

1 Royal decree: gene expression in transgenerationally immune primed

2 bumblebee workers mimics a primary immune response

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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 (*Arthobacter*-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-*Arthobacter* [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 *tol* signaling
151 pathway and downstream antimicrobial peptide production.



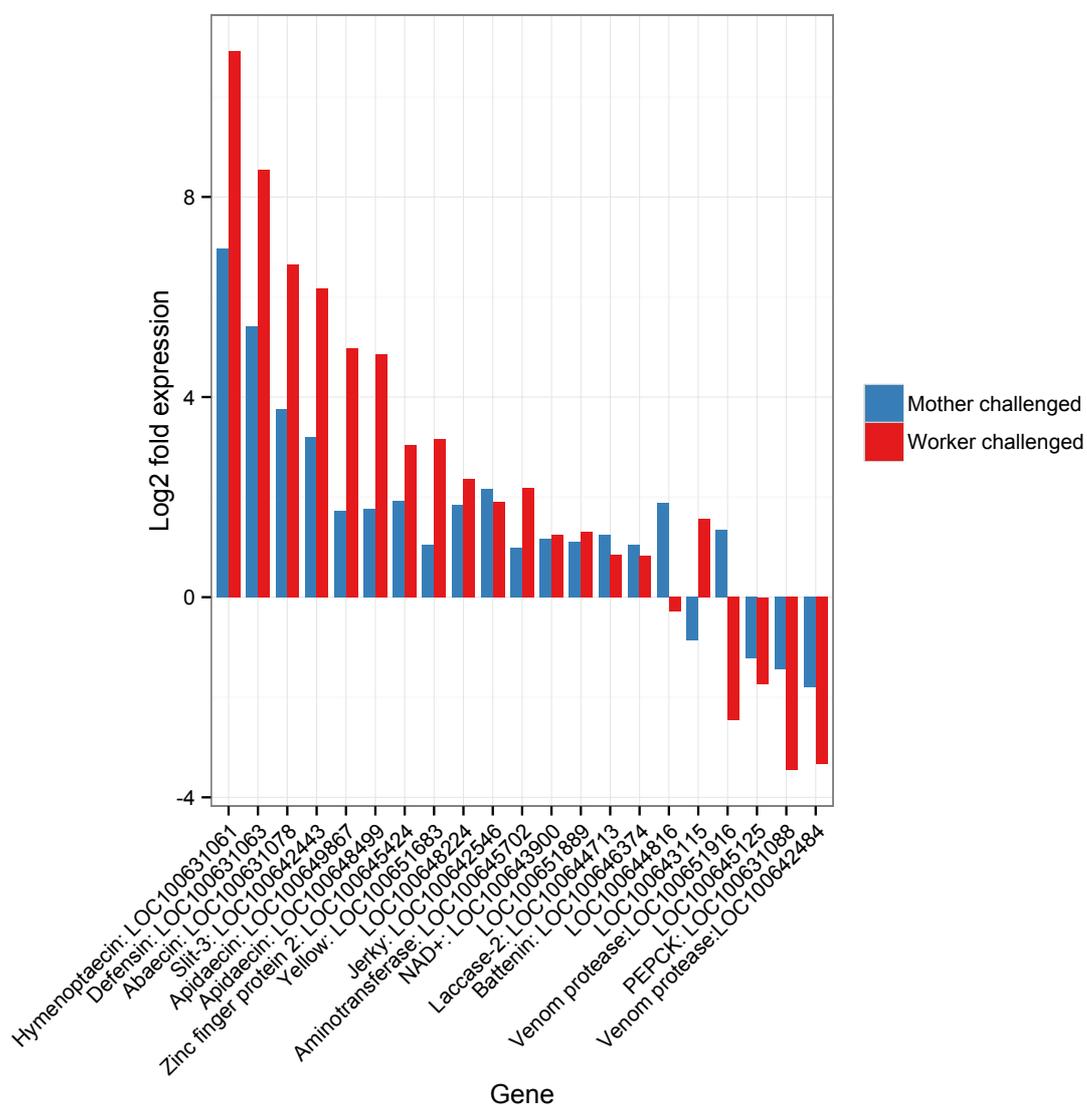
152

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

174 **Figure 2:** Log 2 fold expression based on RNAseq data (relative to naïve
175 worker offspring from naïve mother queens) for all genes that are significantly
176 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 μ l 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 μ l 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 1 α 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

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