Received: 4 October 2021 | Accepted: 9 June 2022

DOI: 10.1111/imb.12796

ORIGINAL ARTICLE

Insect Molecular Biology

Royal Entomological Society

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

Jannik S. Möllmann | **Thomas J. Colgan**

Institute of Organismic and Molecular Evolution, Johannes Gutenberg University Mainz, Mainz, Germany

Correspondence
Jannik S. Möllmann and Thomas J. Colgan, Institute of Organismic and Molecular Evolution, Johannes Gutenberg University Mainz, Hanns-Dieter-Hüsch-Weg 15, 55128 Mainz, Germany.
Email: jmoellma@students.uni-mainz.de and tcolgan@uni-mainz.de

Funding information
Deutsche Bundesstiftung Umwelt, Grant/Award Number: 20021/743

Abstract

Insect pollinators provide crucial ecosystem services yet face increasing environmental pressures. The challenges posed by novel and reemerging pathogens on bee health means we need to improve our understanding of the immune system, an important barrier to infections and disease. Despite the importance of solitary bees, which are ecologically relevant, our understanding of the genomic basis and molecular mechanisms underlying their immune potential, and how intrinsic and extrinsic factors may influence it is limited. To improve our understanding of the genomic architecture underlying immunity of a key solitary bee pollinator, we characterized putative immune genes of the red mason bee, *Osmia bicornis*. In addition, we used publicly available RNA-seq datasets to determine how sexes differ in immune gene expression and splicing but also how pesticide exposure may affect immune gene expression in females. Through comparative genomics, we reveal an evolutionarily conserved set of more than 500 putative immune-related genes. We found genome-wide patterns of sex-biased gene expression, with greater enrichment of immune-related processes among genes with higher constitutive expression in males than females. Our results also suggest an up-regulation of immune-related genes in response to exposure to two common neonicotinoids, thiacloprid and imidacloprid. Collectively, our study provides important insights into the gene repertoire, regulation and expression differences in the sexes of *O. bicornis*, as well as providing additional support for how neonicotinoids can affect immune gene expression, which may affect the capacity of solitary bees to respond to pathogenic threats.

KEYWORDS
immunity, neonicotinoid, *Osmia*, pesticide, sex difference, solitary bee

INTRODUCTION

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

An important barrier to infection and establishment of disease is the invertebrate immune system (Rolff et al., 2009; Sadd et al., 2009). Despite lacking the adaptive immune system found in vertebrates, invertebrates have a dynamic innate immune system consisting of

recognition molecules, signalling pathways and effector molecules, which coordinate the targeting and removal of potentially harmful entities (Hoffmann, 1995). In addition to the evolutionary importance of the immune system (Sadd et al., 2008; Viljakainen, 2015), understanding insect immunity has applied purposes, especially within the fields of biomedical, agricultural and conservation biology. Genomic studies on insects have provided novel insights into the genes and genomic architecture underlying the immune system (Christophides et al., 2002; Evans et al., 2006; Gerardo et al., 2010; Sackton et al., 2007; Waterhouse et al., 2007). Such studies have documented and helped understand the immune potential and capacity of a species through the identification of gene family expansions, contractions, as well as lineage-specific or novel genes that demonstrate immune function (Adams et al., 2000; Barribeau et al., 2015; Evans et al., 2006). Indeed, comparative genomics allow for examining the types of selection pressures acting on immune genes providing important insights into their evolutionary history. Given the enormous selection pressures placed upon hosts by pathogens (Combes, 2001), genes involved in the immune system are expected and have been observed to evolve under strong positive selection. Indeed, in comparative genomic studies of both vertebrates and invertebrates, including insects, immune genes are often identified with signatures of accelerated rates of evolution (Roux et al., 2014; Shultz et al., 2019; Viljakainen et al., 2009).

Functional genomics, such as genome-wide transcriptional profiling ('RNA-seq'), provides a large amount of detailed information on genome-wide changes in gene expression in response to immune or pathogen challenge but also intrinsic differences in expression between different life cycle stages or sexes (Fish, 2008; Klein et al., 2016). Indeed, sexually dimorphic gene expression has been identified across taxonomically diverse groups and may exist due to differences in life histories, hormonal abundance, biochemical reactions or sex-specific genomic architecture (Hill-Burns et al., 2009). Such differences can underlie differences in immune expression and function but also susceptibility to disease and related survival (Ingersoll, 2017). A striking example whereby sex-specific differences in genomic architecture may underlie differences in immune potential, activity and expression are members of the Hymenoptera, which include the bees, ants, wasps and sawflies. Within this group, sexes differ in their ploidy with females developing from fertilized diploid eggs while males develop from unfertilised haploid eggs (Pamilo et al., 1981). The haploid nature of males means that any alleles carried are automatically expressed and open to selection, which can result in the rapid removal of maladaptive deleterious alleles from the gene pool (Joseph et al., 2004). The haploid nature of males has led to predictions that they are more susceptible to environmental challenges, including pathogens (O'Donnell et al., 2004), although empirical evidence to support this view has been conflicting (Baer et al., 2005; Calleri et al., 2006; Colgan et al., 2011; Retschnig et al., 2014; Ruiz-González et al., 2006).

Despite the fact that pathogen exposure and intrinsic differences can affect immune gene expression, other factors can also have an influence, including nutritional status (DeGrandi-Hoffman et al., 2015; Moret et al., 2000), mating (Barribeau et al., 2017; Lawniczak et al., 2007; Peng et al., 2005), periods of senescence or dormancy (Colgan et al., 2019; Kubrak et al., 2014; Nakamura et al., 2011), as well as environmental factors, such as temperature (Chen et al., 2015; Xu et al., 2012). One environmental challenge that has received considerable attention of late is the influence of pesticide exposure on immune expression and function. Chemical pesticides, such as pyrethroids and neonicotinoids, interact with the insect nervous system resulting in paralysis and death (Matsuda et al., 2001). The efficacy of chemicals, such as neonicotinoids, combined with its lower toxicity to vertebrates, their systemic mode of action, as well as the lack of requirement for reapplication has resulted in their increased use in modern agricultural practices (Jeschke et al., 2008). Despite their efficacy in killing agricultural pest species, the ubiquitous distribution of neonicotinoids across tissues means that non-target insects, including beneficial pollinators, may come in contact with such chemicals through food resources, such as nectar and pollen (Blacquière et al., 2012). The concentration of such chemicals can result in sublethal and indirect effects on the insect phenotype and has been identified to adversely affect the behaviour (Arce et al., 2018; Arce et al., 2017; Gill et al., 2012; Siviter et al., 2018; Stanley et al., 2015), neurobiology (Moffat et al., 2016; Moffat et al., 2015) and gene expression of key ecological and commercial pollinators (Beadle et al., 2019; Bebane et al., 2019; Chaimanee et al., 2016; Colgan et al., 2019). In addition, such chemicals have been shown to affect immune expression and function (Brandt et al., 2020; Chmiel et al., 2019; di Prisco et al., 2013; Mason et al., 2013) raising concerns that these effects may influence the ability of an exposed individual to respond to pathogenic threats (James et al., 2012). While studies have identified molecular mechanisms by which bees can metabolize certain neonicotinoids (Beadle et al., 2019; Manjon et al., 2018; Troczka et al., 2019), our understanding of changes in immune expression due to neonicotinoid exposure, especially for solitary bees, is limited.

The mason bees (*Osmia* species) are an important group of solitary bee pollinators but are generally understudied from an immunological perspective. One such species is the red mason bee *Osmia bicornis* (Order Hymenoptera; Family Megachilidae), a common pollinator found across central Europe, which has been increasingly incorporated into modern agricultural practices (Gruber & Eckel, 2011; Splitt et al., 2021). Despite its importance, it faces a number of environmental challenges that can influence the immune system, including pathogens and parasites (Bramke et al., 2019; Schoonvaere et al., 2018; Seidelmann, 2006; Tian et al., 2018), as well as pesticides (Brandt et al., 2020). Similarly, intrinsic differences between the sexes, which differ in morphology, physiology, behaviour and ploidy (Dmochowska-Ślęzak et al., 2015; Rogers et al., 2016; Szentgyörgyi et al., 2017) may result in differences in immune gene expression and splicing and associated susceptibility to pathogenic threats. In particular, higher parental investment and life span as well as an additional set of chromosomes support the hypothesis that females may be more immunocompetent than males (Rolff, 2002;

Zuk et al., 2002). However, at present, this has not yet been investigated in *O. bicornis* and our understanding of its immune gene repertoire and expression is currently limited.

To improve our understanding on the immune potential of *O. bicornis*, we performed a homology-based comparative genomic analysis using publicly available genomic resources to identify the immune gene repertoire of the red mason bee as well as identify potential contractions and expansions of important gene families and determine whether the red mason bee is missing immune genes. Furthermore, to understand how genes underlying immunity may be expressed differently between the sexes, we investigated evidence of sex-biased gene expression and alternative splicing using a published dataset (Beadle et al., 2019). Lastly, as pesticides can negatively affect different aspects of *Osmia* health, including developmental rate (Mokkapati et al., 2021), foraging behaviour (Boff et al., 2021; Straub & Orih, 2021), reproductive output (Ruddle et al., 2018; Sandrock et al., 2014; Woodcock et al., 2017), thermoregulation (Azpiazu et al., 2019), as well as impact immune function in red mason bees (Brandt et al., 2020), we examined whether neonicotinoid-exposed individuals differed in immune gene expression and splicing using an additional dataset from the same published study (Beadle et al., 2019).

EXPERIMENTAL PROCEDURES

Identification of putative immune genes in the red mason bee

To infer homologues for *O. bicornis* genes that are in other insect species, we ran OrthoFinder (v.2.5.2) (Emms et al., 2019) with proteomes of 21 species, comprising 11 bee species from three families (Family Megachilidae: *Osmia bicornis*, *Osmia lignaria* and *Megachile rotundata*; Family Apidae: *Apis mellifera*, *Ceratina calcarata*, *Eufriesea mexicana*, *Habropoda laboriosa* and *Bombus terrestris*; and Family Halicitidae: *Megalopta genalis*, *Nomia melanderi* and *Dufourea novaeangliae*), as well as 10 non-bee insects (*Drosophila melanogaster*, *Aedes aegypti*, *Anopheles gambiae*, *Bombyx mori*, *Tribolium castaneum*, *Acyrthosiphon pisum*, *Nasonia vitripennis*, *Solenopsis invicta*, *Polistes dominula* and *Vespa mandarinia*). All proteomes were obtained from the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database and then reduced to the longest isoform for each protein based on the recommendations of the authors of OrthoFinder. These longest isoforms from then on served as the reference protein isoform for each gene. We then ran OrthoFinder using the default parameters with the inferred species trees forming a consensus with the known phylogeny. Given that model organisms, such as *D. melanogaster*, contain the most comprehensive functional annotation of genes with immune function or potential, we examined the *O. bicornis* predicted proteome for putative homologues of *D. melanogaster* immune genes. To obtain *D. melanogaster* immune-responsive genes, we queried the FlyBase database on July 9, 2021 for any gene associated with the Gene Ontology (GO) term 'immune system process', the highest order GO term associated with the immune system, and inferred the *O. bicornis* homologues via the homologues table generated earlier from OrthoFinder. As an additional approach, to identify immune genes that may be lineage-specific within the Hymenoptera or too divergent between *O. bicornis* and *D. melanogaster* given their evolutionary distance, we examined the presence of *O. bicornis* homologues through comparison with canonical immune genes characterized in the earth bumblebee, *B. terrestris*. The canonical *B. terrestris* immune genes were directly obtained from the earth bumblebee genome papers (Barribeau et al., 2015; Sadd et al., 2015). An additional reason for the inclusion of this social bee species is that it has a curated homologue list with *D. melanogaster*, available through the Ensembl Metazoa database, which provided the ability to compare the orthogroups and homologous pairs generated by OrthoFinder with orthogroups and pairs independently generated by Ensembl, which incorporates additional information on synteny and gene order conservation for the identification of putative homologues between two species. This approach identified a high overlap (86.85% of Ensembl pairs correctly identified by OrthoFinder) between the pairs generated by both analyses providing additional confidence in the orthogroups generated by OrthoFinder. Homologous genes from *D. melanogaster* and *B. terrestris* were obtained by first translating the *O. bicornis* gene-IDs to protein-IDs via the annotation column in the RefSeq gene feature file and then using the homology information from OrthoFinder to translate *O. bicornis* protein-IDs to *D. melanogaster* and *B. terrestris* protein-IDs, respectively, and further translating the protein-IDs to the species-specific gene-IDs, yielding a many-to-many homologue table of *O. bicornis* gene-ID's to *D. melanogaster* and *B. terrestris* gene-ID's, respectively. To infer for each gene family how many homologous genes exist for the set of putative *O. bicornis* immune genes in *A. mellifera*, *B. terrestris*, *D.melanogaster*, *O. lignaria* and *M. rotundata*, we derived annotation of *D. melanogaster* genes with gene families from Flybase on June 2, 2021 and annotated the immune gene homologues in each species with the according gene family description. We then summarized this data by counting the number of genes in each species and for each gene family.

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

In addition to the identification of immune-related genes, we also inferred canonical immune genes from *B. terrestris* or *D. melanogaster* missing in *O. bicornis* and *O. lignaria*. For this, we parsed the output of

OrthoFinder for orthogroups that carried immune-associated genes in *D. melanogaster* or *B. terrestris* but not in both *O. bicornis* and *O.lignaria*. Similarly, we inferred *Osmia*-specific genes by parsing orthogroups containing only *O. bicornis* and *O. lignaria* homologues, which were also absent in the other 19 species.

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

To examine the functional expression of putative immune genes of *O. bicornis*, we obtained publicly available paired-end RNA-seq libraries for two analyses: (a) sex-biased analysis containing males ($n = 3$) and females ($n = 3$); and (b) pooled libraries of unexposed ('control'; $n = 4$) females or those exposed to thiacloprid ($n = 4$) or imidacloprid ($n = 4$). All datasets were obtained from the NCBI (National Center for Biotechnology Information) Short Read Archive (SRA) database (BioProject: PRJNA285788; Beadle et al., 2019, Supporting Information Material S5). The original dataset contained an additional female sample (SRR2895245), which we discarded from the analysis due to its particularly high read count (about five times higher than the average) to avoid the introduction of a library size bias in the splicing analysis. We performed data quality assessment based on per-sample quality evaluations using FastQC (v.0.11.9) (Andrews, 2010), accssd and the calculation of the proportions of reads mapping to the predicted transcriptome of the RefSeq *O. bicornis* reference genome assembly (Obicornis_v3; GCF_004153925.1) using Kallisto (v.0.46.1) (Bray et al., 2016). We then combined and visualized the results for both tools and across all samples with MultiQC (v.1.7) (Ewels et al., 2016). Based on the results of the quality assessment we removed adapter sequences and filtered by quality (phred quality score ≥ 15) and length (minimum length ≥ 50 bp) using fastp (v.0.20.1) (Chen et al., 2018). For each sample, we then aligned the trimmed and filtered reads against the most recent chromosome-level genome assembly (iOsmBic2.1; GCA_907164935.1) using star (v.2.7.8a) (Dobin et al., 2013). As the chromosome-level assembly currently lacks annotations, we first transferred gene coordinates from the annotated reference assembly (Obicornis_v3; GCF_004153925.1) to the new assembly using Liftoff (v.1.6.1) (Shumate et al., 2020). star was ran in two-pass-mode using the inferred splice junctions from the first run to improve the alignment of the second run and with parameter –quantMode GeneCounts used to generate gene-level abundances of aligned reads (Supporting Information Material S6). The mean alignment rate across all samples was 94.09%. For the sex-biased analysis, we used DESeq2 (v.1.30.1) (Love et al., 2014) to correct for library size and infer all differentially expressed genes (DEGs, FDR < 0.05) between sexes with a likelihood-ratio test (LRT; full model: sex; reduced model: intercept). For the pesticide-based analysis, we implemented pairwise Wald tests to determine $\log_2$ fold changes between each pesticide treatment and control, as well as quantify all differences in gene expression between pesticide treatments. For each analysis, we then parsed DEGs for putative immune genes. To determine if we identified more or less immune genes than would be expected, we performed Fisher's exact tests for each analysis.

As a complementary approach to star, we also implemented a pseudoalignment-based differential gene expression analysis using Kallisto as described above in this section. Mean mapping rate across all samples was 87.93%. Similar to the star-based analysis, we implemented the same statistical tests using DESeq2 for both the sex-biased and pesticide-based analysis, respectively. Out of the genes predicted to be differentially expressed using star, 3376 genes (81.78%, $n = 4128$) were also reported to be differentially expressed using Kallisto and inversely, 92.01% ($n = 3669$) of all genes predicted to be differentially expressed using Kallisto were also predicted to be differentially expressed using star.

Splicing of immune genes between sexes and pesticide-treated groups

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

Gene Ontology term enrichment analysis

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

to (a) *D. melanogaster* uniquely; (b) *B. terrestris* uniquely; and (c) to both species. To do so, we implemented the 'classic' algorithm in combination with a Fisher's exact test (node size = 20) using all genes, each marked for presence or absence in the set of putative immune genes. We then tested for enrichment of the most differentially expressed immune genes between sexes and pesticide treatment groups. For this approach, we ranked genes by $\log_2$-fold change value twice, once unadjusted and once with inverted algebraic sign, to allow for differentiation of female- or male-biased expression. Using *p* values instead would only take into account if a gene is significantly differentially expressed, but not which group (sex or exposure treatment) it is associated more strongly with, which likely masks important patterns. We then performed a rank-based analysis (Kolmogorov–Smirnov test with 'weighted01' algorithm and a node size $n = 20$). We also performed a general GO term enrichment analysis where we populated our GO term database with all genes as opposed to putative immune genes only.

RESULTS

Putative expanded immune gene repertoire in the Hymenoptera

To infer putative immune genes in *O. bicornis*, we independently examined the presence of homologues of previously characterized immune genes of the closely related earth bumblebee, *B. terrestris* (Order: Hymenoptera) as well as the more distantly related model organism, *D. melanogaster* (Order: Diptera), in the predicted proteome of the *O. bicornis* reference genome assembly. The *D. melanogaster* canonical immune gene repertoire ($n = 606$) was inferred via a query of the FlyBase database using the GO term 'immune system process', while the *B. terrestris* canonical immune gene repertoire ($n = 160$) was obtained directly from Barribeau and Sadd (2015). Homology between species was inferred using OrthoFinder and 21 insect proteomes, predicted from their reference genome assemblies and publicly available in NCBI RefSeq (full species list in Section 2). We merged resulting *O. bicornis* homologues of known immune genes in *D. melanogaster* and *B. terrestris*, resulting in a total of 541 putative immune gene homologues, of which 99 were only found among the set of *B. terrestris* immune genes, 387 were only found among the set of *D. melanogaster* immune genes and 55 were found in the overlap of both sets (Figure 1a, Supporting Information Material S1). To determine whether such an expanded number was a result of the predicted red mason bee proteome, we also ran the analysis for *M. rotundata*, another Megachilid bee, resulting in a similar number of putative immune genes ($n = 541$) with, however, more genes ($n = 59$) in the overlap and more genes ($n = 108$) with homologues only among the set of *B. terrestris* immune genes and fewer genes ($n = 378$) with homologues only among the set of *D. melanogaster* immune genes. We also ran functional enrichment analyses and found enrichment in immune terms for the putative immune genes derived uniquely via homology to *D. melanogaster* and for the putative immune genes found among both sets while for the putative immune genes derived uniquely via homology to *B. terrestris* we found enrichment in terms only indirectly linked to the immune system such as 'response to stress' and 'response to toxic substance' (Supporting Information Material S2).

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

Our homology-based analysis also revealed immune genes potentially missing in *O. bicornis*. We did not identify homologues for 232 *D. melanogaster* immune genes as well as one canonical *B. terrestris* immune gene, the antimicrobial peptide abaecin, in *O. bicornis* or its sister taxa, *O. lignaria* (Supporting Information Material S1). Inversely, as *Osmia* species may contain lineage-specific genes, including genes with potential immune functions, we identified genes ($n = 78$, split across 48 orthogroups; Supporting Information Material S1) shared between *O. bicornis* and *O. lignaria* that lacked homologues in the predicted proteomes of all other 19 insect species we used to infer homology relations. Among the *Osmia*-specific genes that were annotated with at least one domain ($n = 24$), we find 16 genes annotated with ribonuclease-domains (IPR036397, IPR012337) as well as one gene (LOC114879997) annotated with a rhabdovirus nucleoprotein domain (IPR004902), indicating a potential involvement in immunity for some of the *Osmia*-specific genes.

To understand variation in the immune gene complement of the red mason bee, we used our homology-based approach to compare the number of putative *O. bicornis* immune genes in conserved gene families and pathways to the number of homologues in four closely related hymenopteran species (*O. lignaria*, *M. rotundata*, *B. terrestris* and *A. mellifera*) and one more distantly related fly species (*D. melanogaster*). We found similar numbers of genes in the five hymenopterans for most gene families, however with slightly lower gene counts in the two *Osmia* species compared to the other three hymenopterans for the Immune deficiency (ImD) and Toll pathways

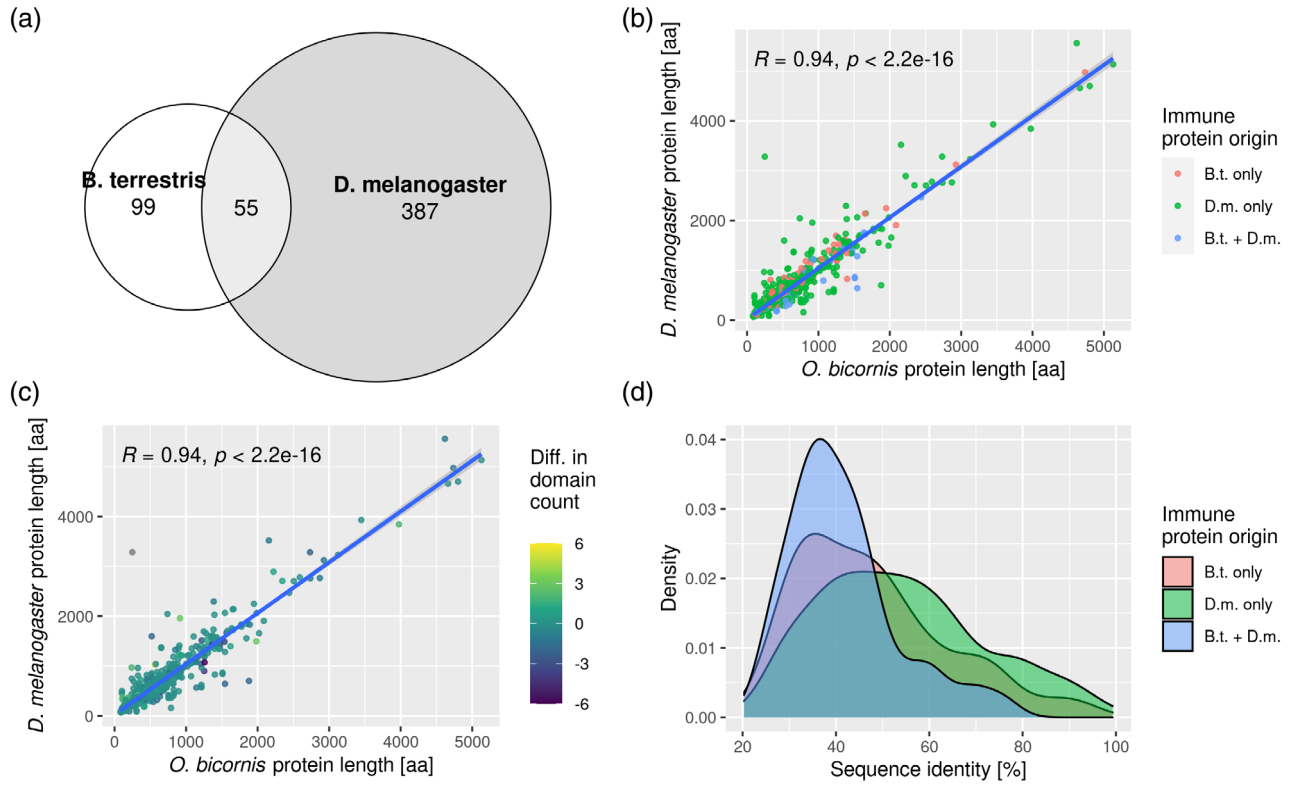

FIGURE 1 Expanded repertoire of putative immune genes in the red mason bee, *Osmia bicornis*. (a) Overlap of putative *O. bicornis* immune genes inferred via homology to *Bombus terrestris* and *Drosophila melanogaster*. (b,c) Correlation of protein lengths (amino acids) and number of functional domains shared between *O. bicornis* and *D. melanogaster* protein homologues with colours indicating (b) immune gene set where homology was found ('B.t. only' = homologues identified through comparison with *B. terrestris*; 'D.m. only' = homologues identified through comparison with *D. melanogaster*; 'B.t. + D.m.' = homologues identified in comparisons with both species) and (c) difference in number of domains (yellow: higher domain count in *D. melanogaster*, purple: higher domain count in *O. bicornis*). (d) Distribution of sequence identity (percentage of identical amino acid positions) of homologous protein pairs between *D. melanogaster* and *O. bicornis* for putative *O. bicornis* immune proteins. Colours indicate the species through which homologues were identified.

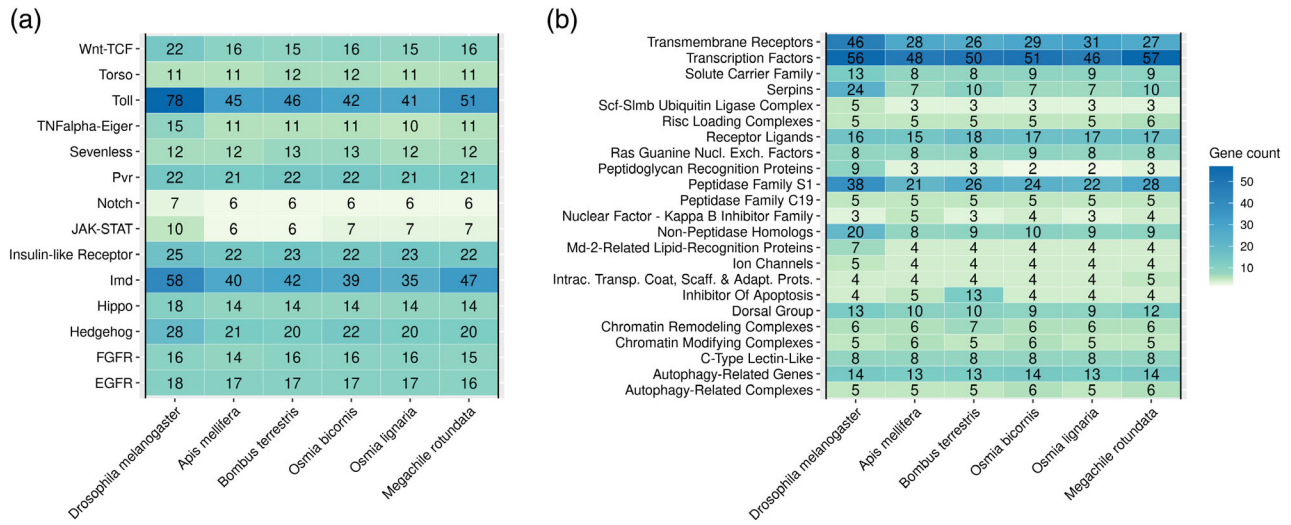

FIGURE 2 Conservation of immune genes and pathways in *Osmia bicornis*. Heatmaps depicting gene counts of homologues of putative *O. bicornis* immune genes for (a) molecular signalling pathways and (b) non-pathway gene families in closely related hymenopterans (*Apis mellifera*, *Bombus terrestris*, *Osmia lignaria*, *Megachile rotundata*) and the more distantly related *Drosophila melanogaster*. For each species, the number of homologues are shown.

(Figure 2a) and with even higher gene counts for these two pathways and transcription factors in *M. rotundata*. In addition, we found higher gene numbers for inhibitors of apoptosis in *B. terrestris* ($n = 13$) compared to the other hymenopterans (median $n = 4$, Figure 2b). When comparing the hymenopterans to *D. melanogaster*, the latter has higher gene counts for six out of 14 signalling pathways and 13 out of 22 non-pathway gene families but similar numbers for all other pathways and gene families. In addition, we checked the number of homologues in *O. bicornis* for *D. melanogaster* immune genes on a gene family level and found a high average conservation of 84.86% but a large difference for antimicrobial peptides (AMPs) with only one *O. bicornis* homologue compared to 22 *D. melanogaster* genes.

Sex-biased differential expression of immune genes

To provide functional information on the expression of putative immune genes in *O. bicornis*, we compared whole-body transcriptomes of 24-h-old male ($n = 3$) and female adults ($n = 3$) which were previously generated by Beadle and Singh (2019). We identified 2957 genes (25.06% of total gene count, $n = 11,799$) as significantly differentially expressed (LRT, FDR < 0.05) between the sexes, of which, 1559 and 1388 had female- and male-biased expression, respectively (Supporting Information Material S3). We quantified expression of 520 putative immune genes (96.11% of total immune genes, $n = 541$) in both sexes, of which 159 were differentially expressed (30.06% of total DEGs) which was significantly more than expected by chance (Fisher's exact test, $p = 0.035$). These DEGs were nearly equally shared between the sexes with slightly more genes ($n = 80$) showing male-biased rather than female-biased expression ($n = 79$), a pattern that did not significantly differ from expectation (Binomial test, $p = 0.428$, Supporting Information Material S1). Among the male-biased differentially expressed immune genes, we found enrichment for 32 biological process (BP), seven cellular component (CC) and seven molecular function (MF) terms while for female-biased differentially expressed immune genes, we found enrichment for 30 BP, 11 CC and six MF terms. The most strongly enriched terms among male-biased immune genes were related to immune processes such as 'response to bacterium' or 'response to toxic substance'; however, for female-biased immune genes, enrichment was related more to general regulation of gene expression with 'larval lymph gland hemopoiesis' as the only observed immunity-related term (Figure 3, Supporting Information Material S2). We also analysed the *Osmia*-specific genes for signatures of differential expression and found seven genes that significantly differed in their expression between the sexes.

Sex-biased alternative splicing of immune genes

We also performed alternative splicing analysis and identified 921 genes (7.81% of total gene count, $n = 11,799$) significantly differentially spliced (LRT, FDR < 0.05) between the sexes (Supporting Information Material S4). Among the differentially spliced genes, we identified 58 putative immune genes (10.72%), which was significantly more than expected (Fisher's exact test; $p = 0.03$). The differentially spliced immune genes were significantly enriched (Kolmogorov–Smirnov test, $p < 0.05$) for 39 BP GO terms, five CC GO terms and five MF GO terms with immune terms such as 'response to bacterium', 'melanotic encapsulation of foreign target' and 'response to organonitrogen compound' among the most significant terms (Supporting Information Material S7, Figures S1-S3). In addition, we found differences between the sexes in the frequency of different splicing events with retained intron events significantly more common in females than in males (Fisher's exact test, BH-adjusted $p = 1.07e{-}8$). We also found a significantly higher occurrence of skipped exon events in females compared to males (Fisher's exact test, BH-adjusted $p = 0.01$). However, we found no significant differences (BH-adjusted $p > 0.05$) for alternative 3′ splice sites, alternative 5′ splice sites or mutually exclusive exons.

Immune gene expression changes in response to pesticide exposure

For the neonicotinoid exposure analysis, we again used RNA-seq datasets generated by Beadle et al. (2019) to compare whole-body transcriptomes of untreated ($n = 4$ replicates, five adult females pooled per replicate), imidacloprid-treated ($n = 4$ replicates, five female pools each) and thiacloprid-treated bees ($n = 4$ replicates, five female pools each), all of them generated from female adults. As outlined by Beadle and Singh (2019), the neonicotinoid-treated individuals were exposed to sublethal doses (LD_{10}) of the compounds via oral application after a 24 h training period and a 16 h starvation period and subsequently all bees were fed with a sucrose solution for 24 h. We identified 617 genes, including 42 putative immune genes, significantly differentially expressed (Wald test, FDR < 0.05) in the group of thiacloprid-exposed females compared to the untreated control group (Figure 4c, Supporting Information Material S3), with a significant difference in numbers of up- and down-regulated genes in the thiacloprid-exposed group (436 genes up-regulated, 181 genes down-regulated, Binomial-test, $p = 9.41e{-}22$). Comparing imidacloprid-exposed females with untreated females, we found 127 genes significantly differentially expressed (Wald test, FDR $p < 0.05$), including seven immune genes (Figure 4a). We also found a significant difference in the number of up-regulated and down-regulated genes in the imidacloprid-exposed group (89 genes up-regulated, 38 genes down-regulated, Binomial test, $p = 6.97e{-}5$). We found more differentially expressed immune genes than expected by chance in the thiacloprid-exposed group ($n = 42$ immune genes; Fisher's exact test, $p = 0.014$) but not in the imidacloprid-exposed group ($n =$ seven immune genes; Fisher's exact test, $p = 0.515$). Five putative immune genes (LOC114878095, LOC114878683, LOC114881181, LOC114874985 and LOC114872156) had increased transcript expression both in response to thiacloprid and imidacloprid with no significant difference between the $\log_2$ fold change values for these five genes between the two pesticide treatment groups (Welch's *t*-test, $p = 0.76$, Supporting

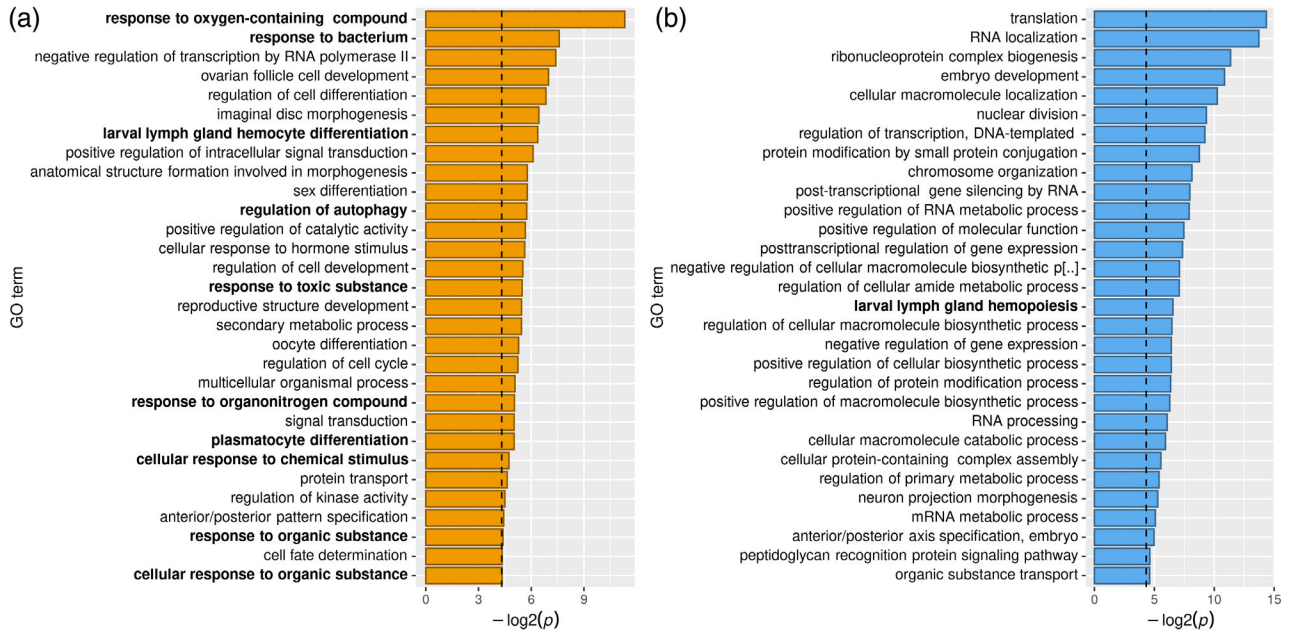

FIGURE 3 Functional enrichment analysis of putative *Osmia bicornis* immune genes that differ between the sexes. Enriched biological process GO terms associated with putative immune genes that are (a) male-biased and (b) female-biased. Each bar on the *y*-axis corresponds to a GO term with a corresponding confidence value. The confidence values on the *x*-axis are negative log-transformed *p* values and the dashed vertical line corresponds to a *p* value confidence threshold of 0.05. Terms highlighted in bold represent immune-related terms.

Information Material S3). In terms of functional enrichment of significantly differentially expressed immune genes, we found 17 BP GO terms enriched among genes up-regulated in response to thiacloprid, as well as six CC and five MF terms with many immune system-related processes such as 'defence response to Gram-positive bacterium' and 'cellular response to stress' among the top enriched terms (Figure 4a). Among genes down-regulated in response to thiacloprid, we found 60 BP, seven CC and nine MF terms significantly enriched with 'negative regulation of immune system process' as the only immune-system-related term and all other terms related to regulation of gene expression or developmental processes (Figure 4a). For the imidacloprid-exposed group, we found 18 BP terms enriched among up-regulated immune genes as well as five CC terms and two MF terms with 'defence response to Gram-negative bacterium' as only immune-related process among the top enriched terms (Figure 4b, Supporting Information Material S2). Among down-regulated immune genes, we found enrichment for 46 BP, seven CC and seven MF terms with 'response to organic substance' as the only immune-related process among the most significantly enriched terms. Among the *Osmia*-specific genes, we identified three genes differentially expressed between the thiacloprid-exposed group and control group but no DEGs between the imidacloprid-exposed group and the control group.

For alternative splicing, we found 142 differentially spliced genes (LRT, FDR < 0.05) for the thiacloprid-exposed group, including six putative immune genes, while 74 genes were differentially spliced for the imidacloprid-exposed group, including four putative immune genes. One immune gene (LOC114880106) was differentially spliced in response to both pesticides (Supporting Information Material S4).

DISCUSSION

Our understanding of the genomic and molecular bases of immunity in the Hymenoptera has largely been informed by studies on social bees while for other species, especially solitary bees, such information is limited. Here, we performed a comparative genomic analysis to characterize genes with potential immune functions in the genome of the red mason bee, *O. bicornis*. Using a homology-based approach, we identify an immune gene repertoire enlarged beyond the canonical immune genes previously described in other hymenopteran genomes. We find extensive differences in immune gene expression between the sexes, both in terms of expression amplitude and splicing, highlighting intrinsic regulatory differences in the molecular basis of immunity between males and females. Lastly, we find immune-related genes differentially expressed in response to neonicotinoid exposure with greater expression differences in bees exposed to thiacloprid than those exposed to imidacloprid demonstrating differences in how the molecular phenotype responds to different neonicotinoid subclasses and how each may influence immune expression.

The insect immune system consists of an innate immune response with the ability to detect and remove a diverse range of pathogens (Beckage, 2008). The earliest genomic studies on the Hymenoptera documented a reduction in canonical immune genes in comparison to other insect orders, most notably in comparison to members of the Diptera (Barribeau et al., 2015; Bonasio et al., 2010; Evans et al., 2006; Werren et al., 2010). Reasons for this reduction ranged from the technical (e.g., fragmented genome assembly, missing or truncated gene models) to the biological level (e.g., novel immune genes and pathways, Albert et al., 2011; Dong et al., 2020; or relaxed selection acting

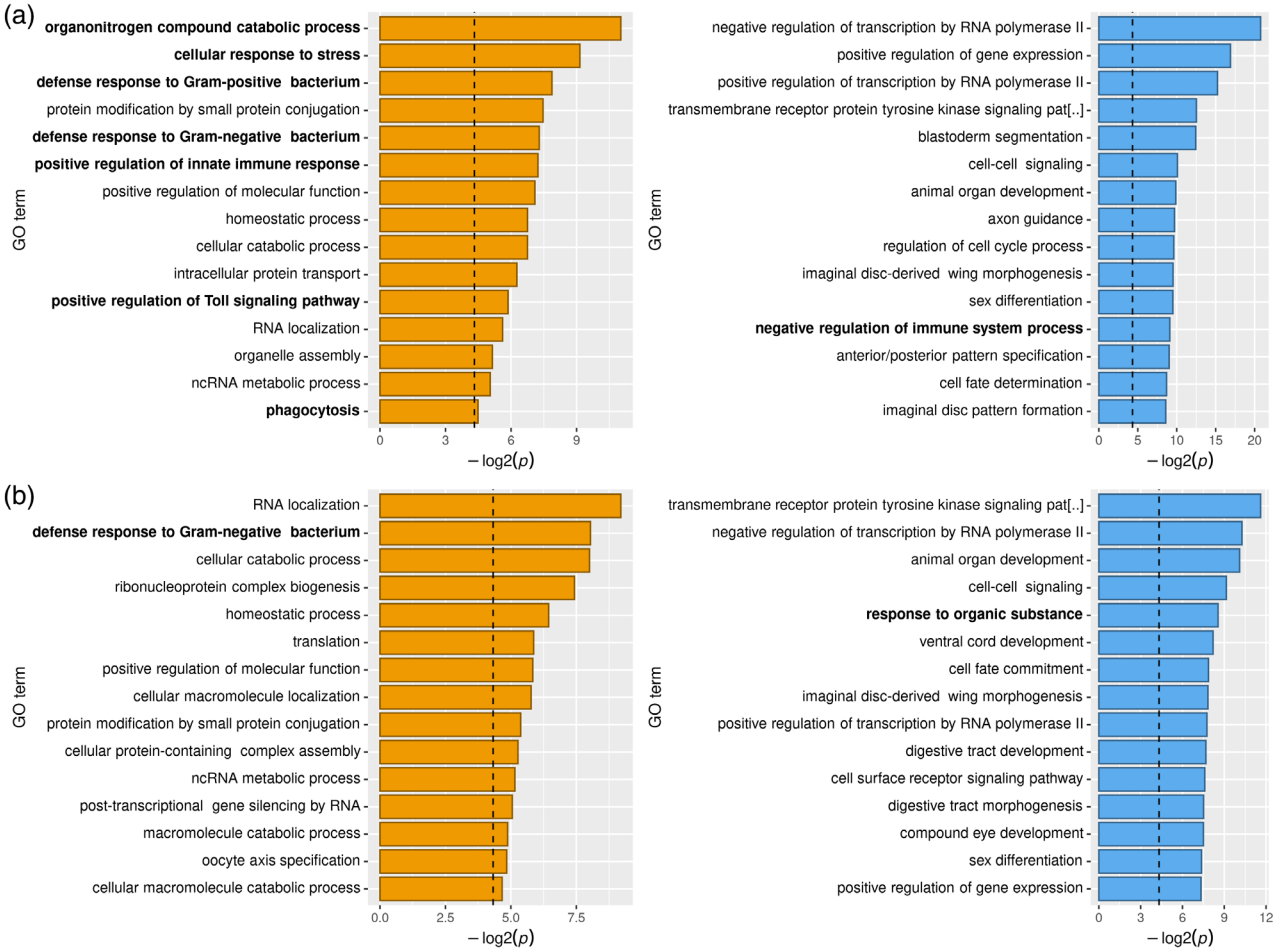

FIGURE 4 Functional enrichment analysis of putative *Osmia bicornis* immune genes that differ in expression in response to neonicotinoid exposure. Enriched biological process GO terms associated with putative immune genes up-regulated (orange) or down-regulated (blue) in response to (a) thiacloprid and (b) imidacloprid. Each bar on the *y*-axis corresponds to a GO term with a corresponding confidence value. The confidence values on the *x*-axis are negative log-transformed *p* values and the dashed vertical line corresponds to a *p* value confidence threshold of 0.05. Terms highlighted in bold represent immune-related terms.

on canonical immune genes due to social immunity; Harpur et al., 2013). Similar levels of reduced immune genes were previously characterized in the alfalfa leafcutting bee *M. rotundata* (Barribeau et al., 2015) yet our understanding for other ecologically important bees is missing. Here, we performed an in-depth investigation of the immune gene repertoire of the red mason bee to expand on our understanding of immune potential in solitary bee species.

Our initial approach for the detection of putative immune genes was based on homology with immune system-associated genes in *D. melanogaster*, where many such immune genes have been experimentally validated. As many immune genes have been previously shown to evolve under strong episodic positive selection (Jiggins et al., 2007; Shultz et al., 2019; Viljakainen, 2015), which can result in divergence beyond detection through homology searches or the appearance of novel lineage-specific immune genes, we also investigated homologues in *O. bicornis* of canonical immune genes from the earth bumblebee, *B. terrestris*, a closely related social insect with an annual life-cycle. As the majority of canonical immune genes identified in *B. terrestris* were generated based on homology searches with other insect genomes, including *D. melanogaster* (Barribeau et al., 2015), we would have expected canonical immune genes in both species to be annotated with immune process GO terms and therefore, we would have expected to see a high overlap in immune gene sets. Surprisingly, we found a weak overlap between *Osmia* homologues identified using both approaches, with only a third of *B. terrestris* canonical immune genes identified also through homology to *D. melanogaster* immune genes and thus annotated with GO terms directly related to immunity. The other two-thirds, however, were still annotated with GO terms associated with aspects of immunity, such as 'response to toxic substance', 'RNA interference' and 'autophagy'. The lack of annotation of immunity-related GO terms for two thirds of the described canonical immune genes in *B. terrestris* may result in the underreporting of immunological changes for genomic studies reliant on GO term-based analyses.

To provide better support for conserved function for putative immune homologues in *Osmia* species, we further assessed *O. bicornis* immune homologues identified through the *D. melanogaster*

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

Among the 541 putative immune genes of *O. bicornis*, we did not find any major patterns of immune gene family expansions or contractions in comparison with that of *B. terrestris* and *A. mellifera*, suggesting that there were no large-scale recent duplications or losses of genes that could be imperative to the functioning of the immune system of bees. However, we did find slight reductions in the number of genes involved in two major immune signalling pathways, Imd and Toll, when we compared *O. bicornis* and *O. lignaria* to *A. mellifera*, *B. terrestris* and *M. rotundata*. Imd and Toll are both involved in the induction of AMPs (de Gregorio et al., 2002), thus, fundamental for insect survival in response to pathogen challenge. Related to this, the biggest difference in immune gene families between *O. bicornis* and *D. melanogaster* was for gene copies of AMPs. This can be expected as *Drosophila* have evolved a number of AMP gene families, such as the cecropins, diptericins and attacins (Imler et al., 2005), which have not been identified in other hymenopteran genomes (Barribeau et al., 2015; Evans et al., 2006). Consequently, we did identify the presence of defensin, an evolutionary conserved AMP that possesses antibacterial properties (Hoffmann et al., 1992), as well as the hymenopteran-specific AMP, hymenoptaecin (Casteels et al., 1993). However, we did not detect a copy of abaecin, a bacterial-inducible AMP described in honeybees (Casteels et al., 1990) and bumblebees (Rees et al., 1997). The conserved lack of abaecin, as well as components of the Imd and Toll signalling pathways, in the genome assemblies of two *Osmia* species, which were sequenced and assembled independently, suggests that such missing genes may be true biological signals rather than technical artefacts. In addition, our observation of higher instead of lower gene counts for the Toll and ImD pathways in the closely related *M. rotundata* suggests that this trend is not shared in all Megachilidae and could be limited to the genus *Osmia*. At present, the evolutionary consequences of such potential losses, if any, are unknown, but it represents evidence for the existence of immune system-specific differences in the genetic makeup of solitary and social bees as well as within the solitary bee group Megachilidae.

For species that sexually reproduce, the genome codes for distinct sexes that can differ dramatically in behaviour, morphology and physiology (Parsch et al., 2013), including immunity (Klein et al., 2016). As immunity can be both energetically costly to maintain and activate (Rolff et al., 2003), it can result in metabolic trade-offs with other processes, such as reproduction (Schwenke et al., 2016). Sexes can also differ in immune investment, which may be reflected in differences in gene expression. Here, we found 159 homology-derived immune genes to be differentially expressed between the sexes with stronger functional enrichment for immunity-related terms among male-biased genes than among female-biased genes. This suggests that the sexes do indeed differ in constitutive expression of immune genes, more specifically in relation to bacterial defence and detoxification, but also in relation to important housekeeping roles, such as RNA and protein turnover. Contrary to our initial hypothesis, however, these results are not supportive of a higher immunocompetence in females compared to males. The case for differences in antiviral defence is further highlighted by genes, such as the endonuclease Dcr-1, which have roles in virus recognition and degradation (Brutscher et al., 2015; Galiana-Arnoux et al., 2006), being differentially expressed between the sexes. We found more putative immune genes than expected by chance to be differentially expressed or differentially spliced between the sexes suggesting that the molecular differences between the sexes are particularly pronounced when it comes to immune system processes. We also looked at alternative splicing with 58 homology-derived immune genes differentially spliced between the sexes and with a strong enrichment for immunity-related terms, suggesting that the molecular differences in immunity between the sexes are not just limited to one mechanism of gene regulation.

Pesticides, including neonicotinoids, act as agonists of the nicotine acetylcholine receptors, resulting in disruption of the neuronal cholinergic signal transduction and excitation of neuronal triggers culminating in paralysis and death (Matsuda et al., 2001). The efficacy of the mode of action of neonicotinoids has led to their increased popularity in modern agriculture practices yet recent studies have highlighted the negative impact sublethal and lethal doses can have on pollinator health (Jeroen et al., 2013), including immune function. Neonicotinoids, such as clothianidin and imidacloprid, have been identified in exposed honeybees to negatively modulate the NF-kappa B signalling pathway and affect the ability to mount effective antiviral defences (di Prisco et al., 2013). Other studies on neonicotinoids have provided additional evidence of the indirect or direct effects of these neurotoxins on immune function or expression (Brandt et al., 2017; Brandt et al., 2020). Here we found changes in gene expression in response to two classes of neonicotinoids with thiacloprid exposure affecting the expression of more genes, including immune genes, than

imidacloprid. This is in line with other studies that have examined thiacloprid exposed red mason bees and observed impairment in immunity (Brandt et al., 2020) or larval development (Claus et al., 2021). For both neonicotinoids, we find significantly more genes up-regulated in response to pesticide exposure than expected, suggesting that overall the exposure to pesticides results in an active response of heightened gene expression as opposed to a mere passive shift in gene expression. Focusing on immune system processes, we find more immune genes differentially expressed than expected only in thiacloprid-treated individuals, suggesting that thiacloprid exposure elicits a stronger immune response than imidacloprid. Interestingly, of the seven DEGs with elevated expression in imidacloprid-exposed bees, five genes were also significantly up-regulated in the thiacloprid-exposed bees which could possibly point to a common set of immune genes that are up-regulated in response to neonicotinoid exposure. In terms of functional enrichment of the differentially expressed immune genes, both pesticide-treated groups share 'defence response to Gram-negative bacterium' as enriched term among the up-regulated genes; however, genes up-regulated in response to thiacloprid show many additional immunity-related terms, once again underlining the difference in the effect of these two neonicotinoids. Interestingly, the enrichment analysis suggests that thiacloprid may lead to an active up-regulation of immune processes, as reflected in 'negative regulation of immune system process' enriched among the down-regulated genes and 'positive regulation of innate immune response' among the up-regulated genes, suggesting a considerable impact of thiacloprid on *O. bicornis'* immune system after exposure to this neonicotinoid. Such a pattern is less clear in the imidacloprid-exposed group, although our results suggest that immunity may play a role in the bees response to exposure since we find 'defence response to Gram-negative bacterium' enriched among the up-regulated genes. Collectively, our findings provide additional support to the impact neonicotinoids can have on bee health.

CONCLUSIONS

The red mason bee, *O. bicornis*, is a commercially and ecologically relevant solitary bee species, whose immune system has been understudied, despite it being integral to its future chances of survival. Here, we utilized a comparative genomic approach to propose a set of genes as part of the immune gene repertoire of *O. bicornis*, and used RNA-seq data to show that the expression and regulation of these putative immune genes differ markedly between sexes and respond with heightened expression to treatment with two neonicotinoid pesticides. Additionally, our findings provide support for the application of a combined approach to inference of gene families, using homology information of more than one species of reference. Future studies on *O. bicornis* immunity will benefit from tissue-specific profiling, as well as tracking gene expression changes in response to different immune challenges. Similarly, the application of population genomics will provide important insights into the recent selection pressures acting on immune genes of mason bees. Collectively, our study provides novel insights into the immune system of an important, yet still understudied solitary bee species and identifies a candidate repertoire of immune genes for future research on the immune system of the red mason bee.

ACKNOWLEDGEMENTS

The authors would like to thank the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt, DBU) for supporting Jannik S. Möllmann with a doctoral scholarship (20021/743). They would also like to thank the ZDV department of the Johannes-Gutenberg University Mainz, in particular for providing access to the high-performance computing cluster MOGON II, but also for the maintenance and installation of software on the cluster, facilitating the research on this thesis project. They would also like to thank Jenny Fuchs for the cartoons included in the graphical abstract. Lastly, the authors would also like to thank the editors and two anonymous reviewers for constructive comments that greatly improved the quality of the manuscript. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors declare that there are no conflict of interest.

DATA AVAILABILITY STATEMENT

Data used for the present analysis originated from datasets generated by Beadle and Singh 2019). Raw sequences for the RNA-seq-based analyses can be obtained from the NCBI Short Read Archive (BioProject PRJNA285788). All scripts are publicly available on GitHub: https://github.com/janniksven/Genomic-architecture-of-immunity-in-Osmia-bicornis.

REFERENCES

Adams, M.D., Celniker, S.D., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G. et al. (2000) The genome sequence of *Drosophila melanogaster*. *Science*, 287, 2185–2195. https://doi.org/10.1126/science.287.5461.2185

Albert, Š., Gätschenberger, H., Azzami, K., Gimple, O., Grimmer, G., Sumner, S. et al. (2011) Evidence of a novel immune responsive protein in the Hymenoptera. *Insect Biochemistry and Molecular Biology*, 41(12), 968–981.

Alexa, A. & Rahnenführer, J. (2009) Gene set enrichment analysis with topGO. *Bioconductor Improv*, 27, 1–26.

Andrews, S. (2010) FastQC: a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Arai, I., Ohta, M., Suzuki, A., Tanaka, S., Yoshizawa, Y. & Sato, R. (2013) Immunohistochemical analysis of the role of hemocytin in nodule formation in the larvae of the silkworm, *Bombyx mori*. *Journal of Insect Science*, 13, 125.

Arce, A.N., David, T.I., Randall, E.L., Rodrigues, A.R., Colgan, T.J., Wurm, Y. et al. (2017) Impact of controlled neonicotinoid exposure on bumblebees in a realistic field setting. *The Journal of Applied Ecology*, 54(4), 1199–1208.

Arce, A.N., Ramos Rodrigues, A., Yu, J., Colgan, T.J., Wurm, Y. & Gill, R.J. (2018) Foraging bumblebees acquire a preference for neonicotinoid-treated food with prolonged exposure. *Proceedings of the Royal*

Society B: Biological Sciences, 285(1885), 20180655. https://doi.org/10.1098/rspb.2018.0655

Azpiazu, C., Bosch, J., Vinuela, E., Medrzycki, P., Teper, D. & Sgolastra, F. (2019) Chronic oral exposure to field-realistic pesticide combinations via pollen and nectar: effects on feeding and thermal performance in a solitary bee. *Scientific Reports*, 9(1), 13770.

Baer, B., Krug, A., Boomsma, J.J. & Hughes, W.O.H. (2005) Examination of the immune responses of males and workers of the leaf-cutting ant *Acromyrmex echinatior* and the effect of infection. *Insectes Sociaux*, 52(3), 298–303.

Barribeau, S.M., Sadd, B.M., Plessis, L. D., Brown, M.J.F., Buechel, S.D., Cappelle, K., Carolan, J.C., Christiaens, O., Colgan, T.K., Erler, S., Evans, J., Helbing, S., Karaus, E., Lattorf, H. M. G., Marxer, M., Meeus, I., Napflin, K., Niu, J., Schmid-Hempel, R.,Smagghe, G., Waterhouse, R.M., Yu, N., Zdobnov, E.M. & Schmid-Hempel, P. (2015) A depauperate immune repertoire precedes evolution of sociality in bees. *Genome Biology*, 16, 83.

Barribeau, S.M. & Schmid-Hempel, P. (2017) Sexual healing: mating induces a protective immune response in bumblebees. *Journal of Evolutionary Biology*, 30(1), 202–209.

Beadle, K., Singh, K.S., Troczka, B.J., Randall, E., Zaworra, M., Zimmer, C.T. et al. (2019) Genomic insights into neonicotinoid sensitivity in the solitary bee *Osmia bicornis*. *PLoS Genetics*, 15(2), e1007903.

Bebane, P.S.A., Hunt, B.J., Pegoraro, M., Jones, A.R.C., Marshall, H., Rosato, E. et al. (2019) The effects of the neonicotinoid imidacloprid on gene expression and DNA methylation in the buff-tailed bumblebee *Bombus terrestris*'. *Proceedings of the Royal Society B: Biological Sciences*, 286(1905), 20190718.

Beckage, N.E. (2008) *Insect immunology*. New York: Academic Press.

Blacquiere, T., Smagghe, G., Gestel, C.A.M. & Mommaerts, V. (2012) Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology*, 21(4), 973–992.

Boff, S., Scheiner, R., Raizer, J. & Lupi, D. (2021) Survival rate and changes in foraging performances of solitary bees exposed to a novel insecticide. *Ecotoxicology and Environmental Safety*, 211, 111869.

Bonasio, R., Zhang, G., Ye, C., Mutti, N.S., Fang, X., Qin, N. et al. (2010) Genomic comparison of the ants *Camponotus floridanus* and *Harpegnathos saltator*. *Science*, 329(5995), 1068–1071.

Bramke, K., Müller, U., McMahon, D. & Rolff, J. (2019) Exposure of larvae of the solitary bee *Osmia bicornis* to the honey bee pathogen *Nosema ceranae* affects life history. *Insects*, 10(11), 380. https://doi.org/10.3390/insects10110380

Brandt, A., Grikscheit, K., Siede, R., Grosse, R., Meixner, M.D. & Büchler, R. (2017) Immunosuppression in honeybee queens by the neonicotinoids thiacloprid and clothianidin. *Scientific Reports*, 7(1), 4673.

Brandt, A., Hohnheiser, B., Sgolastra, F., Bosch, J., Meixner, M.D. & Büchler, R. (2020) Immunosuppression response to the neonicotinoid insecticide thiacloprid in females and males of the red mason bee *Osmia bicornis* L'. *Scientific Reports*, 10(1), 4670.

Bray, N.L., Pimentel, H., Melsted, P. & Pachter, L. (2016) Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology*, 34(5), 525–527.

Brown, M.J.F. & Paxton, R.J. (2009) The conservation of bees: a global perspective. *Apidologie*, 40(3), 410–416.

Brutscher, L.M., Daughenbaugh, K.F. & Flenniken, M.L. (2015) Antiviral defense mechanisms in honey bees. *Current Opinion in Insect Science*, 10, 71–82.

Calderone, N.W. (2012) Insect pollinated crops, insect pollinators and US agriculture: trend analysis of aggregate data for the period, 1992–2009. *PLoS ONE*, 7, e37235. https://doi.org/10.1371/journal.pone.0037235

Calleri, D.V., McGrail Reid, E., Rosengaus, R.B., Vargo, E.L. & Traniello, J. F. (2006) Inbreeding and disease resistance in a social insect: effects of heterozygosity on immunocompetence in the termite *Zootermopsis angusticollis*. *Proceedings of the Royal Society B: Biological Sciences*, 273, 2633–2640. https://doi.org/10.1098/rspb.2006.3622

Casteels, P., Ampe, C., Jacobs, F. & Tempst, P. (1993) Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (Apis mellifera). *The Journal of Biological Chemistry*, 268(10), 7044–7054.

Casteels, P., Ampe, C., Riviere, L., van Damme, J., Elicone, C., Fleming, M. et al. (1990) Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis mellifera*). *European Journal of Biochemistry*, 187, 381–386. https://doi.org/10.1111/j.1432-1033.1990.tb15315.x

Chaimanee, V., Evans, J.D., Chen, Y., Jackson, C. & Pettis, J.S. (2016) Sperm viability and gene expression in honey bee queens (Apis mellifera) following exposure to the neonicotinoid insecticide imidacloprid and the organophosphate acaricide coumaphos. *Journal of Insect Physiology*, 89, 1–8.

Chen, J., Nolte, V. & Schlötterer, C. (2015) Temperature-related reaction norms of gene expression: regulatory architecture and functional implications. *Molecular Biology and Evolution*, 32(9), 2393–2402.

Chen, S., Zhou, Y., Chen, Y. & Gu, J. (2018) fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*, 34(17), i884–i890.

Chmiel, J.A., Daisley, B.A., Burton, J.P. & Reid, G. (2019) Deleterious effects of neonicotinoid pesticides on *Drosophila melanogaster* immune pathways. *mBio*, 10(5), e01395-19. https://doi.org/10.1128/mBio.01395-19

Christophides, G.K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C. et al. (2002) Immunity-related genes and gene families in *Anopheles gambiae*. *Science*, 298(5591), 159–165.

Claus, G., Pisman, M., Spanoghe, P., Smagghe, G. & Eeraerts, M. (2021) Larval oral exposure to thiacloprid: Dose-response toxicity testing in solitary bees, *Osmia* spp. (Hymenoptera: Megachilidae). *Ecotoxicology and Environmental Safety*, 215, 112143.

Colgan, T.J., Carolan, J.C., Bridgett, S.J., Sumner, S., Blaxter, M.L., & Brown, M.J.F. (2011) Polyphenism in social insects: insights from a transcriptome-wide analysis of gene expression in the life stages of the key pollinator, *Bombus terrestris*. *BMC Genomics*, 12, 623.

Colgan, T.J., Finlay, S., Brown, M.J.F. & Carolan, J.C. (2019) Mating induces selective immune priming which is maintained throughout bumblebee queen diapause. *BMC Genomics*, 20, 959. https://europepmc.org/article/ppr/ppr143125

Colinet, D., Cazes, D., Belghazi, M., Gatti, J.L. & Poirié, M. (2011) Extracellular superoxide dismutase in insects: characterization, function, and interspecific variation in parasitoid wasp venom. *The Journal of Biological Chemistry*, 286(46), 40110–40121.

Combes, C. (2001) *Parasitism: The ecology and evolution of intimate interactions*. Chicago: University of Chicago Press.

de Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M. & Lemaitre, B. (2002) The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *The EMBO Journal*, 21(11), 2568–2579.

DeGrandi-Hoffman, G. & Chen, Y. (2015) Nutrition, immunity and viral infections in honey bees. *Current Opinion in Insect Science*, 10, 170–176.

di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F. et al. (2013) Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences of the United States of America*, 110(46), 18466–18471.

Dmochowska-Ślęzak, K., Giejdasz, K., Fliszkiewicz, M. & Żółtowska, K. (2015) Variations in antioxidant defense during the development of the solitary bee *Osmia bicornis*. *Apidologie*, 46(4), 432–444.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S. et al. (2013) STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, 29(1), 15–21.

Dong, J., Wu, J., Han, L., Huang, J. & Wang, D. (2020) Novel characteristics of immune responsive protein IRP30 in the bumble bee *Bombus*

lantschouensis (Hymenoptera: Apidae). *Journal of Insect Science*, 20(2), 11. https://doi.org/10.1093/jisesa/ieaa017

Durinck, S., Spellman, P.T., Birney, E. & Huber, W. (2009) Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. *Nature Protocols*, 4(8), 1184–1191.

Emms, D.M. & Kelly, S. (2019) OrthoFinder: phylogenetic orthology inference for comparative genomics. *Genome Biology*, 20(1), 238.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H. et al. (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Molecular Biology*, 15(5), 645–656.

Ewels, P., Magnusson, M., Lundin, S. & Käller, M. (2016) MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, 32(19), 3047–3048.

Fish, E.N. (2008) The X-files in immunity: sex-based differences predispose immune responses. *Nature Reviews. Immunology*, 8(9), 737–744.

Galiana-Arnoux, D., Dostert, C., Schneemann, A., Hoffmann, J.A. & Imler, J.L. (2006) Essential function *in vivo* for Dicer-2 in host defense against RNA viruses in *Drosophila*. *Nature Immunology*, 7(6), 590–597.

Gallai, N., Salles, J.M., Settele, J. & Vaissière, B.E. (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, 68(3), 810–821.

Gerardo, N.M., Altincicek, B., Anselme, C., Atamian, H., Barribeau, S.M., de Vos, M. et al. (2010) Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. ', *Genome Biology*, 11(2), R21.

Gill, R.J., Ramos-Rodriguez, O. & Raine, N.E. (2012) Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature*, 491(7422), 105–108.

Goulson, D., Nicholls, E., Botías, C. & Rotheray, E.L. (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, 347(6229), 1255957.

Gruber, B., Eckel, K., Everaars, J. & Dormann, C.F. (2011) On managing the red mason bee (*Osmia bicornis*) in apple orchards. *Apidologie*, 42(5), 564. https://doi.org/10.1007/s13592-011-0059-z

Harpur, B.A. & Zayed, A. (2013) Accelerated evolution of innate immunity proteins in social insects: adaptive evolution or relaxed constraint? *Molecular Biology and Evolution*, 30, 1665–1674. https://doi.org/10.1093/molbev/mst061

Hill-Burns, E.M. & Clark, A.G. (2009) X-linked variation in immune response in *Drosophila melanogaster*. *Genetics*, 183(4), 1477–1491.

Hoffmann, J.A. (1995) Innate immunity of insects. *Current Opinion in Immunology*, 7, 4–10. https://doi.org/10.1016/0952-7915(95)80022-0

Hoffmann, J.A. & Hetru, C. (1992) Insect defensins: inducible antibacterial peptides. *Immunology Today*, 268, 411–415. https://doi.org/10.1016/0167-5699(92)90092-l

Imler, J.-L. & Bulet, P. (2005) Antimicrobial peptides in *Drosophila*: structures, activities and gene regulation. *Chemical Immunology and Allergy*, 86, 1–21.

Ingersoll, M.A. (2017) Sex differences shape the response to infectious diseases. *PLoS Pathogens*, 13(12), e1006688.

James, R.R. & Xu, J. (2012) Mechanisms by which pesticides affect insect immunity. *Journal of Invertebrate Pathology*, 109(2), 175–182.

Jeroen, P., Simon-Delso, N., Goulson, D., Maxim, L., Bonmatin, J. & Belzunces, L. (2013) Neonicotinoids, bee disorders and the sustainability of pollinator services. *Current Opinion in Environmental Sustainability*, 5(3-4), 293–305.

Jeschke, P. & Nauen, R. (2008) Neonicotinoids-from zero to hero in insecticide chemistry. *Pest Management Science*, 64(11), 1084–1098.

Jiggins, F.M. & Kim, K.W. (2007) A screen for immunity genes evolving under positive selection in *Drosophila*. *Journal of Evolutionary Biology*, 20(3), 965–970.

Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C. et al. (2014) InterProScan 5: genome-scale protein function classification. *Bioinformatics*, 30(9), 1236–1240.

Joseph, S.B. & Kirkpatrick, M. (2004) Haploid selection in animals. *Trends in Ecology & Evolution*, 19(11), 592–597.

Kinsella, R.J., Kähäri, A., Haider, S., Zamora, J., Proctor, G., Spudich, G. et al. (2011) Ensembl BioMarts: a hub for data retrieval across taxonomic space. *Database: The Journal of Biological Databases and Curation*, 2011, bar030.

Klein, S.L. & Flanagan, K.L. (2016) Sex differences in immune responses. *Nature Reviews. Immunology*, 16(10), 626–638.

Klein, A.-M., Steffan-Dewenter, I. & Tscharntke, T. (2003) Fruit set of highland coffee increases with the diversity of pollinating bees. *Proceedings of the Royal Society B: Biological Sciences*, 270(1518), 955–961.

Korayem, A.M., Fabbri, M., Takahashi, K., Scherfer, C., Lindgren, M., Schmidt, O. et al. (2004) A *Drosophila* salivary gland mucin is also expressed in immune tissues: evidence for a function in coagulation and the entrapment of bacteria. *Insect Biochemistry and Molecular Biology*, 34(12), 1297–1304.

Kubrak, O.I., Kučerová, L., Theopold, U. & Nässel, D.R. (2014) The sleeping beauty: how reproductive diapause affects hormone signaling, metabolism, immune response and somatic maintenance in *Drosophila melanogaster*. *PloS one*, 9(11), e113051.

Lavine, M.D. & Strand, M.R. (2002) Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology*, 32, 1295–1309. https://doi.org/10.1016/s0965-1748(02)00092-9

Lawniczak, M.K.N., Barnes, A.I., Linklater, J.R., Boone, J.M., Wigby, S. & Chapman, T. (2007) Mating and immunity in invertebrates. *Trends in Ecology & Evolution*, 22(1), 48–55.

Losey, J.E. & Vaughan, M. (2006) The economic value of ecological services provided by insects. *Bioscience*, 56(4), 311–323.

Love, M.I., Huber, W. & Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15(12), 550.

Manjon, C., Troczka, B.J., Zaworra, M., Beadle, K., Randall, E., Hertlein, G. et al. (2018) Unravelling the molecular determinants of bee sensitivity to neonicotinoid insecticides. *Current Biology*, 28(7), 1137–1143.

Mason, R., Tennekes, H., Sánchez-Bayo, F. & Jepsen, P.U. (2013) Immune suppression by neonicotinoid insecticides at the root of global wildlife declines. *Journal of Environmental Immunology and Toxicology*, 1(1), 3–12.

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M. & Sattelle, D.B. (2001) Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends in Pharmacological Sciences*, 22(11), 573–580.

Moffat, C., Buckland, S.T., Samson, A.J., McArthur, R., Pino, V.C., Bollan, K.A. et al. (2016) Neonicotinoids target distinct nicotinic acetylcholine receptors and neurons, leading to differential risks to bumblebees. *Scientific Reports*, 6, 24764.

Moffat, C., Pacheco, J.G., Sharp, S., Samson, A.J., Bollan, K.A., Huang, J. et al. (2015) Chronic exposure to neonicotinoids increases neuronal vulnerability to mitochondrial dysfunction in the bumblebee (*Bombus terrestris*). *FASEB Journal*, 29(5), 2112–2119.

Mokkapati, J.S., Bednarska, A.J. & Laskowski, R. (2021) The development of the solitary bee *Osmia bicornis* is affected by some insecticide agrochemicals at environmentally relevant concentrations. *The Science of the Total Environment*, 775, 145588.

Moret, Y. & Schmid-Hempel, P. (2000) Survival for immunity: the price of immune system activation for bumblebee workers. *Science*, 290(5494), 1166–1168.

Nakamura, A., Miyado, K., Takezawa, Y., Ohnami, N., Sato, M., Ono, C. et al. (2011) Innate immune system still works at diapause, a physiological state of dormancy in insects. *Biochemical and Biophysical Research Communications*, 410(2), 351–357.

Ni, W., Bao, J., Mo, B., Liu, L., Li, T., Pan, G. et al. (2020) Hemocytin facilitates host immune responses against *Nosema bombycis*. *Developmental and Comparative Immunology*, 103, 103495.

O'Donnell, S. & Beshers, S.N. (2004) The role of male disease susceptibility in the evolution of haplodiploid insect societies. *Proceedings of the Royal Society B: Biological Sciences*, 271(1542), 979–983.

Pace, K.E. & Baum, L.G. (2002) Insect galectins: roles in immunity and development. *Glycoconjugate Journal*, 19(7-9), 607–614.

Pamilo, P. & Crozier, R.H. (1981) Genic variation in male haploids under deterministic selection. *Genetics*, 98(1), 199–214.

Parsch, J. & Ellegren, H. (2013) The evolutionary causes and consequences of sex-biased gene expression. *Nature Reviews Genetics*, 14(2), 83–87.

Peng, J., Zipperlen, P. & Kubli, E. (2005) Drosophila sex-peptide stimulates female innate immune system after mating via the Toll and Imd pathways. *Current Biology*, 15(18), 1690–1694.

Rao, X.-J., Wu, P., Shahzad, T., Liu, S., Chen, L., Yang, Y.F. et al. (2016) Characterization of a dual-CRD galectin in the silkworm *Bombyx mori*. *Developmental and Comparative Immunology*, 60, 149–159.

Rees, J.A., Moniatte, M. & Bulet, P. (1997) Novel antibacterial peptides isolated from a European bumblebee, *Bombus pascuorum* (Hymenoptera, Apoidea). *Insect Biochemistry and Molecular Biology*, 27(5), 413–422.

Retschnig, G., Williams, G.R., Mehmann, M.M., Yañez, O., de Miranda, J.R. & Neumann, P. (2014) Sex-specific differences in pathogen susceptibility in honey bees (*Apis mellifera*). *PLoS One*, 9(1), e85261.

Rogers, L.J., Frasnelli, E. & Versace, E. (2016) Lateralized antennal control of aggression and sex differences in red mason bees, *Osmia bicornis*. *Scientific Reports*, 6, 29411.

Rolff, J. (2002) Bateman's principle and immunity. *Proceedings of the Royal Society B: Biological Sciences*, 269(1493), 867–872.

Rolff, J. & Reynolds, S. (2009) *Insect infection and immunity: Evolution, ecology, and mechanisms*. Oxford: Oxford University Press.

Rolff, J. & Siva-Jothy, M.T. (2003) Invertebrate ecological immunology. *Science*, 301(5632), 472–475.

Roux, J., Privman, E., Moretti, S., Daub, J.T., Robinson-Rechavi, M. & Keller, L. (2014) Patterns of positive selection in seven ant genomes. *Molecular Biology and Evolution*, 31(7), 1661–1685.

Ruddle, N., Elston, C., Klein, O., Hamberger, A. & Thompson, H. (2018) Effects of exposure to winter oilseed rape grown from thiamethoxam-treated seed on the red mason bee *Osmia bicornis*. *Environmental Toxicology and Chemistry/SETAC*, 37(4), 1071–1083.

Ruiz-González, M.X. & Brown, M.J.F. (2006) Males vs workers: testing the assumptions of the haploid susceptibility hypothesis in bumblebees. *Behavioral Ecology and Sociobiology*, 60(4), 501–509.

Sackton, T.B., Lazzaro, B.P., Schlenke, T.A., Evans, J.D., Hultmark, D. & Clark, A.G. (2007) Dynamic evolution of the innate immune system in *Drosophila*. *Nature Genetics*, 39(12), 1461–1468.

Sadd, B.M., Barribeau, S.M., Bloch, G., de Graaf, D.C., Dearden, P., Elsik, C.G. et al. (2015) The genomes of two key bumblebee species with primitive eusocial organization. *Genome Biology*, 16, 76.

Sadd, B.M. & Schmid-Hempel, P. (2008) PERSPECTIVE: principles of ecological immunology. *Evolutionary Applications*, 2(1), 113–121.

Sadd, B.M. & Schmid-Hempel, P. (2009) Ecological and evolutionary implications of specific immune responses. In: *Insect Infection and Immunity*. Oxford: Oxford University Press, pp. 225–240. https://doi.org/10.1093/acprof:oso/9780199551354.003.0014

Sandrock, C., Tanadini, L.G., Pettis, J.S., Biesmeijer, J.C., Potts, S.G. & Neumann, P. (2014) Sublethal neonicotinoid insecticide exposure reduces solitary bee reproductive success. *Agricultural and Forest Entomology*, 16(2), 119–128.

Schoonvaere, K., Smagghe, G., Francis, F. & de Graaf, D.C. (2018) Study of the metatranscriptome of eight social and solitary wild bee species reveals novel viruses and bee parasites. *Frontiers in Microbiology*, 9, 177.

Schwenke, R.A., Lazzaro, B.P. & Wolfner, M.F. (2016) Reproduction-immunity trade-offs in insects. *Annual Review of Entomology*, 61, 239–256.

Seidelmann, K. (2006) Open-cell parasitism shapes maternal investment patterns in the red mason bee *Osmia rufa*. *Behavioral Ecology*, 17, 839–848. https://doi.org/10.1093/beheco/arl017

Shen, S., Park, J.W., Lu, Z.X., Lin, L., Henry, M.D., Wu, Y.N. et al. (2014) rMATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proceedings of the National Academy of Sciences of the United States of America*, 111(51), E5593–E5601.

Shultz, A.J. & Sackton, T.B. (2019) Immune genes are hotspots of shared positive selection across birds and mammals. *eLife*, 8, e41815. https://doi.org/10.7554/eLife.41815

Shumate, A. & Salzberg, S.L. (2020) Liftoff: accurate mapping of gene annotations. *Bioinformatics*, 37, 1639–1643. https://doi.org/10.1093/bioinformatics/btaa1016

Siviter, H., Koricheva, J., Brown, M.J.F. & Leadbeater, E. (2018) Quantifying the impact of pesticides on learning and memory in bees. *The Journal of Applied Ecology*, 19, 915.

Splitt, A., Schulz, M. & Skórka, P. (2021) Current state of knowledge on the biology and breeding of the solitary bee – *Osmia bicornis*. *Journal of Apicultural Research*, https://doi.org/10.1016/j.jip.2018.09.005.

Stanley, D.A., Smith, K.E. & Raine, N.E. (2015) Bumblebee learning and memory is impaired by chronic exposure to a neonicotinoid pesticide. *Scientific Reports*, 5, 16508.

Straub, F., Orih, I.J., Kimmich, J. & Ayasse, M. (2021) Negative effects of the neonicotinoid clothianidin on foraging behavior and antennal sensitivity in two common pollinator species, *Osmia bicornis* and *Bombus terrestris*. *Frontiers in Ecology and Evolution*, 9, 697355. https://doi.org/10.3389/fevo.2021.697355

Szentgyörgyi, H., Moroń, D., Nawrocka, A., Tofilski, A. & Woyciechowski, M. (2017) Forewing structure of the solitary bee *Osmia bicornis* developing on heavy metal pollution gradient. *Ecotoxicology*, 26(8), 1031–1040.

Tian, T., Piot, N., Meeus, I. & Smagghe, G. (2018) Infection with the multi-host micro-parasite *Apicystis bombi* (Apicomplexa: Neogregarinorida) decreases survival of the solitary bee *Osmia bicornis*. *Journal of Invertebrate Pathology*, 158, 43–45. https://doi.org/10.1016/j.jip.2018.09.005

Troczka, B.J., Homem, R.A., Reid, R., Beadle, K., Kohler, M., Zaworra, M. et al. (2019) Identification and functional characterisation of a novel N-cyanoamidine neonicotinoid metabolising cytochrome P450, CYP9Q6, from the buff-tailed bumblebee *Bombus terrestris*. *Insect Biochemistry and Molecular Biology*, 111, 103171.

Vanengelsdorp, D. & Meixner, M.D. (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of Invertebrate Pathology*, 103-(Suppl 1), S80–S95.

Viljakainen, L. (2015) Evolutionary genetics of insect innate immunity. *Briefings in Functional Genomics*, 14, 407–412. https://doi.org/10.1093/bfgp/elv002

Viljakainen, L., Evans, J.D., Hasselmann, M., Rueppell, O., Tingek, S. & Pamilo, P. (2009) Rapid evolution of immune proteins in social insects. *Molecular Biology and Evolution*, 26(8), 1791–1801.

Waterhouse, R.M., Kriventseva, E.V., Meister, S., Xi, Z., Alvarez, K.S., Bartholomay, L.C. et al. (2007) Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. *Science*, 316(5832), 1738–1743.

Werren, J.H., Richards, S., Desjardins, C.A., Niehuis, O., Gadau, J., Colbourne, J.K. et al. (2010) Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. *Science*, 327(5963), 343–348.

Woodcock, B.A., Bullock, J.M., Shore, R.F., Heard, M.S., Pereira, M.G., Redhead, J. et al. (2017) Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. *Science*, 356(6345), 1393–1395.

Xu, J. & James, R.R. (2012) Temperature stress affects the expression of immune response genes in the alfalfa leafcutting bee, *Megachile rotundata*. *Insect Molecular Biology*, 21(2), 269–280.

Zuk, M. & Stoehr, A.M. (2002) Immune defense and host life history. *The American Naturalist*, 160(Suppl 4), S9–S22.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Supporting Information Material

Appendix S2. Supporting Information Material

Appendix S3. Supporting Information Material

Appendix S4. Supporting Information Material

Appendix S5. Supporting Information Material

Appendix S6. Supporting Information Material

Figure S1. Supporting Information Material.

Figure S2. Supporting Information Material.

Figure S3. Supporting Information Material.

How to cite this article: Möllmann, J.S. & Colgan, T.J. (2022) Genomic architecture and sexually dimorphic expression underlying immunity in the red mason bee, *Osmia bicornis*. *Insect Molecular Biology*, 31(6), 686–700. Available from: https://doi.org/10.1111/imb.12796