

OPEN

# *Spodoptera frugiperda* transcriptional response to infestation by *Steinernema carpocapsae*

Louise Huot<sup>1</sup>, Simon George<sup>1</sup>, Pierre-Alain Girard<sup>1</sup>, Dany Severac<sup>2</sup>, Nicolas Nègre<sup>1</sup> & Bernard Duvic<sup>1</sup> 

*Steinernema carpocapsae* is an entomopathogenic nematode (EPN) used in biological control of agricultural pest insects. It enters the hemocoel of its host via the intestinal tract and releases its symbiotic bacterium *Xenorhabdus nematophila*. In order to improve our knowledge about the physiological responses of its different hosts, we examined the transcriptional responses to EPN infestation of the fat body, the hemocytes and the midgut in the lepidopteran pest *Spodoptera frugiperda*. The tissues poorly respond to the infestation at an early time post-infestation of 8 h with only 5 genes differentially expressed in the fat body of the caterpillars. Strong transcriptional responses are observed at a later time point of 15 h post-infestation in all three tissues. Few genes are differentially expressed in the midgut but tissue-specific panels of induced metalloprotease inhibitors, immune receptors and antimicrobial peptides together with several uncharacterized genes are up-regulated in the fat body and the hemocytes. Among the most up-regulated genes, we identified new potential immune effectors, unique to Lepidoptera, which show homology with bacterial genes of unknown function. Altogether, these results pave the way for further functional studies of the responsive genes' involvement in the interaction with the EPN.

There is a growing desire in Europe to reduce the use of chemical pesticides on agricultural land, because of their toxicity for the environment and human health (European Directive EC91/414). Also, the development of alternative methods for the control of crop pests is encouraged. These methods include the use of predators and pathogens of insect pests such as viruses, bacteria, fungi, parasitoid wasps and entomopathogenic nematodes (EPNs).

EPNs of the genus *Steinernema* associated with the symbiotic bacterium *Xenorhabdus* are among the most widely used and studied biological control agents<sup>1</sup>. They pose little threat to human health and non-target species<sup>2</sup>, but are capable of killing a broad spectrum of insect pests including the moth *Spodoptera frugiperda* (Lepidoptera: Noctuidae)<sup>3,4</sup>. The infestation cycle of the nematode *Steinernema carpocapsae* has been well described. It starts with the entry of infective juvenile larvae (IJ) into the insect intestinal tract via the natural orifices<sup>5</sup>. Once the intestinal epithelium is crossed, EPNs are found in the hemocoel where they release their symbiotic bacteria *Xenorhabdus nematophila*. There, both the nematodes and their symbiotic bacteria cause the death of the insect in less than 48 h, thanks to a multitude of immunosuppressive virulence factors<sup>6–15</sup>. Then, EPNs multiply by feeding on the insect tissues, reassociate with their symbiotic bacteria<sup>16</sup> and finally go back to the environment in search for a new prey<sup>17</sup>.

Insects live in environments containing microorganisms (bacteria, fungi or viruses) potentially pathogenic for them. To defend themselves against these aggressors, insects have developed a powerful and diversified immune system essentially based on innate immunity but also on an adaptive-like immunity<sup>18,19</sup>. While the adaptive-like immunity is essentially directed against viruses, the innate immunity is the main mechanism involved in defense against bacteria and eukaryotic pathogens. Innate immunity has been well described in the *Drosophila* model<sup>20</sup>. Its main mechanisms and pathways seem to be conserved in different orders of insects<sup>21–23</sup> even though some insects, such as *Apis mellifera* or *Acyrtosiphon pisum*, have reduced immune repertoire<sup>24,25</sup>. The first line of

<sup>1</sup>DGIMI, Univ Montpellier, INRA, Montpellier, France. <sup>2</sup>MGX, Univ Montpellier, CNRS, INSERM, Montpellier, France. Correspondence and requests for materials should be addressed to N.N. (email: [nicolas.negre@umontpellier.fr](mailto:nicolas.negre@umontpellier.fr)) or B.D. (email: [bernard.duvic@umontpellier.fr](mailto:bernard.duvic@umontpellier.fr))

defense are physical barriers, including the cuticle, which covers almost all insects' interfaces with the environment, and the peritrophic matrix, which replaces the cuticle in the midgut<sup>26</sup>. The cuticle is a thick exoskeleton made of wax, chitin and sclerotized proteins that confers mechanical protection against wounds and invaders<sup>27</sup>, while the midgut peritrophic matrix is a thinner network of chitin and proteins that allows the uptake of nutrients but which has a low permeability to microorganisms and toxins<sup>28</sup>. The intestinal epithelium can produce several immune molecules such as antimicrobial peptides (AMP) or reactive oxygen species depending on the location<sup>26</sup>. Once into the hemolymph, parasites are facing circulating hemocytes, which are the immune blood cells of insects. Hemocytes participate in sclerotization, coagulation as well as in the elimination of small pathogens (bacteria and yeasts) by phagocytosis and nodulation, and of large pathogens (parasitoid wasp eggs, nematodes) by encapsulation<sup>29</sup>. Encapsulation, nodulation and coagulation also involve a process of melanisation, which results in the formation of melanin and toxic chemicals that help to sequester and to kill the pathogen<sup>30</sup>. Melanisation is activated by an extracellular proteolytic cascade, the pro-phenoloxidase system, following the recognition of microbial determinants or danger signals<sup>31</sup>. Finally, the systemic response of insects relies mainly on the massive secretion of AMP by the fat body into the hemolymph, after activation of the IMD and/or Toll pathways. The IMD pathway is primarily activated by peptidoglycan recognition proteins (PGRP) in response to Gram-negative bacteria and the Toll pathway by PGRP and Gram negative binding proteins (GNBP) in response to Gram-positive bacteria and fungi<sup>32</sup>. The Toll pathway may also be activated by exogenous proteases from pathogens<sup>33</sup>.

Recently, different studies performed in the model insect, *Drosophila melanogaster*, described the responses of this insect to two EPNs, *Steinernema carpocapsae* or *Heterorhabditis bacteriophora*<sup>34–36</sup>. By the use of transcriptomic approaches on whole larvae or adult flies, the authors showed the overexpression of a large number of immune-related genes involved in defense responses such as coagulation, melanisation and the production of antimicrobial peptides, and in several immune and stress-reponse pathways (Toll, Imd, Jak/Stat or JNK).

Since EPNs can be used in biological control, we wished to study their effect in a crop pest against which they could be directed. *S. frugiperda* is an important pest of cultures such as maize, sorghum, rice and cotton and has recently become a worldwide menace since its invasion of Africa in 2016<sup>37</sup>. In this study, we carried out a transcriptomic analysis of the fat body, hemocytes and the midgut of 6<sup>th</sup> instar *S. frugiperda* larvae at two time points after infestation with the EPN *S. carpocapsae*. At 8 hours after infestation (hpi), we found only 5 genes that were differentially expressed in the fat body. However, at 15 hpi, we detected the significant expression modulation of 271 genes. Few genes were differentially expressed in the midgut whereas strong transcriptional responses were observed in the fat body and the hemocytes. These responses consisted mainly in the overexpression of induced metalloprotease inhibitors (IMPI), immune receptors (mostly PGRP) and AMP, indicating that the fat body as well as the hemocytes produce potent immune responses. Among the most up-regulated genes, we identified a cluster of new potential immune effectors, unique to Lepidoptera, which show homology with bacterial genes of unknown function. Finally, we identified a cluster of genes that are overexpressed in all tissues, but whose function is unknown and which are restricted to some noctuid species.

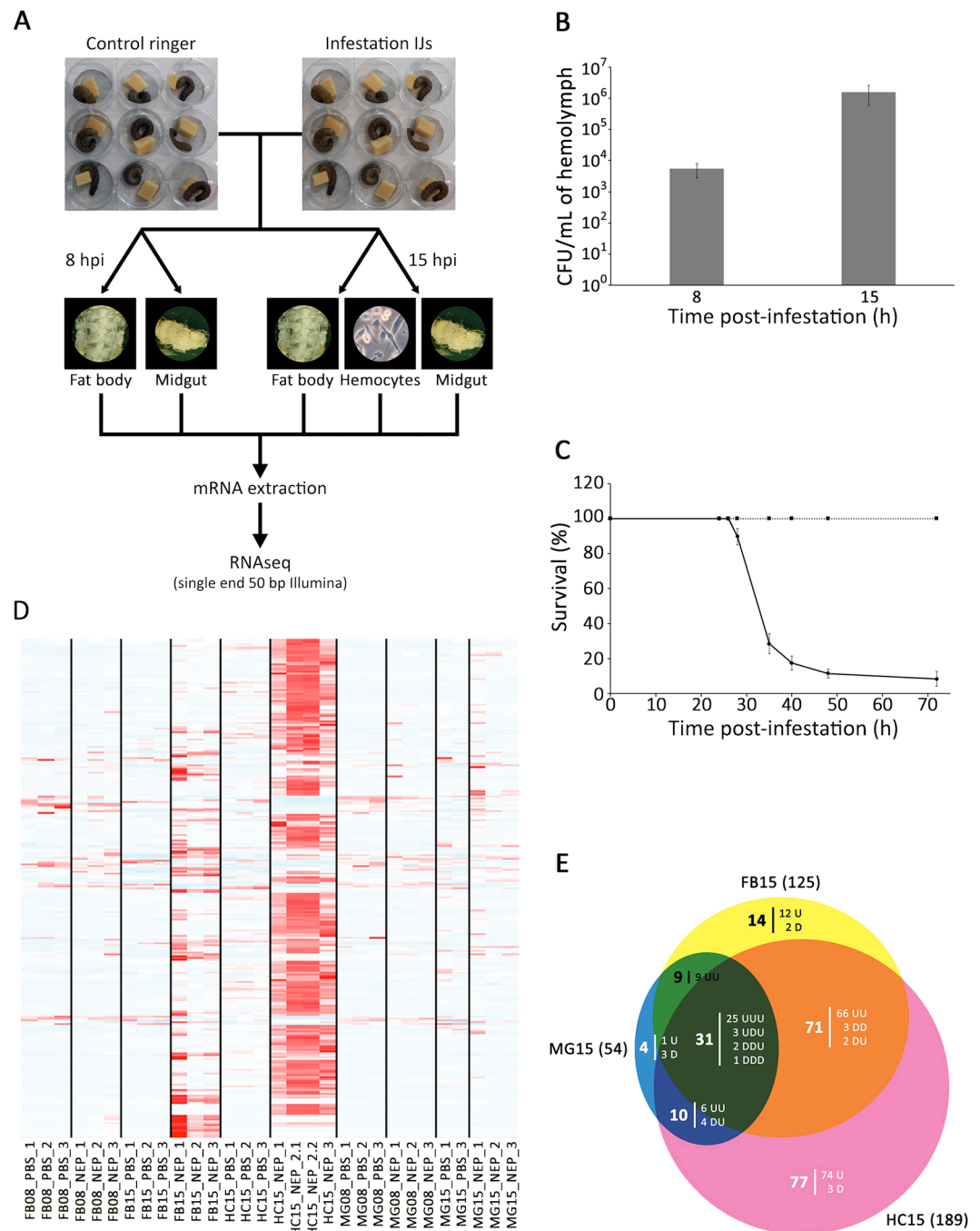
## Results and Discussion

**EPNs infestation and pathogenicity.** To measure how *S. frugiperda* larvae respond to entomopathogenic nematodes (EPNs), we performed infestation experiments where 6<sup>th</sup> instar larvae were individually put in contact with either Ringer solution (control experiment) or a solution of EPNs in Ringer (infestation condition) at time T = 0 (see Methods and Fig. 1A). We targeted two time points after infestation based on our previous knowledge of the mode of infestation<sup>16</sup>. Eight hours post infestation (hpi), nematodes are supposed to have travelled in the intestinal tract of *S. frugiperda* larvae, crossed the intestinal barrier and started releasing their symbiotic bacteria, *Xenorhabdus nematophila*, within the hemocoel of the caterpillar<sup>16</sup>. At 15 hpi, bacteria have multiplied and septicemia is supposed to be reached. In order to verify these assertions, we quantified the *X. nematophila* cells into the hemolymph by CFU counting on a selective medium. In parallel, the survival of the caterpillar to the EPNs infestation was monitored for 72 h. Our data show that bacterial growth has already started at 8 hpi, from few bacterial cells released to 10<sup>4</sup> cells/mL of hemolymph and up to 10<sup>6</sup> cells/mL at 15 hpi, which is considered septicemia (Fig. 1B). When we measured survival of the larvae following infestation, we observed that the first deaths occur at 28 hpi. At 72 hpi, 92% of treated larvae were dead (Fig. 1C) whereas no death was observed in the control experiments.

At 8 hpi and 15 hpi, we removed the larvae from either the control or the infestation plates and dissected three tissues: the midgut (MG), the fat body (FB) and the hemocytes (HC at 15 hpi only). From these tissues, RNA was extracted and processed to perform single end 50 bp Illumina sequencing on three biological replicates (Fig. 1A). We recovered between 10 and 100 million reads per sample, of which between 50 and 70% align onto the reference transcriptome for *S. frugiperda*<sup>38</sup> (see Methods and Table S1). After normalization with DESeq2<sup>39</sup>, we observed that gene expression datasets cluster by tissue (Supplementary Fig. S1) and that within tissues, condition replicates correlate well with each other.

**Overview of transcriptional response.** In midgut (MG) and fat body (FB), at 8 hpi, we detected a very small transcriptional response with only 5 statistically significant differentially expressed (DE) genes in FB and none in MG (Supplementary Fig. S2). From these, 4 genes are overexpressed in response to infestation and are also retrieved overexpressed at later time-points in all 3 tissues (Supplementary Fig. S3). They are annotated in the genome as unknown transcripts.

At 15 hpi, there is a dramatic transcriptional response with thousands of DE genes in all three tissues at padj < 0.01 (Supplementary Fig. S2), with in each case, more genes overexpressed than underexpressed. In order to detect the most significant genes responding to EPNs infestation at 15 h, we modeled the EPN effect across all datasets and identified 271 DE genes (Fig. 1D) which we intersected with all previous 1 on 1 comparison per conditions (see Methods). Of these, we identified a total of 216 DE genes at 15 hpi in the three different tissues



**Figure 1.** Tissue specific transcriptional response time series of *Spodoptera frugiperda* larvae to EPN infestation. **(A)** Overview of the experimental design. In three independent experiments, 9 infested and 9 control larvae from culture plates were dissected at 8 hpi and 15 hpi. Hemocytes, fat bodies and midguts were extracted and pooled for each time and condition. Polyadenylated RNAs were purified from these pools and corresponding cDNA libraries were built. cDNA were sequenced on a single end by Illumina and RNAseq data were analyzed to identify the genes that are differentially expressed during *Steinernema carpocapsae* infestation. **(B)** Growth of *Xenorhabdus nematophila* following *S. frugiperda* infestation with 150 symbiotic *S. carpocapsae*. At 8 hpi and 15 hpi, the number of CFU per mL of hemolymph was estimated from three independent experiments with three technical replicates (three larvae per technical replicate). Error bars indicate standard errors of the means. **(C)** Survival curve. Larvae were infested with 150 nematobacterial IJ. Data represent means  $\pm$  SEs of four independent experiments, each containing 12 larvae. **(D)** Significantly differentially expressed genes in response to EPN. This heatmap shows z-score of expression variation across all RNAseq samples (red being overexpressed and blue under-expressed) for 271 genes with significant variations to EPN infestation. **(E)** Venn diagram showing the tissue specificity of the EPN responsive genes at 15 hpi. The response can be overexpression (Up:U) or under-expression (Down:D). For example, there are 71 genes varying significantly upon EPN infestation in both the fat body (FB) and the hemocytes (HC). Of these, 66 are up-regulated in both tissues (UU), 3 are down-regulated in both tissues (DD) and 2 are down-regulated in FB and up-regulated in HC (DU). By convention, the order of the U and D letters represent respectively MG, FB and HC tissues.

Tissue	Midgut (MG)	Fat body (FB)	Hemocytes (HC)
Differentially expressed genes (padj < 0.01)	54	125	189
Overexpressed genes (padj < 0.01, Log2FC > 0)	41 (75.9%)	115 (92.0%)	182 (96.3%)
Underexpressed genes (padj < 0.01, Log2FC < 0)	13 (24.1%)	10 (8.0%)	7 (3.7%)

**Table 1.** Differentially expressed genes per condition at 15 hpi.

(Fig. 1E). Most of the response occurred in FB and HC tissues (Fig. 1E, Table 1) with, again, a vast majority of overexpressed genes (Fig. 1D,E, Table 1).

The data obtained by RNAseq were confirmed by quantitative RT-PCR on a selection of DE genes in the three tissues (Supplementary Fig. S4).

**Functional response of the midgut at 15 hpi.** The first line of defense against *S. carpocapsae* EPN is the midgut barrier, which is also the main entry point of *Steinernema* nematodes in *S. frugiperda*<sup>6</sup>. This organ is known for its immune activity through the production of reactive oxygen species and anti-microbial peptides in response to pathogens<sup>40</sup>. It is not supposed to be directly confronted to *X. nematophila*<sup>16,41</sup> and therefore, we hypothesized that the genes, which would be overexpressed specifically in the midgut may identify anti-nematode factors. However, we did not find any DE genes in the midgut at 8 hpi and only 4 genes that were DE specifically in the midgut tissue at 15 hpi, with only 1 being overexpressed and 3 under-expressed (Fig. 2A). The overexpressed gene encodes a heat-shock protein of the hsp70 family (Supplementary Data S1). This superfamily of genes is usually upregulated in response to oxidative stress and in the midgut of *Drosophila*, Hsp68 promotes the proliferation of intestinal stem cells, and thus its regeneration<sup>42</sup>.

One of the under-expressed genes is an E3 ubiquitin-protein ligase of the Seven In Absentia family (SIAH). The mammalian homologue Siah1 cooperates with SIP (Siah-interacting protein), the F-box protein Ebi and the adaptor protein Skp1, to target beta-catenin, a multifunctional protein that plays an important role in the transduction of Wnt signals and in the intercellular adhesion by linking the cytoplasmic domain of cadherin, for ubiquitination and degradation via a p53-dependent mechanism. Thus, down-regulation of SIAH might increase levels of  $\beta$ -catenin, which favors proliferation of intestinal stem cells in *Drosophila*<sup>26</sup>.

The two other under-expressed genes are an ABC family transporter and an NT-C2 domain protein, for which no obvious link to infestation or to intestinal homeostasis can be established from the literature.

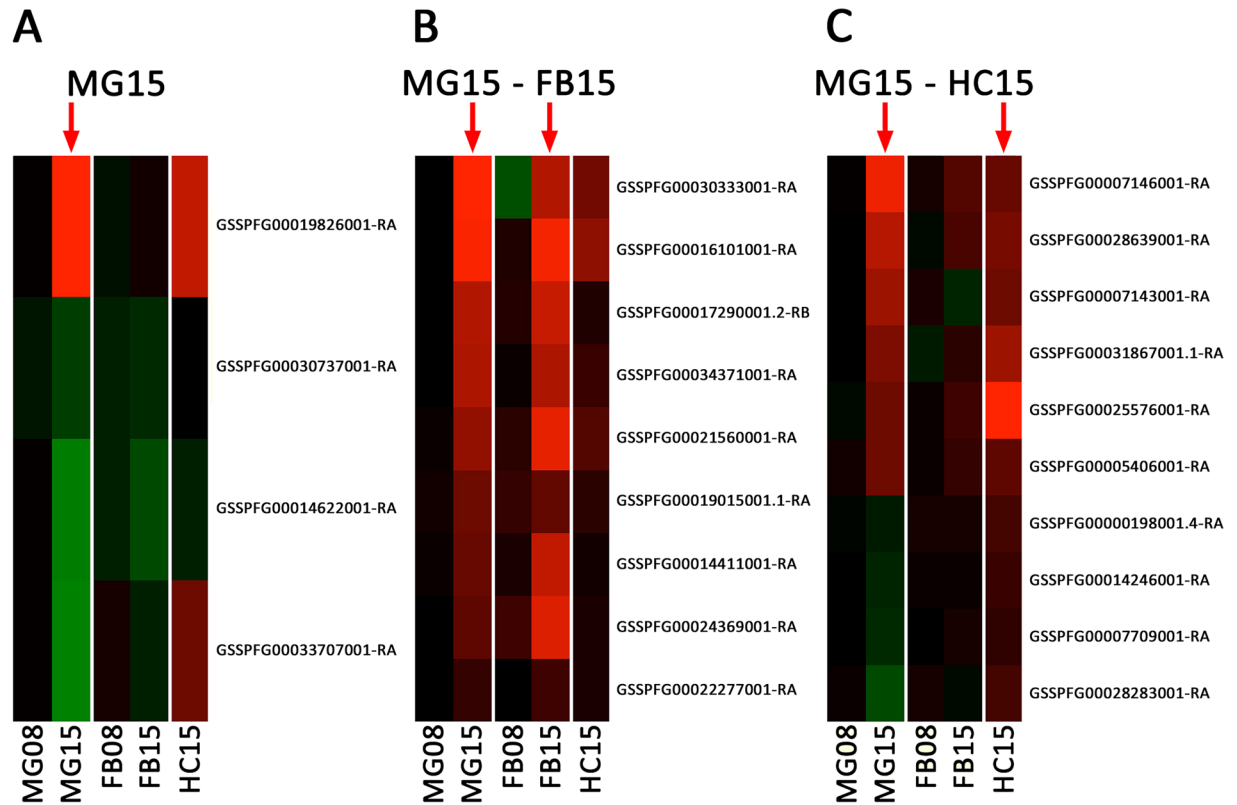
While we found no evidence of a direct response to EPNs specifically by the midgut, we investigated whether this tissue may share a common immune response with the fat body or the hemocytes.

We found nine common DE genes between the midgut and the fat body, which are all overexpressed in both tissues (Fig. 2B). Of those, three genes have no annotated structure or function. Four other genes with no clear homology have domains that can be associated to regulation (protein-kinase domain, calcium binding domain, amino-acid transporter and MADF domain transcription factor) (Supplementary Data S1). Interestingly, we found a cytochrome P450 gene encoding the CYP340L16. CYP genes are usually involved in detoxification of foreign chemicals such as plant xenobiotics and pesticides<sup>43</sup>. Finally, one trypsin inhibitor-like cysteine rich domain proteinase inhibitor was also overexpressed. There is no enrichment for a specific molecular function or biological process among those nine genes.

Similarly, 10 genes were found significantly differentially expressed in both the midgut and hemocytes, with 6 of them being overexpressed in both tissues and 4 being under-expressed in MG and upregulated in HC (Fig. 2C). Of the 6 overexpressed genes, 4 are small solute transporters of the Major Facilitator Superfamily (MFS), 1 is an antennal carboxylesterase and 1 is a proteinase inhibitor (Supplementary Data S1). No particular function of note has been identified for the 4 genes that were under-expressed in MG but overexpressed in HC, except for *Iap2*, which is an inhibitor of apoptosis and a member of the IMD pathway<sup>44</sup>.

From this comparison, it seems likely that the midgut is not specifically involved in the defense of the *S. frugiperda* larvae against EPNs infestation. There are few DE genes in this tissue, whether specific or in common with other tissues, and no specific functional pathway can be clearly identified. Rather, some of the genes identified may be reacting to oxidative stress and homeostasis maintenance of the intestinal epithelium, which might be consequences of the host infestation. The question remains whether the midgut is normally immunocompetent against EPNs in *S. frugiperda* or if this response is altered by EPNs. Indeed, several studies show that EPN can either evade detection by immune systems<sup>45–47</sup> or modulate it<sup>6,48</sup>. However, in this latter hypothesis, we would still observe an immune reaction at the transcriptional level, which is not the case.

**Specific response of the fat body at 15 hpi.** In insects, the adipocytes that compose the fat body are in direct contact with the hemolymph. The physiological function of this organ is to store energetic reserves, in the form of glycogen and lipids, and release them if needed<sup>49</sup>. It is also the main tissue involved in systemic immunity, since it produces AMP during immune challenge<sup>20</sup>. We found 14 DE genes specifically in the fat body, with 12 overexpressed and 2 under-expressed genes. Among the latter are 1 uncharacterized protein and 1 putative transposable element (TE). Among the 12 fat body-specific up-regulated genes, we found one major actor of immunity, the Toll receptor<sup>50</sup>, which recognizes the cleaved circulating cytokine Spätzle to induce the production of anti-microbial peptides<sup>51</sup>. It is noteworthy that Toll receptor is expressed in all three tissues, but overexpressed in response to EPNs in the fat body only (Fig. 3A). Among the 11 other overexpressed genes, we found one



**Figure 2.** Midgut Associated Response. Heatmaps of differential expression (in log<sub>2</sub>FoldChange - green, under-expression, red, overexpression according to values in Supplementary Data S1) across all experimental conditions of genes found significantly differentially expressed (A) specifically in the MG at 15 hpi (MG15 - 4 genes) (B) common to MG15 and FB15 (9 genes) and (C) common to MG15 and HC15 (10 genes).

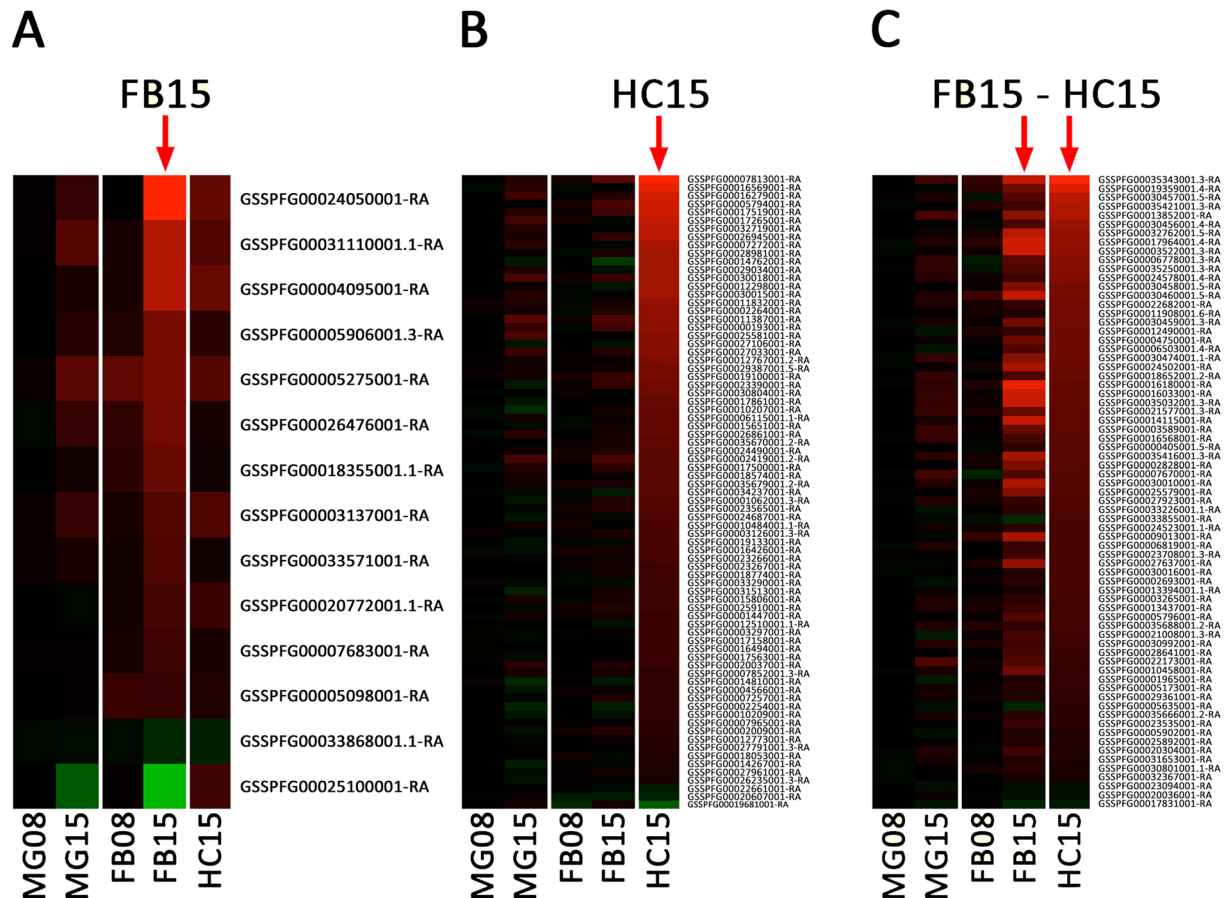
potential receptor of the arrestin family, several enzymes (lipase, carboxypeptidase and a GTPase co-factor), again a MADF-domain transcription factor and a dynein (Supplementary Data S1).

**Specific response of the hemocytes at 15 hpi.** In insects, hemocytes are the main actors of the cellular immune responses like phagocytosis or encapsulation<sup>52</sup>. They are also involved in other defense mechanisms such as coagulation<sup>53</sup>, and melanization<sup>54,55</sup>. In addition, different reports have shown that hemocytes are, as the fat body, capable of synthesis of AMP<sup>56–58</sup>. Therefore, we hypothesized that genes specifically induced in the hemocytes may be involved in coagulation and/or melanization along with cellular immune responses.

The largest number of DE genes is found specifically in the hemocytes with 77 genes (Fig. 1E), of which 74 are overexpressed in response to EPNs infestation (Fig. 3B). No enriched GO categories have been detected in this list. However, we noticed several categories of genes of interest. The most overexpressed gene encodes a serine protease without CLIP-domain (Supplementary Data S1) that is homologous to hemolymph proteinase 7 (HP7) and 10 (HP10) in *Manduca sexta*<sup>59</sup>. In the insect immune system, serine proteases participate in the activation of Toll-dependent response to infection as well as in the prophenoloxidase-dependent melanization cascade<sup>60</sup>. However, many serine-proteases, in particular without CLIP-domains such as HP7 & HP10, still have unknown function. They are regulated by protease inhibitors, a large family of small peptides, one of which is also found highly induced in our list (Supplementary Data S1) (log<sub>2</sub>FoldChange = 8.06). This induction suggests a role of HP7/HP10 in the specific activation of hemocytes after infestation by EPNs.

The molecular functions we found in the hemocytes specific gene list include MFS transporters, ubiquitin-conjugating enzymes, sina-like, antennal esterases, heat-shock proteins and several protein kinases. We also noticed several genes that may play a role in vacuolar trafficking and signaling, with several transmembrane domain proteins. The general molecular function of these genes makes it hard to link them to any biological process.

Surprisingly, we noticed that very few genes linked to immunity were present in this list. In particular, we have not found a deregulation of genes linked to the activation of the PPO pathway besides the above-mentioned serine proteases. The only gene that we could relate to melanization is homologous to the L-dopachrome tautomerase Yellow-f2 that is responsible for the conversion of DOPA into dopamine, a precursor of melanin. This observation suggests that the humoral immune response is not cellular autonomous in the hemocytes and implies a cooperation between the fat body and the hemocytes. However, the cellular response seems to be restricted to hemocytes.



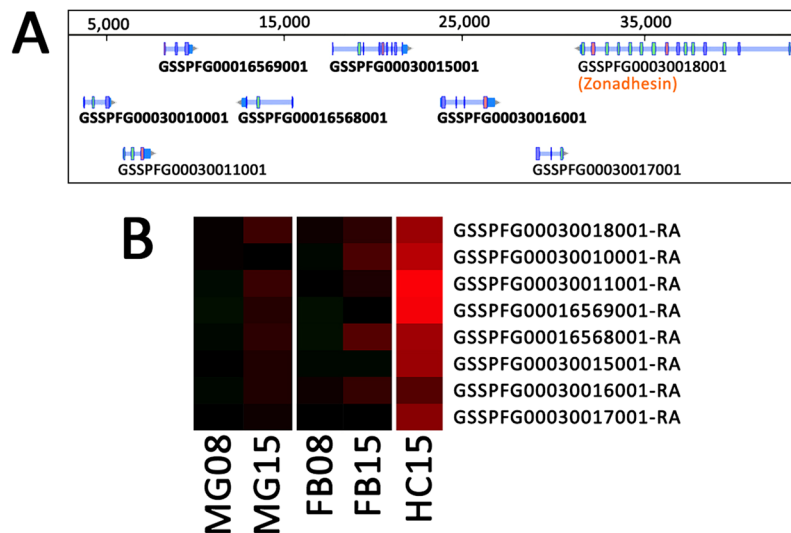
**Figure 3.** Fat body and hemocytes associated responses. As in Fig. 2, heatmaps of differential expression for genes (A) specific to FB15 (14 genes), (B) specific to HC15 (77 genes) and (C) common to FB15 and HC15 (71 genes).

Three transcription factors are also found overexpressed in HC, including *Vrille*. *Vrille* is known to activate the serine protease *Easter* that, in turns, cleaves the *Spätzle* protein that is the ligand of Toll receptor, which we found overexpressed in the fat body (see above). Other genes involved in immunity are *Pellino* which might be either a negative regulator<sup>61</sup> or an enhancer<sup>62</sup> of the Toll pathway, and *IMD* which is a member of the IMD pathway.

**Common response of the fat body and the hemocytes at 15 hpi.** We identified 71 genes differentially expressed upon EPNs infestation in both FB and HC tissues (Fig. 1E), 66 of them being overexpressed in both tissues. The “immunity” ontology is the most enriched GO category (Supplementary Fig. S5). The most overexpressed genes correspond to a battery of anti-microbial peptides (attacins, cecropins, defensins, gloverins and moricins) (Supplementary Data S1). We identified by homology a repertoire of 40 AMP in the genome of *S. frugiperda*, classified in 7 different families<sup>38</sup>. The majority of AMP production is performed by the FB tissue with members of the attacins, cecropins, gloverins and lebecins strongly overexpressed (Fig. 3C). These AMP are also significantly overexpressed in the HC but to a lesser extent (Fig. 3C). Defensins such as the *gallerimycin*<sup>63</sup> and *Spod-x-tox*<sup>64</sup> are overexpressed in both tissues. Remarkably, of the 10 moricins present in *S. frugiperda* genome, only *Moricin 10* is strongly overexpressed in both tissues. The diapausin overexpression is less clear with low levels of expression.

Among the most overexpressed genes in both FB and HC tissues, we also identified several members of the peptidoglycan recognition proteins (PGRP) (Supplementary Data S1), a family of receptors, which are involved in the recognition of pathogens associated molecular patterns (PAMP) and in the subsequent activation of the Toll, Imd and PPO system pathways<sup>31,32</sup>. In the genome of *S. frugiperda*, we identified 10 PGRP (Supplementary Fig. S6) that were named according to *Bombyx mori* nomenclature<sup>21</sup>. Upon EPNs infestation, PGRP-S2, -S6 and -L3 are overexpressed in both FB and HC, with PGRP-S2 being the highest overexpressed (Supplementary Data S1). A phylogenetic analysis (Supplementary Fig. S6) shows that PGRP-S2 is closely related to *Drosophila melanogaster* PGRP-SA, which is involved in the induction of the Toll pathway<sup>65</sup>. Recently, it has also been reported that a PGRP-SA homolog may be responsible for the activation of the phenoloxidase system in the Chinese tussar moth *Antheraea pernyi*<sup>66</sup>.

This overexpression of AMP and PGRP upon EPNs infestation has been similarly observed in transcriptomic studies of *Drosophila melanogaster* larvae infested by *Heterorhabditis* sp. or *Steinernema* sp. nematodes<sup>34–36</sup>.



**Figure 4.** Inducible MetalloProtease Inhibitor. (A) WebApollo viewer showing the annotation of inducible metalloprotease inhibitor genes in cluster on the scaffold\_1741 in the genome of *Spodoptera frugiperda*. (B) As in Fig. 2, heatmap of differential expression for the identified IMPI genes.

Other immunity genes overexpressed in both tissues are implicated in the Toll pathway. This pathway is activated by PAMP recognition proteins or by proteases from pathogens<sup>33,67</sup>. Signal is transduced by an intracellular complex (MyD88/Tube/Pelle) that binds to the intracytoplasmic domain of Toll and is regulated by Pellino<sup>61,62</sup>. Signal transduction results in the phosphorylation of the ankyrin-repeat containing protein Cactus, which allows its dissociation from the transcription factor Dorsal. This dissociation promotes the translocation of Dorsal to the nucleus where it activates the production of AMP. Several members of this pathway are overexpressed in both FB and HC at 15 hpi, including Pelle, Pellino, and Cactus. Altogether, these results suggest that the Toll pathway is activated in both the fat body and the hemocytes.

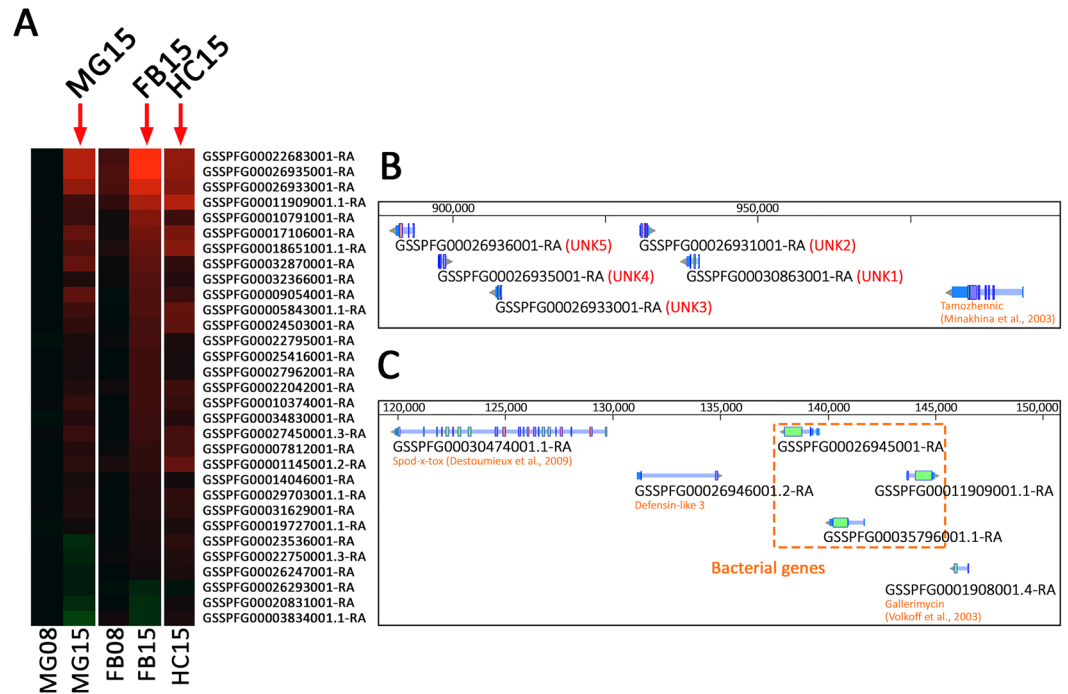
We also observed the overexpression of Hdd23 which mediates PPO activation<sup>68</sup> and of 3 putative transcription factors, one of them containing a zinc-finger domain (GATA4-like) known to mediate immune response in *Drosophila*<sup>69</sup>.

In addition to immune-related genes, this list contains 7 protease inhibitors. One is a serine protease inhibitor, which may be involved in the regulation of serine proteases cascades, such as the prophenoloxidase activating cascade<sup>60</sup> or the Toll activating cascade<sup>20</sup>, while another has homology with a tissue inhibitor of metalloproteases (TIMP). More interestingly, the 5 remaining protease inhibitors belong to the family of inducible metalloprotease inhibitors also called IMPI<sup>70</sup>. These 5 IMPI are present as a cluster of genes in the genome of *S. frugiperda* (Fig. 4A) and their expression is upregulated especially in the hemocytes (Fig. 4B). The first IMPI was purified from the hemolymph of the greater wax moth *Galleria mellonella*<sup>71</sup> and further cloned<sup>72</sup>. The expression of IMPI, along with antimicrobial peptides/proteins, is induced by metalloproteases released by damage tissue or metalloproteases from pathogens during the humoral immune response of *G. mellonella*<sup>70</sup>. It has been shown that nematodes of the genus *Steinernema* as well as *Xenorhabdus* bacteria establish their pathogenesis by secreting virulence factors among which metalloproteases<sup>7,9,73–76</sup>. Therefore, we may hypothesize that *S. frugiperda* induces the expression of IMPI to counteract the metalloproteases produced by nemato-bacterial complexes.

**Common response of the midgut, the fat body and the hemocytes at 15 hpi.** Finally, we identified 31 genes that are significantly differentially expressed in all three tissues (MG, FB and HC), 25 of them being overexpressed in all tissues (Fig. 1E, Supplementary Data S1). There is no enrichment for a specific molecular function or biological process among these 25 genes and only 3 of them could be related to the caterpillar defenses. They encode the previously cited Hdd23 and Cactus, plus Relish, the transcription factor of the Imd pathway, suggesting that this anti-Gram negative bacteria immune pathway<sup>77</sup> could also take part in the previously described humoral responses.

During the annotation of these genes, we noticed that the 4 most differentially expressed genes had no known function. Three of them were also among the few that were overexpressed in the FB at 8 hpi (Fig. 5A). We pursued the investigation on the potential origin of these genes, which led us to the identification of 2 previously uncharacterized clusters (Fig. 5B,C).

The first cluster (Fig. 5B) is composed of 5 genes for which we could not find any homology in sequence databases at the protein nor at the nucleotide level in any other organism than *Spodoptera frugiperda*. However, we could find the whole cluster in the Sf9 and Sf21 cell lines genomes recently published by other labs<sup>78,79</sup>. In addition, after careful exploration of the syntenic regions, this cluster was also identified in the genomes of two other noctuid species, *Spodoptera litura* and *Helicoverpa armigera*<sup>80,81</sup>. Interestingly, the cluster is located close to a gene homolog of *D. melanogaster* tamozhennic, which has been reported to be involved in Dorsal nuclear translocation<sup>82</sup>. Their genomic localization, their organization in cluster, the presence of eukaryotic signal peptides in their predicted amino acid sequences, the fact that they were not only the most differentially expressed genes upon



**Figure 5.** Expression for the 31 genes common to midgut, the fat body and the hemocytes. **(A)** As in Figs 2 and 3, heatmap of differential expression for the 31 genes common to MG15, FB15 and HC15. **(B)** WebApollo viewer showing the annotation of unknown genes in cluster on the scaffold\_520. **(C)** WebApollo viewer showing the annotation of clustered genes of bacterial origin within a defensin cluster.

EPNs infestation but also the earliest differentially expressed, led us to the hypothesis that they might encode a new class of immune effectors restricted to some noctuid species.

A second intriguing category of genes in this list has homology to bacterial proteins of unknown function. They are a set of three genes in cluster (Fig. 5C), localized between several defensin encoding genes. They possess a eukaryotic signal peptide and two introns but their main coding sequence is homologous to genes from the *Lactococcus lactis* bacteria (Supplementary Fig. S7) and shares a homology with a cysteine peptidase domain of the papain family. Among insects, these genes are found only in the genomes of other Lepidoptera (Supplementary Fig. S7). This homology to bacteria could be due to convergence, if the bacterial and insect proteins share common molecular functions, or could indicate an ancient event of horizontal gene transfer (HGT). Acquisition of antimicrobial activity from bacteria to eukaryotes by HGT has been documented before<sup>83</sup>, but not in insects. We think it will be interesting to characterize further the function of these two clusters of genes and their role in immunity against EPNs.

## Conclusions

In this work, we have conducted a time-series analysis of tissue-specific transcriptomic response of the Lepidoptera *Spodoptera frugiperda* larvae to the infestation by the EPN complex *Steinernema carpocapsae/Xenorhabdus nematophila*. We show that at 8 hours after infestation only a few genes are differentially expressed in the fat body and none in the midgut, despite the presence and amplification of the EPN symbiont *X. nematophila* in the hemocoel. However, we observed a strong response of the larvae at 15 hours post infestation. This response corresponds to a complementary activation of the immune system by the fat body and the hemocytes, resulting in the production of a large repertoire of humoral effectors and receptors.

When we compare our data to RNAseq analyses performed by other laboratories, albeit on different interaction systems, we observe a very similar response. For example, Castillo *et al.* (2015) report an activation of the immune system of *Drosophila melanogaster* larvae by the EPN complex *Heterorhabditis bacteriophora/Photorhabdus luminescens*, represented by several AMPs, several peptidase and protease inhibitors and also Yellow-F. This suggests that all potential hosts possess a conserved ability to fight EPNs complexes.

Despite this powerful response, *S. carpocapsae/X. nematophila* complex will be successful regardless of the system they will be confronted with. Anatomies of their host might differ but IJs will find their way inside the midgut and pierce it to enter the hemocoel. There, they will survive the inflammatory response of their host for a sufficient amount of time in order for their released bacterial symbionts to multiply within the insect and kill it<sup>16,17,84</sup>. It was proposed that the nematodes were able to camouflage themselves from the insect immune system<sup>85</sup>. The facts that we found very few responsive genes at the early time point of 8 hpi and that very few immune-related genes were found differentially expressed in the midgut at any time point support this idea. However, despite a previous study suggesting that *X. nematophila* could resist to the humoral immune responses by transcriptional



down-regulation<sup>11</sup>, our study and recent others<sup>36,86</sup> clearly show that, regardless of the host insect, the immune system is triggered and will react to the EPNs infestation.

Nothing in our data suggests a mechanism by which the EPNs bypass the insect defenses at 15 hpi. Indeed, at this time point, all signaling pathways seem activated in both the fat body and the hemocytes and 16 different AMP are produced. Several studies have evidenced a loss of hemolymph AMP and antimicrobial activity during infestation by the EPN or infection by *X. nematophila*<sup>10,87</sup>. A likely hypothesis might be that these AMP are degraded by *X. nematophila* virulence factors, as shown for the *X. nematophila* protease II in *Galleria mellonella* and *Pseudaletia unipuncta*<sup>73</sup>.

## Materials and Methods

**Insect rearing.** Corn variant *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae were reared on a corn-based artificial diet<sup>88</sup>. They were maintained at 23 °C +/- 1 °C with a photoperiod of 16h/8h (light/dark) and a relative humidity of 40% +/- 5%. *Galleria mellonella* (Lepidoptera: pyralidae) larvae were reared on honey and pollen at 28 °C in dark.

**Nematode production and storage.** Symbiotic *Steinernema carpocapsae* (strain SK27 isolated from Plougastel, France) were renewed on White traps<sup>89</sup> after infestation of the wax moth *Galleria mellonella* last larval stages. They were maintained in aerated Ringer sterile solution with 0.1% formaldehyde at 8 °C for two to four weeks to ensure optimal pathogenicity.

**Infestation.** Infestation experiments were processed at 23 °C in 12-well culture plates. In each well, one second-day sixth instar larva of *S. frugiperda* was placed on a filter paper (Whatman) with artificial corn-based medium. Nine larvae were used per experimental condition. For infested larvae, 150 µL of Ringer sterile solution containing 150 *S. carpocapsae* IJs were introduced in each well. For control larvae, 150 µL of Ringer sterile solution was used. In order to control the pathogenicity of the nematodes, 12 larvae were treated as described above, with or without nematodes, and their survival was monitored for 48 hours. The percentage of survival was the ratio of live larvae on the total larvae used in the experiment.

**Xenorhabdus nematophila quantification.** The concentration of *X. nematophila* in *S. frugiperda* hemolymph after infestation was estimated by CFU counting on NBTA (nutrient agar supplemented with 25 mg of bromothymol blue per liter and 40 mg of triphenyltetrazolium chloride per liter) with 15 µg/mL of erythromycin. For 3 independent experiments and 3 technical replicates, hemolymph was collected by bleeding of 3 caterpillars in 200 µL PBS buffer supplemented with phenylthiourea. The volumes of hemolymph were then estimated and serial dilutions of the samples were plated. CFU were counted after 48 h incubation at 28 °C and CFU numbers were then reported to the estimated hemolymph volumes in order to calculate the bacterial concentrations. The hemolymph of naive caterpillars was also plated to verify the absence of bacterial growth.

**RNA extraction.** *Spodoptera frugiperda* larvae were bled and hemolymph was collected in anti-coagulant buffer<sup>90</sup>. Hemocytes were recovered by a short centrifugation at 800 g for 1 min at 4 °C. The hemocyte pellet was immediately flash-frozen. Then, larvae were dissected and fat bodies and midguts were extracted, rinsed with PBS, flash-frozen with liquid nitrogen in eppendorf tubes and conserved at -80 °C until use. After thawing, 1 mL of Trizol (Life technologies) was added and pooled organs were grounded with a TissueLyzer 85210 Rotator (Qiagen) with one stainless steel bead (3 mm diameter) at 30 Hz for 3 min. Grounded tissues were transferred in new eppendorf tubes and left at room temperature for 5 min then 200 µL of chloroform (Interchim) were added. The preparations were homogenized and left at room temperature for 2 min. After a centrifugation at 15,000 g and 4 °C for 15 min, the aqueous phase was transferred in new eppendorf tubes. Four hundred µL of 70% ethanol were added and nucleic acid extraction was immediately done with the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Contaminating DNA was removed by the use of a Turbo DNA-free™ kit (Life Technologies) according to the manufacturer's protocol.

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

**Library preparation and Illumina sequencing.** Library preparation and RNA sequencing were conducted by MGX GenomiX (IGE, Montpellier, France). Libraries were prepared with the TruSeq Stranded mRNA Sample preparation kit (Illumina). In brief, after a purification step with oligo(dT) magnetic beads, polyadenylated RNAs were chemically fragmented. A first cDNA strand was synthesized with random primers and SuperScript IV Reverse Transcriptase (Life Technologies) and the second strand was then synthesized. After the addition of single adenine nucleotides, indexed adapters were ligated to the cDNA ends. Adapters-ligated cDNAs were then amplified by PCR and libraries were validated on Fragment Analyzer with a Standard Sensitivity NGS kit (Advanced Analytical Technologies, Inc) and quantified by qPCR with a Light Cycler 480 thermal cycler (Roche Molecular diagnostics).

cDNAs were sequenced with the HiSeq 2500 system (Illumina) on 50 base pairs with a single-end protocol. In brief, libraries were equimolarly pooled and cDNAs were denatured, diluted to 8 pM and injected in the flow cell. The samples were multiplexed by 6 and a PhiX spike control was used. Clusters were generated with a cluster generation kit (Illumina), cDNAs were sequenced by synthesis. Image analysis and base calling were realized with the HiSeq Control Software (Illumina) and the RTA software (Illumina), respectively. After a demultiplexing step, the sequences quality and the absence of contaminant were verified with the FastQC software and the FastQ Screen

software, respectively. Raw data were submitted to a Purity Filter (Illumina) to remove overlapping clusters. FastQ files are available at ArrayExpress accession E-MTAB-7976.

**Alignment and counting.** For each sample, the reads were pseudoaligned on the *S. frugiperda* reference transcriptome version OGS2.2<sup>38</sup> using Bowtie2.2.3<sup>91</sup>. Processing of alignment files (.sam files) into sorted.bam files was performed by samtools view and samtools sort commands<sup>92</sup>. Read counts for each gene were obtained using samtools idxstats command.

**Differential expression analysis.** Differential expression was analyzed with the R package DESeq2<sup>39</sup>. Treated versus untreated samples of the same tissue + time conditions were analyzed using a classical method. An example is shown in Supplementary Data S2 for the analysis of differential expression in the fat body at 8 hpi identifying 5 DE genes at a p-value adjusted of 0.01, equivalent to 1% false discovery rate (Supplementary Fig. S2).

For the global analysis of the EPN effect, we used the Likelihood Ratio test function of DESeq2 as presented in Supplementary Data S3. At an adjusted p-value of 0.1, this method identified 271 DE genes associated to EPN treatment. We overlapped this list with the pair-wise comparisons above to define tissue-specific or common responses as shown in Fig. 1E.

Each sub list of DE genes has been analyzed with Blast2GO Pro software<sup>93</sup> to identify homolog sequences by blastx as well as GO categories. By using the full list of *S. frugiperda* OGS2.2 transcripts as reference<sup>38</sup>, the enriched GO terms were identified with a Fisher's exact test (one-tailed, FDR < 0.05).

Heatmaps were generated using the heatmap.2 function of the gplots R package such as this presented in Supplementary Data S4 that generated Fig. 1D.

**qPCR and primers.** Differential expression data were verified with control RT-qPCR on selected upregulated and downregulated genes on independently performed EPN infestation experiments. cDNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen) from 1 µg of RNA sample, according to the manufacturer's protocol.

The primers (Supplementary Table S1) were designed with the Primer3Web tool<sup>94</sup>. Their efficiency was estimated by using serial dilutions of pooled cDNA samples and their specificity was verified with melting curves analysis. Amplification and melting curves were analyzed with the LightCycler 480 software (Roche Molecular diagnostics) version 1.5.0.

RT-qPCR were carried out in triplicate for each biological sample, with the LightCycler 480 SYBR Green I Master kit (Roche Molecular diagnostics). For each couple of sample and primer, 1.25 µL of sample containing 50 ng/µL of cDNA and 1.75 µL of Master mix containing 0.85 µM of primers were distributed in multiwell plates by the Echo 525 liquid handler (Labcyte). After an enzyme activation step of 95 °C for 15 min, the amplification was monitored in the LightCycler 480 (Roche) thermal cycler for 45 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 15 s.

Crossing points were determined using the Second Derivative Maximum method with the LightCycler 480 software (Roche Molecular diagnostics) version 1.5.0. Relative expression quantifications were then processed with the REST 2009 software<sup>95</sup>, using the pairwise fixed randomization test with 2,000 permutations. Targets relative levels were normalized to RpL32 housekeeping gene relative levels and the EF1 gene was used as an internal control<sup>96–98</sup>.

## References

- Lacey, L. A. *et al.* Insect pathogens as biological control agents: Back to the future. *Journal of invertebrate pathology* **132**, 1–41, <https://doi.org/10.1016/j.jip.2015.07.009> (2015).
- Ehlers, R. U. & Hokkanen, H. M. T. Insect biocontrol with non-endemic entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* spp): Conclusions and recommendations of a combined OECD and COST Workshop on Scientific and Regulatory Policy Issues. *Biocontrol Science and Technology* **6**, 295–302, <https://doi.org/10.1080/09583159631280> (1996).
- Negrisoni, A. S., Garcia, M. S., Negrisoni, C. R. C. B., Bernardi, D. & da Silva, A. Efficacy of entomopathogenic nematodes (Nematoda: Rhabditida) and insecticide mixtures to control *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae) in corn crops. *Crop Protection* **29**, 677–683, <https://doi.org/10.1016/j.cropro.2010.02.002> (2010).
- Viteri, D. M., Linares, A. M. & Flores, L. Use of the entomopathogenic nematode *Steinernema carpocapsae* in combination with low-toxicity insecticides to control fall armyworm (Lepidoptera: Noctuidae) Larvae. *Florida Entomologist* **101**, 327–329, <https://doi.org/10.1653/024.101.0228> (2018).
- Koppenhofer, A. M., Grewal, P. S. & Fuzy, E. M. Differences in penetration routes and establishment rates of four entomopathogenic nematode species into four white grub species. *Journal of invertebrate pathology* **94**, 184–195, <https://doi.org/10.1016/j.jip.2006.10.005> (2007).
- Balasubramanian, N., Toubarro, D. & Simoes, N. Biochemical study and *in vitro* insect immune suppression by a trypsin-like secreted protease from the nematode *Steinernema carpocapsae*. *Parasite Immunology* **32**, 165–175, <https://doi.org/10.1111/j.1365-3024.2009.01172.x> (2010).
- Chang, D. Z., Serra, L., Lu, D., Mortazavi, A. & Dillman, A. R. A core set of venom proteins is released by entomopathogenic nematodes in the genus *Steinernema*. *PLoS Pathogens* **15**, e1007626, <https://doi.org/10.1371/journal.ppat.1007626> (2019).
- Crawford, J. M., Portmann, C., Zhang, X., Roeflaers, M. B. J. & Clardy, J. Small molecule perimeter defense in entomopathogenic bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 10821–10826, <https://doi.org/10.1073/pnas.1201160109> (2012).
- Dillman, A. R. *et al.* Comparative genomics of *Steinernema* reveals deeply conserved gene regulatory networks. *Genome Biology* **16**, <https://doi.org/10.1186/s13059-015-0746-6> (2015).
- Duvic, B. *et al.* Cecropins as a marker of *Spodoptera frugiperda* immunosuppression during entomopathogenic bacterial challenge. *Journal of insect physiology* **58**, 881–888, <https://doi.org/10.1016/j.jinsphys.2012.04.001> (2012).
- Ji, D. & Kim, Y. An entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibits the expression of an antibacterial peptide, cecropin, of the beet armyworm, *Spodoptera exigua*. *Journal of insect physiology* **50**, 489–496, <https://doi.org/10.1016/j.jinsphys.2004.03.005> (2004).
- Kim, I. H. *et al.* The insect pathogenic bacterium *Xenorhabdus innexi* has attenuated virulence in multiple insect model hosts yet encodes a potent mosquitocidal toxin. *BMC genomics* **18**, <https://doi.org/10.1186/s12864-017-4311-4> (2017).

13. Lu, D. *et al.* Activated entomopathogenic nematode infective juveniles release lethal venom proteins. *PLoS Pathog* **13**, e1006302, <https://doi.org/10.1371/journal.ppat.1006302> (2017).
14. Toubarro, D. *et al.* A serpin released by an entomopathogen impairs clot formation in insect defense system. *PLoS one* **8**, e69161, <https://doi.org/10.1371/journal.pone.0069161> (2013).
15. Walter, T. N., Dunphy, G. B. & Mandato, C. A. *Steinernema carpocapsae* DD136: metabolites limit the non-self adhesion responses of haemocytes of two lepidopteran larvae, *Galleria mellonella* (F. Pyralidae) and *Malacosoma disstria* (F. Lasiocampidae). *Experimental Parasitology* **120**, 161–174, <https://doi.org/10.1016/j.exppara.2008.07.001> (2008).
16. Sicard, M. *et al.* Stages of infection during the tripartite interaction between *Xenorhabdus nematophila*, its nematode vector, and insect hosts. *Applied and environmental microbiology* **70**, 6473–6480, <https://doi.org/10.1128/AEM.70.11.6473-6480.2004> (2004).
17. Dowds, B. C. A. & Peters, A. In *Entomophthogenic Nematology* (ed R. Gaugler) 69–96 (CABI Publishing, 2002).
18. Sadd, B. M. & Schmid-Hempel, P. Insect immunity shows specificity in protection upon secondary pathogen exposure. *Current Biology* **16**, 1206–1210, <https://doi.org/10.1016/j.cub.2006.04.047> (2006).
19. Tassetto, M., Kunitomi, M. & Andino, R. Circulating immune cells mediate a systemic RNAi-based adaptive antiviral response in *Drosophila*. *Cell* **169**, 314–325, <https://doi.org/10.1016/j.cell.2017.03.033> (2017).
20. Lemaitre, B. & Hoffmann, J. A. The host defense of *Drosophila melanogaster*. *Annual Review of Immunology* **25**, 697–743, <https://doi.org/10.1146/annurev.immunol.25.022106.141615> (2007).
21. Tanaka, H. *et al.* A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori*. *Insect biochemistry and molecular biology* **38**, 1087–1110, <https://doi.org/10.1016/j.ibmb.2008.09.001> (2008).
22. Zou, Z. *et al.* Comparative genomic analysis of the *Tribolium* immune system. *Genome Biology* **8**, R177, <https://doi.org/10.1186/Gb-2007-8-8-R177> (2007).
23. Cao, X. *et al.* The immune signaling pathways of *Manduca sexta*. *Insect biochemistry and molecular biology* **62**, 64–74, <https://doi.org/10.1016/j.ibmb.2015.03.006> (2015).
24. Evans, J. D. *et al.* Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect molecular biology* **15**, 645–656, <https://doi.org/10.1111/j.1365-2583.2006.00682.x> (2006).
25. Gerardo, N. M. *et al.* Immunity and other defenses in pea aphids, *Acyrtosiphon pisum*. *Genome Biology* **11**, R21, <https://doi.org/10.1186/gb-2010-11-2-r21> (2010).
26. Ferrandon, D. The complementary facets of epithelial host defenses in the genetic model organism *Drosophila melanogaster*: from resistance to resilience. *Current opinion in immunology* **25**, 59–70, <https://doi.org/10.1016/j.coi.2012.11.008> (2013).
27. Kristensen, N. & Chauvin, G. In *Handbook of Zoology Vol. IV* (ed W. de Gruyter) 1–8 (W. de Gruyter, 2012).
28. Lehane, M. J. Peritrophic matrix structure and function. *Annual Review of Entomology* **42**, 525–550, <https://doi.org/10.1146/annurev.ento.42.1.525> (1997).
29. Strand, M. R. The insect cellular immune response. *Insect Science* **15**, 1–14, <https://doi.org/10.1111/j.1744-7917.2008.00183.x> (2008).
30. Jiravanichpaisal, P., Lee, B. L. & Soderhall, K. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* **211**, 213–236, <https://doi.org/10.1016/j.imbio.2005.10.015> (2006).
31. Nakhleh, J., El Moussawi, L. & Osta, M. A. Chapter Three - The Melanization Response in Insect Immunity. In *Advances in Insect Physiology* Vol. 52 (ed Petros Ligoxygakis) 83–109 (Academic Press, 2017).
32. Ferrandon, D., Imler, J. L., Hetru, C. & Hoffmann, J. A. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. *Nature reviews immunology* **7**, 862–874, <https://doi.org/10.1038/nri2194> (2007).
33. Issa, N. *et al.* The circulating protease Persephone is an immune sensor for microbial proteolytic activities upstream of the *Drosophila* Toll pathway. *Molecular Cell* **69**, 539–550 e536, <https://doi.org/10.1016/j.molcel.2018.01.029> (2018).
34. Arefin, B. *et al.* Genome-wide transcriptional analysis of *Drosophila* larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins. *Journal of Innate Immunity* **6**, 192–204, <https://doi.org/10.1159/000353734> (2014).
35. Castillo, J. C. *et al.* *Drosophila* anti-nematode and antibacterial immune regulators revealed by RNA-Seq. *BMC genomics* **16**, 519, <https://doi.org/10.1186/s12864-015-1690-2> (2015).
36. Yadav, S., Daugherty, S., Shetty, A. C. & Eleftherianos, I. RNAseq analysis of the *Drosophila* response to the entomopathogenic nematode *Steinernema*. *G3 (Bethesda)* **7**, 1955–1967, <https://doi.org/10.1534/g3.117.041004> (2017).
37. Goergen, G., Kumar, P. L., Sankung, S. B., Togola, A. & Tamo, M. First Report of Outbreaks of the Fall Armyworm Spodoptera frugiperda (J E Smith) (Lepidoptera, Noctuidae), a new alien invasive pest in West and Central Africa. *PLoS one* **11**, e0165632, <https://doi.org/10.1371/journal.pone.0165632> (2016).
38. Gouin, A. *et al.* Two genomes of highly polyphagous lepidopteran pests (*Spodoptera frugiperda*, Noctuidae) with different host-plant ranges. *Scientific reports* **7**, 11816, <https://doi.org/10.1038/s41598-017-10461-4> (2017).
39. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**, 550, <https://doi.org/10.1186/s13059-014-0550-8> (2014).
40. Zhang, L., Wang, Y. W. & Lu, Z. Q. Midgut immune responses induced by bacterial infection in the silkworm, *Bombyx mori*. *Journal of Zhejiang University. Science. B* **16**, 875–882, <https://doi.org/10.1631/jzus.B1500060> (2015).
41. Poinar, G. O. & Himsforth, P. T. *Neoplectana* parasitism of larvae of the greater wax moth *Galleria mellonella*. *Journal of invertebrate pathology* **9**, 241–246 (1967).
42. Biteau, B. *et al.* Lifespan extension by preserving proliferative homeostasis in *Drosophila*. *PLoS Genetics* **6**, e1001159, <https://doi.org/10.1371/journal.pgen.1001159> (2010).
43. Feyereisen, R. Insect P450 enzymes. *Annual Reviews of Entomology* **44**, 507–533, <https://doi.org/10.1146/annurev.ento.44.1.507> (1999).
44. Kleino, A. *et al.* Inhibitor of apoptosis 2 and TAK1-binding protein are components of the *Drosophila* Imd pathway. *EMBO Journal* **24**, 3423–3434, <https://doi.org/10.1038/sj.emboj.7600807> (2005).
45. Brivio, M. F., Mastore, M. & Moro, M. The role of *Steinernema feltiae* body-surface lipids in host-parasite immunological interactions. *Molecular and Biochemical Parasitology* **135**, 111–121 (2004).
46. Liu, H. *et al.* *Steinernema glaseri* surface enolase: molecular cloning, biological characterization, and role in host immune suppression. *Molecular and Biochemical Parasitology* **185**, 89–98, <https://doi.org/10.1016/j.molbiopara.2012.06.006> (2012).
47. Toubarro, D., Avila, M. M., Montiel, R. & Simoes, N. A pathogenic nematode targets recognition proteins to avoid insect defenses. *PLoS one* **8**, <https://doi.org/10.1371/journal.pone.0075691> (2013).
48. Balasubramanian, N., Hao, Y. J., Toubarro, D., Nascimento, G. & Simoes, N. Purification, biochemical and molecular analysis of a chymotrypsin protease with prophenoloxidase suppression activity from the entomopathogenic nematode *Steinernema carpocapsae*. *International Journal for Parasitology* **39**, 975–984, <https://doi.org/10.1016/j.ijpara.2009.01.012> (2009).
49. Arrese, E. L. & Soulages, J. L. Insect fat body: energy, metabolism, and regulation. *Annual Review of Entomology* **55**, 207–225, <https://doi.org/10.1146/annurev-ento-112408-085356> (2010).
50. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. & Hoffmann, J. A. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* **86**, 973–983 (1996).
51. Weber, A. N. *et al.* Binding of the *Drosophila* cytokine Spatzle to Toll is direct and establishes signaling. *Nature immunology* **4**, 794–800, <https://doi.org/10.1038/ni955> (2003).

52. Lavine, M. D. & Strand, M. R. Insect hemocytes and their role in immunity. *Insect biochemistry and molecular biology* **32**, 1295–1309 (2002).
53. Bidla, G., Lindgren, M., Theopold, U. & Dushay, M. S. Hemolymph coagulation and phenoloxidase in *Drosophila* larvae. *Developmental and Comparative Immunology* **29**, 669–679, <https://doi.org/10.1016/j.dci.2004.11.007> (2005).
54. Kanost, M. R. & Gorman, M. J. Phenoloxidases in insect immunity. In *Insect Immunology* (ed Nancy E. Beckage) 69–96 (Academic Press, 2008).
55. Lu, A. *et al.* Insect prophenoloxidase: the view beyond immunity. *Frontiers in Physiology* **5**, 252–252, <https://doi.org/10.3389/fphys.2014.00252> (2014).
56. Bartholomay, L. C. *et al.* Description of the transcriptomes of immune response-activated hemocytes from the mosquito vectors *Aedes aegypti* and *Armigeres subalbatus*. *Infection and immunity* **72**, 4114–4126, <https://doi.org/10.1128/iai.72.7.4114-4126.2004> (2004).
57. Dimopoulos, G. *et al.* Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection. *Proceedings of the National Academy of Sciences USA* **99**, 8814–8819, <https://doi.org/10.1073/pnas.092274999> (2002).
58. Irving, P. *et al.* New insights into *Drosophila* larval haemocyte functions through genome-wide analysis. *Cell Microbiology* **7**, 335–350, <https://doi.org/10.1111/j.1462-5822.2004.00462.x> (2005).
59. Jiang, H. *et al.* Molecular identification of a bevy of serine proteinases in *Manduca sexta* hemolymph. *Insect biochemistry and molecular biology* **35**, 931–943, <https://doi.org/10.1016/j.ibmb.2005.03.009> (2005).
60. Kanost, M. R. & Jiang, H. Clip-domain serine proteases as immune factors in insect hemolymph. *Current Opinion in Insect Science* **11**, 47–55, <https://doi.org/10.1016/j.cois.2015.09.003> (2015).
61. Haghayeghi, A., Sarac, A., Czerniecki, S., Grosshans, J. & Schock, F. Pellino enhances innate immunity in *Drosophila*. *Mechanisms of Development* **127**, 301–307, <https://doi.org/10.1016/j.mod.2010.01.004> (2010).
62. Ji, S. *et al.* Cell-surface localization of Pellino antagonizes Toll-mediated innate immune signalling by controlling MyD88 turnover in *Drosophila*. *Nature Communications* **5**, 3458, <https://doi.org/10.1038/ncomms4458> (2014).
63. Volkoff, A. N. *et al.* Characterization and transcriptional profiles of three *Spodoptera frugiperda* genes encoding cysteine-rich peptides. A new class of defensin-like genes from lepidopteran insects? *Gene* **319**, 43–53 (2003).
64. Destoumieux-Garzon, D. *et al.* *Spodoptera frugiperda* X-tox protein, an immune related defensin rosary, has lost the function of ancestral defensins. *PLoS one* **4**, e6795, <https://doi.org/10.1371/journal.pone.0006795> (2009).
65. Michel, T., Reichhart, J. M., Hoffmann, J. A. & Royet, J. *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature* **414**, 756–759, <https://doi.org/10.1038/414756a> (2001).
66. Zhao, S. *et al.* A novel peptidoglycan recognition protein involved in the prophenoloxidase activation system and antimicrobial peptide production in *Antheraea pernyi*. *Developmental and Comparative Immunology* **86**, 78–85, <https://doi.org/10.1016/j.dci.2018.04.009> (2018).
67. Krautz, R., Arefin, B. & Theopold, U. Damage signals in the insect immune response. *Frontiers in Plant Science* **5**, <https://doi.org/10.3389/fpls.2014.00342> (2014).
68. Qiao, C. *et al.* SRP gene is required for *Helicoverpa armigera* prophenoloxidase activation and nodulation response. *Developmental and Comparative Immunology* **44**, 94–99, <https://doi.org/10.1016/j.dci.2013.11.016> (2014).
69. Senger, K., Harris, K. & Levine, M. GATA factors participate in tissue-specific immune responses in *Drosophila* larvae. *Proceedings of the National Academy of Sciences USA* **103**, 15957–15962, <https://doi.org/10.1073/pnas.0607608103> (2006).
70. Vilcinskis, A. & Wedde, M. Insect inhibitors of metalloproteinases. *IUBMB life* **54**, 339–343, <https://doi.org/10.1080/15216540216040> (2002).
71. Wedde, M., Weise, C., Kopacek, P., Franke, P. & Vilcinskis, A. Purification and characterization of an inducible metalloprotease inhibitor from the hemolymph of greater wax moth larvae, *Galleria mellonella*. *European journal of biochemistry* **255**, 535–543 (1998).
72. Clermont, A. *et al.* Cloning and expression of an inhibitor of microbial metalloproteinases from insects contributing to innate immunity. *The Biochemical Journal* **382**, 315–322, <https://doi.org/10.1042/bj20031923> (2004).
73. Caldas, C., Cherqui, A., Pereira, A. & Simões, N. Purification and characterization of an extracellular protease from *Xenorhabdus nematophila* involved in insect immunosuppression. *Applied and environmental microbiology* **68**, 1297–1304, <https://doi.org/10.1128/AEM.68.3.1297-1304> (2002).
74. Jing, Y. J., Toubarro, D., Hao, Y. J. & Simoes, N. Cloning, characterisation and heterologous expression of an astacin metalloprotease, Sc-AST, from the entomoparasitic nematode *Steinernema carpocapsae*. *Molecular and Biochemical Parasitology* **174**, 101–108, <https://doi.org/10.1016/j.molbiopara.2010.07.004> (2010).
75. Massaoud, M. K., Marokhazi, J. & Venekei, I. Enzymatic characterization of a serralsin-like metalloprotease from the entomopathogen bacterium, *Xenorhabdus*. *Biochimica et biophysica acta* **1814**, 1333–1339, <https://doi.org/10.1016/j.bbapap.2011.05.008> (2011).
76. Schmidt, T. M., Bleakley, B. & Neelson, K. H. Characterization of an extracellular protease from the insect pathogen *Xenorhabdus luminescens*. *Applied and environmental microbiology* **54**, 2793–2797 (1988).
77. Myllymaki, H., Valanne, S. & Ramet, M. The *Drosophila* imd signaling pathway. *Journal of immunology* **192**, 3455–3462, <https://doi.org/10.4049/jimmunol.1303309> (2014).
78. Kakumani, P. K., Malhotra, P., Mukherjee, S. K. & Bhatnagar, R. K. A draft genome assembly of the army worm, *Spodoptera frugiperda*. *Genomics* **104**, 134–143, <https://doi.org/10.1016/j.ygeno.2014.06.005> (2014).
79. Nandakumar, S., Ma, H. & Khan, A. S. Whole-genome sequence of the *Spodoptera frugiperda* Sf9 insect cell line. *Genome announcements* **5**, <https://doi.org/10.1128/genomeA.00829-17> (2017).
80. Cheng, T. *et al.* Genomic adaptation to polyphagy and insecticides in a major East Asian noctuid pest. *Nature ecology & evolution* **1**, 1747–1756, <https://doi.org/10.1038/s41559-017-0314-4> (2017).
81. Pearce, S. L. *et al.* Genomic innovations, transcriptional plasticity and gene loss underlying the evolution and divergence of two highly polyphagous and invasive *Helicoverpa* pest species. *BMC biology* **15**, 63, <https://doi.org/10.1186/s12915-017-0402-6> (2017).
82. Minakhina, S., Yang, J. & Steward, R. Tamo selectively modulates nuclear import in *Drosophila*. *Genes to Cells* **8**, 299–310, <https://doi.org/10.1046/j.1365-2443.2002.00634.x> (2003).
83. Chou, S. *et al.* Transferred interbacterial antagonism genes augment eukaryotic innate immune function. *Nature* **518**, 98–101, <https://doi.org/10.1038/nature13965> (2015).
84. Aymeric, J. L., Givaudan, A. & Duvic, B. Imd pathway is involved in the interaction of *Drosophila melanogaster* with the entomopathogenic bacteria, *Xenorhabdus nematophila* and *Photorhabdus luminescens*. *Molecular Immunology* **47**, 2342–2348, <https://doi.org/10.1016/j.molimm.2010.05.012> (2010).
85. Mastore, M., Arizza, V., Manachini, B. & Brivio, M. F. Modulation of immune responses of *Rhynchophorus ferrugineus* (Insecta: Coleoptera) induced by the entomopathogenic nematode *Steinernema carpocapsae* (Nematoda: Rhabditida). *Insect Science* **22**, 748–760, <https://doi.org/10.1111/1744-7917.12141> (2015).
86. Pena, J. M., Carrillo, M. A. & Hallem, E. A. Variation in the susceptibility of *Drosophila* to different entomopathogenic nematodes. *Infection and Immunity* **83**, 1130–1138, <https://doi.org/10.1128/IAI.02740-14> (2015).
87. Binda-Rossetti, S., Mastore, M., Protasoni, M. & Brivio, M. F. Effects of an entomopathogen nematode on the immune response of the insect pest red palm weevil: Focus on the host antimicrobial response. *Journal of invertebrate pathology* **133**, 110–119, <https://doi.org/10.1016/j.jip.2015.11.001> (2016).

88. Poitout, S. Elevage de plusieurs espèces de Lépidoptères Noctuidae sur milieu artificiel riche et sur milieu artificiel simplifié. *Annales de Zoologie et Ecologie Animale* **2**, 79–91 (1970).
89. White, G. F. A method for obtaining infective nematode larvae from cultures. *Science* **66**, 302–303, <https://doi.org/10.1126/science.66.1709.302-a> (1927).
90. van Sambeek, J. & Wiesner, A. Successful parasitisation of locusts by entomopathogenic nematodes is correlated with inhibition of insect phagocytes. *Journal of invertebrate pathology* **73**, 154–161, <https://doi.org/10.1006/jipa.1998.4823> (1999).
91. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nature methods* **9**, 357–359, <https://doi.org/10.1038/nmeth.1923> (2012).
92. Li, H. *et al.* The sequence alignment/map format and SAMtools. *Bioinformatics (Oxford, England)* **25**, 2078–2079, <https://doi.org/10.1093/bioinformatics/btp352> (2009).
93. Conesa, A. *et al.* Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics (Oxford, England)* **21**, 3674–3676, <https://doi.org/10.1093/bioinformatics/bti610> (2005).
94. Untergasser, A. *et al.* Primer3 - New capabilities and interfaces. *Nucleic acids research* **40**, e115, <https://doi.org/10.1093/nar/gks596> (2012).
95. Pfaffl, M. W., Horgan, G. W. & Dempfle, L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic acids research* **30**, e36 (2002).
96. Le, S. Q. & Gascuel, O. An improved general amino acid replacement matrix. *Molecular Biology and Evolution* **25**, 1307–1320, <https://doi.org/10.1093/molbev/msn067> (2008).
97. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution; international journal of organic evolution* **39**, 783–791, <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x> (1985).
98. Kanost, M. R. *et al.* Multifaceted biological insights from a draft genome sequence of the tobacco hornworm moth, *Manduca sexta*. *Insect biochemistry and molecular biology* **76**, 118–147, <https://doi.org/10.1016/j.ibmb.2016.07.005> (2016).

## Acknowledgements

This work was supported by grants from the French Institut National de la Recherche Agronomique to BD & NN, of Institut Universitaire de France to NN. DS acknowledge financial support from France Génomique National infrastructure, funded as part of “Investissement d’avenir” program managed by Agence Nationale pour la Recherche (contract ANR-10-INBS-09). We thank the quarantine insect platform (PIQ), member of the Vectopole Sud network, for providing the infrastructure needed for pest insect experimentations. We are also grateful to Clotilde Gibard and Gaëtan Clabots for maintaining the insect collections of the DGIMI laboratory in Montpellier.

## Author Contributions

N.N. and B.D. conceived and directed this study. L.H. and P.-A.G. performed the infestation experiments. L.H., P.-A.G., N.N. and B.D. performed dissections. L.H. and P.-A.G. extracted and purified the RNA. D.S. performed the RNAseq. L.H., S.G. and N.N. analyzed the data. L.H. and S.G. performed the qPCRs. L.H., N.N. and B.D. wrote the manuscript. All authors have read and approved the manuscript.

## Additional Information

**Supplementary information** accompanies this paper at <https://doi.org/10.1038/s41598-019-49410-8>.

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

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2019