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10   **A gene for social immunity in the burying beetle *Nicrophorus vespilloides*?**
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30 **Abstract**
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32 Some group-living species exhibit social immunity, where the immune system of one

33 individual can protect others in the group from infection. In burying beetles this is

34 part of parental care. Larvae feed on vertebrate carcasses which their parents smear

35 with exudates that inhibit microbial growth. We have sequenced the transcriptome of

36 the burying beetle *Nicrophorus vespilloides* and identified six genes that encode

37 lysozymes – a type of antimicrobial enzyme that has previously been implicated in

38 social immunity in burying beetles. When females start breeding and producing

39 antimicrobial anal exudates, we found that the expression of one of these genes was

40 increased by ~1000 times to become one of the most abundant transcripts in the

41 transcriptome. We conclude that we have likely identified a gene for social immunity,

42 and that it was recruited during evolution from a previous function in personal

43 immunity.
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<p>46</p>

Introduction

<p>47</p>

48 Insects occupy some of the most microbe-rich environments in nature and have

49 evolved diverse immunological defences to overcome the challenge that microbes

50 pose to their fitness [1,2]. In some group-living species, individuals are selected to

51 defend other individuals, as well as themselves, from potential pathogens. This is

52 social immunity in the broad sense, and it is seen in transient animal societies such as

53 animal families as well as more permanent animal societies such as the eusocial

54 insects and group-living primates [3]. Social immunity can take a range of forms,

55 from the collective behaviour that causes social fever in bees, to the production of

56 antibacterial substances by parents to defend offspring or a breeding resource [2–4].

57 Yet, while the mechanisms underlying personal immunity in insects are increasingly

58 well-described [5,6], relatively little is known about the mechanisms underlying social

59 immunity. Nor is it clear whether social immune function might have originally been

60 derived from personal immune function.

<p>61</p>

62   In burying beetles (*Nicrophorus* spp), social immunity is a vital part of

63 parental care. These insects breed on small vertebrate carcasses which they shave, roll

64 into a ball and smear with anal exudates. These exudates have strong antimicrobial

65 properties [7,8] and promote larval survival [9]. The strength of antimicrobial activity

66 in anal exudates is proportional to the perceived microbial threat but increasing levels

67 of antimicrobial activity comes at a fitness cost to adults [10] and trades-off against

68 personal immunity [11]. Antimicrobial activity in the anal exudates is thus carefully

69 modulated. It is virtually non-existent in non-breeding individuals [8], is induced by

70 reproduction and the presentation of a carcass [8] and reaches its maximum strength

71 when the larvae arrive at the carcass shortly after hatching in the soil surrounding the

72 carcass [11].

<p>73</p>

74   How has social immunity evolved in the burying beetle? One hypothesis is

75 that elements of the personal immune response have been recruited to control the

76 microbiota in the wider environment. Lysozymes, which are enzymes that can kill

77 bacteria by hydrolysing structural polysaccharides in their cell walls, are a likely

78 candidate because they are ubiquitous in nature and have key roles in personal

79 immunity [5,12]. In insects that feed on microbe-rich resources (e.g. *Drosophila*,

<p style="text-align:center">3</p>

80   house-fly), lysozymes in the gut are thought not only to have an immune function but

81   also to digest bacteria [13,14]. Perhaps in burying beetles, lysozymes that were

82   originally confined to the gut are now exuded and applied to the carcass to limit

83   microbial growth during reproduction. Supporting this hypothesis is the finding that a

84   key active antimicrobial substance in the burying beetle's anal exudates has

85   lysozyme-like properties [8,9]

86

87   Here our aim is to test whether lysozyme genes are upregulated during the

88   mounting of a social immune response in the burying beetle *N. vespilloides*. We

89   sequence the *N. vespilloides* transcriptome, identify the lysozymes within it, and

90   compare the transcriptional response in the gut of breeding and non-breeding female

91   burying beetles to identify upregulated genes.

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Methods

Beetle rearing and dissecting

The beetles used in this experiment were bred in 2014 and descended from field-collected beetles trapped earlier that year from two sites in Cambridgeshire, UK. The field-collected beetles were interbred to create a large, genetically diverse population. This population was maintained with full parental care and no inbreeding for several generations before the start of this experiment.

We examined the transcriptional response to breeding in *N. vespilloides* by comparing the transcriptional profiles of a breeding female beetle and a non-breeding female of the same age. We focused on females alone, because our previous work suggests that they contribute more to social immunity than males [1]. Prior to each treatment, beetles were given a small meal of minced beef as part of the usual protocol for beetle husbandry in the lab. The "breeding" treatment initially consisted of 3 female-male pairs of beetles. Each pair was placed in a breeding box with soil and a thawed mouse carcass (10-16 g). These boxes were then put in a dark cupboard to simulate underground conditions and the beetles were allowed to mate and begin preparing the carcass. Forty-eight hours after pairing, at peak antimicrobial activity in the anal exudates [2], we removed the female from each breeding box and placed them individually in small plastic boxes (box dimensions, length x width x depth: 12 cm x 8 cm x 2 cm) where they remained for approximately 1 hour before being killed and dissected. The "non-breeding" treatment consisted of 3 females that were treated in exactly the same way as the "breeding" treatment except that the non-breeding females were placed individually in a breeding box that did not contain a mouse carcass. This was repeated on two occasions to generate 6 breeding and 6 non-breeding beetles. Individual beetles were euthanized with CO_2 and immediately dissected to remove the gut. We focused on gut tissue because this is where the anal exudates are produced.

Transcriptome Sequencing

The dissected gut was immediately homogenised in TRIzol® Reagent, (Life Technologies) and frozen in liquid nitrogen. RNA was extracted following the

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128   standard protocol. Illumina sequencing libraries were constructed with poly-A

129   enrichment and sequenced in a single lane of Illumina HiSeq 2500® (version 3

130   chemistry, 100bp paired-end reads) by BGI (Hong Kong). Raw reads were initially

131   checked for quality using FastQC [3]. Having been found to be satisfactory, they were

132   then trimmed using Trimmomatic [4], removing trailing and leading bases with a

133   quality below q15, cutting reads where quality fell below q20 in a 4 base sliding

134   window, and only retaining reads of minimum length 30. The raw sequence data has

135   been submitted to the NCBI sequence read archive under the accession number

136   xxxxxxxxx.

137

138   *Transcriptome assembly*

139

140   The RNAseq reads from a single breeding and a single non-breeding beetle gut were

141   combined and the transcriptome *de novo* assembled (Supplementary Figure S1). The

142   assembly was performed using Trinity, a compact and fast transcript assembly

143   program for Illumina RNA-seq data [5]. Briefly, a single Trinity assembly was built

144   using forward and reverse reads from both libraries and default parameters. The full

145   recommended protocol "Identification and Analysis of Differentially Expressed

146   Trinity Genes and Transcripts" was applied

147   (http://trinityrnaseq.sourceforge.net/analysis/diff_expression_analysis.html, accessed

148   10/04/15). The assembled transcriptome has been submitted to the NCBI short read

149   archive (accession xxxxxxxxxxxx) and predicted peptides are available in Dryad

150   xxxxxxxxxx.

151

152   *Differential expression analysis*

153

154   To estimate transcript abundance, we aligned reads separately from each library onto

155   the combined-read transcriptome assembly using the short read aligner `bowtie`

156   `[6]`. Abundance estimates were then produced using `RSEM [7]`. These steps are

157   combined into a single perl script bundled with Trinity,

158   `align_and_estimate_abundance.pl`. In further analyses we used estimates

159   of transcript abundance for each gene (as opposed to each isoform). Finally, we

160   estimated levels of differential expression using EdgeR, an R Bioconductor package

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161 for differential expression analysis. Differentially expressed transcripts were

162 identified using the Trinity scripts `run_DE_analysis.pl` and

163 `analyze_diff_expr.pl` with default settings. In a very small number of cases

164 it was clear that alternative haplotypes of a gene had been split into two genes during

165 the assembly and this gave a false signal of differential expression. The avoid this we

166 identified all the genes whose predicted peptides were >98% identical to another gene

167 using CD-HIT [8] and excluded them from the analysis.

168

169 *Peptide and domain prediction*

170

171 Trinity assembles nucleotide reads into nucleotide transcripts, and as such candidate

172 peptide sequences must be predicted post-hoc (Supplementary Figure S1). Peptide

173 predictions were generated from the combined read assembly using `Transdecoder`

174 `[5]` and the standard protocol for peptide prediction. Any transcript that did not

175 encode a predicted peptide was removed from our assembly.

176

177 The resulting peptide predictions were then run through the `NCBI Batch`

178 `Conserved Domain Search` [9] to annotate domains. Putative lysozymes were

179 then identified by presence of the LYZ1 C-type lysozyme domain (cd00119), which is

180 found in *Drosophila* lysozymes and expected to be required for *Drosophila*-like

181 function of the protein. Full domain annotations can be found in the Dryad

182 xxxxxxxxxx. The predicted lysozyme sequences have been submitted to genbank

183 under the accession numbers xxxxxxxxxxxx.

184

185 *Quantitative PCR*

186 Differential expression of lysozymes was verified by quantitative PCR. We

187 synthesised cDNA using Promega Go-script® reverse transcriptase following the

188 standard conditions using 1ul RNA template and incubating at 25C for 5 minutes, 50C

189 for 50 minutes and 70C for 15 minutes. PCR primers were designed that amplify the

190 six lysozymes (all three *lys1* isoforms were amplified by a single primer pair) and the

191 reference gene *actin5C* (Table S1). The quantitative PCR was performed using SYBR

192 green using the SensiFAST SYBR Hi-ROX Kit with a 10 ul reaction volume (2 ul

193 template cDNA diluted 1:10 from original cDNA synthesis).

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197 **Results**

198 *The burying beetle transcriptome*

199 To allow us to investigate the transcriptional response in the guts of burying beetle

200 when they breed, we first sequenced their transcriptome from the gut of a single

201 breeding and non-breeding beetle, combined the sequence reads and then assembled

202 them *de novo*. This process resulted in 11290 genes that encoded 26378 different

203 transcripts. This suggests that we sequenced the majority of genes in the genome, as

204 the exceptionally well-annotated *Drosophila* genome contains 13920 protein coding

205 genes encoding 30443 transcripts (Flybase release 6).   As the guts we used for the

206 RNA extraction might contain RNA from the mouse the beetles were feeding on, or

207 nematode parasites, we used Blast to search for the most similar sequence in the *Mus*

208 *musculus, C. elegans, Drosophila melanogaster* and *Tribolium castaneum* genomes.

209 The top hit of 91% of the genes was another insect (*Drosophila* or the beetle

210 *Tribolium*), suggesting the levels of contamination were low (Figure 1A).

211

212 *Many genes are strongly upregulated in the guts of breeding beetles*

213 By mapping reads from the breeding and non-breeding beetles to the transcriptome,

214 we found that there was a strong transcriptional response in the breeding beetles

215 (Figure 1B). Among the most significantly differentially expressed genes ($p<10^{-20}$),

216 90% were upregulated in the breeding beetles (Figure 1B; N=42, 95% binomial CI:

217 77-97%). The magnitude of these changes in transcription was often large – on

218 average the expression of these 42 most significantly differentially expressed genes

219 changed by nearly 1000 times (mean $\log_2$ (fold change)=9.96). Furthermore, some of

220 the most strongly differentially expressed genes also had the highest total levels of

221 expression in our transcriptome (Figure 1B).

222

223 *A lysozyme is highly expressed in breeding females*

224 We identified lysozymes by searching for the conserved LYZ1 domain, which

225 contains the active site of C-type lysozymes. Using this approach we identified six

226 lysozymes (Figure 2A). These ranged in size from 103-214 amino acids, which is

227 within the typical size range of insect lysozymes.

228

229 To identify the gene that may be responsible for the antimicrobial activity of the anal

230 exudate of breeding females we compared the expression of the six lysozyme genes in

9

231   breeding and non-breeding females in the whole transcriptome data. Five of the genes

232   had similar expression levels in breeding and non-breeding beetles, while *Lys6* was

233   massively upregulated – the expression level in breeding females was 1409 times

234   greater than in non-breeding females (Figure 1B; $\log_2$ (fold change)=10.46, $p<10^{-26}$).

235   In the breeding beetles *Lys6* was the 14th most abundant transcript in the entire

236   transcriptome, while in the non-breeding beetles it was only the 5967th most abundant.

237

238   We replicated this result using quantitative PCR to measure the expression of the

239   lysozymes across six breeding and six non-breeding females (Figure 2B). In the non-

240   breeding females the different lysozymes all had similar levels of expression. As was

241   the case in the transcriptome analysis, *Lys6* was strongly upregulated in breeding

242   females, with an average expression level that was 860 times than non-breeding

243   beetles ($t =12.6$, df = 9.99, $p <10^{-07}$). The expression of the five remaining lysozymes

244   was unaltered in the breeding females.

245

246   **Discussion**

247

248   Our analyses indicate that breeding induces a very strong transcriptional response in

249   female burying beetles, causing substantial upregulation of just one lysozyme gene

250   (*Lys6*) in their gut tissues relative to non-breeding females. This correlates with our

251   earlier phenotypic observations that the antimicrobial properties of the anal exudates

252   in females are dramatically elevated after the presentation of a carcass and the onset

253   of reproduction [8, 11]. Since none of the other 5 lysozyme genes that are expressed

254   in the gut were upregulated during reproduction, it suggests that upregulation of *Lys6*

255   causes at least some of the change in the exudates' antimicrobial properties during

256   breeding. In addition, it provides further evidence to support the hypothesis that

257   burying beetles have recruited a component of their personal immune system, namely

258   lysozyme, to play a major role in social immunity.

259

260   Does this mean we have therefore found a gene for social immunity in the burying

261   beetle *N. vespilloides*? We cautiously suggest that the answer to this question is likely

262   to be yes. Previous work has identified lysozyme-like activity in the antimicrobial

263   anal exudates [8,9]. Given the very high levels of *Lys6* expression were only detected

264   in breeding females, it seems most likely that this gene functions primarily in social

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265   immunity. The finding that a lysozyme has a role in social immunity would be

266   unsurprising. These enzymes provide a broad-spectrum defence against microbes in

267   their environment, and they are secreted onto external surfaces that are vulnerable to

268   infection, such as the gut, eyes, mucous membranes and respiratory tract [12]. It may

269   therefore be straightforward to recruit lysozymes to social immune functions.

270   Nevertheless, further experiments are now needed to confirm that the protein encoded

271   by this gene is present in the anal exudates and that it is indeed functioning in social

272   immunity.

273

274   It might be argued that bacteria form a key part of the diet of breeding burying beetles

275   or their larvae, but not of non-breeding burying beetles. Thus, a possible alternative

276   interpretation of our data is that *Lys6* primarily serves a digestive function, rather than

277   an immune function, as has been suggested for the lysozymes expressed in housefly

278   or *Drosophila* guts. However, we think this alternative interpretation is unlikely as

279   behavioural evidence suggests that beetles prefer to feed on meat rather than on the

280   microbes living on the meat [7]. Furthermore, beetles in both treatments were given a

281   similar meat-based diet, whether they bred or not, which suggests that upregulation of

282   *Lys6* in the breeding beetles was not induced simply by consuming mouse flesh. Thus,

283   although at this stage we cannot rule out the possibility that *Lys6* plays some minor

284   role in digestion, this is unlikely to be its sole or even primary function.

285

286   In summary, we suggest that we have found a gene (*Lys 6*) for social immunity in the

287   burying beetle and that it was recruited from personal immune function in the

288   evolutionary past. The challenge for future work is to determine how this gene's

289   function is integrated with other components of the social immune system to influence

290   the microbial community on the burying beetle's carcass breeding resource.

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299　**References**

300　1.　Schmid-Hempel, P. 2005 Evolutionary ecology of insect immune defenses.
301　　　*Annu. Rev. Entomol.* **50**, 529–551.
302　　　(doi:10.1146/annurev.ento.50.071803.130420)

303　2.　Siva-Jothy, M. T., Moret, Y. & Rolff, J. 2005 Insect Immunity: An Evolutionary
304　　　Ecology Perspective. *Adv. In Insect Phys.* **32**, 1–48. (doi:10.1016/S0065-
305　　　2806(05)32001-7)

306　3.　Cotter, S. C. & Kilner, R. M. 2010 Personal immunity versus social immunity.
307　　　*Behav. Ecol.* **21**, 663–668. (doi:10.1093/beheco/arq070)

308　4.　Cremer, S., Armitage, S. A. O. & Schmid-Hempel, P. 2007 Social Immunity.
309　　　*Curr. Biol.* **17**. (doi:10.1016/j.cub.2007.06.008)

310　5.　Hoffmann, J. A. 1995 Innate immunity of insects. *Curr. Opin. Immunol.* **7**, 4–10.
311　　　(doi:10.1016/0952-7915(95)80022-0)

312　6.　Hultmark, D. 2003 Drosophila immunity: Paths and patterns. *Curr. Opin.*
313　　　*Immunol.* **15**, 12–19. (doi:10.1016/S0952-7915(02)00005-5)

314　7.　Rozen, D. E., Engelmoer, D. J. P. & Smiseth, P. T. 2008 Antimicrobial strategies
315　　　in burying beetles breeding on carrion. *Proc. Natl. Acad. Sci. U. S. A.* **105**,
316　　　17890–17895. (doi:10.1073/pnas.0805403105)

317　8.　Cotter, S. C. & Kilner, R. M. 2010 Sexual division of antibacterial resource
318　　　defence in breeding burying beetles, Nicrophorus vespilloides. *J. Anim. Ecol.*
319　　　**79**, 35–43. (doi:10.1111/j.1365-2656.2009.01593.x)

320　9.　Arce, A. N., Johnston, P. R., Smiseth, P. T. & Rozen, D. E. 2012 Mechanisms and
321　　　fitness effects of antibacterial defences in a carrion beetle. *J. Evol. Biol.* **25**,
322　　　930–937. (doi:10.1111/j.1420-9101.2012.02486.x)

323　10.　Cotter, S. C., Topham, E., Price, A. J. P. & Kilner, R. M. 2010 Fitness costs
324　　　associated with mounting a social immune response. *Ecol. Lett.* **13**, 1114–
325　　　1123. (doi:10.1111/j.1461-0248.2010.01500.x)

326　11.　Cotter, S. C., Littlefair, J. E., Grantham, P. J. & Kilner, R. M. 2013 A direct
327　　　physiological trade-off between personal and social immunity. *J. Anim. Ecol.*
328　　　**82**, 846–853. (doi:10.1111/1365-2656.12047)

329　12.　Hultmark, D. 1996 Insect lysozymes. *EXS* **75**, 87–102.

330　13.　Daffre, S., Kylsten, P., Samakovlis, C. & Hultmark, D. 1994 The lysozyme locus
331　　　in Drosophila melanogaster: an expanded gene family adapted for expression
332　　　in the digestive tract. *Mol. Gen. Genet.* **242**, 152–162.

333   14.   Ito, Y., Nakamura, M., Hotani, T. & Imoto, T. 1995 Insect lysozyme from house
334   fly (Musca domestica) larvae: possible digestive function based on sequence
335   and enzymatic properties. *J. Biochem.* **118**, 546–551.
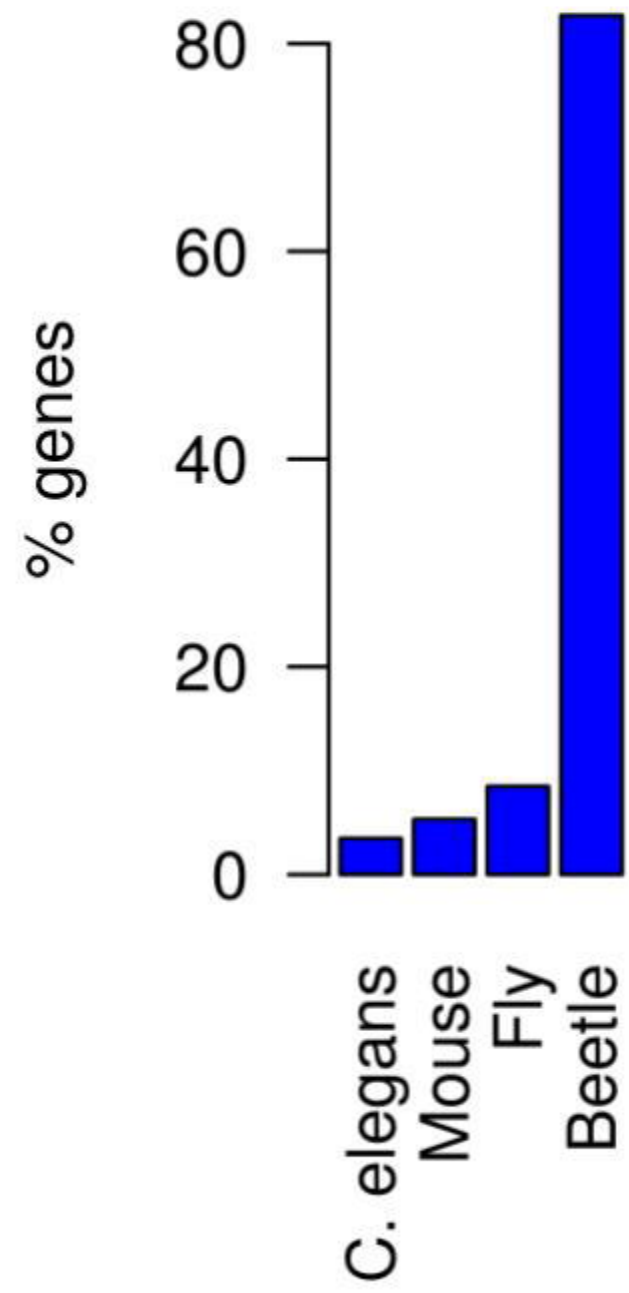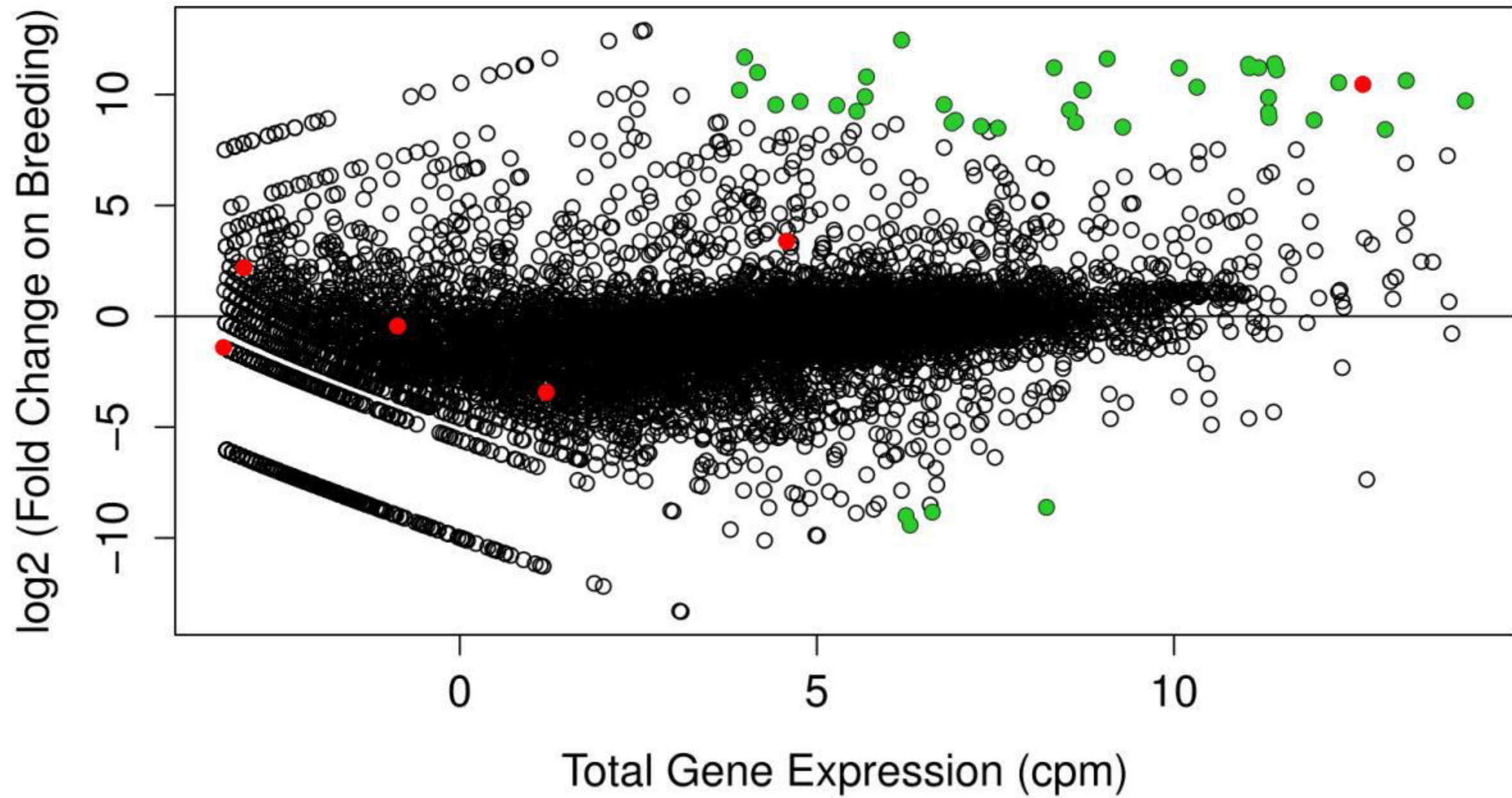
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337

338   **Figure 1.** The transcriptome of *N. vespilloides.* (A) The percentage of peptides whose
339   most similar sequence was in the genome of the mouse *Mus musculus,* the nematode
340   *Caenorhabditis elegans,* the fly *Drosophila melanogaster* or the beetle *Tribolium*
341   *castaneum.*   A single isoform of each gene in the differential expression analysis is
342   included. (B) Total gene expression (counts per million) and $\log_2$ (fold change) in
343   gene expression in the guts of breeding versus non-breeding females. Lysozymes are
344   shown in red. The most significantly differentially expressed genes ($P<10^{-20}$) are in
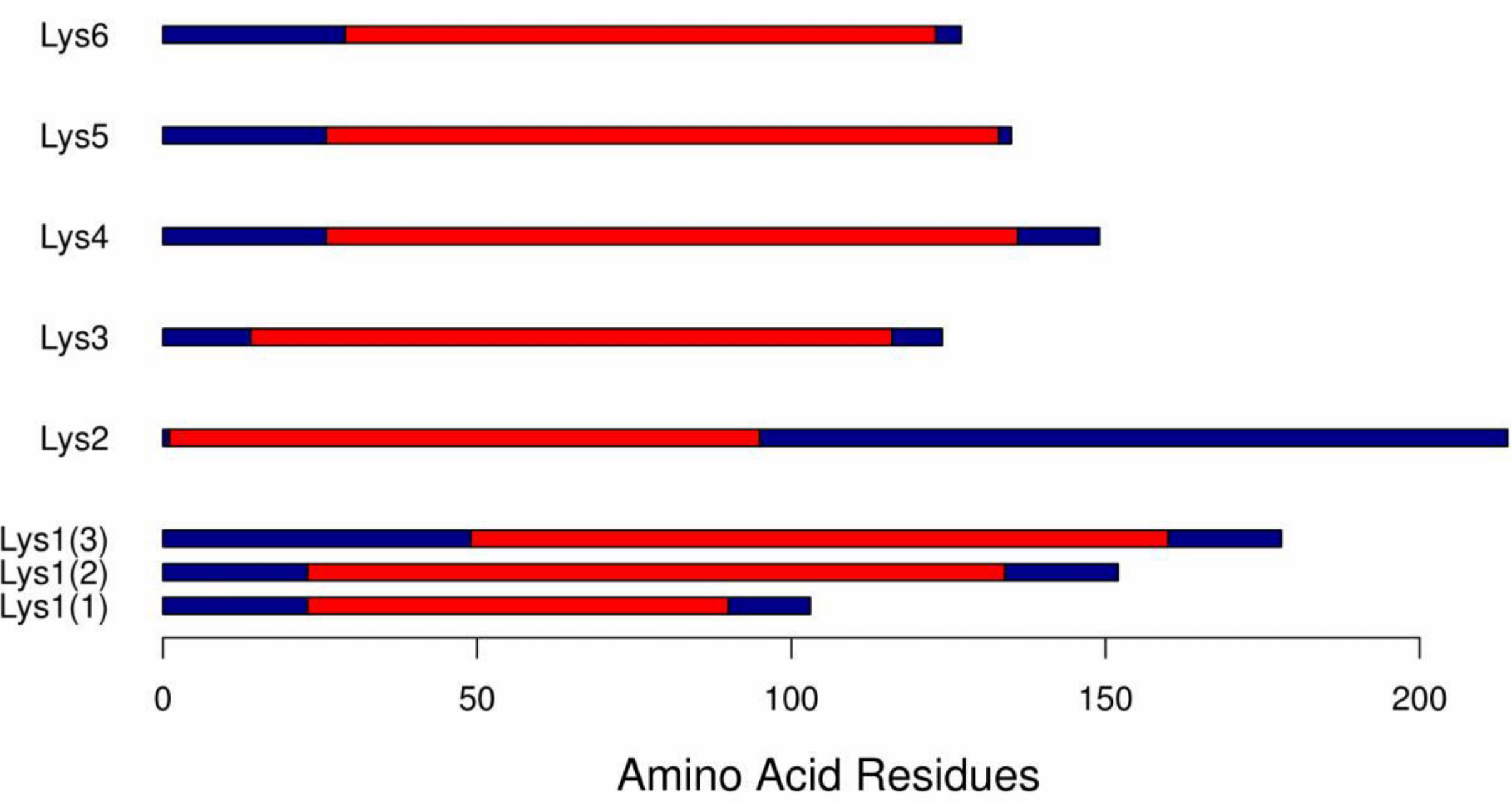345   green.

346

347   **Figure 2.** Lysozymes and their expression. (A) The six predicted lysozymes in the
348   transcriptome of *N. vespilloides.* The LYZ1 C-type lysozyme domain (cd00119) is
349   shown in red. There are three alternative isoforms of *Lys1.* (B) The expression of the
350   lysozyme genes in the guts of six breeding (red) and six non-breeding (blue) females.
351   Expression was measured by quantitative PCR relative to *Actin5C* (scale shifted so
352   begin at zero). Each point is the mean of three technical replicates and the horizontal
353   bars are means.
354