



Swansea University
Prifysgol Abertawe



Cronfa - Swansea University Open Access Repository

This is an author produced version of a paper published in :

Virulence

Cronfa URL for this paper:

<http://cronfa.swan.ac.uk/Record/cronfa26752>

Paper:

Dubovskiy, I., Grizanova, E., Whitten, M., Mukherjee, K., Greig, C., Alikina, T., Kabilov, M., Vilcinskas, A., Glupov, V. & Butt, T. (2016). Immuno-physiological adaptations confer wax moth *Galleria mellonella* resistance to *Bacillus thuringiensis*. *Virulence*, 00-00.

<http://dx.doi.org/10.1080/21505594.2016.1164367>

This article is brought to you by Swansea University. Any person downloading material is agreeing to abide by the terms of the repository licence. Authors are personally responsible for adhering to publisher restrictions or conditions. When uploading content they are required to comply with their publisher agreement and the SHERPA RoMEO database to judge whether or not it is copyright safe to add this version of the paper to this repository.

<http://www.swansea.ac.uk/iss/researchsupport/cronfa-support/>

1 **Immuno-physiological adaptations confer wax moth *Galleria mellonella***
2 **resistance to *Bacillus thuringiensis***

3 **Dubovskiy^{a1}, I.M., Grizanova^a, E.V., Whitten^b, M.M.A., Mukherjee^c, K.,**
4 **Greig^c, C., Alikina^f, T., Kabilov^f, M., Vilcinskas^d, A., Glupov^a, V.V., Butt^c, T.M.**

- 5 a. Institute of Systematics and Ecology of Animals, Siberian Branch of
6 Russian Academy of Science, Novosibirsk 630091, Russia
7 b. Institute of Life Science, College of Medicine, Swansea University,
8 Singleton Park, Swansea SA2 8PP, UK.
9 c. Department of Biosciences, College of Science, Swansea University,
10 Singleton Park, Swansea SA2 8PP, UK.
11 d. Institute for Insect Biotechnology, Justus-Liebig University, 26-32 Heinrich-
12 Buff-Ring, Giessen 35392, Germany
13 e. Fraunhofer Institute for Molecular Biology and Applied Ecology,
14 Department of Bioresources, Winchester Str. 2, 35394 Giessen, Germany
15 f. Institute of Chemical Biology and Fundamental Medicine, Siberian Branch
16 of Russian Academy of Science, Novosibirsk, Russia

17

18 ¹ Corresponding author: Ivan Dubovskiy, e-mail: dubovskiy2000@yahoo.com

19

20 **Competing Interests:** The authors have declared that no competing interests exist.

21

22

23 **ABSTRACT**

24 Microevolutionary mechanisms of resistance to a bacterial pathogen were explored
25 in a population of the Greater wax moth, *Galleria mellonella*, selected for an 8.8-
26 fold increased resistance against the entomopathogenic bacterium *Bacillus*
27 *thuringiensis* (Bt) compared with a non-selected (susceptible) line. Defence

28 strategies of the resistant and susceptible insect lines were compared to uncover
29 mechanisms underpinning resistance, and the possible cost of those survival
30 strategies. In the uninfected state, resistant insects exhibited enhanced basal
31 expression of genes related to regeneration and amelioration of Bt toxin activity in
32 the midgut. In addition, these insects also exhibited elevated activity of genes
33 linked to inflammation/stress management and immune defence in the fat body.
34 Following oral infection with Bt, the expression of these genes was further
35 elevated in the fat body and midgut of both lines and to a greater extent some of
36 them in resistant line than the susceptible line. This gene expression analysis
37 reveals a pattern of resistance mechanisms targeted to sites damaged by Bt with the
38 insect placing greater emphasis on tissue repair as revealed by elevated expression
39 of these genes in both the fat body and midgut epithelium. Unlike the susceptible
40 insects, Bt infection significantly reduced the diversity and richness (abundance) of
41 the gut microbiota in the resistant insects. These observations suggest that the
42 resistant line not only has a more intact midgut but is secreting antimicrobial
43 factors into the gut lumen which not only mitigate Bt activity but also affects the
44 viability of other gut bacteria. Remarkably the resistant line employs multifactorial
45 adaptations for resistance to Bt without any detected negative trade off since the
46 insects exhibited higher fecundity.

47

48 **Key index words or phrases**

49 Insect, experimental evolution, Bt, resistance, microevolution, immune response

50

51

52 **Introduction**

53 Both the pathogen and insect host are participants in a highly dynamic co-
54 evolutionary arms race where the insect's defences are continuously evolving to
55 keep pace with the corresponding infection adaptations of the pathogen. The
56 selective pressures driving these processes are strong and often require some form

57 of trade off. For example, resistant and susceptible insects may differ in their
58 colour, development time and fecundity ^{1,2}.

59

60 *Bacillus thuringiensis* (Bt) is a widespread Gram positive bacterium that has been
61 developed as a biopesticide to control insect pests attacking crops as well as
62 disease vectors such as mosquitoes ³. Bt must be ingested in order to infect and kill
63 its host. Bt virulence factors include enterotoxins, hemolysins, phospholipases and
64 metalloproteases, which are transcribed in the vegetative cells and play an
65 important role in the infection process ⁴. These factors are activated by the
66 quorum-sensing system PlcR-PapR ⁵. The insecticidal activity of Bt is primarily
67 due to proteinaceous crystal endotoxins (Cry), which are produced during
68 sporulation and activated by the host's gut fluids ⁶. Cry toxins can act alone (as
69 seen in genetically modified plants) but spores can also contribute to virulence ⁷.
70 The binding of toxins to receptors in the midgut epithelial cell membrane either
71 creates pores that subsequently lead to cell lysis, or they activate intracellular
72 signalling pathways that result in cell death by oncosis ^{8 9}.

73

74 There are increasing reports of resistance in insect populations to Bt; this is
75 particularly evident with crops genetically modified with the Cry toxin genes ^{10 11}.
76 The mechanisms of resistance to Bt endotoxins has been studied extensively and
77 appears to be multifaceted ⁶. Even in those cases that seem to fit a monogenic
78 model, resistance is rarely completely recessive, suggesting that resistant
79 phenotypes contain major and minor genes contributing to overall resistance ¹².
80 This fact is particularly relevant where virulence factors such as the bacterial spore
81 play a vital role in the overall toxicity of Bt -based insecticides in which case
82 development of resistance is likely to be multigenic. Indeed, disparate mechanisms
83 for resistance to Bt have been reported. The most commonly reported mechanism
84 involves reduced binding of the toxins through the alteration or loss of midgut
85 toxin-binding proteins ¹³⁻¹⁵. Other insect resistance mechanisms include
86 sequestration of the toxin by lipophorin ^{16,17}, esterases ¹⁸ or alkaline phosphatase ¹⁹,

87 absence of enzymes or environment to activate pro-toxin ²⁰, and increased stem
88 cell production in the gut to replace damaged epithelial cells ²¹. The insect gut
89 biota can also influence Bt efficacy either by degrading the toxin or initiating
90 septicaemia ^{22,23}. Resistance to Bt is also linked to the host's immune response, but
91 the role of the different defence components is often inconclusive, contradictory or
92 variable. For example, some researchers report a correlation between
93 phenoloxidase (PO) activity and Bt efficacy ²⁴, whereas others noted no differences
94 between Bt-resistant and Bt-susceptible insects ²⁵. Furthermore, no differences were
95 noted for haemocyte populations and nitric oxide levels ²⁶. Bt mediated
96 suppression of key immune components will increase the host's susceptibility to Bt
97 infections and exacerbate secondary infections by opportunistic pathogens ²⁷⁻³⁰.

98

99 This paper focuses on an artificial selection experiment designed to explore the
100 evolution of resistance of Greater wax moth *Galleria mellonella* to natural peroral
101 infections by Bt. The goal was to identify traits in the selected insects that could
102 account for their increased resistance when challenged with a Bt spore-crystal
103 mixture, and to assess any corresponding "trade-offs". Since Bt resistance is
104 multifaceted, the current study examined specific parameters: humoral immunity,
105 stress management, resource re-allocation and changes to the gut microbiome in
106 selected and non-selected lines.

107

108 **Results**

109 **Selection with *B. thuringiensis* leads to enhanced resistance of wax moth**

110 Wax moth, *G. mellonella*, were selected for resistance to *B. thuringiensis* over 20
111 generations, but the first indication of increased resistance to Bt was observed after
112 five generations when larval survival was significantly higher ($p < 0.05$) for the
113 selected resistant (R) line than for the non-selected susceptible (S) control line (SI
114 Fig 1). By the 20th generation resistance was observed at three different Bt
115 concentrations tested, and was most striking at the highest dose where mortality

116 was <40% for the R line fourth instar larvae compared with 100% for the S line
117 fourth instar larvae (SI Fig 2). At the 20th generation, the resistance ratio (RR) to
118 Bt of R line larvae relative to the S line was 8.8. In a separate study using a cohort
119 of 18th generation R line insects, no reversal of resistance was observed in three
120 successive generations reared on a Bt-free diet (SI Fig 3).

121

122 **High basal (uninfected) expression of immunity/stress-related genes in** 123 **resistant insects**

124 The expression of fifteen immunity, stress and inflammatory management genes,
125 inducible metalloproteases inhibitor (IMPI) and two growth factor genes was
126 measured in the midgut and fat body of uninfected control insects of the 20th
127 generation R and S lines. Several important trends were observed. The most
128 notable differences in gene expression between the R and S lines arose in the
129 midgut. Expression of IMPI, and the growth factors Contig 703 and Contig 233
130 was significantly higher in the midgut of R larvae compared with S larvae (13
131 ($p<0.05$), 23 ($p<0.05$) and 489 ($p<0.001$) fold higher, respectively) (Fig 1 A). Also
132 notable was the comparatively lower expression of HSP90 in the midgut of the R
133 line compared with the S line larvae (Fig 2 A). Relative to the S line, the basal
134 expression of most of the other immune, inflammatory and stress management
135 genes in R larvae was slightly higher in the midgut of R line larvae (2 fold change)
136 (Fig 1 A, SI Table 1). When the fat body genes of R line insects are examined as
137 functional clusters there is a trend towards increased expression of AMPs, IMPI,
138 stress and inflammation management genes compared with the S larvae (3-6 fold
139 change) (Fig 1 B, SI Table 1). Furthermore, in comparing the midgut with the fat
140 body, the R line expression of growth factors was significantly higher ($p<0.01$) but
141 AMPs / immunity and stress management significantly lower ($p<0.05$) (SI Fig 4).

142

143 **Enhanced expression of immunity/stress-related genes in infected resistant**
144 **insects**

145 Tissue-specific differences in gene expression were noted for both the R and S
146 lines following infection (Fig 1). Whereas expression of most genes increased
147 relative to basal expression, particularly in the fat body, others appeared unchanged
148 and a few were downregulated (Fig 1). Genes coding for growth factors, ROS and
149 inflammation management, which were already highly expressed in the fat body of
150 uninfected R insects, were further elevated following infection with Bt (10-80 fold
151 $p < 0.05$ and 5-100 fold $p < 0.01$, respectively; Fig 1B; SI Fig 5). Although Bt
152 infection stimulates upregulation of immune genes in both lines (SI Fig. 5), the
153 critical difference separating these lines is that immune gene expression is of a
154 higher magnitude in the R line before infection and for the majority after infection
155 (Fig. 1); this mirrors the pattern of expression observed for all other genes
156 examined (Fig.1, SI Fig.5). Susceptible insects do show an increase in expression
157 of growth factor genes (particularly Contig 233; 364 fold following infection; SI
158 Fig 5), but this is overshadowed by the significantly higher expression in the R
159 insects, which even under basal conditions was 489 fold higher than the S insects
160 (Fig 1A). Similarly, infection triggered increased expression of IMPI in the midgut
161 of both R and S lines (10 and 70 fold, respectively) but basal (uninfected)
162 expression was higher in R larvae (Fig 1A, SI Fig 5).

163

164 **Lysozyme activity in midgut elevated under Bt treatment in R and S lines**

165 Lysozyme activity was elevated 1.5 times in the midgut of infected R ($p < 0.05$) and
166 S ($p < 0.01$) lines compared with uninfected larvae from the same lines 48 hrs post
167 infection (Fig 2), however, there was no statistical difference in the level of activity
168 of R and S line insects in either the basal or infected state (Fig 2).

169

170 **Alkaline phosphatase (ALP) and aminopeptidase N (AMN) activity is lower in**
171 **Bt resistant lines**

172 ALP and AMN activity in the brush border membrane of uninfected R line insects
173 were ca. 82% and 31% lower than those of the S larvae, respectively ($p < 0.001$, Fig
174 3).

175

176 **Midgut bacterial community changes following Bt infection**

177 Taxonomic classification based on 16S rRNA gene sequencing of bacteria in the
178 midgut of S and R line larvae revealed that bacterial communities were dominated
179 by only a few phyla, with over 99.5% of the community being represented by four
180 phyla (average relative abundance values averaged across all uninfected larvae):
181 Firmicutes ($80.7 \pm 6.3\%$), Proteobacteria ($11.8 \pm 4.5\%$), Actinobacteria (3.9 ± 1.6) and
182 Bacteroidetes (3.1 ± 1.1) (Fig 4A).

183

184 Infection of both lines with Bt led to a shift in dominance from the Firmicutes
185 ($80.7 \pm 6.3\%$) to the Proteobacteria ($86.3 \pm 2.6\%$) ($p < 0.001$) (Fig 4A, 4B).
186 Uninfected R line had significantly more *Enterobacter* than the S larvae, however,
187 upon infection with Bt the levels were both much elevated but to the same degree
188 (SI, Fig. 6). *Pseudomonas* was present at similar but low levels in uninfected R and
189 S larvae, but Bt infection resulted in opposite effects on the two lines. In the case
190 of the R line no *Pseudomonas* was detected, while there was an increase in the S
191 line relative to the uninfected insects ($p < 0.05$, SI Fig 6). Phenomena common to
192 both lines were the disappearance of several genera (e.g. Micrococcineae) post-
193 infection and a huge shift in dominance from *Enterococcus* (Gram +ve) in
194 uninfected to *Enterobacter* (Gram -ve) in infected insects (Fig 4; SI Fig 6). No
195 Bacillaceae were detected in uninfected R and S lines but small amounts (2-3%)
196 were detected post-infection (SI Fig 6). Most striking was the significant reduction
197 in richness and diversity of bacterial communities in the midgut of the infected R
198 line, because such changes were not observed in the S line (Fig 5). In the infected
199 R line, there was a significant ($p < 0.01$) depletion of the community quantitative

200 index (richness) i.e. there was a decrease in the number of detectable bacterial
201 phylotypes (Fig 5A). Similarly, the Shannon (diversity) index revealed a
202 significant decline in abundance and species evenness of each phylotype in the
203 infected R line ($p < 0.05$, Fig 5B).

204

205 **Life history traits of R and S line insects**

206 There was no difference in survival rate between uninfected R and S insects.
207 Interestingly, uninfected R line insects had significantly greater pupal biomass for
208 both males (15%) and females (18%) compared with S line insects (both $p < 0.05$)
209 (Fig 6A). Adult fecundity was also significantly enhanced (up to 25%) with the
210 average R moth laying more eggs than the S counterpart ($p < 0.001$) (Fig 6B).

211

212

213 **Discussion**

214 This study shows that laboratory populations of wax moth larvae developed
215 resistance to Bt in a relatively short time, and that this was retained even after
216 removal of the selective pressure. The R line implemented several complementary
217 strategies, maintained even in the uninfected state, but which could be further
218 activated upon infection. These included cellular repair, antimicrobial activity,
219 limiting Cry toxin and toxin receptor sites, mitigating inflammation and stress.
220 Besides midgut repair and reduced receptor sites, which are well known
221 mechanisms, this study is the first to implicate the possible role of antimicrobial
222 peptides (AMPs) and inflammation/stress management in evolution of resistance to
223 Bt, and to demonstrate the importance of their elevated, constitutive activity. In
224 addition, it reports an unusual positive trade-off resulting in increased fecundity.

225 It can be hypothesized that elevated basal expression of defence and repair
226 genes enables the R line to pre-empt infection or rapidly mitigate the damage
227 caused by Bt. This rarely reported phenomenon was also described as a strategy for
228 resistance to entomopathogenic fungi in melanic wax moth larvae^{31, 32}. It appears

229 that insects resistant to pathogens also adapt their response according to the
230 pathogen's route of entry. Thus, the focal point of fungus-resistant melanic wax
231 moth larvae is the integument³¹ whereas in the current study, the foci are the gut
232 and fat body. It is likely that resistant insects balance energy allocation between the
233 midgut and fat body defences. Activity in the midgut appears to be directed
234 towards repair and limiting toxin damage, while additional support is provided by
235 the fat body in secreting AMPs that could combat microbial breaches of the midgut
236 barrier, thereby preventing septicaemia. Elevated expression of selected AMPs was
237 also observed in *Spodoptera exigua* larvae in response to Bt Cry and Vip toxins,
238 however, the study was limited to local midgut responses in a susceptible line³³. In
239 the current study, it is unclear if the fat body is responding to signals generated by
240 and transmitted by the injured midgut and/or direct exposure to bacteria that
241 subsequently breach the gut barrier. Systemic immune responses are well
242 documented in other insects following exposure to ingested bacteria or topical
243 infections by fungal pathogens³⁴. The present study shows that not only is the R
244 line much more responsive than the S line but its expression profile, especially that
245 of AMPs, is different and deserves further investigation. Moreover, it also
246 highlights the importance of the contribution of midgut immunity to larval
247 resistance to Bt. Lysozyme was induced by both R and S lines and appears to be a
248 generic response in most insects to injury, infection or stress³⁵. Lysozyme is,
249 therefore, not a reliable indicator of insect resistance to Bt.

250 Central to Bt pathogenicity is activation of Cry proteins, of which the
251 earliest stages are mediated by the host proteases and bacterial metalloproteases³⁶.
252 Here the R larvae had enhanced basal expression of an inducible metalloprotease
253 inhibitor (IMPI), with its expression increasing during Bt infection in both R and S
254 larvae. Thus, R line insects would be in the position to limit proteolysis of the Cry
255 protein and subsequent damage to the midgut, whereas the S line would first have
256 to synthesize IMPI and this delay could profoundly influence their survival.
257 Moreover, IMPI could inactivate the Bt zinc immune inhibitor metalloproteases
258 (e.g. *InhA*), which are known to digest the hosts AMPs³⁷. Elevated IMPI is

259 complemented in the R line by reduced Cry toxin binding receptors (alkaline
260 phosphatase and aminopeptidase N) and strong inflammation and repair responses.
261 Together these activities could contribute to damage limitation by Bt toxins.

262 Bt toxins can disrupt the redox-regeneration balance in insects³⁸. In the
263 current study, the patterns of gene expression suggest that R line insects have the
264 capacity to ameliorate oxidative/inflammation damage caused by later stages of Bt
265 infection i.e. invasion of the gut epithelium. Consequently, the greatest
266 upregulation of oxidative/inflammation genes is in post infection R line insects. In
267 contrast, S line insects are incapable of mounting a similar response. Although
268 differences were observed in the expression of stress management genes in R and S
269 lines, suggesting a role for these genes in resistance, it was unclear exactly how
270 they mitigated Bt damage. Interestingly, the constitutive expression of growth
271 factor genes was higher in R than S lines but elevated upon infection with Bt,
272 which corroborates the findings of others that repair of the midgut epithelium was
273 one of the mechanisms insects resisted Bt^{21, 39}.

274 The gut of the infected R line appears to offer a hostile environment to
275 microbes as reflected in the Shannon index, which is an indicator of richness and
276 diversity. This would have significant benefit by reducing the risk of secondary
277 infections and septicaemia. The latter is one mechanism by which Bt successfully
278 kill and colonise their hosts^{4, 40, 41}. The exact mechanisms altering the gut
279 environment have not been identified but may include changes in pH, secretion of
280 AMPs into the gut lumen, and removal of antagonistic microbes. There are minor
281 fluctuations in the representation of certain bacterial groups which are specific for
282 the R line e.g. complete loss of *Pseudomonas* in Bt infected R insects. The
283 pathological significance of these changes is hard to determine without further
284 investigation of the role of the specific bacteria involved.

285 A striking feature of the R line was their larger pupal mass and higher
286 fecundity than the S line. This positive trade-off is a rare and unusual phenomenon
287 since most micro-evolutionary trade-offs are negative, such as small size and
288 reduced fecundity, which compensate for beneficial traits such as increased

289 resistance to pathogens or insecticides^{25, 31, 42}. The success of the R line may partly
290 be linked to contig 233, a growth-blocking peptide, that not only controls cell
291 proliferation and blocks juvenile hormone (JH) esterase activity¹⁸, but may also
292 elevate immune responses⁴³. Thus, contig 233 would not only prevent the onset of
293 metamorphosis from larva to pupa but also influence body size. Contig 233 has
294 high constitutive expression in the R line relative to the S line but after infection
295 expression is highly elevated in the fat body, which presumably allows the insect
296 to retain juvenility until it has attained sufficient body mass or reserves to progress
297 to the next development stage.

298

299 **Acknowledgements**

300 The authors gratefully acknowledge funding from the RFBR (Grant Number 15-
301 34-20488 mol_a_ved (IMD), 14-04-31507 mol_a (EVG), BBSRC LWEC PHABI
302 (TMB), and D.F.G Priority Programme 1399 ‘Host–parasite-coevolution—rapid
303 reciprocal adaptation and its genetic basis’, and the Hessian Ministry for Science
304 and Art via the LOEWE research focus Insect Biotechnology VI 219/3-1 (AV).
305 VVG gratefully acknowledge funding from the Russian Science Foundation
306 (project №15-14-10014) for support in study of bacterial community.

307 MATERIALS AND METHODS

308 Insects

309 For artificial selection we used insects from a laboratory population of the Greater
310 wax moth, *Galleria mellonella*, from the Institute of Systematics and Ecology of
311 Animals (ISEA), Siberian Branch Russian Academy of Sciences. The starting
312 population was separated into two lines the first was exposed to *B. thuringiensis*
313 (Bt), and selected for increased resistance to the pathogen (R line) while the second
314 consisted of the untreated susceptible control (S line). The 20th generation R and S
315 insects were compared to elucidate the resistance mechanism(s) to Bt. Also a group
316 of 400 larvae from the 18th generation R line was reared over 3 generations without
317 Bt (non-selected, NS line) to determine if resistance was reversible. The resistance
318 ratio was calculated based on the LC₅₀ of R and S lines. Fourth instar larvae have
319 been used in all experiments. Full details of insect rearing and selection are
320 provided in the Supplementary Information Experimental Procedures.

321

322 Bacterial infection

323 The insect pathogen, *Bacillus thuringiensis* ssp. *galleria*, H-serotype V, strain 69-6
324 was provided by the ISEA bacterial collection. Insects from the 20th generation
325 were Bt naïve until initiation of the experiments whereupon the susceptibility of R
326 and S lines to Bt was determined by natural peroral application of a spore-crystal
327 mixture. To quantify the differential susceptibility of the R and S lines, a cohort of
328 fourth instar larvae were starved for 2 h before being exposed to different doses of
329 Bt. The R and S larvae received predetermined sub-lethal, half-lethal, and lethal
330 doses corresponding to 5×10^8 , 1×10^9 and 5×10^9 per ml which result in 15%, 50%
331 and 100% mortality of S larvae within 5 days, respectively. To determine the
332 resistance ratio (RR) of 20th generation S and R line larvae, the LC₅₀ of R line
333 was divided by the LC₅₀ of the S line. In a parallel study, infected fourth instar
334 insects from both lines were collected 48 h post-exposure to Bt to: (1) determine
335 the bacterial content of the midgut (n=20 larvae per treatment), (2) quantify genes

336 expression in the midgut and fat body (n=9 larvae per treatment) and (3) determine
337 haemolymph lysozyme activity (n=40 larvae per treatment) in control and half-
338 lethal treatments. Experiments were carried out in triplicate. Full details of
339 bacterial culture and inoculation methods are provided in the Supplementary
340 Information Experimental Procedures.

341

342 **QRT-PCR analysis of insect immunity-related gene expression**

343 To identify resistance factors, a comparison was made in the R and S larvae of the
344 expression of genes operational under basal conditions (uninfected) and during Bt
345 infection in both fat body and midgut samples. Eighteen genes previously detected
346 as part of immune response, repair, regeneration and stress regulation in wax moth
347 were investigated^{32, 44}. These were the genes coding for the antimicrobial peptides
348 gallerimycin, galiomicin, gloverin, cecropin D and 6-tox, the siderophore
349 transferrin, the insect metalloproteinase inhibitor (IMPI), three coding for heat-
350 shock proteins (HSP-90, contig 21310 and 1489) whose activities ameliorate stress
351^{45, 46}, four coding for enzymes dealing with oxidative stress (Contigs 17373, 14880,
352 20582 and 15362), and two involved with cell proliferation (Contigs 704 and 233).
353 Gene expression was measured by real-time quantitative RT-PCR using
354 normalised cDNA samples with the Rotor-Gene 6000 (Corbett Research), with
355 Rotor-Gene SYBR Green PCR mix (Qiagen), relative to two reference genes, *18S*
356 *rRNA* (AF286298) and *Elongation Factor 1-alpha* (EF1; AF423811). Full details
357 are provided in SI Table 2.

358

359 **Midgut lysozyme-like activity**

360 Antibacterial activity in midgut was determined by a zone-of-clearance assay using
361 freeze-dried *Micrococcus lysodeikticus* as a substrate suspended in agarose. The
362 radius of the digested zone was compared with a standard curve made with egg
363 white lysozyme (EWL) and expressed as an EWL equivalent per mg of protein in

364 the samples. The experiment was repeated independently three times. Full details
365 are provided in the Supplementary Information Experimental Procedures.

366

367 **Quantification of alkaline phosphatase (ALP) and aminopeptidase-N (APN)** 368 **activities**

369 Brush border membrane vesicles (BBMV) were prepared by Mg^{2+} precipitation.
370 Specific alkaline phosphatase (ALP) and N-aminopeptidase (APN) enzymatic
371 activities of BBMV proteins were measured using p-nitrophenyl phosphate
372 disodium (pNPP) and leucine-p-nitroanilide (Sigma, St. Louis, MO, USA). One
373 enzymatic unit was defined as the amount of enzyme that would hydrolyze 1.0
374 μ mole of substrate to chromogenic product per min per mg of protein at the
375 specific reaction pH and temperature. Sixty larvae were examined for each enzyme
376 per insect line. Midguts from three insects were pooled in one sample. Data are
377 presented as the mean specific activities from 20 independent BBMV samples. The
378 experiment was repeated independently three times. Full details are provided in the
379 Supplementary Information Experimental Procedures.

380

381 **16S rRNA bacterial diversity analysis**

382 The bacterial community in the midgut of Bt infected (48 hrs post exposure) and
383 uninfected R and S larvae was analysed by 16S pyrosequencing with a MiSeq
384 Illumina sequencer. Midguts with intact contents were frozen in liquid nitrogen
385 before being homogenized using a pestle and mortar. DNA was extracted from
386 midguts using the MoBIO PowerSoil-htp 96 Well DNA Isolation kit (Carlsbad,
387 California). Each sample was amplified with bacterial 16S rRNA gene primers that
388 amplify the V3-V4 region. The experiment was repeated independently four times.
389 The mean number of analyzed sequences for each variant was 10701 sequences
390 (min 3730, max 24303) for the non-infected S line, 21027 sequences (min 9047,
391 max 48050) for the S line infected with Bt, 12670 sequences (min 4267, max
392 33005) for the non-infected R line, and 16902 sequences (min 8508, max 29322)

393 for the R line infected with Bt. Profile of the bacterial community and comparison
394 were made with CloVR-16S version 1.0 package ⁴⁷.

395 Additional processing of sequence data was performed using the “Rarefied”
396 datasets (with equivalent sampling depths) generated in QIIME by randomly
397 subsampling 3700 (high quality, chimera-free) sequences from each sample. The
398 Shannon diversity index and Chao1 richness estimates were calculated for
399 “Rarefied” datasets with CLoVR. Diversity metrics computed for OTUs for each
400 sample. Full details are provided in the Supplementary Information Experimental
401 Procedures.

402

403 **Life history traits**

404 The following life history traits were monitored in the 20th generation uninfected R
405 and S insects: survival rate of insects over a period of whole ontogenesis (300
406 individuals per line), pupal weight (200 individuals per line) and adult fecundity
407 (mean fertile egg production over 5 days per female) with 30 pairs per line. Full
408 details are provided in the Supplementary Information Experimental Procedures.

409

410 **Data analyses**

411 Data was analyzed using GraphPad Prism v4.0 (GraphPad Software Inc, USA) and
412 Statistic v6.0 (StatSoft Inc., USA). Data were checked for normal (Gaussian)
413 distribution using the Agostino-Pearson omnibus test, and if abnormally distributed
414 a more conservative non-parametric analysis was applied. In Q-RT-PCR data with
415 a Gaussian distribution, Grubbs’ extreme studentized deviate (ESD) test was used
416 to exclude extreme outliers. In order to assess overall trends associated with
417 selection for Bt resistance in basal and induced gene expression, the data from
418 three independently repeated experiments were pooled for different gene clusters:
419 immunity / AMPs (Gallerimycin, Galiomycin, Gloverin, Cecropin-D, 6-tox,
420 Contig 19932, Transferrin, 2GM Contig 20004), IMPI, growth factors (Contig 233,
421 Contig 704), ROS / inflammatory management (Contig 17373, Contig 14880,

422 Contig 20582, Contig 15362), and stress management (6GM Contig, 7GM Contig,
423 HSP 90). Individual, clustered gene and bacterial diversity (Chao and Shannon)
424 comparisons were made with t-test and non-parametric one-way ANOVA
425 (Kruskall-Wallis with Dunn's post test) respectively. Cox's proportional hazards
426 survival regression was used to quantify differences in mortality rates after
427 bacterial infections between R and S larvae. No mortality was recorded for
428 uninfected control larvae in dose mortality studies, therefore, it was unnecessary to
429 compare R and S controls. One-way ANOVA (with Tukey's post test) was used to
430 assess differences between lysozyme responses , and life history traits in R and S
431 insects. Differences between R and S larvae, or between treated and control
432 samples, were considered significant when $P < 0.05$. DNA sequence data from gut
433 bacterial communities (profiles of the microbiota) were analysed using CLoVR
434 (metastats)⁴⁷.
435

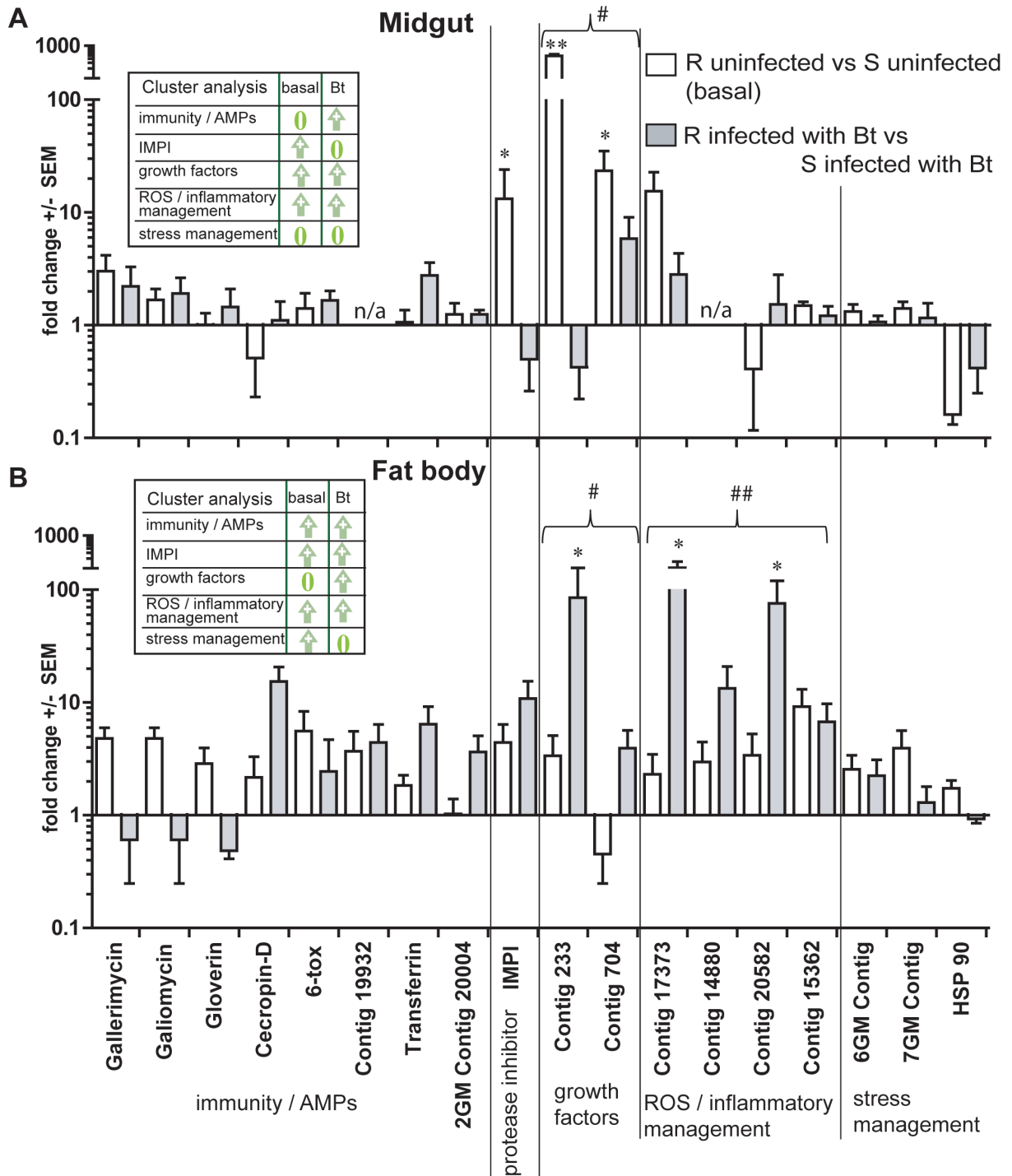
436 **References**

- 437 1. Janmaat AF, Bergmann L, Ericsson J. Effect of low levels of *Bacillus thuringiensis*
438 exposure on the growth, food consumption and digestion efficiencies of *Trichoplusia ni* resistant
439 and susceptible to Bt. *Journal of invertebrate pathology* 2014; 119:32-9.
- 440 2. Paris M, David JP, Despres L. Fitness costs of resistance to Bti toxins in the dengue
441 vector *Aedes aegypti*. *Ecotoxicology* 2011; 20:1184-94.
- 442 3. Raymond B, Johnston PR, Nielsen-LeRoux C, Lereclus D, Crickmore N. *Bacillus*
443 *thuringiensis*: an impotent pathogen? *Trends Microbiol* 2010; 18:189-94.
- 444 4. Nielsen-LeRoux C, Gaudriault S, Ramarao N, Lereclus D, Givaudan A. How the insect
445 pathogen bacteria *Bacillus thuringiensis* and *Xenorhabdus/Photorhabdus* occupy their hosts.
446 *Curr Opin Microbiol* 2012; 15:220-31.
- 447 5. Slamti L, Perchat S, Huillet E, Lereclus D. Quorum Sensing in *Bacillus thuringiensis* Is
448 Required for Completion of a Full Infectious Cycle in the Insect. *Toxins* 2014; 6:2239-55.
- 449 6. Bravo A, Gill SS, Soberon M. *Bacillus thuringiensis*: mechanisms and use. In: Gilbert LI,
450 Kostas I, Gill SS, eds. *Comprehensive Molecular Insect Science*. Amsterdam: Elsevier,
451 2005:175-205.
- 452 7. Li RS, Jarrett P, Burges HD. Importance of spores, crystals, and δ -endotoxins in the
453 pathogenicity of different varieties of *Bacillus thuringiensis* in *Galleria mellonella* and *Pieris*
454 *brassicae*. *Journal of invertebrate pathology* 1987; 50:277-84.
- 455 8. Zhang X, Candas M, Griko NB, Taussig R, Bulla LA, Jr. A mechanism of cell death
456 involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of
457 *Bacillus thuringiensis*. *Proc Natl Acad Sci U S A* 2006; 103:9897-902.
- 458 9. Soberon M, Gill SS, Bravo A. Signaling versus punching hole: How do *Bacillus*
459 *thuringiensis* toxins kill insect midgut cells? *Cell Mol Life Sci* 2009; 66:1337-49.

- 460 10. Tabashnik BE, Brevault T, Carriere Y. Insect resistance to Bt crops: lessons from the first
461 billion acres. *Nat Biotechnol* 2013; 31:510-21.
- 462 11. Griffiths JS, Aroian RV. Many roads to resistance: how invertebrates adapt to Bt toxins.
463 *BioEssays : news and reviews in molecular, cellular and developmental biology* 2005; 27:614-
464 24.
- 465 12. Ferre J, Van Rie J. Biochemistry and genetics of insect resistance to *Bacillus*
466 *thuringiensis*. *Annu Rev Entomol* 2002; 47:501-33.
- 467 13. Gahan LJ, Gould F, Heckel DG. Identification of a gene associated with Bt resistance in
468 *Heliothis virescens*. *Science* 2001; 293:857-60.
- 469 14. Darboux I, Pauchet Y, Castella C, Silva-Filha MH, Nielsen-LeRoux C, Charles JF, et al.
470 Loss of the membrane anchor of the target receptor is a mechanism of bioinsecticide resistance.
471 *Proc Natl Acad Sci U S A* 2002; 99:5830-5.
- 472 15. Bravo A, Soberon M. How to cope with insect resistance to Bt toxins? *Trends in*
473 *biotechnology* 2008; 26:573-9.
- 474 16. Ma G, Sarjan M, Preston C, Asgari S, Schmidt O. Mechanisms of inducible resistance
475 against *Bacillus thuringiensis* endotoxins in invertebrates. *Insect Science* 2005; 12:319-30.
- 476 17. Ma G, Rahman MM, Grant W, Schmidt O, Asgari S. Insect tolerance to the crystal toxins
477 Cry1Ac and Cry2Ab is mediated by the binding of monomeric toxin to lipophorin glycolipids
478 causing oligomerization and sequestration reactions. *Dev Comp Immunol* 2012; 37:184-92.
- 479 18. Gunning RV, Dang HT, Kemp FC, Nicholson IC, Moores GD. New resistance
480 mechanism in *Helicoverpa armigera* threatens transgenic crops expressing *Bacillus thuringiensis*
481 Cry1Ac toxin. *Applied and environmental microbiology* 2005; 71:2558-63.
- 482 19. Caccia S, Moar WJ, Chandrashekhar J, Oppert C, Anilkumar KJ, Jurat-Fuentes JL, et al.
483 Association of Cry1Ac toxin resistance in *Helicoverpa zea* (Boddie) with increased alkaline
484 phosphatase levels in the midgut lumen. *Applied and environmental microbiology* 2012;
485 78:5690-8.
- 486 20. Oppert B, Kramer KJ, Johnson DE, MacIntosh SC, McGaughey WH. Altered protoxin
487 activation by midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia*
488 *interpunctella*. *Biochem Biophys Res Commun* 1994; 198:940-7.
- 489 21. Martinez-Ramirez AC, Gould F, Ferre J. Histopathological effects and growth reduction
490 in a susceptible and a resistant strain of *Heliothis virescens* (Lepidoptera : Noctuidae) caused by
491 sublethal doses of pure Cry1A crystal proteins from *Bacillus thuringiensis*. *Biocontrol Sci Techn*
492 1999; 9:239-46.
- 493 22. Broderick NA, Robinson CJ, McMahon MD, Holt J, Handelsman J, Raffa KF.
494 Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary across a range of
495 Lepidoptera. *BMC biology* 2009; 7:11.
- 496 23. Patil CD, Borase HP, Salunke BK, Patil SV. Alteration in *Bacillus thuringiensis* toxicity
497 by curing gut flora: novel approach for mosquito resistance management. *Parasitology research*
498 2013; 112:3283-8.
- 499 24. Valadez-Lira JA, Alcocer-Gonzalez JM, Damas G, Nunez-Mejia G, Oppert B,
500 Rodriguez-Padilla C, et al. Comparative evaluation of phenoloxidase activity in different larval
501 stages of four lepidopteran pests after exposure to *Bacillus thuringiensis*. *Journal of insect*
502 *science* 2012; 12:80.
- 503 25. Gassmann AJ, Carriere Y, Tabashnik BE. Fitness costs of insect resistance to *Bacillus*
504 *thuringiensis*. *Annu Rev Entomol* 2009; 54:147-63.
- 505 26. Ribeiro LMD, Wanderley-Teixeira V, da Cunha FM, Teixeira AAC, de Siqueira HAA.
506 Immunological response of resistant and susceptible *Plutella xylostella* (Lepidoptera: Plutellidae)
507 to *Bacillus thuringiensis*. *Rev Colomb Entomol* 2012; 38:208-14.
- 508 27. Richards EH, Dani MP. A recombinant immunosuppressive protein from *Pimpla*
509 *hypochondriaca* (rVPr1) increases the susceptibility of *Lacanobia oleracea* and *Mamestra*
510 *brassicae* larvae to *Bacillus thuringiensis*. *Journal of invertebrate pathology* 2010; 104:51-7.

- 511 28. Shrestha S, Hong YP, Kim Y. Two chemical derivatives of bacterial metabolites suppress
512 cellular immune responses and enhance pathogenicity of *Bacillus thuringiensis* against the
513 diamondback moth, *Plutella xylostella*. J Asia-Pac Entomol 2010; 13:55-60.
- 514 29. Broderick NA, Raffa KF, Handelsman J. Chemical modulators of the innate immune
515 response alter gypsy moth larval susceptibility to *Bacillus thuringiensis*. BMC Microbiol 2010;
516 10:129.
- 517 30. Hwang J, Kim Y. RNA interference of an antimicrobial peptide, gloverin, of the beet
518 armyworm, *Spodoptera exigua*, enhances susceptibility to *Bacillus thuringiensis*. Journal of
519 invertebrate pathology 2011; 108:194-200.
- 520 31. Dubovskiy IM, Whitten MM, Kryukov VY, Yaroslavtseva ON, Grizanova EV, Greig C,
521 et al. More than a colour change: insect melanism, disease resistance and fecundity. P Roy Soc
522 B-Biol Sci 2013; 280:20130584.
- 523 32. Dubovskiy IM, Whitten MMA, Yaroslavtseva ON, Greig C, Kryukov VY, Grizanova
524 EV, et al. Can Insects Develop Resistance to Insect Pathogenic Fungi? Plos One 2013; 8.
- 525 33. Crava CM, Jakubowska AK, Escrache B, Herrero S, Bel Y. Dissimilar Regulation of
526 Antimicrobial Proteins in the Midgut of *Spodoptera exigua* Larvae Challenged with *Bacillus*
527 *thuringiensis* Toxins or Baculovirus. Plos One 2015; 10:e0125991.
- 528 34. Ferrandon D, Imler JL, Hetru C, Hoffmann JA. The *Drosophila* systemic immune
529 response: sensing and signalling during bacterial and fungal infections. Nature reviews
530 Immunology 2007; 7:662-74.
- 531 35. Wojda I, Kowalski P, Jakubowicz T. Humoral immune response of *Galleria mellonella*
532 larvae after infection by *Beauveria bassiana* under optimal and heat-shock conditions. J Insect
533 Physiol 2009; 55:525-31.
- 534 36. Oppert B. Protease interactions with *Bacillus thuringiensis* insecticidal toxins. Arch
535 Insect Biochem Physiol 1999; 42:1-12.
- 536 37. Lovgren A, Zhang M, Engstrom A, Dalhammar G, Landen R. Molecular characterization
537 of immune inhibitor A, a secreted virulence protease from *Bacillus thuringiensis*. Molecular
538 microbiology 1990; 4:2137-46.
- 539 38. Dubovskiy IM, Martemyanov VV, Vorontsova YL, Rantala MJ, Gryzanova EV, Glupov
540 VV. Effect of bacterial infection on antioxidant activity and lipid peroxidation in the midgut of
541 *Galleria mellonella* L. larvae (Lepidoptera, Pyralidae). Comparative biochemistry and
542 physiology Toxicology & pharmacology : CBP 2008; 148:1-5.
- 543 39. Loeb MJ, Martin PA, Hakim RS, Goto S, Takeda M. Regeneration of cultured midgut
544 cells after exposure to sublethal doses of toxin from two strains of *Bacillus thuringiensis*. J Insect
545 Physiol 2001; 47:599-606.
- 546 40. Broderick NA, Raffa KF, Handelsman J. Midgut bacteria required for *Bacillus*
547 *thuringiensis* insecticidal activity. Proc Natl Acad Sci U S A 2006; 103:15196-9.
- 548 41. Raymond B, Johnston PR, Wright DJ, Ellis RJ, Crickmore N, Bonsall MB. A mid-gut
549 microbiota is not required for the pathogenicity of *Bacillus thuringiensis* to diamondback moth
550 larvae. Environ Microbiol 2009; 11:2556-63.
- 551 42. Rivero A, Magaud A, Nicot A, Vezilier J. Energetic cost of insecticide resistance in
552 *Culex pipiens* mosquitoes. J Med Entomol 2011; 48:694-700.
- 553 43. Tsuzuki S, Ochiai M, Matsumoto H, Kurata S, Ohnishi A, Hayakawa Y. *Drosophila*
554 growth-blocking peptide-like factor mediates acute immune reactions during infectious and non-
555 infectious stress. Scientific reports 2012; 2:210.
- 556 44. Dubovskiy IM, Whitten MA, Kryukov VY, Yaroslavtseva ON, Grizanova EV, Greig C,
557 et al. More than a colour change: insect melanism, disease resistance and fecundity. P Roy Soc
558 B-Biol Sci 2013; 280.
- 559 45. Semighini CP, Heitman J. Dynamic duo takes down fungal villains. Proc Natl Acad Sci U
560 S A 2009; 106:2971-2.

- 561 46. Freitak D, Schmidtberg H, Dickel F, Lochnit G, Vogel H, Vilcinskas A. The maternal
562 transfer of bacteria can mediate trans-generational immune priming in insects. *Virulence* 2014;
563 5:547-54.
- 564 47. Angiuoli SV, Matalaka M, Gussman A, Galens K, Vangala M, Riley DR, et al. CloVR: A
565 virtual machine for automated and portable sequence analysis from the desktop using cloud
566 computing. *Bmc Bioinformatics* 2011; 12.
567
- 568



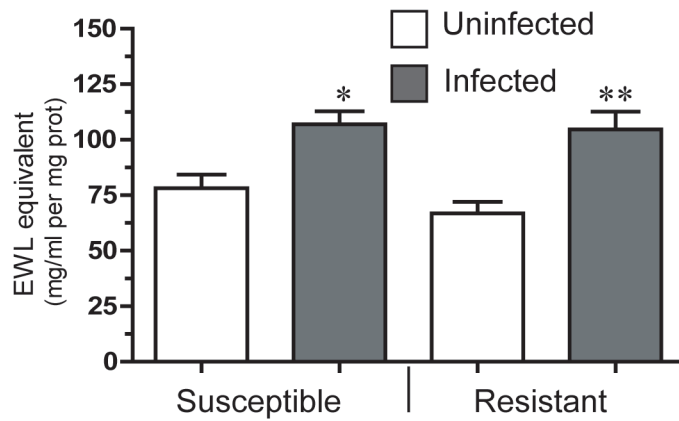
570

571 **Fig 1 Basal (uninfected) and Bt induced (48 pi) expression of defence genes in**
 572 **the midgut (A) and fat body (B) of *G. mellonella* larvae.**

573 Expression of antimicrobial peptide genes and other immunity /stress-management
 574 genes in the fat body and midgut of resistant (R) and susceptible (S) fourth instar
 575 larvae. Expression was assessed under basal (uninfected) conditions and Bt-treated
 576 (infected) conditions 48h post-infection. The y-axis represents basal expression in

577 uninfected/infected R larvae as a fold change relative to S uninfected/infected
578 larvae. Na = not assayed in midgut tissue; * = $p < 0.05$; ** = $p < 0.01$; *** =
579 $p < 0.001$ significant change in fold expression compared with S larvae; #- $p < 0.05$,
580 ## = $p < 0.01$ show significant changes in expression of genes grouped in functional
581 clusters in R vs S insects under Bt infection compared with uninfected R vs S. Data
582 presented as mean \pm SE and analysed by one-way ANOVA (Kruskall-Wallis with
583 Dunn's post test). Tables (cluster analysis) present trends in expression of defence
584 genes grouped in clusters (arrow indicates significant upregulation, fold change
585 cutoff ≥ 2.0). Additional information is presented in SI Table 1.
586

587

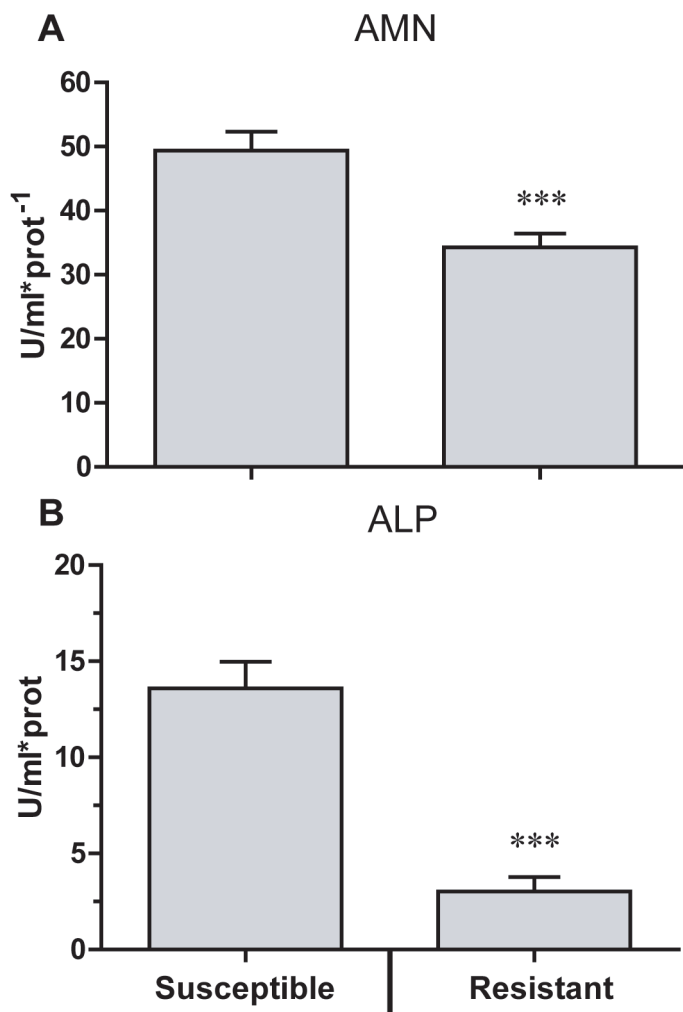


588

589 **Fig 2 Lysozme activity in infected and uninfected R and S line larvae.**

590 Lysozyme-like activity in midgut of fourth instar larvae from both susceptible and
591 resistant wax moth lines 48 h following ingestion with Bt (data presented as mean
592 +/-SEM; *P<0.05, **P<0.01, compared with uninfected larvae from the same
593 line).

594

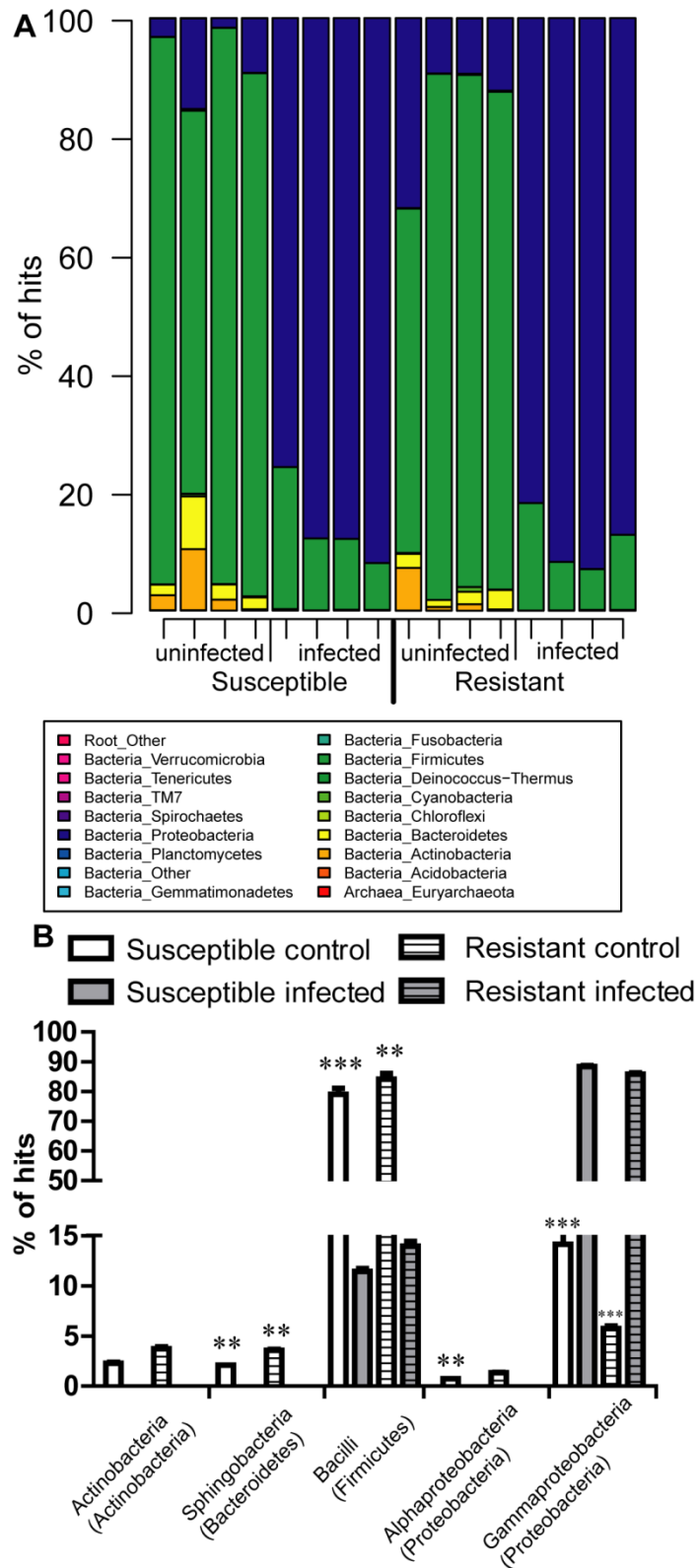


596

597 **Fig 3. Midgut receptors of infected and uninfected R and S line larvae**

598 Aminopeptidase-N (AMN) (A) and alkaline phosphatase (ALP) (B) activity in the
599 midgut of fourth instar uninfected larvae from both the susceptible and resistant
600 lines (***) $p < 0.001$ compared with susceptible).

601



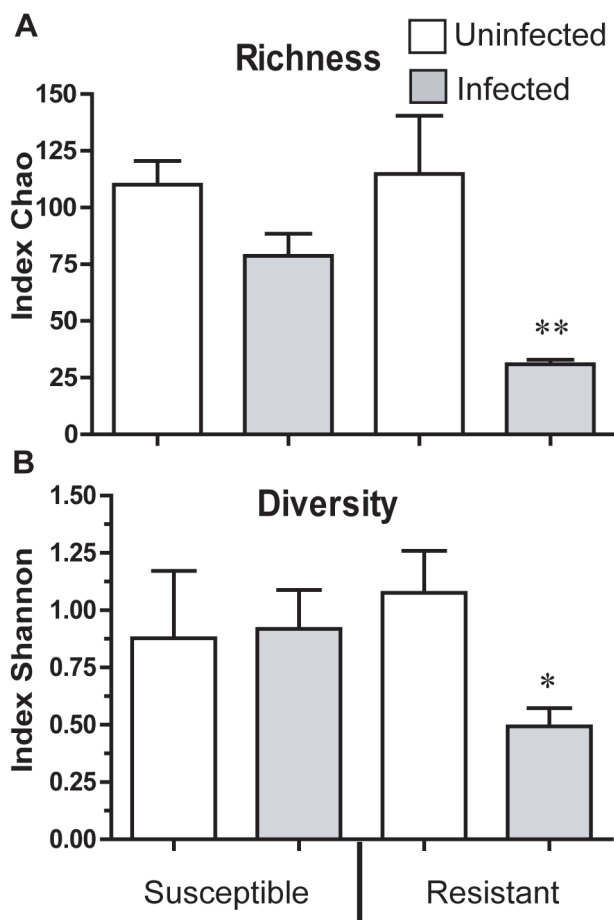
603

604 **Fig 4 Gut biota profiles in Bt infected and uninfected R and S line larvae.**

605 Profile of the bacterial community in midguts from fourth instar larvae from both
 606 resistant and susceptible lines on the second day post Bt infection. Values are
 607 averaged across four independent control (uninfected) and four infected samples of

608 each line. (A) Bacteria classified by phylum and (B) Comparison of community,
609 classified by phylum and class, from infected and uninfected R and S line larvae
610 ($p < 0.01$, $p < 0.001$ compared with infected insects from the corresponding line).
611

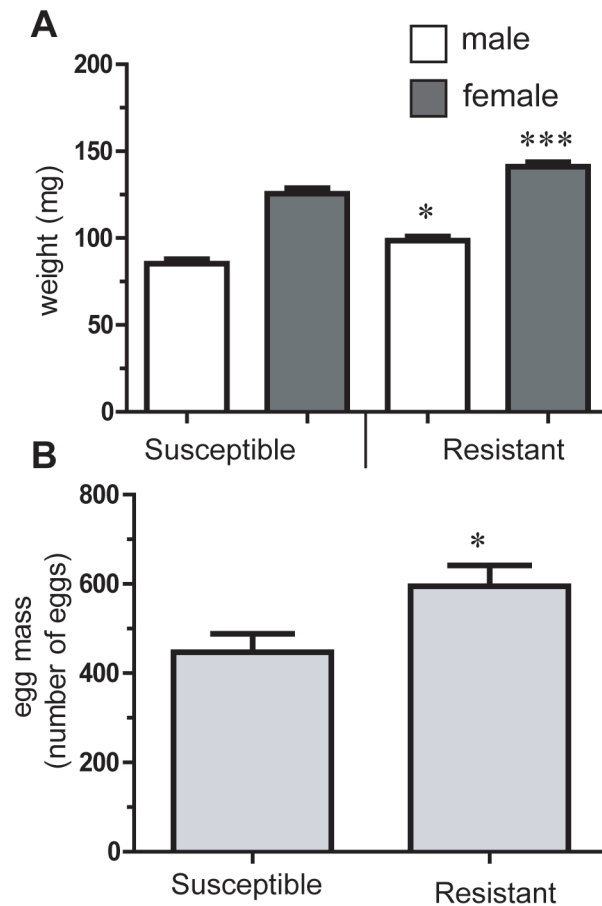
612



613

614 **Fig 5 Richness and diversity of bacterial communities in infected and**
615 **uninfected R and S lines** (A) Chao community quantitative index reflecting
616 richness (i.e. different bacterial phylotypes) in a dataset. (B) Shannon index
617 reflecting diversity of bacterial communities for resistant and susceptible lines
618 following infection with Bt (**p < 0.01, compared with other variants; *p < 0.05
619 compared with same non-infected line). This index quantifies how evenly the basic
620 entities (such as phylotypes) are distributed. To prevent bias due to sampling
621 depth, all samples were first rarefied (randomly standardized) to 3 700 sequences
622 per sample.

623



625

626 **Fig 6. Increased fecundity: a positive trade-off in wax moth resistant to Bt**

627 Life-history traits in uninfected susceptible and resistant lines of 20th generation
 628 wax moth. (A) Pupal weights and (B) adult fecundity as measured by mean egg
 629 production over 5 days per female (* $p < 0.05$, *** $p < 0.001$ compared with
 630 susceptible line).