


Figure 1



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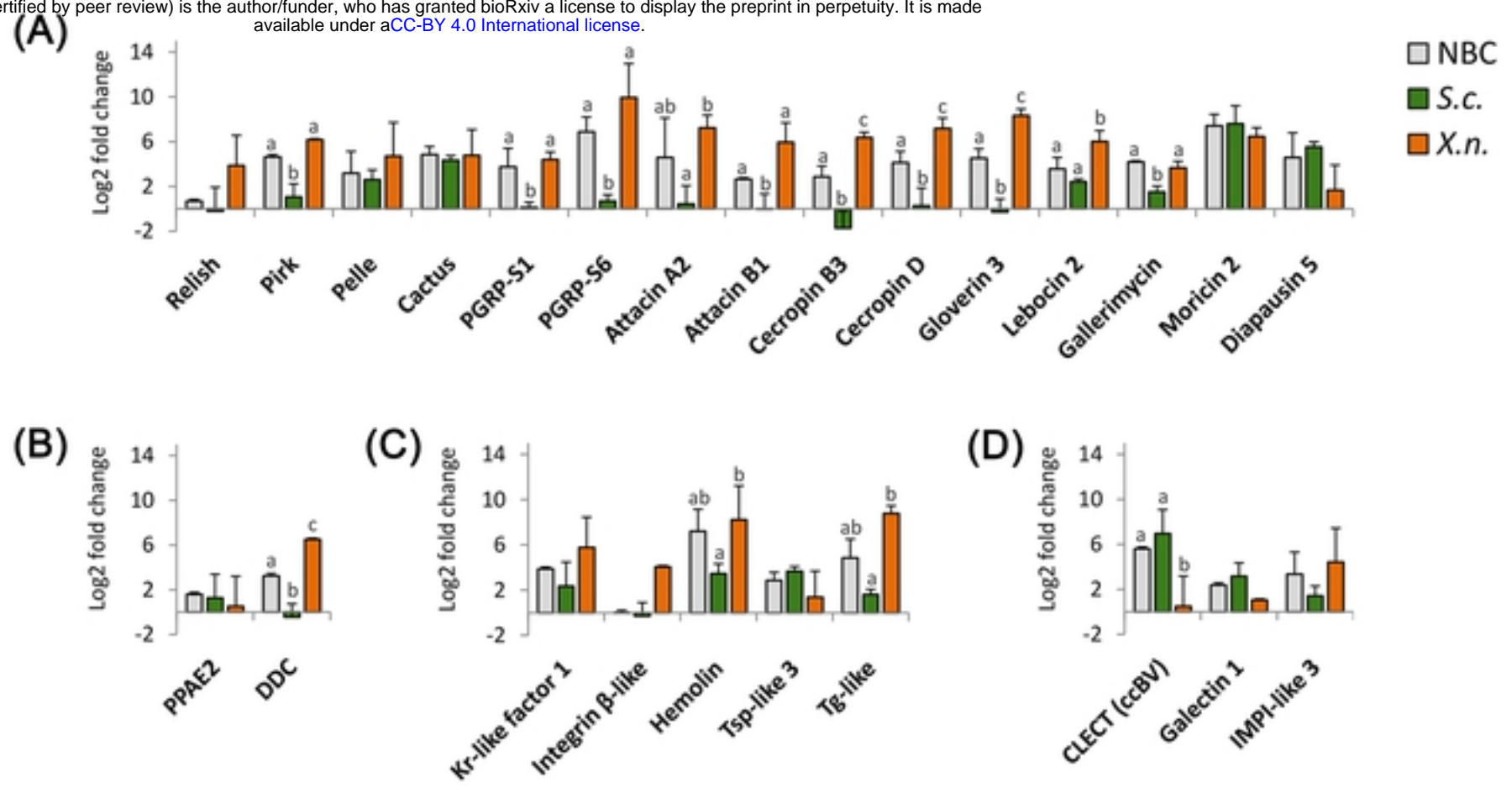


Figure 3

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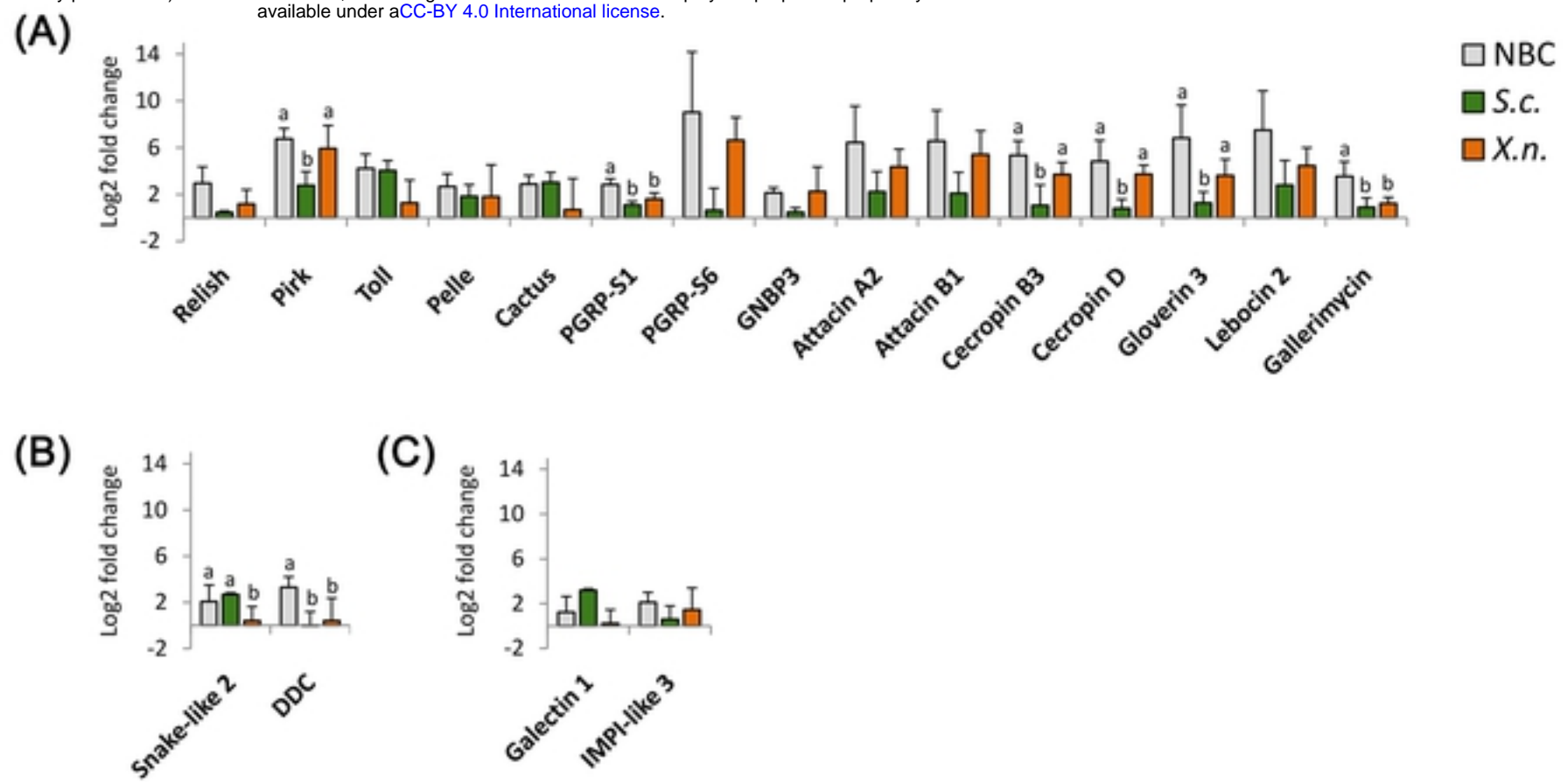
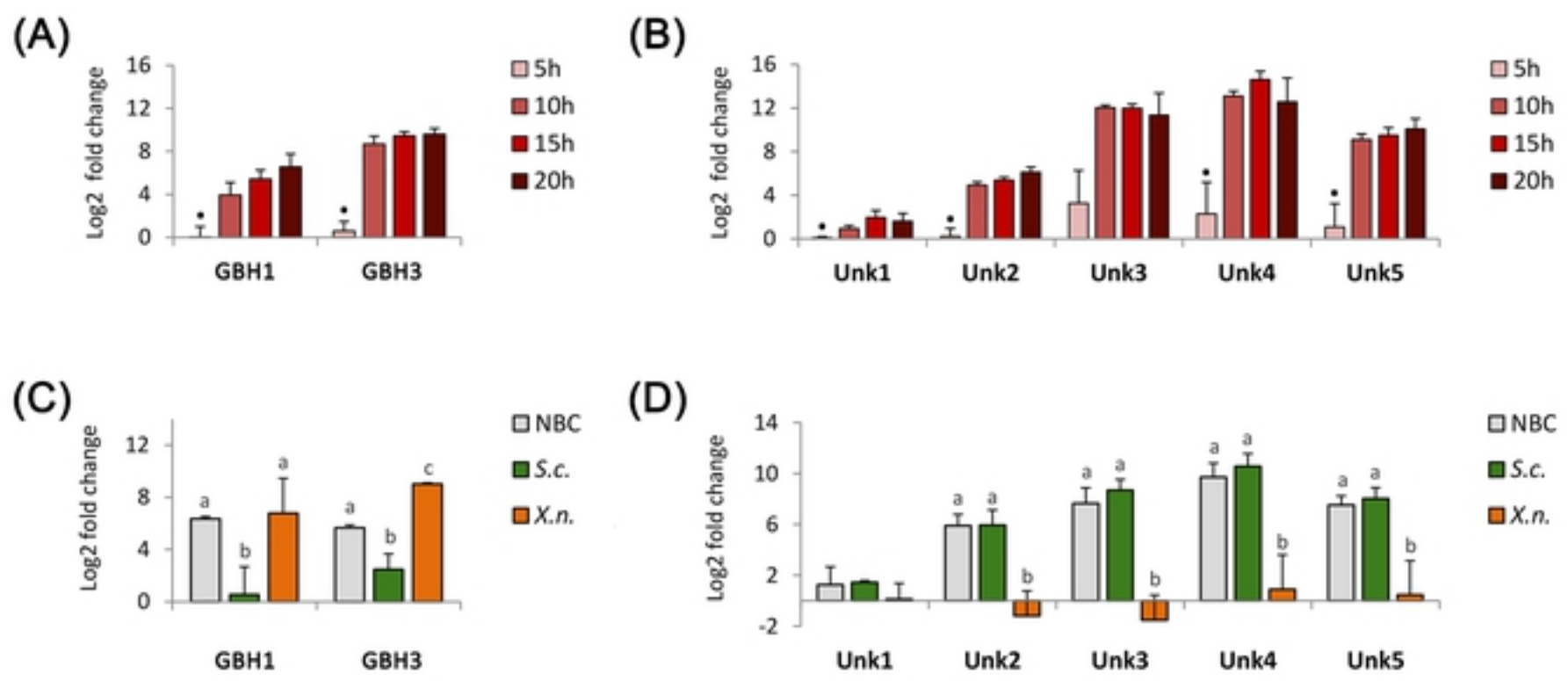


Figure 4



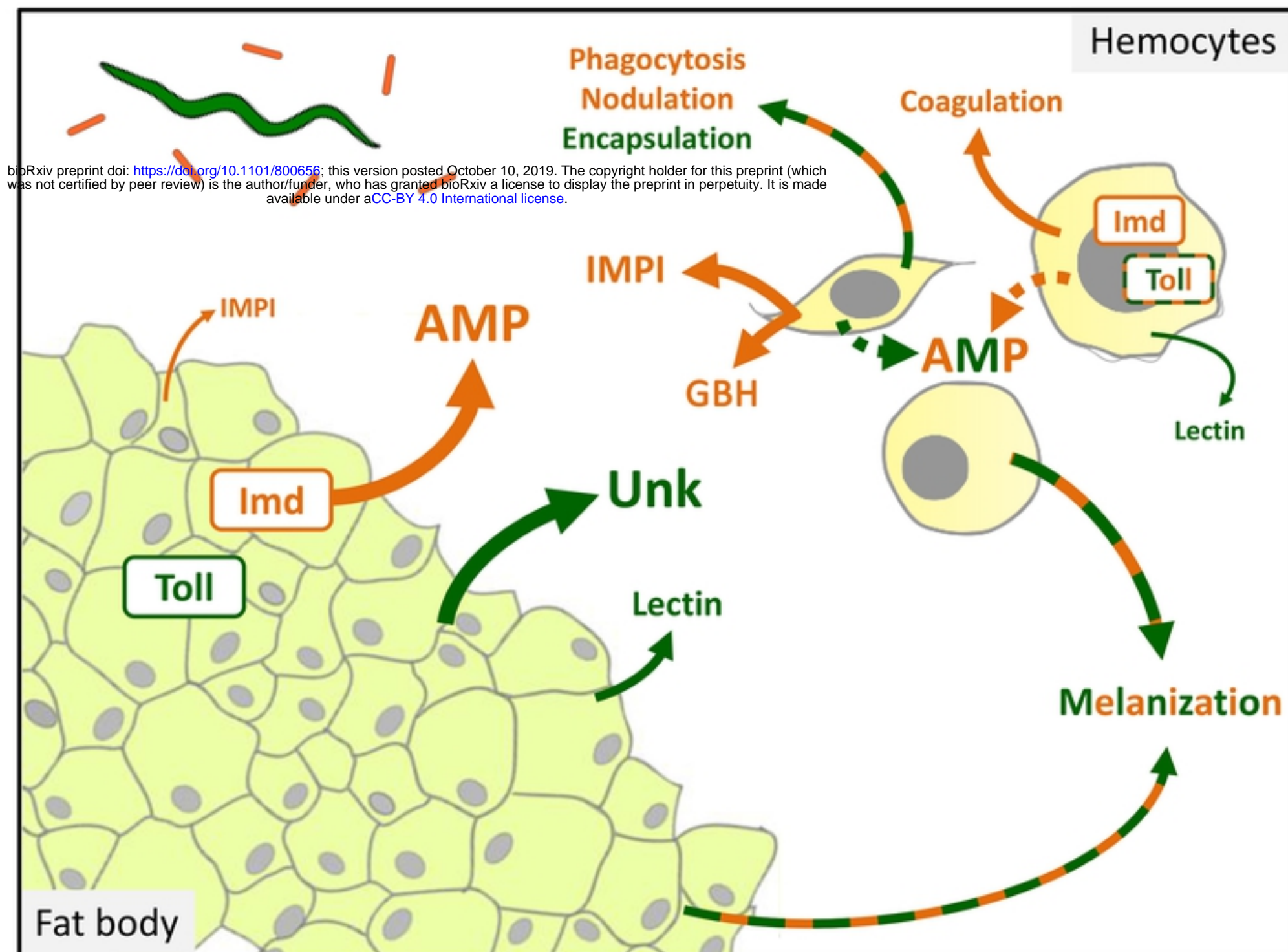


Figure 6