1   Royal decree: gene expression in transgenerationally immune primed

2   bumblebee workers mimics a primary immune response

3

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11

12   Abstract

13   Invertebrates lack the cellular and physiological machinery of the adaptive

14   immune system, but show specificity in their immune response [1, 2] and

15   immune priming [3-11]. Functionally, immune priming is comparable to

16   immune memory in vertebrates. Individuals that have survived exposure to a

17   given parasite are better protected against subsequent exposures. Protection

18   may be cross-reactive (e.g. [12]), but demonstrations of persistent and

19   specific protection in invertebrates are increasing [3, 5]. This immune priming

20   can cross generations ("trans-generational" immune priming) [4, 8], preparing

21   offspring for the prevailing parasite environment. While these phenomena

22   gain increasing support, the mechanistic foundations underlying such immune

23   priming, both within and across generations, remain largely unknown. Using a

24   transcriptomic approach, we show a bacterial challenge to bumblebee

25   queens, known to induce trans-generational immune priming, alters daughter

26   (worker) gene expression. Daughters, even when unchallenged themselves,

27   constitutively express a core set of the genes induced upon direct bacterial

28   exposure, including high expression of antimicrobial peptides, a beta-glucan

29   receptor protein implicated in bacterial recognition and the induction of the *toll*

30   signaling pathway[13], and *slit-3* which is important in honeybee immunity[14].

31   Maternal challenge results in a distinct upregulation of their daughters'

32   immune system, with a signature overlapping with the induced individual

33   response to a direct immune challenge. This will mediate mother-offspring

34   protection, but also associated costs related to reconfiguration of constitutive

35   immune expression. Identification of conserved immune pathways in memory-

36   like responses has important implications for our understanding of the innate

37   immune system, including the innate components in vertebrates, which share

38   many of these pathways[15].

39

40   Author Summary

41   Invertebrate individuals surviving exposure to an infectious disease can

42   become better at fighting future infection by that same disease. This

43   protection, known as immune priming, can even be transferred to the

44   individuals' offspring. The functional outcome is very similar to that

45   of vertebrate immune memory, but the mechanisms of how invertebrates

46   achieve immune priming within an individual or across generations remain

47   enigmatic. We found that bumblebee daughters of mothers exposed to a

48   simulated bacterial infection express strongly many of the genes that they

49   would activate if they were themselves infected. Our results show how

50   immune priming across generations might be produced in bumblebees. Many

51   parts of the invertebrate immune system are shared with us, and thus our

52   study also sheds a light on how diverse immune memory-like effects could be

53   achieved.

54

55   **Introduction**

56   Parasites, broadly construed to include both macro- and microparasites, are

57   ubiquitous and can cause significant damage to their hosts. As a

58   consequence, parasites represent a major selective force for any organism.

59   Hosts, in turn, have adaptations that prevent parasite establishment and

60   reduce the costs of having an infection. These adaptations, which can be

61   broadly viewed as elements of a defense system, notably including the

62   immune response, range in their specificity, their mode of action, and the

63   nature of regulation. As investment into immunity is costly on multiple

64   levels[16], the most efficient investment into immunity will be a function of the

65   prevailing pressure from parasites (likelihood of encounter and virulence) and

66   demands imposed by other life-history traits. On an ecological scale, there will

67   therefore be a benefit to a plastic adjustment of immune investment relevant

68   for sufficiently accurate "perceived" risk of parasitism. This perception may be

69   related to ecological conditions, such as crowding[17], but may also result

70   from prior immunological experience with parasites. In particular, hosts can

71   encounter the same parasites multiple times within their lifetime, and across

72   generations. If hosts encounter the same parasite repeatedly, some form of

73   memory, which would improve resistance to that same parasite upon re-

74   exposure, will be adaptive.

75

76   The best-studied and classic example of an adjustment in immune responses

77   in relation to a prior parasite exposure is the adaptive immune system of

78   vertebrates. The adaptive immune system, which produces specific and long-

79   lasting protection against subsequent exposure to the same parasite, is based

80   on a repertoire of specialized lymphocytes and its molecular underpinnings

81   are well characterized[18]. There is growing evidence that functionally

82   comparable processes may exist in other organisms[19, 20]. To avoid

83   mechanism-based confusion in terms, these phenomena so far described for

84   several invertebrates[19], plants[20, 21] or even bacteria[22], are frequently

85   referred to as 'immune priming'. Astonishingly, induced protection against

86   parasites in these systems can traverse generations, a phenomenon known

87   as trans-generational immune priming [23-25].

88

89   The molecular understanding of immune priming outside of the adaptive

90   immune system of vertebrates is still in its infancy. Some progress has been

91   made in understanding these mechanisms insects[26, 27] and plants[28].

92   Invertebrates are particularly important to understand in this regard as they

93   share a number of conserved characteristics of the innate immune system

94   with vertebrates, including humans[29]. The potential for these innate immune

95   components to exhibit a memory-like response is an intriguing possibility[30,

96   31]. While invertebrates may serve as a valuable model for understanding

97   memory-like phenomena produced solely by innate immune system, the

98   mechanisms remain enigmatic. Studies have identified the roll of the *toll*

99   pathway and phagocytosis within an individual's life[10] in *Drosophila*

100 *melanogaster*; and cellular mechanisms are suspected for mosquitoes [9].

101

102   Here we investigate patterns of gene expression underlying the phenomenon

103   of trans-generational immune priming in a social insect, the bumblebee

104   *Bombus terrestris*. In social insects, such as bumblebees, temporal and

105   spatial overlap of worker offspring and their mothers will mean that they are

106   faced with a parasite threat that can, with a high probability, be predicted from

107   the mother's prior immunological experience. *B. terrestris*, is a model of

108   ecological host-parasite interactions that shows a specific immune response

109   [1, 2, 32, 33], and within-individual [5, 34] and trans- generational [4, 6]

110   immune priming. Daughters of bacterial-challenged queens show elevated

111   antibacterial responses, but pay costs in terms of resistance to distinct

112   parasites[5, 7]. The mechanisms underlying these responses are unknown.

113

114   We injected *B. terrestris* queens with a heat-inactivated inoculum of the Gram-

115   positive bacterium *Arthobacter globiformis*, in the same manner as previous

116   studies demonstrating trans-generational immunity[6, 7], To gain some insight

117   into the molecular foundations of observed trans-generational immunity in this

118   system we measured genome-wide expression of subsequently produced

119   naïve daughters (*Arthrobacter*-Naïve [AN] treatment) relative to the

120   expression of naïve daughters born from unchallenged mothers (Naïve-Naïve

121   [NN] treatment). We further contrast this memory response with the immune

122   response of daughters that are exposed to the bacterial challenge, but whose

123   mothers were naïve (Naïve-*Arthrobacter* [NA] treatment).

124

125   **Results**

126   Whole genome expression, as measured by mRNA sequencing on the

127   Illumina HiSeq platform revealed that when workers from unchallenged,

128   naïve, mothers were directly challenged with the bacterial inoculum (NA) they

129   responded with significant differential expression of 327 genes (Table S1).

130   Naïve workers from challenged mothers (AN) significantly altered the

131   expression of only 21 genes (Table S2), but 20 of these are shared with the

132   direct induced response (NA) (Fig 1). These shared genes (Fig 2) include all

133   known bumblebee antimicrobial peptides (*abaecin*, two *apidaecins*, *defensin,*

134   *hymenoptaecin*) and a number of additional known immune genes such as

135   *battenin, laccase-2, slit-3*, and *yellow*. The only gene differentially expressed

136   in the primed condition, but not under direct induction, codes for

137   LOC100644816, a 53aa hydrophobic (58.49% of residues) peptide with

138   homology to Mast Cell Degranulating Peptide (MCDP) from another

139   bumblebee, *B. pennsylvanicus*.

140

141   We confirmed the patterns determined by the whole genome transcriptome

142   approach (Fig 2) by targeted qPCR of a suite of immune genes (Table S3).

143   Our qPCR results agree with our transcriptomic results for all tested genes.

144   This included the high constitutive expression of the antimicrobial peptides

145   and additionally of a beta-glucan receptor protein (BGRP, Fig S1, Table S4) in

146   naïve offspring of immune-challenged mothers (AN). There was a trend for

147   higher BGRP expression in the transcriptome of workers from challenged

148   mothers, but this was not significant after correction for multiple testing ($P <$

149   0.01 before correction, 0.072 after correction). Differential expression of this

150   receptor may be particularly relevant as it can trigger the *toll* signaling

151   pathway and downstream antimicrobial peptide production.
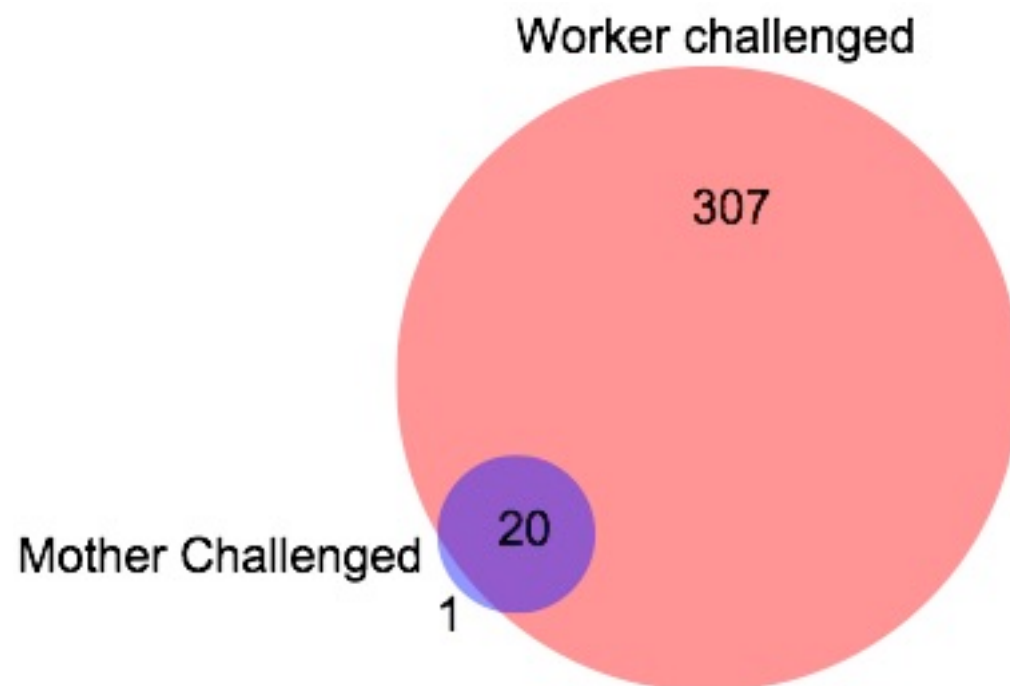
152

153   **Figure 1**: The number of differentially expressed genes in naïve worker

154   offspring of mother queens that were injected with heat killed Gram-positive

155   bacterium (*Arthrobacter globiformis*) (trans-generational immunity treatment;

156   AN), and worker offspring from naïve mother queens but themselves exposed

157   to an immune challenge of *A. globiformis* (induced immune response

158   condition; NA). The expression of these genes is measured relative to that of

159   naïve worker offspring of naïve mothers (NN).

160

161   We identified a number of different isoforms for putative immune response

162   genes, including for antimicrobial peptides (Fig S2-17: *abaecin*

163   [LOC100631078], 4 isoforms; both *apidaecins* [LOC100649867], 2 isoforms,

164   and [LOC100648499], 3 isoforms, aminopeptidase [LOC100645702],

165   tetraspannin [LOC100651747], a venom protease [LOC100651916], an

166   uncharacterized protein shared only within honeybees and bumblebees

167   [LOC100645125], and a novel gene [NC_015763.1:3848320-3855802] with

168   sequence homology to *A. mellifera* cuticular protein 14. We also identified two

169   *dscam* like genes with multiple isoforms (Supplemental Fig S18-21;

170   LOC100644003, 12 isoforms; LOC100649765, 9 isoforms). Among the

171   significantly differentially expressed genes, isoform transcript abundance did

172   not vary significantly among conditions.

173
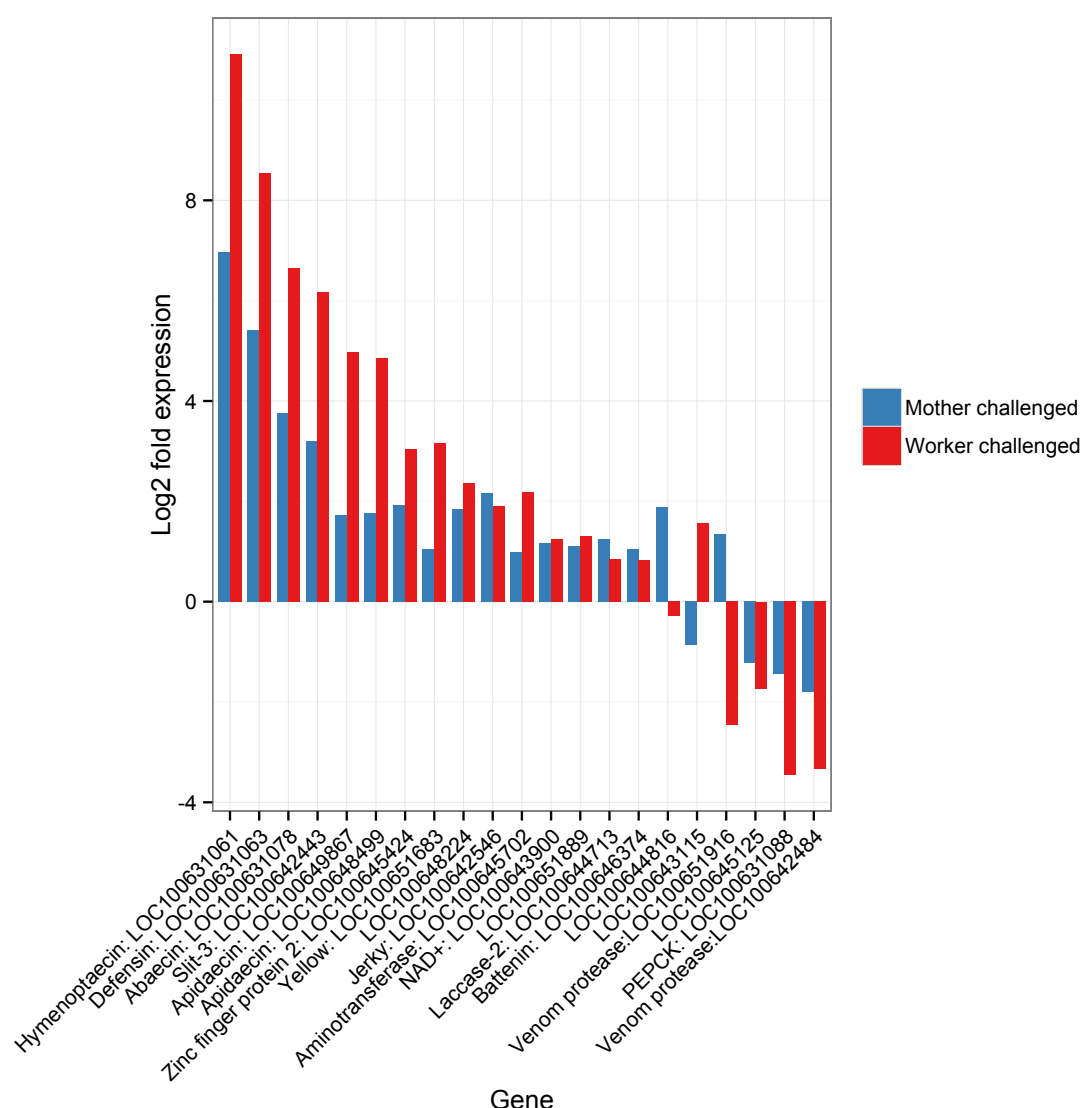
Figure 2: Log 2 fold expression based on RNAseq data (relative to naïve

worker offspring from naïve mother queens) for all genes that are significantly

differentially expressed in the trans-generational priming condition (naïve

177   offspring of challenged mothers, blue). We also show the expression of

178   challenged workers from naïve mothers (red) to demonstrate the similarity of

179   the induced response to a direct challenge to the signature of trans-

180   generational immunity. All differentially expressed genes here are also

181   significantly differentially expressed upon direct challenge, except for

182   LOC100644816, which encodes for mast cell degranulating peptide. qPCR

183   confirmation of these results can be found in Fig. S1.

184

185

186   **Discussion**

187   We found that offspring workers which had never been exposed themselves

188   ("naive workers") but whose mothers were exposed to a bacterial immune

189   challenge express a strikingly pathogen exposure-like immune response, as

190   compared to offspring workers from naïve mothers. In fact, all but one of the

191   differentially expressed genes in this priming condition were shared with

192   workers that were directly immune challenged with the same bacterium. This

193   indicates a major reconfiguration of the constitutively expressed immune gene

194   profile, and is one that will likely confer appropriate benefits in the face of

195   specific parasites, but may also result in the costs previously described when

196   there is mismatch between the maternal parasite environment and the

197   offspring parasite environment[7]. These results give us an insight into the

198   innate immune-related molecular pathways at the base of invertebrate

199   immune priming across generations.

200

201   Among the differentially expressed genes, all of the antimicrobial peptides are

202   upregulated. It is particularly noteworthy that these are the end-point of the

203   immune response, indicating an immediate readiness of defense in trans-

204   generationally primed individuals. We also find increased expression in a

205   number of other immunologically important genes including *yellow* and

206   *laccase-2*, which are involved in the melanization response [35, 36], *battenin,*

207   the *D. melanogaster* homolog of which (*CLN3*) regulates *JNK* signaling[37],

208   and *slit-3* which is induced upon bacterial challenge in honeybees and the

209   leaf-cutting ant *Atta cephalotes* (a.k.a. *IRP30*)[14]. We also found that a beta-

210   glucan receptor protein (BGRP) was more highly expressed in naïve workers

211   of challenged queens. BGRPs induce the *toll* signaling pathway in

212   invertebrates[13]. The only gene that was differentially expressed in the

213   primed condition, but not in directly challenge workers, was the mast cell

214   degranulating peptide (MCDP), which is found in venom[38]. MCDP, neuro-

215   and immunotoxic, is named for its degranulating effect on vertebrate

216   granulocytes[38, 39]. Whether this peptide also has the same effect on

217   invertebrate granulocytes (a class of haemocytes) that are important for

218   phagocytosis[40] is unknown. Intra-generationally primed *Drosophila*

219   *melanogaster,* utilize the *toll* pathway and phagocytosis, but not antimicrobial

220   peptides[10] that appear to play an important role here.

221

222   The down syndrome cell adhesion molecule (*dscam*) is implicated in immune

223   defenses, and because of its ability to produce prodigious numbers of

224   isoforms[25] has been proposed as a possible mechanism for specific

225   immune memory[41]. We detect two *dscam* like genes that produce multiple

226   isoforms. These genes nor their isoforms are not, however, differentially

227   expressed in the priming condition or in workers that are directly exposed to

228   the bacterium. While this does not rule out a role for *dscam* isoforms in

229   immune priming, it suggests that differential expression of isoforms is not a

230   major component of trans-generational antibacterial immune priming in this

231   system.

232

233   The elevated constitutive gene expression into adulthood of a holometabolous

234   insect, with its associated tissue rearrangements, is testament to the

235   persistence of the trans-generational priming in the innate immune system.

236   Evidence in insects of elevated constitutive expression of immune-related

237   genes that is precipitated by immune experiences in prior generations is

238   important beyond a demonstration of the underlying mechanistic foundations

239   of trans-generational immunity. It will also have important consequences for

240   the fitness costs associated with this phenomenon, which will influence the

241   conditions under which it may be expected to evolve and be maintained by

242   selection. Elevated immune investment comes at a cost to an organism

243   through resource trade-offs and other routes [42]. Higher constitutive

244   expression of immunity in naïve offspring may constrain their investment into

245   other life-history traits, especially under conditions where resources are

246   scarce. The striking signature of gene expression related to trans-generational

247   immunity is also likely to underpin other related costs, including increased

248   susceptibility to a distinct parasite infection, as has been demonstrated in this

249   system [7].

250

251 Evidence is mounting that the evolutionarily ancient innate immune system is

252 able to retain information about immune history in both vertebrates and

253 invertebrates[43], which translates to better defenses upon subsequent

254 exposure. This priming effect is observed both within the lifetime of an

255 individual and between parents and offspring. Trans-generational immune

256 priming likely evolved as a part of parental care and investment into offspring.

257 This may be particularly important in social insects, such as *B. terrestris*,

258 where generations overlap and related individuals very intimately share an

259 environment - including parasites - in a closed, populous, highly interactive

260 colony. While our study does not attempt to identify the mechanisms involved

261 in transfer of immune compounds to the offspring, a recent paper in

262 honeybees identified the yolk protein vitellogenin as playing a role in binding

263 and transferring bacterial cell components to eggs[44]. Here we find that

264 trans-generationally primed workers - even if not infected themselves -

265 increase transcription of antimicrobial peptides (that in part are under the

266 control of the *toll* signaling pathway) and a key recognition protein that

267 induces *toll* signaling. This transcriptional signature resembles an abridged

268 version of the normal response to infection, suggesting that *B. terrestris*

269 achieves trans-generational protection by sequestering the existing induced

270 responses into prophylactic constitutive expression to prevent parasite

271 establishment. A recent study in moths found elevated ovary expression of

272 some immune genes in daughters of challenged mothers, hinting that these

273 responses may even be transmitted across multiple generations[45]. The

274 conserved nature of these innate immune pathways suggests that the

275 patterns detected here may also underlie immune experience based immune

276 system plasticity not only invertebrates, but also in the innate immune system

277 of vertebrates.

278

279

280 **Materials and Methods**

281 Experimental methods

282 We collected queens as they emerged from hibernation in spring 2013 in

283 northern Switzerland and maintained them under standard colony

284 establishment conditions[1]. All of the colonies used for this experiment were

285 microscopically checked for common infections twice and found to be clear of

286 identifiable infection. On their production by the colonies, young queens

287 (gynes) were removed and mated to males from unrelated colonies. We

288 designed the matings such that sister queens from one colony were mated to

289 males all derived from a single colony to produce comparable genetic

290 backgrounds for matching across treatments. Five days after mating, we

291 hibernated the queens for 48 days at 4C. Seven-days after removal from

292 hibernation we either injected queens to the challenged with 2 µl of 10^8

293 colony-forming-units/mL of *Arthrobacter globiformis* (DSM 20124) that had

294 been heat inactivated by heating at 95C for 5 min, washed three times and

295 resuspended in ringer saline solution. Naive, unchallenged queens were

296 handled similarly but not injected. We then allowed queens to found colonies

297 in the lab. We used three sister queen colony sets. We deliberately used

298 these second generation colonies to exclude unknown maternal effects

299 outside of our treatments. Emerging adult worker offspring from naïve queens

300 were uniformly distributed to a naive group (NN) or an induced treatment

301   (NA). In the induced treatment, daughters five-days post-eclosion received an

302   injection of 2 µl of 10^8 colony-forming-units/mL of *A. globiformis* prepared as

303   above replicated twice per condition, per colony, (NA: naïve queens, *A.*

304   *globiformis* exposed worker daughters) and were snap frozen in liquid

305   nitrogen 24hrs after injection. Naïve group workers (NN) were handled

306   similarly, but not injected, and frozen at the same time. Similarly, we took

307   workers from queens that were exposed to the bacterial challenge and

308   handled and froze them as above (AN). We extracted RNA from the workers

309   following the same protocols as in [1] but using whole abdomens. For RNA

310   sequencing we pooled the RNA from two individual workers per queen and

311   treatment combination. The sequencing used the Illumina HiSeq 2000

312   platform and was done at the Beijing Genomics Institute.

313

314   After removing adapters and poor quality reads we mapped the reads to the

315   *B. terrestris* genome[46] with Tophat2[47] in two ways. First, using the

316   annotated transcripts (-G option) to assess differential expression of known

317   genes, and second, without this restriction to assess isoform variation. We

318   identified differentially expressed genes using Cuffdiff[48, 49]. In both cases

319   we used the current version of the *B. terrestris* genome (Bterr_1.0) with the

320   accompanying gtf annotation file. We limited the maximum intron size to 50kb.

321   The analyses compared the expression of naïve daughters of challenged

322   mothers (AN) vs naïve daughters of naïve mothers (NN), and separately

323   compared the induced response (NA) to the baseline expression of NN

324   workers.

325

326   From un-pooled offspring samples described above, we also synthesized

327   cDNA using the QuantiTect Reverse Transcription Kit (Qiagen) following the

328   manufacturers instructions. In addition, cDNA was synthesized from offspring

329   of queens from three additional matched genetic background providing further

330   AN, NA and NN samples. One of these colonies had duplicate offspring for

331   each treatment, while the other two colonies had one replicate offspring. Prior

332   to cDNA synthesis potential DNA contamination was removed from all RNA

333   samples using the Turbo DNA-free kit (Ambion) according to the

334   manufacturers instructions. In the reverse transcribed samples, we quantified

335   the expression of 25 immune genes relative to two invariant housekeeping

336   genes (elongation factor 1α and ribosomal protein L13 based on their scores

337   in geNorm, qBase plus, biogazelle) and analyzed as in[1]. Full details of these

338   genes and their primers are in Table S5. We used the mean difference in

339   expression of the target gene from the composite housekeeping gene (dCt)

340   from each colony for subsequent analyses. We transformed the mean dCt

341   value for each gene using Yeo-Johnson transformations to improve normality

342   and homoscedasticity and used paired t-tests within colony genetic

343   background to assess statistical differences between NN and AN treatments

344   and between NN and NA treatments (Table S6).

345

346   **Acknowledgements**

351   hymenopteragenome.org/beebase/) for providing genomic resources that

352   were used for this study.

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