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1 # Genomic architecture and sexually dimorphic expression underlying
2 # immunity in the red mason bee, *Osmia bicornis*

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9 ## Abstract

10 Insect pollinators provide crucial ecosystem services yet face increasing environmental pressures.
11 The challenges posed by novel and reemerging pathogens on bee health means we need to
12 improve our understanding of the immune system, an important barrier to infections and disease.
13 Despite its importance, for certain ecologically important species, such as solitary bees, our
14 understanding of the genomic basis and molecular mechanisms underlying immune potential, and
15 how intrinsic and extrinsic factors may influence immune gene expression is lacking. Here, to
16 improve our understanding of the genomic architecture underlying immunity of a key solitary bee
17 pollinator, we characterised putative immune genes of the red mason bee, *Osmia bicornis*. In
18 addition, we used publicly available RNA-seq datasets to determine how sexes differ in immune
19 gene expression and splicing but also how pesticide exposure may affect immune gene expression
20 in females. Through comparative genomics, we reveal an evolutionary conserved set of more than
21 500 putative immune-related genes. We found genome-wide patterns of sex-biased gene
22 expression, including immune genes involved in antiviral-defence. Interestingly, the expression of
23 certain immune genes were also affected by exposure to common neonicotinoids, particularly
24 genes related to haemocyte proliferation. Collectively, our study provides important insights into
25 the gene repertoire, regulation and expression differences in the sexes of *O. bicornis*, as well as
26 providing additional support for how neonicotinoids can affect immune gene expression, which may
27 affect the capacity of solitary bees to respond to pathogenic threats.

28 **Keywords:** immunity, solitary bees, *Osmia*, sex differences, pesticide, gene expression

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29 **Short title:** Immune gene expression in the red mason bee.

30 ## Introduction

31 Insect pollinators provide key ecosystem services that are essential for the maintenance of
32 agricultural crop yields, as well as natural biodiversity (Klein, Steffan-Dewenter and Tscharntke,
33 2003; Losey and Vaughan, 2006; Gallai *et al.*, 2009). Pollination by insects, including social and
34 solitary bees, is estimated to contribute $15.2 billion to the US economy demonstrating the
35 economic benefits provided by such services (Calderone, 2012). Despite the importance of such
36 services, recent documented declines in bee populations have raised concerns over the continued
37 provision of such services and related issues with food security (Vanengelsdorp and Meixner,
38 2010; *European Red List of Bees*, 2014; Goulson *et al.*, 2015). Both abiotic and biotic factors have
39 been highlighted as contributing factors to decline, including habitat loss and fragmentation,
40 climate change, increased pesticide usage in modern agriculture, as well as pathogens and
41 disease (Brown and Paxton, 2009; Goulson *et al.*, 2015).

42 An important barrier to infection and establishment of disease is the invertebrate immune system
43 (Rolff and Reynolds, 2009; Sadd and Schmid-Hempel, 2009). Despite lacking the adaptive immune
44 system found in vertebrates, invertebrates have a dynamic innate immune system consisting of
45 recognition molecules, signalling pathways and effector molecules, which coordinate the targeting
46 and removal of potentially harmful entities (Hoffmann, 1995). In addition to the evolutionary
47 importance of the immune system (Sadd and Schmid-Hempel, 2008; Viljakainen, 2015),
48 understanding insect immunity has applied purposes, especially within the fields of biomedical,
49 agricultural and conservation biology. Genomic studies on insects have provided novel insights into
50 the genes and genomic architecture underlying the immune system (Christophides *et al.*, 2002;
51 Evans *et al.*, 2006; Sackton *et al.*, 2007; Waterhouse *et al.*, 2007; Gerardo *et al.*, 2010). Such
52 studies have documented and helped understand the immune potential and capacity of a species
53 through the identification of gene family expansions, contractions, as well as lineage-specific or
54 novel genes that demonstrate immune function (Adams, 2000; Evans *et al.*, 2006; Barribeau *et al.*,

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55 2015). Indeed, comparative genomics allows for examining the types of selection pressures acting
56 on such genes providing important insights into their evolutionary history. Given the enormous
57 selection pressures placed upon hosts by pathogens (Combes, 2001), genes involved in the
58 immune system are expected and have been observed to evolve under strong positive selection.
59 Indeed, in comparative genomic studies of both vertebrates and invertebrates, including insects,
60 immune genes are often identified with signatures of accelerated rates of evolution (Viljakainen *et*
61 *al.*, 2009; Roux *et al.*, 2014; Shultz and Sackton, 2019).

62 Functional genomics, such as genome-wide transcriptional profiling ("RNA-seq"), provide high-
63 scale resolution of genome-wide changes in gene expression in response to immune or pathogen
64 challenge but also intrinsic differences in expression between different life cycle stages or sexes
65 (Fish, 2008; Klein and Flanagan, 2016). Indeed, sexually dimorphic gene expression has been
66 identified across taxonomically diverse groups and may exist due to differences in life histories,
67 hormonal abundance, biochemical reactions or sex-specific genomic architecture (Hill-Burns and
68 Clark, 2009). Such differences can underlie differences in immune expression and function but
69 also susceptibility to disease and related survival (Ingersoll, 2017). A striking example whereby
70 sex-specific differences in genomic architecture may underlie differences in immune potential,
71 activity and expression are members of the Hymenoptera, which include the bees, ants and wasps.
72 Within this group, sexes differ in their ploidy with females developing from fertilised diploid eggs
73 while males develop from unfertilised haploid eggs (Pamilo and Crozier, 1981). The haploid nature
74 of males means that any alleles carried are automatically expressed and open to selection, which
75 can result in the rapid removal of maladaptive deleterious alleles from the gene pool (Joseph and
76 Kirkpatrick, 2004). The haploid nature of males has led to predictions that they are more
77 susceptible to environmental challenges, such as pathogens (O'Donnell and Beshers, 2004),
78 although empirical evidence to support this view has been conflicting (Baer *et al.*, 2005; Calleri *et*
79 *al.*, 2006; Ruiz-González and Brown, 2006; Colgan *et al.*, 2011; Retschnig *et al.*, 2014).

80 Despite the fact that pathogen exposure and intrinsic differences can affect or influence immune
81 gene expression, other factors can also have an influence, including nutritional status (Moret and

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82 Schmid-Hempel, 2000; DeGrandi-Hoffman and Chen, 2015), mating (Peng, Zipperlen and Kubli,
83 2005; Lawniczak *et al.*, 2007; Barribeau and Schmid-Hempel, 2017), periods of senescence or
84 dormancy (Nakamura *et al.*, 2011; Kubrak *et al.*, 2014; Colgan *et al.*, 2019), as well as
85 environmental factors, such as temperature (Xu and James, 2012; Chen, Nolte and Schlötterer,
86 2015). One environmental challenge that has received considerable attention of late is the
87 influence of pesticide exposure on immune expression and function. Chemical pesticides, such as
88 pyrethroids and neonicotinoids, interact with the insect nervous system resulting in paralysis and
89 death (Matsuda *et al.*, 2001). The efficacy of chemicals, such as neonicotinoids, combined with its
90 lower toxicity to vertebrates, their systemic mode of action, as well as the lack of requirement for
91 reapplication has resulted in their increased use in modern agricultural practices (Jeschke and
92 Nauen, 2008). Despite their efficacy in killing agricultural pest species, the ubiquitous distribution of
93 neonicotinoids across tissues means that non-target insects, including beneficial pollinators, may
94 come in contact with such chemicals through food resources, such as nectar and pollen
95 (Blacquière *et al.*, 2012). The concentration of such chemicals can result in sublethal and indirect
96 effects on the insect phenotype and has been identified to adversely affect the behaviour (Gill,
97 Ramos-Rodriguez and Raine, 2012; Stanley, Smith and Raine, 2015; Arce *et al.*, 2017, 2018;
98 Siviter *et al.*, 2018), neurobiology (Moffat *et al.*, 2015, 2016) and gene expression of key ecological
99 and commercial pollinators (Chaimanee *et al.*, 2016; Beadle *et al.*, 2019; Bebane *et al.*, 2019;
100 Colgan *et al.*, 2019). In addition, such chemicals have been shown to affect immune expression
101 and function (Di Prisco *et al.*, 2013; Mason *et al.*, 2013; Chmiel *et al.*, 2019; Brandt *et al.*, 2020)
102 raising concerns that these effects may influence the ability of an exposed individual to respond to
103 pathogenic threats (James and Xu, 2012). While studies have identified molecular mechanisms by
104 which bees can metabolise certain neonicotinoids (Manjon *et al.*, 2018; Beadle *et al.*, 2019;
105 Troczka *et al.*, 2019), our understanding of changes in immune expression due to neonicotinoid
106 exposure, especially for solitary bees, is limited.

107 The mason bees (*Osmia* species) are an important group of solitary bee pollinators but are
108 generally understudied from an immunological perspective. One such species is the red mason

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109 bee *Osmia bicornis* (Order Hymenoptera; Family Megachilidae), a common pollinator found across
110 central Europe, which has been increasingly incorporated into modern agricultural practices
111 (Gruber *et al.*, 2011). Despite its importance, it faces a number of environmental challenges that
112 can influence the immune system, including pathogens and parasites (Seidelmann, 2006;
113 Schoonvaere *et al.*, 2018; Tian *et al.*, 2018; Bramke *et al.*, 2019), as well as pesticides (Brandt *et*
114 *al.*, 2020). Similarly, intrinsic differences between the sexes, which differ in morphology,
115 physiology, behaviour and ploidy (Dmochowska-Ślęzak *et al.*, 2015; Rogers, Frasnelli and
116 Versace, 2016; Szentgyörgyi *et al.*, 2017), may result in differences in immune expression and
117 associated susceptibility to pathogenic threats. However, at present, our understanding of the
118 immune gene repertoire and expression in *O. bicornis* is currently limited.

119 To improve our understanding on the immune potential of *O. bicornis*, we performed a comparative
120 genomic analysis to identify the immune gene repertoire of the red mason bee as well as identify
121 potential contractions and expansions of important gene families and determine whether the red
122 mason bee is missing immune genes. Furthermore, to understand how genes underlying immunity
123 may be expressed differently between the sexes, we investigated evidence of sex-biased gene
124 expression and alternative splicing. Lastly, as pesticides can negatively affect different aspects of
125 *Osmia* health, including developmental rate (Mokkapati, Bednarska and Laskowski, 2021), foraging
126 behaviour (Boff *et al.*, 2021; Straub *et al.*, 2021), reproductive output (Sandrock *et al.*, 2014;
127 Woodcock *et al.*, 2017; Ruddle *et al.*, 2018), thermoregulation (Azpiazu *et al.*, 2019), as well as
128 impact immune function in red mason bees (Brandt *et al.*, 2020), we examined whether
129 neonicotinoid-exposed individuals differed in immune gene expression.

130 Results

131 Putative expanded immune gene repertoire in the Hymenoptera

132 To infer putative immune genes in *O. bicornis*, we independently examined the presence of
133 homologues of previously characterised immune genes of the fruit fly, *Drosophila melanogaster*,
134 and the closely related earth bumblebee, *Bombus terrestris* in the *O. bicornis* predicted proteome.

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135 We merged resulting *O. bicornis* homologues of known immune genes in *D. melanogaster* and *B.*
136 *terrestris*, resulting in a total of 541 putative immune gene homologues, of which 99 were only
137 found among the set of *B. terrestris* immune genes, 387 were only found among the set of *D.*
138 *melanogaster* immune genes and 55 were found among both sets (Figure 1A, Supplemental File
139 S1). We also ran functional enrichment analyses and found enrichment in immune terms for the
140 putative immune genes derived uniquely via homology to *D. melanogaster* and for the putative
141 immune genes found among both sets while for the putative immune genes derived uniquely via
142 homology to *B. terrestris* we found enrichment in terms only indirectly linked to the immune system
143 such as “response to stress” and “response to toxic substance” (Supplemental File S2).

144 Given that previous genomic studies on immune gene repertoires in hymenopterans have
145 described lower gene counts, and since *O. bicornis* is more evolutionarily distantly related to *D.*
146 *melanogaster* than *B. terrestris*, we further examined immune genes identified only through
147 homology to *D. melanogaster* to determine confidence in homology. For this, we examined the
148 sequence identity of homologous immune proteins as calculated by OrthoFinder between *O.*
149 *bicornis* and *D. melanogaster* revealing higher percentage sequence identity for putative immune
150 proteins found only in the set of *D. melanogaster* immune proteins compared to immune proteins
151 found uniquely in the set of canonical *B.terrestris* immune proteins or immune proteins found in
152 both sets (Figure 1D). As metrics of sequence identity can be influenced by protein length, we
153 compared predicted protein lengths between homologous pairs, revealing a strong positive
154 correlation (Pearson’s Product Moment Correlation Coefficient, $R=0.94$, $p< 2.2e-16$, Figure 1B-C).
155 Lastly, we examined if homologous sequences shared the same type and number of functional
156 domains, which would potentially suggest conserved function. We found identical domain
157 annotations for a high percentage (71.01%, n=722) of all homologous pairs between *O. bicornis*
158 and *D. melanogaster* with on average 90.05% of the domains shared between pairs.

159 Our analysis also revealed immune genes potentially missing in *O. bicornis*. We did not identify
160 homologues for 232 *D. melanogaster* immune genes as well as one canonical *B. terrestris* immune
161 gene, the antimicrobial peptide abaecin, in *O. bicornis* or its sister taxa, *O. lignaria* (Supplemental

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162 File S1). Inversely, as *Osmia* may contain lineage specific genes, including genes with potential
163 immune functions, we identified genes (n=78, split across 48 orthogroups; Supplemental File S1)
164 shared between *O. bicornis* and *O. lignaria* that lacked homologues in the predicted proteomes of
165 all other 19 insect species we used to infer homology relations (for species list see Experimental
166 Procedures). Among the *Osmia*-specific genes that were annotated with at least one domain
167 (n=24), we find 16 genes annotated with ribonuclease-domains (IPR036397, IPR012337) as well
168 as one gene (LOC114879997) annotated with a rhabdovirus nucleoprotein domain (IPR004902).

169 To understand variation in the immune gene complement of the red mason bee, we compared the
170 number of putative *O. bicornis* immune genes in conserved gene families and pathways to the
171 number of homologues in three closely-related hymenopteran species (*O. lignaria*, *B. terrestris* and
172 *A. mellifera*) and one more distantly-related fly species (*D. melanogaster*). We found similar
173 numbers of genes in the four hymenopterans for most signaling pathways or non-pathway gene
174 families, with slightly lower gene numbers in the two *Osmia* species compared to the other two
175 hymenopterans for the Immune deficiency (ImD) and Toll pathways (Figure 2A) and higher
176 numbers for inhibitors of apoptosis in *B. terrestris* (n=13) compared to the other hymenopterans
177 (n=4 to 5, Figure 2B). When comparing the hymenopterans to *D. melanogaster*, the latter has
178 higher gene counts for six out of 14 signaling pathways and 13 out of 22 non-pathway gene
179 families but similar numbers for all other pathways and gene families. In addition, we checked the
180 number of homologues in *O. bicornis* for *D. melanogaster* immune genes on a gene family level
181 and found a high average conservation of 84.86% but a large difference for antimicrobial peptides
182 with only one *O. bicornis* homologue compared to 22 *D. melanogaster* genes.

183 Sex-biased differential expression of immune genes

184 To provide functional information on the expression of putative immune genes in *O. bicornis*, we
185 compared whole-bodied transcriptomes of male (n=3) and female adults (n=4). We identified 4,128
186 genes (34.99% of total gene count, n=11,799) as significantly differentially expressed (Likelihood-
187 ratio test, BH-adjusted $p<0.05$) between the sexes, of which, 2,087 and 2,041 had female- and
188 male-biased expression, respectively (Supplemental File S3). Among the differentially expressed

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189 genes, we found a significant enrichment or depletion (Kolmogorov-Smirnov test, $p<0.05$) of 125
190 biological process-associated GO terms, 47 cellular component GO terms and 29 molecular
191 function GO terms with “cytoplasmic translation”, “cytosolic large ribosomal subunit” and “structural
192 constituent of ribosome” as the most enriched terms for each of the three ontologies, respectively
193 (Supplemental File S2). We quantified expression of 520 putative immune genes (96.11% of total
194 immune genes, n=541) in both sexes, of which 222 were differentially expressed (42.69% of total
195 DEGs) which was significantly more than expected by chance (Fisher’s Exact test, $p=0.017$).
196 These differentially expressed genes were nearly equally shared between the sexes with slightly
197 more genes (n=118) showing male-biased rather than female-biased expression (n=104), a pattern
198 which did not significantly differ from expectation (Fisher’s Exact test, $p=0.554$, Supplemental File
199 S1). Among the differentially expressed immune genes, we found enrichment or depletion for 28
200 biological process GO terms, five cellular component GO terms and five molecular function GO
201 terms with “RNA localization”, “intracellular non-membrane-bounded organelle” and “RNA binding”
202 as top enriched terms of each of the ontologies, respectively (Figure 3B, Supplemental File S2).
203 We also analysed the *Osmia*-specific genes for signatures of differential expression and found 15
204 genes that significantly differed in their expression between the sexes (Supplemental File S1).

205 **Sex-biased alternative splicing of immune genes**

206 We also performed alternative splicing analysis and identified 1,019 genes (8.64% of total gene
207 count, n=11,799) significantly differentially spliced (Likelihood-ratio test, FDR < 0.05) between the
208 sexes (Supplemental File S4). These sex-biased genes were significantly enriched or depleted
209 (Kolmogorov-Smirnov test, $p<0.05$) for 107 biological process GO terms, 24 cellular component
210 GO terms and 18 molecular function GO terms with “sarcomere organisation”, “Z disc” and “actin
211 binding” as the top enriched terms for each ontology, respectively, as well as “immune system
212 process”, significantly enriched among the biological process GO terms (Supplemental File S2).
213 We identified 71 putative immune genes as differentially spliced (13.12%) between the sexes,
214 which is significantly more than expected (Fisher’s exact test; $p=0.002$). In addition, we found
215 differences between the sexes in the frequency of different splicing events with retained intron

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216 events significantly more common in females than in males (Fisher's exact test, BH-adjusted
217 *p*=0.036). For other splicing events, we found a borderline significant male-bias for alternative 3'
218 splice sites (Fisher's exact test, BH-adjusted *p*=0.051) while we found no significant differences
219 (BH-adjusted *p*>0.05) for skipped exons, alternative 5' splice sites and mutually exclusive exons.

220 Immune gene expression changes in response to pesticide exposure

221 For the neonicotinoid exposure analysis we identified 617 genes, including 42 putative immune
222 genes, significantly differentially expressed (Wald-test, BH-adjusted *p*<0.05) in the group of
223 thiacloprid-exposed females compared to the untreated control group (Figure 4C, Supplemental
224 File S3), with a significantly unequal partitioning of 436 genes up-regulated and 181 genes down-
225 regulated in the thiacloprid-exposed group (Binomial-test, *p*=9.41e-22). Comparing imidacloprid-
226 exposed females with untreated females, we found 127 genes significantly differentially expressed
227 (Wald-test, BH-adjusted *p*<0.05), including seven immune genes (Figure 4A). We also found a
228 significantly unequal partitioning of 89 genes up-regulated and 38 genes down-regulated in the
229 imidacloprid-exposed group (Binomial-test, *p*=6.97e-5). We found more differentially expressed
230 immune genes than expected by chance in the thiacloprid-exposed group (n=42 immune genes;
231 Fisher's exact test, *p*=0.014) but not in the imidacloprid-exposed group (n=seven immune genes;
232 Fisher's exact test, *p*=0.515). Five putative immune genes (LOC114878095, LOC114878683,
233 LOC114881181, LOC114874985, LOC114872156) had increased transcript expression both in
234 response to thiacloprid and imidacloprid with no significant difference between the log2 fold change
235 values for these five genes between the two pesticide treatment groups (Welch's t-test, *p*=0.76,
236 Supplemental Files S1, S3). In terms of functional enrichment of significantly differentially
237 expressed immune genes, we found 45 biological process-associated GO terms enriched or
238 depleted for the thiacloprid-exposed group, as well as five cellular component and eight molecular
239 function terms with "regulation of hemocyte proliferation", "integral component of plasma
240 membrane" and "signaling receptor activity" as top enriched terms for each ontology, respectively
241 (Figure 4D). For the imidacloprid-exposed group, we found 30 biological process GO terms
242 enriched or depleted among the differentially expressed immune genes as well as four cellular

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243 component terms and seven molecular function terms with "transmembrane receptor protein
244 tyrosine kinase signaling pathway", "integral component of plasma membrane" and "signaling
245 receptor activity" as top terms of each of the ontologies, respectively (Figure 4B, Supplemental File
246 S2). Among the *Osmia*-specific genes we identified three genes differentially expressed between
247 the thiacloprid-exposed group and control group but no differentially expressed genes between the
248 imidacloprid-exposed group and the control group (Supplemental File S1).

249 For alternative splicing, we found 142 differentially spliced genes (Likelihood-ratio test, FDR<0.05)
250 for the thiacloprid-exposed group, including six putative immune genes, while 74 genes were
251 differentially spliced for the imidacloprid-exposed group, including four putative immune genes.
252 One immune gene (LOC114880106) was differentially spliced in response to both pesticides
253 (Supplemental File S4).

254 Discussion

255 The insect immune system represents an important barrier against infections and disease and thus
256 provides a physiological function vital to an individual's success. While our understanding of the
257 genomic and molecular bases of immunity in the Hymenoptera has largely been informed by
258 studies on social bees, for other species, especially solitary bees, such information is limited. Here,
259 we performed a comparative genomic analysis to characterise genes with potential immune
260 functions in the genome of the red mason bee, *O. bicornis*. Using a homology-based approach, we
261 identify an immune gene repertoire enlarged beyond the canonical immune genes previously
262 described in other hymenopteran genomes. We find extensive differences in immune gene
263 expression between the sexes, both in terms of expression amplitude and splicing, highlighting
264 intrinsic regulatory differences in the molecular basis of immunity between males and females.
265 Lastly, we find immune-related genes differentially expressed in response to neonicotinoid
266 exposure with greater expression differences in bees exposed to thiacloprid than those exposed to
267 imidacloprid demonstrating differences in how the molecular phenotype responds to different
268 neonicotinoid subclasses and how each may influence immune expression.

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269 The insect immune system consists of an innate immune response with the ability to detect and
270 remove a diverse range of pathogenic entities (Beckage, 2008). In addition to behavioural, physical
271 and chemical defences, it is an important barrier to infection and disease development. The
272 earliest genomic studies on the Hymenoptera documented a reduction in canonical immune genes
273 in comparison to other insect orders, most notably in comparison to members of the Diptera
274 (Evans *et al.*, 2006; Bonasio *et al.*, 2010; Werren *et al.*, 2010; Barribeau *et al.*, 2015). Reasons for
275 this reduction ranged from the technical (e.g., fragmented genome assembly, missing or truncated
276 gene models) to the biological level (e.g., novel immune genes and pathways ((Albert *et al.*, 2011;
277 Dong *et al.*, 2020) or relaxed selection acting on canonical immune genes due to social immunity
278 (Harpur and Zayed, 2013)). Here we performed one of the first investigations of the immune gene
279 repertoire of a solitary bee species.

280 Our initial approach for the detection of putative immune genes was based on homology with
281 genes annotated with roles in immune system function based on Gene Ontology in the model
282 organism, *D. melanogaster*, where many such genes have been experimentally validated with
283 roles in immune function. As many immune genes have been previously shown to evolve under
284 strong episodic positive selection (Jiggins and Kim, 2007; Viljakainen, 2015; Shultz and Sackton,
285 2019), which can result in divergence beyond detection through homology searches or the
286 appearance of novel lineage-specific immune genes, we also investigated homologues in *O.*
287 *bicornis* of canonical immune genes from the earth bumblebee, *B. terrestris*, a closely related
288 social insect with an annual life-cycle. As the majority of canonical immune genes identified in *B.*
289 *terrestris* were generated based on homology searches with other insect genomes, including *D.*
290 *melanogaster* (Barribeau *et al.*, 2015), we would have expected canonical immune genes in both
291 species to be annotated with immune process GO terms and therefore, we would have expected to
292 see a high overlap in immune gene sets. Surprisingly, we found a weak overlap between *Osmia*
293 homologues identified using both approaches, with only a third of *B. terrestris* canonical immune
294 genes identified also through homology to *D. melanogaster* immune genes and thus annotated
295 with immune system GO terms. The other two thirds were annotated with GO terms associated

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296 only more indirectly with immunity, such as “response to toxic substance”, “RNA interference” and
297 “autophagy”. The lack of annotation of immune GO terms for two thirds of the described canonical
298 immune genes in *B. terrestris* may result in the underreporting of immunological changes for
299 genomic studies reliant on GO term based analyses.

300 To provide better support for conserved function for putative immune homologues in *Osmia*, we
301 further assessed *O. bicornis* immune homologues identified through the *D. melanogaster*
302 comparison, which lacked a described homologue in *B. terrestris* canonical immune genes. If such
303 homologues were spurious or low quality matches, we predicted such protein homologues may
304 have lower percentage sequence identity, greater differences in sequence length, as well as
305 variation or lack of structural features, such as abundance and diversity of functional domains
306 compared to canonical immune genes. However, for the majority of homologous pairs, we found
307 the same or greater percentage sequence identity as canonical immune genes, as well as the
308 presence and conservation of functional domains, suggesting that potential immune functions may
309 be conserved and perform similar roles in bees. Identified through homology with *D. melanogaster*
310 immune genes only, we found homologs of many immune relevant genes like defensins,
311 hemocytin and sickie known to be important to the immune system across different insect species
312 (Hoffmann and Hetru, 1992; De Gregorio *et al.*, 2002; Lavine and Strand, 2002; Arai *et al.*, 2013;
313 Ni *et al.*, 2020). Through homology with *B. terrestris* immune genes only, however, we found
314 homologues of immune genes known to be involved in the insect immune system, like mucins,
315 galectin-like proteins and superoxide-dismutases (Pace and Baum, 2002; Korayem *et al.*, 2004;
316 Colinet *et al.*, 2011; Rao *et al.*, 2016), demonstrating the importance of using more than one
317 species to infer undescribed gene sets via homology. While future experimental studies on their
318 function will elucidate potential roles in immunity, if any, for these additional candidate genes, their
319 high number could also speak to the ever-increasing completeness of functional annotation in
320 insect model organisms, like *D. melanogaster*.

321 Among the 541 putative immune genes of *O. bicornis*, we did not find any major patterns of
322 immune gene family expansions or contractions in comparison with that of *B. terrestris* and *A.*

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323 *mellifera*, suggesting that there were no large-scale recent duplications or losses of genes that
324 could be imperative to the functioning of the immune system of bees. However, we did find slight
325 reductions in the number of genes involved in two major immune signaling pathways, Imd and Toll,
326 when we compared *O. bicornis* and *O. lignaria* to *A. mellifera* and *B. terrestris.* Imd and Toll are
327 both involved in the induction of antimicrobial peptides (AMPs) (De Gregorio *et al.*, 2002), thus,
328 fundamental for insect survival in response to pathogen challenge. Related to this, the biggest
329 difference in immune gene families between *O. bicornis* and *D. melanogaster* was for gene copies
330 of AMPs. This can be expected as *Drosophila* have evolved a number of AMP gene families, such
331 as the cecropins, diptericins and attacins (Imler and Bulet, 2005), which have not been identified in
332 other hymenopteran genomes (Evans *et al.*, 2006; Barribeau *et al.*, 2015). Consequently, we did
333 identify the presence of defensin, an evolutionary conserved AMP that possesses antibacterial
334 properties (Hoffmann and Hetru, 1992), as well as the hymenopteran-specific AMP,
335 hymenoptaecin (Casteels *et al.*, 1993). However, we did not detect a copy of abaecin, a bacterial-
336 inducible AMP described in honeybees (Casteels *et al.*, 1990) and bumblebees (Rees, Moniatte
337 and Bulet, 1997). The conserved lack of abaecin, as well as components of the Imd and Toll
338 signaling pathways, in the genome assemblies of two *Osmia* species, which were sequenced and
339 assembled independently, suggests that such missing genes may be true biological signals rather
340 than technical artefacts. At present the evolutionary consequences of such potential losses, if any,
341 are unknown, but it suggests at least that differences in the molecular structure of the immune
342 system do indeed exist for these two solitary bee species compared to these other social bee
343 species.

344 For species that sexually reproduce, the genome codes for distinct sexes that can differ
345 dramatically in behaviour, morphology and physiology (Parsch and Ellegren, 2013), including
346 immunity (Klein and Flanagan, 2016). As immunity can be both energetically costly to maintain and
347 activate (Rolff and Siva-Jothy, 2003), it can result in metabolic trade-offs with other processes,
348 such as reproduction (Schwenke, Lazzaro and Wolfner, 2016). Sexes can also differ in immune
349 investment, which may be reflected in differences in gene expression. Here we found 222 putative

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350 immune genes to be differentially expressed between the sexes with the strongest functional
351 enrichment for terms related to localization and breakdown of RNA and general macromolecules.
352 This suggests that the sexes may differ in important housekeeping roles, such as RNA and protein
353 turnover, but also how they respond to challenging macromolecules that need to be localized and
354 decomposed, such as RNA-viruses and toxins. The case for differences in antiviral defence is
355 further highlighted by genes, such as endonuclease Dcr-1 or defensin, which have roles in virus
356 recognition and degradation (Galiana-Arnoux *et al.*, 2006; Brutscher, Daughenbaugh and
357 Flenniken, 2015), being differentially expressed between the sexes. We found more putative
358 immune genes than expected by chance to be differentially expressed or differentially spliced
359 between the sexes suggesting that the molecular differences between the sexes are particularly
360 pronounced when it comes to immune system processes. We also looked at the expression
361 differences between sexes in general and found the most striking functional enrichment of
362 differentially expressed genes to be related to translational and cell division processes, underlining
363 the generality of molecular differences between the sexes while the most striking enrichment of
364 alternatively spliced genes is mostly related to muscle activity and regulation of muscle excitation
365 which might reflect the different life strategies of male and female mason bees where male bees
366 emerge prior to female bees or thermoregulatory differences.

367 Pesticides, including neonicotinoids, act as agonists of the nicotine acetylcholine receptors,
368 resulting in disruption of the neuronal cholinergic signal transduction and excitation of neuronal
369 triggers culminating in paralysis and death (Matsuda *et al.*, 2001). The efficacy of the mode of
370 action of neonicotinoids has led to their increased popularity in modern agriculture practices yet
371 recent studies have highlighted the negative impact sublethal and lethal doses can have on
372 pollinator health ('Neonicotinoids, bee disorders and the sustainability of pollinator services', 2013),
373 including immune function. Neonicotinoids, such as clothianidin and imidacloprid, have been
374 identified in exposed honeybees to negatively modulate the NF-kappaB signaling pathway and
375 affect the ability to mount effective antiviral defences (Di Prisco *et al.*, 2013). Other studies on
376 neonicotinoids have provided additional evidence of the indirect or direct effects of these

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377 neurotoxins on immune function or expression (Brandt *et al.*, 2017, 2020). Here we found changes
378 in gene expression in response to two classes of neonicotinoids with thiacloprid exposure affecting
379 the expression of more genes, including immune genes, than imidacloprid. This is in line with other
380 studies that have looked at thiacloprid exposed red mason bees and observed impairment in
381 immunity (Brandt *et al.,* 2020) or larval development (Claus *et al.*, 2021). For both neonicotinoids,
382 we find significantly more genes up-regulated in response to pesticide exposure than expected,
383 suggesting that overall the exposure to pesticides results in an active response of heightened gene
384 expression as opposed to a mere passive shift in gene expression. Focusing on immune system
385 processes, we find more immune genes differentially expressed than expected only in thiacloprid-
386 treated individuals, suggesting that thiacloprid elicits a stronger immune response than
387 imidacloprid. Interestingly, of the seven differentially expressed genes with elevated expression in
388 imidacloprid-exposed bees, five genes were also significantly up-regulated in the thiacloprid-
389 exposed bees which could possibly point to a common set of immune genes that are up-regulated
390 in response to neonicotinoid exposure. In terms of functional enrichment of the differentially
391 expressed immune genes, both pesticide-treated groups share similar terms, related to signaling of
392 transmembrane receptors, suggesting that the immune genes play a role in signaling in response
393 to pesticide exposure. In addition, in the thiacloprid-treated group the term “regulation of hemocyte
394 proliferation” is the most significantly enriched term. Haemocytes fulfill an important role in the
395 ingestion and break-down of foreign cells and substances in the insect immune system (Lavine
396 and Strand, 2002). Brandt *et al.* found a reduction in haemocyte density in red mason bees after
397 exposure to thiacloprid, which is in line with our finding of haemocyte proliferation being
398 differentially regulated between thiacloprid treated and untreated individuals, suggesting that the
399 effect of pesticide exposure on haemocyte function may extend down to the molecular level.

400 Conclusions

401 The red mason bee, *O. bicornis*, is a commercially and ecologically relevant solitary bee species,
402 whose immune system has not been well-studied yet, albeit being integral to its future chances of
403 survival when faced with increasing environmental challenges. Here, we utilised a comparative

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404 genomic approach to propose a set of genes as part of the immune gene repertoire of *O. bicornis*,
405 and used RNA-seq data to show that the expression and regulation of these putative immune
406 genes differs markedly between sexes and responds with heightened expression to treatment with
407 two neonicotinoid pesticides. Additionally, our findings provide support for the application of a
408 combined approach to inference of gene families, using homology information of more than one
409 species of reference. Future studies on *O. bicornis* immunity will benefit from tissue-specific
410 profiling, as well as tracking gene expression changes in response to different immune challenges.
411 Similarly the application of population genomics will provide important insights into the recent
412 selection pressures acting on immune genes of mason bees. Collectively, our study provides novel
413 insights into the immune system of an important, yet still understudied solitary bee species and
414 identifies a candidate repertoire of immune genes for future research on the immune system of the
415 red mason bee.

416 Experimental Procedures

417 Identification of putative immune genes in the red mason bee

418 To infer homologues for *O.bicornis* genes that are in other insect species, we ran OrthoFinder
419 [v.2.5.2](Emms and Kelly, 2019) with proteomes of 21 species, comprising 11 bee species from
420 three families (Family Megachilidae: *Osmia bicornis*, *Osmia lignaria*, and *Megachile rotundata*;
421 Family Apidae: *Apis mellifera*, *Ceratina calcarata*, *Eufriesea mexicana*, *Habropoda laboriosa* and
422 *Bombus terrestris*; and Family Halicitidae: *Megalopta genalis*, *Nomia melanderi* and *Dufourea*
423 *novaeangliae*), as well as 10 non-bee insects (*Drosophila melanogaster*, *Aedes aegypti*,
424 *Anopheles gambiae*, *Bombyx mori*, *Tribolium castaneum*, *Acyrthosiphon pisum, Nasonia*
425 *vitripennis*, *Solenopsis invicta*, *Polistes dominula* and *Vespa mandarinia*). All proteomes were
426 obtained from the National Center for Biotechnology Information (NCBI) Reference Sequence
427 (RefSeq) database. We ran OrthoFinder using the default parameters with the inferred species
428 trees forming a consensus with the known phylogeny. Given that model organisms, such as *D.*
429 *melanogaster*, contain the most comprehensive functional annotation of genes with immune

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430 function or potential, we examined the *O. bicornis* predicted proteome for putative homologues of
431 *D. melanogaster* immune genes. To obtain *D. melanogaster* immune-responsive genes, we
432 queried the FlyBase on the 9th of July, 2021 for any gene associated with the GO term "immune
433 system process", the highest order Gene Ontology term associated with the immune system, and
434 inferred the *O. bicornis* homologues via the homologues table generated earlier. As an additional
435 approach, to identify immune genes that may be lineage-specific within the Hymenoptera or too
436 divergent between *O. bicornis* and *D. melanogaster* given their evolutionary distance, we examined
437 the presence of *O. bicornis* homologues from comparison with canonical immune genes
438 characterised in the earth bumblebee, *Bombus terrestris*. The canonical *B. terrestris* immune
439 genes were directly obtained from the most recent earth bumblebee genome papers (Barribeau *et*
440 *al.*, 2015; Sadd *et al.*, 2015). An additional reason for the inclusion of this social bee species is that
441 it has a curated homologue list with *D. melanogaster*, available through the Ensembl Metazoa
442 database, which provided the ability to compare the orthogroups and homologous pairs generated
443 by OrthoFinder with orthogroups and pairs independently generated by Ensembl, which
444 incorporates an additional information on synteny and gene order conservation for the identification
445 of putative homologues between two species. This approach identified a high overlap (86.85% of
446 Ensembl pairs correctly identified by OrthoFinder) between the pairs generated by both analyses
447 providing additional confidence in the orthogroups generated by OrthoFinder. Homologous genes
448 from *D. melanogaster* and *B. terrestris* were obtained by first translating the *O.bicornis* gene-IDs to
449 protein-IDs via the annotation column in the RefSeq gene feature file (GFF) and then using the
450 homology information from OrthoFinder to translate *O. bicornis* protein-IDs to *D. melanogaster* and
451 *B. terrestris* protein-IDs respectively and further translating the protein-IDs to the species-specific
452 gene-IDs, yielding a many-to-many homologue table of *O. bicornis* gene-ID's to *D. melanogaster*
453 and *B. terrestris* gene-ID's, respectively. To infer for each gene family how many homologous
454 genes exist for the set of putative *O. bicornis* immune genes in *A. mellifera*, *B. terrestris*,
455 *D.melanogaster* and *O. lignaria*, we derived annotation of *D. melanogaster* genes with gene
456 families from Flybase on the 02nd of June, 2021 and annotated the immune gene homologues in

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457 each species with the according gene family description. We then summarised this data by
458 counting the number of genes in each species and for each gene family.

459 For *O. bicornis* genes that shared homology with *D. melanogaster* immune-responsive genes but
460 did not overlap with known canonical immune genes in *B. terrestris* ,we further examined homology
461 based on the following criteria: 1) similarity of predicted protein length between homologous pairs;
462 2) high percentage of protein sequence identity between homologous pairs as inferred via
463 Diamond searches performed by OrthoFinder; and 3) the number of shared functional protein
464 domains between homologous as inferred via InterProScan [v5.52-86.0](Jones *et al.*, 2014). The
465 prediction here is that if two homologous proteins shared similar protein length, high sequence
466 identity and the same or similar number and types of functional protein domains, potential
467 functional immune roles may be conserved.

468 In addition to identification of immune-related genes, we also inferred canonical immune genes
469 from *B. terrestris* or *D. melanogaster* missing in *O. bicornis and O. lignaria*. For this, we parsed the
470 output of OrthoFinder for orthogroups that carried immune-associated genes in *D. melanogaster* or
471 *B. terrestris* but not in both *O. bicornis* and *O.lignaria.* Similarly, we inferred *Osmia*-specific genes
472 by parsing orthogroups containing only *O. bicornis* and *O. lignaria* homologues, which were also
473 absent in the other 19 species.

474 *Quality assessment, transcript abundance estimation and differential expression*
475 *analysis of immune genes between sexes and pesticide-treated groups*

476 To examine the functional expression of putative immune genes of *O. bicornis*, we obtained
477 publicly available paired-end RNA-seq libraries for two analyses: a) sex-biased analysis containing
478 males (n=3) and females (n=4); and b) pooled libraries of unexposed ("control"; n=4) females or
479 those exposed to thiacloprid (n=4) or imidacloprid (n=4). All datasets were obtained from the NCBI
480 (National Center for Biotechnology Information) Short Read Archive (SRA) database (BioProject:
481 PRJNA285788; (Beadle *et al.*, 2019), Supplemental File S5). We performed data quality
482 assessment based on per-sample quality evaluations using FastQC [v.0.11.9](Andrews, 2010)

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483 calculation of the proportions of reads mapping to the predicted transcriptome of the RefSeq *O.*
484 *bicornis* reference genome assembly [Obicornis_v3; GCF_004153925.1] using Kallisto [v.0.46.1]
485 (Bray *et al.*, 2016). We then combined and visualized the results for both tools and across all
486 samples with MultiQC [v.1.7](Ewels *et al.*, 2016). Based on the results of the quality assessment
487 we removed adapter sequences and filtered by quality (phred quality score >= 15) and length
488 (minimum length >= 50 bp) using fastp [v.0.20.1](Chen *et al.*, 2018). For each sample, we then
489 aligned the trimmed and filtered reads against the most recent chromosome-level genome
490 assembly [iOsmBic2.1; GCA_907164935.1] using STAR [v.2.7.8a](Dobin *et al.*, 2013). As the
491 chromosome-level assembly currently lacks annotations, we first transferred gene coordinates
492 from the annotated reference assembly [Obicornis_v3; GCF_004153925.1] to the new assembly
493 using Liftoff [v.1.6.1](Shumate and Salzberg, 2020). STAR was ran in two-pass-mode using the
494 inferred splice junctions from the first run to improve the alignment of the second run and with
495 parameter --quantMode GeneCounts used to generate gene level abundances of aligned reads
496 (Supplemental File S6). The mean alignment rate across all samples was 94.09%. For the sex-
497 biased analysis, we used DESeq2 [v.1.30.1](Love, Huber and Anders, 2014) to correct for library
498 size and infer all differentially expressed genes ("DEG", FDR < 0.05) between sexes with a
499 likelihood-ratio-test (LRT; full model: sex; reduced model: intercept). For the pesticide-based
500 analysis, we implemented pairwise Wald tests to determine log2 fold changes between each
501 pesticide treatment and control, as well as quantify all differences in gene expression between
502 pesticide treatments. For each analysis, we then parsed DEGs for putative immune genes. To
503 determine if we identified more or less immune genes than would be expected, we performed
504 Fisher's exact tests for each analysis.

505 As a complementary approach to STAR, we also implemented a pseudoalignment-based
506 differential gene expression analysis using Kallisto [v.0.46.1] as described above in the quality
507 assessment section (mean mapping rate across samples 87.93%). Similar to the STAR-based
508 analysis, we implemented the same statistical tests using DESeq2 for both the sex-biased and
509 pesticide-based analysis, respectively. Out of the genes predicted to be differentially expressed

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510 using STAR, 3376 genes (81.78%, n = 4128) were also reported to be differentially expressed
511 using Kallisto and inversely, 92.01% (n = 3669) of all genes predicted to be differentially expressed
512 using Kallisto were also predicted to be differentially expressed using STAR.

513 Splicing of immune genes between sexes and pesticide-treated groups

514 To determine differentially spliced genes between the sexes, as well as in response to pesticide
515 exposure, we ran rMATS turbo [v.4.1.1](Shen *et al.*, 2014) using the STAR generated alignment
516 files. Similar to our differential expression analyses, we performed two independent analyses: first,
517 we compared males and females to identify significant sex-biased differences in splicing; second,
518 we performed a pesticide-based analysis, comparing thiacloprid- and imidacloprid-exposed
519 females against the control group, respectively. A specific feature of rMATS is that it outputs event-
520 level instead of gene-level results, distinguishing between different event types such as exon-
521 skipping, intron-retainment and 3'- or 5'-splicing and allowing multiple splicing events to be
522 recognized per gene. We used this event-based information to investigate whether there were
523 incidences of alternative splicing where a specific splicing event appears preferentially in one of the
524 experimental groups over the other. To do this we compared the number of times a differential
525 splicing event had higher inclusion levels in one group over the other against the total number of
526 incidences of differential splicing for that event using a Binomial-test with the null hypothesis being
527 that we would see higher inclusion levels equally often for both groups. We then concatenated and
528 summarised the event-level results by grouping the events by gene-ID and summarising down to
529 the lowest *p* value per gene, yielding a gene-level output table.. We then used this information to
530 rank genes by *p* value and investigate functional enrichment of Gene Ontology (GO) terms in
531 alternatively spliced genes (below).

532 Gene Ontology term enrichment analysis

533 As most genes in the *O. bicornis* genome lack functional information, we assigned GO terms to
534 genes from homologues found in *D. melanogaster*, which were obtained from Ensembl Metazoa
535 via biomaRt [v.2.46.3](Durinck *et al.*, 2009; Kinsella *et al.*, 2011). We then ran functional
536 enrichment analyses using topGO [v.2.42.0; (Alexa and Rahnenführer, 2009)] on the sets of

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537 immune genes derived via homology to A) *D. melanogaster* uniquely, B) *B. terrestris* uniquely and
538 C) to both species. To do so we implemented the “classic” algorithm in combination with a Fisher
539 test (node size=20) using all genes, each marked for presence or absence in the set of putative
540 immune genes. We then tested for enrichment of the most differentially expressed genes between
541 sexes and pesticide treatment groups. For this approach, we ranked genes by unadjusted raw *p*
542 values to avoid edge-effects introduced by correction and performed a rank-based analysis
543 (Kolmogorov-Smirnov test with “weighted01” algorithm and a node size=20) . We also performed
544 an immune-focused GO term enrichment analysis where we populated our GO term database only
545 with genes annotated with immune-related GO terms.

546 Data and Code Availability

547 Data used for the present analysis originated from datasets generated by (Beadle *et al.*, 2019).
548 Raw sequences for the RNA-seq based analyses can be obtained from the NCBI Short Read
549 Archive (BioProject PRJNA285788).

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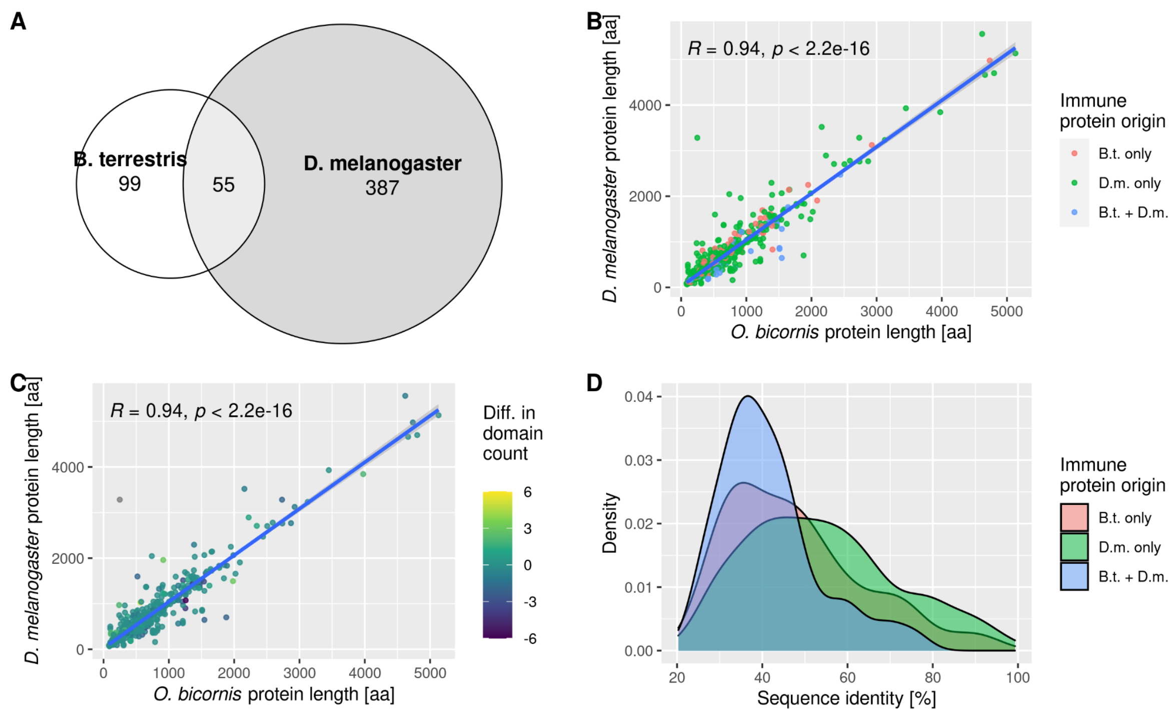
831 # Figures



832 **Figure 1. Expanded repertoire of putative immune genes in the red mason bee, *Osmia bicornis*. (A)**
833 Overlap of putative *O. bicornis* immune genes inferred via homology to *B. terrestris* and *D. melanogaster*. **(B-**
834 **C)** Correlation of protein lengths (amino acids) and number of functional domains shared between *O.*
835 *bicornis* and *D. melanogaster* protein homologues with colours indicating **(B)** immune gene set where
836 homology was found ("B.t. only" = homologues identified through comparison with *B. terrestris*; "D.m. only" =
837 homologues identified through comparison with *D. melanogaster*; "B.t.+D.m." = homologues identified in
838 comparisons with both species) and **(C)** difference in number of domains (yellow: higher domain count in *D.*
839 *melanogaster,* purple: higher domain count in *O. bicornis)*. **(D)** Distribution of sequence identity (percentage
840 of identical amino acid positions) of homologous protein pairs between *D. melanogaster* and *O. bicornis* for
841 putative *O. bicornis* immune proteins. Colours indicate the species through which homologues were
842 identified.

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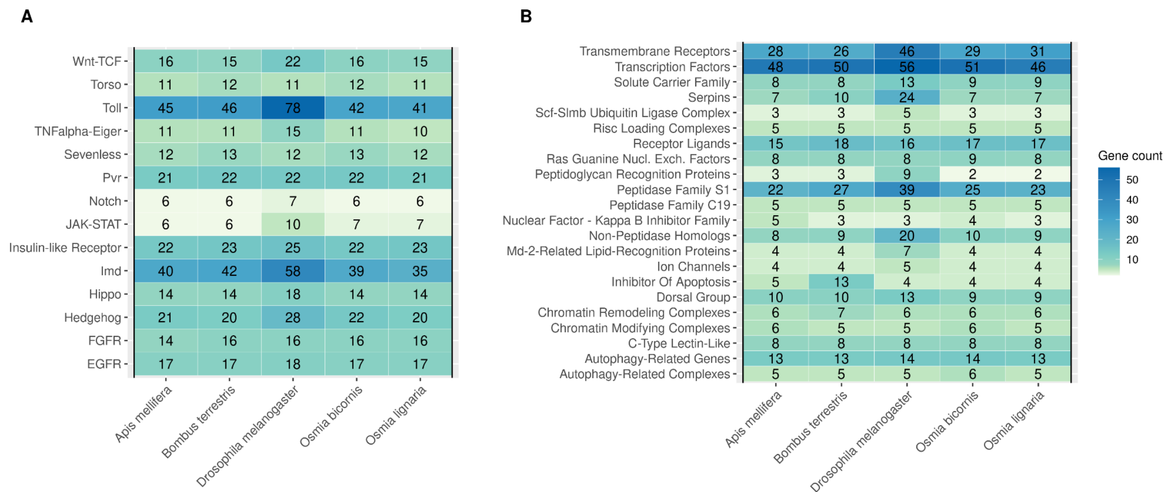

843 **Figure 2. Conservation of immune genes and pathways in *Osmia bicornis*.** Heatmaps depicting gene
844 counts of homologues of putative *O. bicornis* immune genes for **(A)** molecular signaling pathways and **(B)**
845 non-pathway gene families in closely related hymenopterans (*Apis mellifera*, *Bombus terrestris*, *Osmia*
846 *lignaria*) and the more distantly related *Drosophila melanogaster*. For each species the number of
847 homologues are shown.

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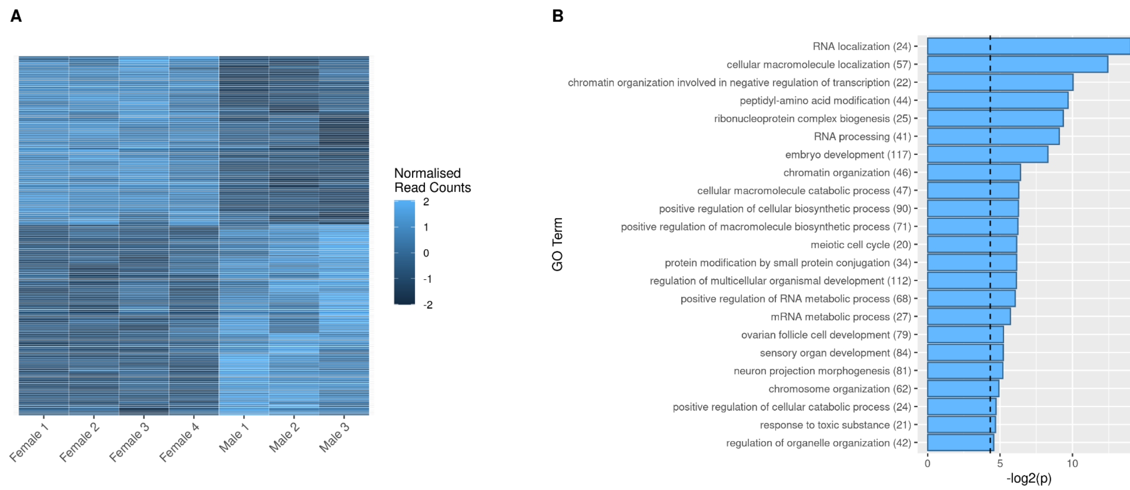

848 **Figure 3. Differential expression of putative *Osmia bicornis* immune genes between males and**
849 **females. (A)** Heatmap showing normalised (variance stabilisation transformed) read counts per sample for
850 significantly differentially expressed (BH adjusted $p < 0.05$) immune genes between males and females. **(B)**
851 Functional enrichment of biological process GO terms of significantly differentially expressed putative *O.*
852 *bicornis* immune genes. The x axis depicts negative log-transformed p values with the dashed vertical line
853 corresponding to a p value confidence threshold of 0.05. Each line on the y axis corresponds to a GO term
854 description with the numbers in brackets indicating the number of genes annotated with that specific term in
855 the GO term database.

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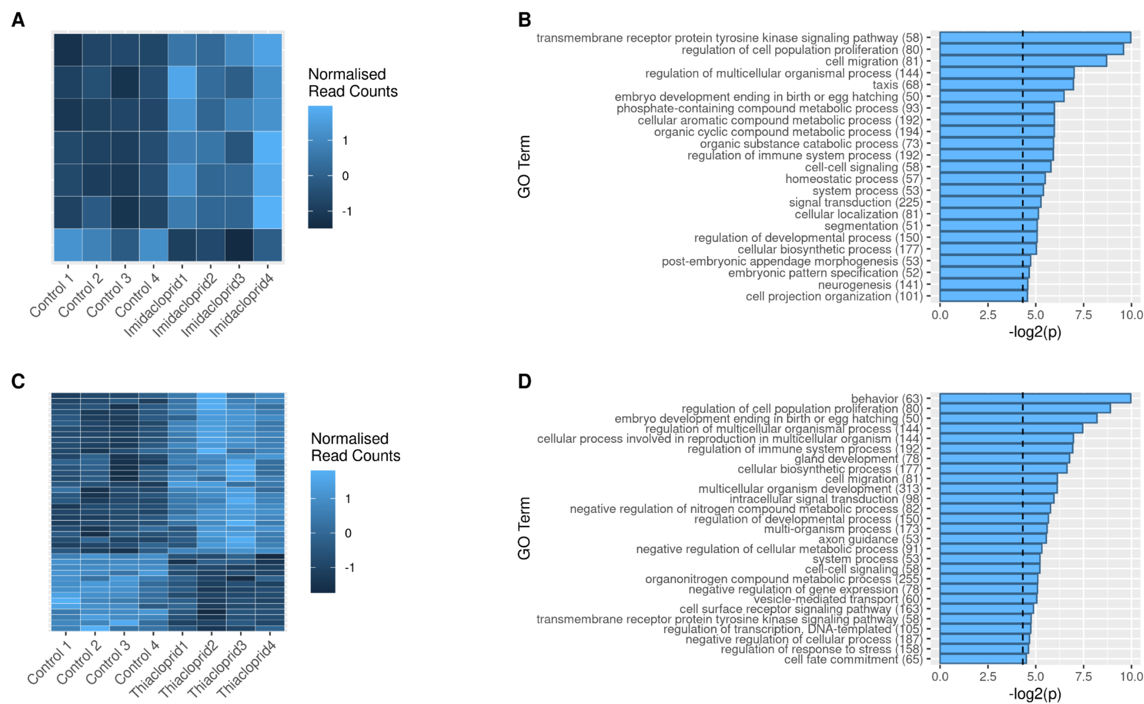

856 **Figure 4. Differential expression of putative *Osmia bicornis* immune genes between pesticide-
857 exposed and control individuals. (A,C)** Heatmap showing normalised (variance stabilisation transformed)
858 read counts per sample for significantly differentially expressed (BH adjusted $p < 0.05$) immune genes
859 between control individuals and **(A)** imidacloprid-treated or **(C)** thiacloprid-treated individuals. **(B,D)**
860 Functional enrichment of biological process GO terms of significantly differentially expressed putative *O.*
861 *bicornis* immune genes between control individuals and **(B)** imidacloprid-treated individuals or **(D)** thiacloprid-
862 treated individuals. The x axis depicts negative log-transformed p values with the dashed vertical line
863 corresponding to a p value confidence threshold of 0.05. Each line on the y axis corresponds to a GO term
864 description with the numbers in brackets indicating the number of genes annotated with that specific term in
865 the GO term database.