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Conserved and Unique Protein Expression Patterns Across Reproductive Stage Transitions in Social Hymenopteran Queens

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ABSTRACT

Hymenopteran queens are collectively highly fecund, often long-lived individuals that undergo dramatic physiological changes after they mate and establish a nest. However, the degree to which these changes are conserved among species with different life histories is not well-defined. We conducted a comparative proteomic study investigating differences between reproductive stages (virgin, mated and established queens) of *Apis mellifera*, *Bombus impatiens*, *B. terrestris* and *Lasius niger*. We analysed haemolymph for all species except *L. niger*, for which a whole-body analysis was performed due to the small size of these queens. We identified conserved upregulation of proteins involved in anatomical and system development as queens transition to establishing a nest in all species except *B. terrestris*. We also identified conserved patterns of vitellogenin, vitellogenin receptor and immune-responsive protein (IRP)30, all of which are proteins typically associated with oviposition. However, expression patterns of other immune proteins, heat-shock proteins (HSPs), detoxification enzymes and antioxidant enzymes were more dissimilar, with some species exhibiting similar trends and co-occurrence through reproductive stages, while others exhibited variable or opposite patterns. These conserved and unique profiles likely in part reflect similarities and differences in selective pressure on reproductive stages of each species and may indicate differing abilities to respond to emergent pathogens or environmental change.

1 | Introduction

Order Hymenoptera includes tens of thousands of species of ants (Formicidae), bees (Anthophila clade; Henríquez-Piskulich, Hugall, and Stuart-Fox 2024) and wasps (non-bee and non-ant members of suborder Apocrita) (Mason and Huber 1993). Within this order, eusociality is a widespread phenomenon which is characterised by pre-imaginal caste differentiation that is normally fixed for life between fertile queens and (typically) sterile workers (Boomsma and

Gawne 2018). While all ant species are eusocial, only a fraction of bee species—including highly eusocial honey bees (*Apis*) and stingless bees (*Melipona*), as well as primitively eusocial bumble bees (*Bombus*), some orchid bees (Euglossini tribe), and some species within the Halictinae subfamily—and some wasp species (some, but not all vespids) exhibit this level of sociality (da Silva 2021).

All eusocial hymenopteran queens mate early in life and must transition to establishing a colony; however, their life histories are not necessarily similar. Different species establish new colonies through different mechanisms; for example, honey bee colonies reproduce via colony fission and mated queens thus begin laying eggs in active nests containing workers derived from the previous queen (Winston 1991), whereas mated bumble bee queens in temperate regions must endure a period of winter diapause and initiate nests independently the following spring (or possibly usurp existing nests) (Heinrich 2004). Ant queens may initiate colonies independently during the summer, but colonies may also be produced by fission, depending on the species (Peeters and Ito 2001). This means that while queens establishing colonies dependently (i.e., honey bee queens and ant queens that found nests via fission) benefit from attendance, foraging and brood incubation by existing workers, those founding independently (i.e., bumble bee queens and some ant queens) must work to incubate and provision their first young on their own. Thus, while all queens are highly fecund, engage in a single mating period and are long-lived relative to workers, the environmental and nutritional constraints they experience before and after initiation of oviposition are not the same.

During mate-finding, foraging and nest establishment, queens must execute a series of tasks to be successful, and they experience different conditions and selective pressures while doing so. For example, honey bee and some ant queens must be capable of the metabolic demands of sustained flight to find mates, and may become exposed to fluctuations in weather conditions. Mating can also expose queens to sexually transmitted infections (Amiri, Meixner, and Kryger 2016; Roberts et al. 2015). This pathogen exposure risk, in combination with selection for sustained longevity, could be why mating tends to induce immune activation in queens of several species (Kocher, Tarpy, and Grozinger 2010; Kocher et al. 2008; Manfredini et al. 2015; Barribeau and Schmid-Hempel 2017; Colgan et al. 2019; McAfee, Chapman, et al. 2024; Guo et al. 2021; Chérasse, Dacquin, and Aron 2019; Castella, Christe, and Chapuisat 2009; Baer, Armitage, and Boomsma 2006). Diapausing and independent founding queens must also find or excavate suitable chambers where they can either overwinter or begin to lay eggs and incubate brood, at which time they are subject to intense energetic demands associated with ovary activation and thermoregulation. Moreover, foraging queens or queens living in already-provisioned nests may have elevated risk of exposure to xenobiotics in the environment compared to fully claustral founding queens. Given that colony success and genetic propagation is solely dependent on the queen's successful establishment, one might expect intense selection for queen resilience to stressors—possibly via investment in high baseline expression levels of stress-mitigating proteins—especially during the mate-searching and nest-founding stages, when they are most vulnerable.

However, reproduction itself is an energetically demanding process and some data suggest that trade-offs between reproductive functions and other capabilities can occur (Baer, Armitage, and Boomsma 2006; Chérasse and Aron 2018; McAfee et al. 2021; Schwenke, Lazzaro, and Wolfner 2016), though not always (Pamminger, Treanor, and Hughes 2016; Shih et al. 2020), and clearly not with lifespan (at least among queens; Blacher, Huggins, and Bourke 2017). Therefore, while a naïve hypothesis might be for queens to strongly express proteins supporting immunity and longevity (such as immune effectors and antioxidant enzymes, which help mitigate molecular damage from oxidising agents), and for proteins supporting abiotic stress tolerance—such as heat-shock proteins (HSPs, which mitigate temperature stress) and detoxification enzymes (which mitigate impacts of xenobiotic toxins)—to be highly expressed during the more environmentally variable virgin and sometimes nest-founding stages, differences in social context, food availability and endocrine network signalling complicate these predictions. In particular, signalling involving insulin-like/IGF, juvenile hormone (JH), and vitellogenin is thought to govern some hymenopteran queens' escape from the reproduction-longevity trade-off (Rodrigues and Flatt 2016; Séguret, Bernadou, and Paxton 2016); thus, proteins involved in these networks might govern other trade-offs with reproduction as well.

Even if a reproduction-based trade-off is assumed, predictions are still difficult to make. For example, ant queens with fully claustral nest founding might not invest in expressing proteins involved in mitigating temperature or xenobiotic stress during the founding phase, as these queens are both resource-limited (not eating) and not contacting the external environment through foraging. Conversely, bumble bee queens are less resource-limited, with their ability to forage and store food, while also being exposed to more variable environmental conditions; therefore, these queens may be expected to maintain high levels of stress-mitigating proteins during colony initiation. However, actual resource availability is difficult to ascertain because it depends on spring weather conditions and residual fat and protein reserves after diapause. Honey bee queens, on the other hand, mate and return to bustling colonies, so they might experience higher resource availability and lower risk of extreme temperature exposure, but with higher contact with xenobiotics (Traynor et al. 2016, 2021; Traynor, vanEngelsdorp, and Lamas 2021) and exposure risk to pathogens (Evans and Spivak 2010). Higher resource availability implies that honey bee queens may be less judicious in their investment in non-reproductive processes, but, unlike independent nest-founders, they immediately begin laying a constant, high daily volume of eggs (Winston 1991), and therefore also have a more demanding resource output.

Barring more precise knowledge of the resource economy in each queen's situation, an informative approach may be to measure expression levels of genes or proteins associated with reproduction, stress and pathogen tolerance as a cumulative read-out of inputs and selection. While many studies have investigated genes or proteins putatively involved in queen longevity, reproduction, immunity or stress tolerance in individual hymenopteran species (Kocher, Tarpy, and Grozinger 2010; Kocher et al. 2008; Colgan et al. 2019; McAfee, Chapman, et al. 2024; Chérasse, Dacquin, and Aron 2019; Pamminger,

Treanor, and Hughes 2016; Gonzalez, Ing, and Rangel 2018; Rangel et al. 2021; Jedlička et al. 2016; Kim et al. 2008; Chen et al. 2021; Liu et al. 2022; Lucas, Romiguier, and Keller 2017; Chérasse and Aron 2017; Liberti et al. 2019; Richard, Tarpy, and Grozinger 2007), a concerted comparative study has not, to our knowledge, been conducted on multiple species and their reproductive stages. Such a study is necessary to determine the degree to which protein profiles associated with resilience to biological and environmental perturbations are conserved or unique among queens with different modes of colony founding, and may eventually allow us to predict differing abilities to tolerate pathogens or changing environmental conditions.

Here, we investigated the haemolymph (or, in the case of *L. niger*, the whole body) proteomes of *A. mellifera*, *L. niger*, *B. impatiens* and *B. terrestris* queens transitioning from virgin emergence to mating and nest establishment in order to assess constitutive levels of proteins linked to immunity, lipid trafficking and adverse environmental exposure (toxins and temperature stress). *L. niger* is phylogenetically distant from the three bee species; however, our goal was to conduct comparative proteomics and interpret the data with respect to life history strategies, rather than phylogenetic relatedness. The two bumble bee species have similar nest founding strategies (summer mating followed by winter diapause and independent nest founding in the spring), whereas *A. mellifera* queen establishment occurs in the presence of workers, and *L. niger* nest establishment is fully claustral and occurs immediately after mating in early summer; therefore, these species represent three broadly different nest-founding strategies. We analysed previously published *B. impatiens* data (McAfee, Chapman, et al. 2024) along with new datasets of *A. mellifera*, *B. terrestris* and *L. niger* to report patterns of protein expression across similar reproductive stage transitions (Figure 1A). We hypothesised that (1) since all queens are at risk of pathogen exposure through mating, all species would exhibit mating-induced immune protein upregulation, (2) since all species seek mates outside the nest in the summer (when extreme heat may occur), strong HSP expression in virgins would be conserved, (3) proteins linked to xenobiotic exposure would reflect the exposure risk of different nest founding strategies (higher in non-claustral mated *Bombus* and *A. mellifera* and lower in claustral *L. niger*, relative to virgins), (4) antioxidant protein abundance in mated queens would be linked to respective queen longevity (highest in *L. niger* and lowest in *Bombus*) and (5) expression patterns of core proteins involved in ovary and egg development would be conserved. These rich datasets provide a window into the physiological changes occurring in queens of diverse social hymenopteran species.

2 | Methods

We acquired proteomics data from *A. mellifera*, *B. impatiens*, *B. terrestris* and *L. niger* queens at different reproductive stages, as described in detail below for each species. We sampled haemolymph for all species except for *L. niger*, which was analysed as whole body extracts due to its small body size. Since we were primarily interested in examining groups of proteins associated with biotic and abiotic stress (immune proteins, HSPs, detoxification enzymes and antioxidant enzymes) alongside proteins generally involved in female reproduction (e.g., vitellogenin orthologues), haemolymph is a suitable choice, as it is a functionally relevant tissue for proteins involved in stress responses (McAfee et al. 2022; Negri et al. 2016) and concurrently circulates vitellogenin ahead of deposition at the ovaries (Piulachs et al. 2003).

2.1 | Honey Bee Queen Sampling

Queen honey bees were produced by Scandia Honey Company (Scandia, AB, Canada) as previously described (McAfee, Magaña, et al. 2024), according to standard commercial queen production practices. Briefly, young (≤1-day old) larvae from mixed genetic sources were grafted into queen cell cups and placed into cell builder colonies managed specifically for queen rearing. Capped queen cells were placed into mating nucleus colonies in which the adult queens emerged. 1–2 days after queens emerged, $n = 10$ were sampled ('virgin' group). 10–12 days after emergence, $n = 10$ queens confirmed to have initiated egg laying were sampled ('mated' group). One month after emergence, a further $n = 10$ laying queens with all brood stages (eggs, larvae and capped brood) were sampled ('established' group).

2.2 | *Bombus impatiens* Queen Sampling

The *B. impatiens* queens are the same samples as described in McAfee, Chapman, et al. (2024). Briefly, unmated queens were collected from four colonies ($n = 5$ each, $n = 20$ total) purchased from Biobest (Langley, BC, Canada) in the summer of 2022 ('virgin' group). The virgins were collected as they attempted to exit the nest on their first flight. The foundress queen had been removed from these colonies when the worker population surpassed ~150–200 individuals, stimulating the colonies to rear new queens, which were collected approximately 30 days later in an enclosed outdoor flight house. Post-diapause mated queens ($n = 10$) were collected outdoors in April, before nest establishment, in Delta, BC, Canada ('mated' group). Laying queens ($n = 10$) were collected from colonies with worker populations of ~150–200 individuals purchased from Biobest ('established' group).

2.3 | *Bombus terrestris* Queen Sampling

Copulation of *B. terrestris* queens was performed at the Université Libre de Bruxelles, Belgium, following methods originally described by Lhomme et al. (2013), and are essentially as reported in Przybyla et al. (2021). We used virgin queens (2-day old) provided by Biobest (Westerlo, Belgium). Virgins ($n = 10$) were sampled by decapitation with a scalpel and stored in microfuge tubes at −80°C ("virgin" group), then, each remaining queen was placed in a flight cage (35 cm × 35 cm × 60 cm) exposed to natural light at room temperature with three different males to stimulate the mating. Once mating initiated, the other males were removed from the device to avoid any disruption to copulation. After a successful mating (±30 min), the queen was placed in a plastic box

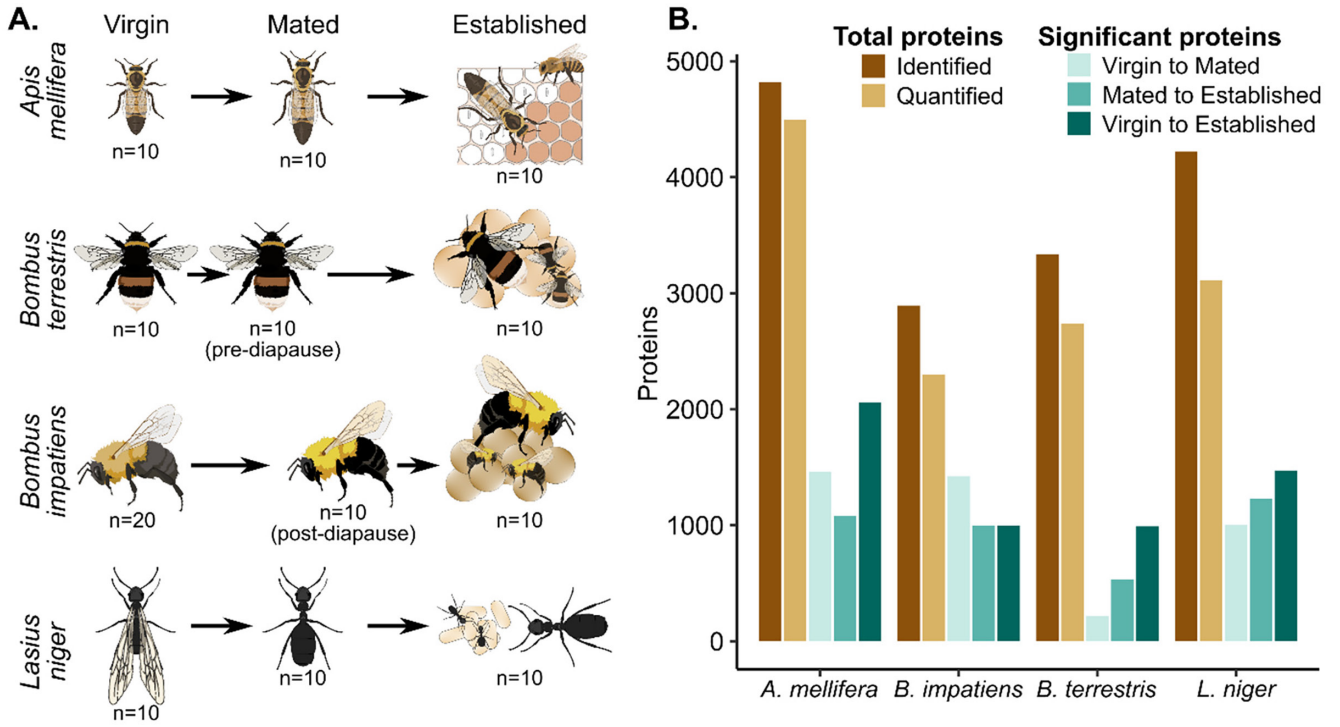

FIGURE 1 | Summary of the datasets. (A) We acquired label-free quantification proteomics data for queens of four social hymenopteran species (*Apis mellifera*, *Lasius niger*, *Bombus terrestris* and *B. impatiens*) and three reproductive stages for each. Sample sizes are indicated below each icon. Bee samples were made up of haemolymph protein extracts, whereas ant samples were composed of whole-body extracts, and all samples were analysed individually (not pooled). Mated *B. terrestris* queens were collected before diapause, whereas mated *B. impatiens* queens were collected after diapause. *A. mellifera* and *L. niger* do not undergo diapause before nest establishment. Bee and ant icons were partially derived from Biorender.com. (B) Summary of differential expression datasets. Identified protein groups (proteins) represent totals after contaminant proteins and reverse sequences removed. Quantified proteins represent proteins with < 30% valid values removed. Significant proteins represent those surviving significance at 5% FDR (Benjamini–Hochberg). Virgin to mated, mated to established, and virgin to established categories indicate the number of proteins differentially expressed in contrasts of the specified stages (corresponding to those depicted in panel A).

(16 cm × 11 cm × 9 cm) with ad libitum *Salix* (willow) pollen and sugar syrup (Biogluc, from Biobest). After 1 week, $n = 10$ queens ('mated' group) were sampled before initiating diapause and stored at −80°C.

To induce diapause, mated queens were placed individually in a match box with a moist cotton to maintain humidity, which were maintained at 5°C for a period of 2 months (Beekman, Van Stratum, and Lingeman 1998). Then, the queens were placed in a flight cage (Yoon et al. 2010) (30 cm × 30 cm × 30 cm) for 1 week at room temperature with ad libitum pollen and sugar syrup. They were subjected to a photoperiod of 8 h of light and 16 h of dark for 3 days and then a phase of 24 h of full light to exit hibernation (Yoon et al. 2010). Finally, all the queens were placed individually in a rearing box (16 cm × 11 cm × 9 cm) to initiate their colony.

Queens were kept in their nesting boxes with optimum temperature conditions (26°C) and constant humidity (50%–60%) in complete darkness. They were fed ad libitum with *Salix* pollen (Ruchers de Lorraine), a diet of optimal quality (Vanderplanck et al. 2019) with sugar syrup (Biogluc, from Biobest). Prior to bumblebee feeding, pollen loads were crushed and mixed with Biogluc. Seven days after emergence of the first workers, $n = 10$ queens were sampled ('established' group). All tissue samples were then shipped on dry ice to the University of British Columbia for further processing.

2.4 | *Lasius niger* Queen Sampling

Virgin and mated *L. niger* queens were collected on the day of the nuptial flight in Brussels (Belgium) in June of 2021. Virgin queens were sampled at the entrance of their natal colonies before they took off to mate, while mated (dealated) queens were collected after the nuptial flight. The queens were placed in 16 × 150 mm laboratory nesting tubes with ad libitum water and maintained under constant laboratory conditions (22°C ± 1°C, relative humidity of 30%–40% and a light/dark cycle of 12 h/12 h), with $n = 10$ queens sampled after 7 days ('mated' group). Queens did not require feeding as colony founding in *L. niger* is claustral: queens seal themselves in small burrows and rear their first brood by metabolising their wing muscles and stored fat. The first workers emerged approximately 2 months after the nuptial flight, with $n = 10$ queens sampled 7 days later ('established' group). Queens were killed by decapitation and stored at −80°C until shipment to the University of British Columbia for further processing.

2.5 | Sample Preparation

Bombus terrestris, *B. impatiens* and *A. mellifera* haemolymph was extracted from previously frozen thoraxes by allowing the tissue to thaw on ice, then removing one hindleg where the coxa meets the

thorax (as described previously) (McAfee, Chapman, et al. 2024). The thorax was gently squeezed and ~0.5–1 μL of haemolymph was extracted from the socket and dispensed into a tube containing 25 μL of 50 mM ammonium bicarbonate. This method avoids potential contamination of haemolymph with fluid from other tissues, as can occur when sampled from the abdomen. We have previously demonstrated that haemolymph data from fresh and previously frozen specimens are comparable (McAfee et al. 2022).

Due to their small size, haemolymph was not sampled from *L. niger*. Rather, whole body extracts (minus the spermatheca, which was previously removed for other purposes) were prepared by homogenising each ant in extraction buffer (6 M guanidinium chloride, 100 mM Tris, pH 8) using a Precellys-24 tissue homogeniser (Bertin Technologies; 3×30 s at 5000 rpm, 2 min incubation on ice in between repetitions). Samples were then transferred to new tubes, debris was removed by centrifugation (16,000 *g*, 4°C), and the supernatants were again transferred to new tubes.

To prepare the samples for proteomics, the diluted haemolymph (for bee samples) and whole body extract supernatant (for *L. niger* samples) were precipitated by adding ice cold acetone to a final concentration of 80% following methods as previously described (McAfee, Chapman, et al. 2024). Samples were incubated overnight at −20°C to allow protein to fully precipitate. Protein was pelleted by centrifugation at 10,000 *g* for 15 min (4°C), the supernatant was discarded, and the pellet was washed twice with cold 80% acetone, discarding the wash. The pellet was then air-dried for 15 min, at which time it was resuspended in 25 μL of digestion buffer (8 M urea, 2 M thiourea, 100 mM Tris, pH 8.0) and sonicated in an ice water bath for 10 min. 10 μg of protein was then reduced (0.2 μg of dithiothreitol, 30 min), alkylated (1 μg of iodoacetamide, dark, 30 min) and digested (0.4 ug of Lys-C/Trypsin mix, Promega). After 4 h of digestion in the urea buffer, 125 μL of 50 mM ammonium bicarbonate buffer was added and samples were allowed to digest overnight at room temperature. The samples were acidified to pH < 2.0 using 20% formic acid and desalted using in-house made C18 STAGE tips (Rappsilber, Ishihama, and Mann 2003). After loading the sample, the STAGE tips were washed with 3×250 μL buffer A (0.5% acetonitrile, 0.5% formic acid), then eluted with 200 μL buffer B (40% acetonitrile, 0.5% formic acid). Samples were evaporated to dryness using a speed-vac (2 h, room temperature) and suspended in 11 μL buffer A. Every sample was checked using a nanodrop to determine final peptide concentration and verify the absence of absorbance at 240 nm (which indicates residual digestion buffer contamination). Samples were diluted to a final concentration of 10 ng/μl in buffer A.

2.6 | Liquid Chromatography and Mass Spectrometry

For the *B. terrestris* haemolymph (50 ng), *B. impatiens* haemolymph (75 ng) and *L. niger* body (75 ng) peptide samples were analysed using previously described LC and mass spectrometry settings (McAfee, Chapman, et al. 2024), whereas the *A. mellifera* samples (50 ng) were analysed using slightly different settings described subsequently. The peptides were injected in randomised orders onto the LC system comprised of a NanoElute2 UHPLC system (Bruker Daltonics) with Aurora Series Gen2 (CSI) analytical column (25 cm × 75 μm 1.6 μm FSC C18, with Gen2 nanoZero and CSI fitting; Ion Opticks, Parkville, Victoria, Australia) heated to 50°C. A standard 30-min gradient was run from 2% buffer B to 12% B over 15 min, then to 33% B from 15 to 30 min, then to 95% B over 0.5 min, and held at 95% B for 7.72 min. Before each run, the analytical column was conditioned with four column volumes of buffer A. Buffer A consisted of 0.1% aqueous formic acid and 0.5% acetonitrile in water, and buffer B consisted of 0.1% formic acid in 99.4% acetonitrile. The NanoElute thermostat temperature was set at 7°C. The analysis was performed at 0.3 μL/min flow rate.

The LC system is coupled to a trapped ion mobility-time of flight mass spectrometer (timsTOF Pro2; Bruker Daltonics, Germany) operated in data independent acquisition, parallel accumulation-serial fragmentation (DIA-PASEF) mode, scanning from 100 to 1700 m/z. The LC and MS instruments were controlled using HyStar 6.2 (Bruker) and timsControl 4.1.8 (Bruker) software. The capillary voltage was set to 1800 V, drying gas to 3 L/min, and drying temperature to 180°C. The MS1 scan was followed by 17 consecutive PASEF ramps containing 22 non-overlapping 35 m/z isolation windows, covering the 319.5–1089.5 m/z range. As for the TIMS settings, the ion mobility range ($1/k_0$) was set to 0.70–1.35 V·s/cm^2, a 100-ms ramp time and accumulation time (100% duty cycle), and a ramp rate of 9.42 Hz, resulting in 1.91 s of total cycle time. The collision energy was ramped linearly as a function of mobility from 27 eV at $1/k_0 = 0.7$ V·s/cm^2 to 55 eV at $1/k_0 = 1.35$ V·s/cm^2. The target intensity was 10,000 counts, with a minimum intensity of 1000 counts. Mass accuracy is typically within 3 ppm and is not allowed to exceed 7 ppm. For calibration of ion mobility, the ions of Agilent ESI-Low Tuning Mix ions are selected (m/z [Th], $1/k_0$ [Th]: 622.0290, 0.9915; 922.0098, 1.1986; 1221.9906, 1.3934).

The *A. mellifera* samples were analysed as described above, except as follows, due to general method optimisation at the facility: The Captive Spray ionisation source was operated at 1700 V capillary voltage and 200°C drying temperature. The MS spectra were collected in positive mode from 100 to 1700 m/z range. The TIMS was operated with equal ramp and accumulation time of 85 ms (100% duty cycle). For each TIMS cycle, seven DIA-PASEF scans were used, each with three to four steps, with a total of 25 DIA-PASEF windows spanning from 299.5 Th 1200.5 Th and from ion mobility range ($1/k_0$) 0.7 V·s/cm^2 to 1.3 V·s/cm^2. Variable isolation width from 36 Th to 61 Th was used with an overlap of 1 Th between two neighbouring windows. The collision energy was ramped linearly as a function of mobility value from 20 eV at $1/k_0 = 0.6$ V·s/cm^2 to 65 eV at $1/k_0 = 1.6$ V·s/cm^2. Quality control testing during instrument optimisation showed that these methodological differences generally improved instrument sensitivity and data richness (number of proteins identified). Such differences do not affect the magnitude or direction of differentially expressed proteins by relative quantification and are unlikely to impact GO term enrichment results with respect to the quantifiable proteome.

2.7 | Raw Data Processing

The data were searched using DIA-NN version 1.8.1 (Demichev et al. 2020) with the default parameters except that the options 'FASTA digest for library-free search', 'Deep learning-based spectra, RTs and IMs prediction', 'unrelated runs' and 'MBR'

were selected, 'Protein inference' was set to protein names from FASTA, two missed cleavages were allowed and 'Neural network classifier' was set to double-pass mode. FASTA databases for *L. niger* and *B. impatiens* were downloaded from Uniprot on December 5, 2022 (19,174 entries for *B. impatiens* and 18,141 entries for *L. niger*), whereas the *A. mellifera* database was downloaded on February 2, 2023 (19,054 entries) and the *B. terrestris* database was downloaded on November 28, 2023 (19,621 entries). To each database, a comprehensive list (Frankenfield et al. 2022) of 381 potential protein contaminants was appended. The *A. mellifera* database also contained all viral and bacterial honey bee pathogen sequences available on Uniprot. All supplementary file contents are described in Data S1, and protein quantification data and sample metadata are available in Data S2.

2.8 | Differential Expression Analysis

Differential expression analysis was performed on data from each species separately in R (version 4.3.0) (R Core Team 2023) via R Studio (build 494) using the limma package (Ritchie et al. 2015). The data were log2 transformed and filtered to remove contaminant sequences and sequences identified in fewer than 30% of samples. For each species, the design matrix used for limma first considered sample injection order as a covariate along with reproductive stage (two levels for honey bees and three levels for all other species) and subsequently dropped if it was not significantly influential. False discoveries were controlled at 5% within each species by the Benjamini–Hochberg method, and proteins were considered differentially expressed if their p values satisfied the 5% FDR threshold in at least one contrast (pairwise comparisons between reproductive stages). All differential expression statistics are reported in Data S3.

2.9 | GO Term Enrichment

Gene ontology term enrichments were conducted using ErmineJ (Lee et al. 2005) for up- and down-regulated proteins separately for each differential expression contrast. GO term associations were downloaded from Uniprot.org for each respective species at the time of downloading the protein FASTA files. We used the gene score resampling (GSR) method, which, unlike typical over-representation analyses, is unaffected by arbitrary significance thresholds chosen for the differentially expressed proteins. It uses raw p values as scores, and tests for enrichment of GO terms along the p value continuum, instead of comparing a 'hit-list' to 'background'. This is particularly useful in our case, as the number of differentially expressed proteins varied greatly between species and contrasts. False discoveries for enrichment tests were controlled to 10% within each test using the Benjamini–Hochberg method. We believe this somewhat relaxed threshold is appropriate for our study, as we also rely on additional evidence to interpret the data (e.g., GO terms that are enriched in contrasts across two or more species, and those with low multifunctionality scores, are considered most reliable). All enrichment statistics are reported in Data S4.

3 | Results

We quantified 2297, 3334, 4219, and 4494 protein groups (proteins with shared peptides that cannot be unambiguously distinguished by unique peptides; hereafter referred to as proteins) in *B. terrestris*, *B. impatiens*, *L. niger* and *A. mellifera* respectively (Figure 1B; Data S2; $N = 10$ each, except *B. impatiens* virgins, for which $N = 20$ individuals were analysed). We most commonly observed the largest number of differentially expressed proteins (5% FDR, Benjamini–Hochberg method) when contrasting virgin and established queens, which is sensible given that these reproductive stages have the largest separation in developmental time and the largest difference in reproductive investment. The exception is in *B. impatiens*, where the largest number of differences were observed in the virgin-to-mated contrast. Complete results of differential expression analyses are in Data S3.

3.1 | GO Term Enrichment Analysis

Next, we tested for functional enrichments among the differentially expressed proteins across all reproductive stages for each species to identify broad patterns of functional changes in expressed proteins. We found 34 GO terms that were significantly enriched at 10% false discovery rate (FDR; Benjamini–Hochberg method) in contrasts among at least two species (Figure 2, Table 1) and 243 enriched GO terms overall (across all 12 contrasts, that is, significant at 10% FDR in at least one contrast within one species; Figure 3). Among the 34 GO terms enriched in at least two species' contrasts, the majority (Séguret, Bernadou, and Paxton 2016) were also significant at 5% FDR in at least one contrast, supporting our use of multiple layers of evidence to help determine significance. Notably, 11 GO terms (Figure 2, indicated in red) were significant at 5% FDR in contrasts within at least two species, which we consider to be highly reliable enrichments.

While some of the multi-species enriched terms related to carbohydrate, amino acid, and nucleotide metabolism are comprised of highly multifunctional proteins (multifunctionality score > 0.90), and are thus more likely to be enriched by chance, other metabolic GO terms are highly specific (such as those related to nicotinamide adenine dinucleotide (NAD) binding: GO:0051287; oxidoreductase activity: GO:0016491, GO:0016614 and GO:0016616; and lyase activity: GO:0016829 and GO:0016835). Though some of these GO terms encompass up to 49 unique protein members (from which meaning is difficult to fully derive) these GO terms do contain enzymes that are linked to different aspects of carbohydrate metabolism (e.g., glyceraldehyde-3-phosphate dehydrogenase, L-galactose dehydrogenase, aldose reductase), lipid metabolism (e.g., malic enzyme, aldo-keto reductase, fatty acid synthase) or prostaglandin processing (e.g., prostaglandin reductase, 15-hydroxyprostaglandin reductase, 15-oxoprostaglandin 13-reductase). The importance of carbohydrate and fatty acid metabolism is already reflected by additional significant GO terms falling under these functional umbrellas (shown in Table 1 and Figure 2) and is unsurprising given the major metabolic changes that must occur with life stage transitions. The contribution of prostaglandin processing enzymes to significant GO terms in multiple species is intriguing, as we

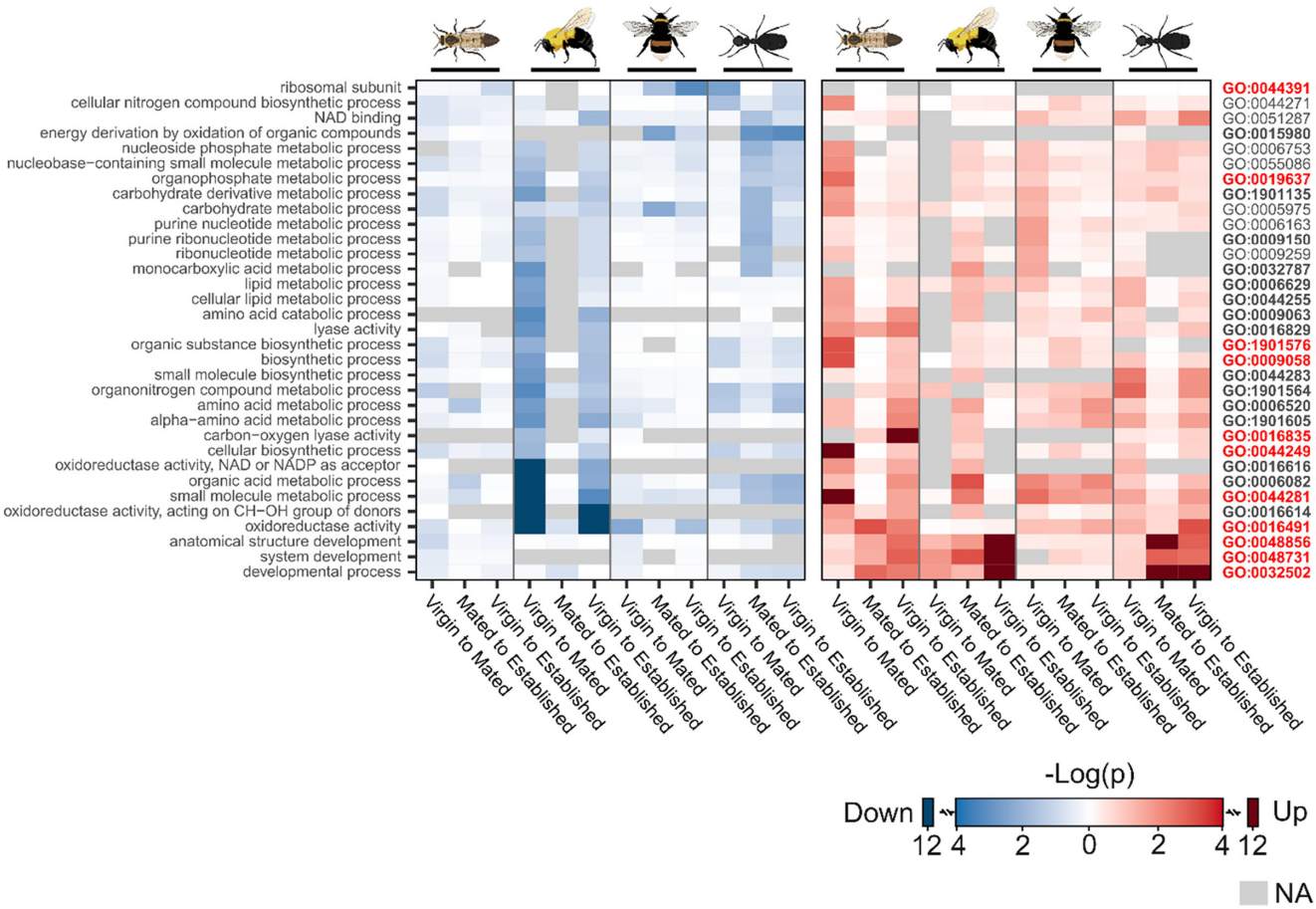

FIGURE 2 | Enriched GO terms across reproductive stages in two or more species. Each GO term depicted was significantly enriched at 10% FDR (Benjamini–Hochberg) in at least one reproductive stage transition across at least two species. Statistical reporting for each GO term is available in Table 1. NA = GO term not available. Bolded GO terms indicate those that were also significant at 5% FDR in at least one species, and red GO terms indicate those that were significant at 5% FDR in contrasts within at least two species. Bee and ant icons were partially derived from Biorender.com.

recently recorded prostaglandins as some of the most strongly differentially abundant metabolites in an analysis of queen honey bees (McAfee, Magaña, et al. 2024).

GO terms related to development (system development: GO:0048731; anatomical structure development: GO:0048856 and developmental process: GO:0032502), all of which have low multifunctionality scores and are upregulated as virgin *A. mellifera*, *B. impatiens* and *L. niger* queens transition to reproductive states, were among the 11 GO terms passing the highest level of significance testing (significant at 5% FDR in contrasts within three species). Curiously, few GO terms that were significantly enriched in *B. terrestris* transitions overlapped with those in other species. The proteins belonging to each GO term as well as all enrichment statistics can be found in Data S4.

3.2 | Analysis of Putative Stress-Mitigating Proteins

GO term enrichments are useful high-level analyses but may fail to identify patterns associated with specific proteins of interest. We were particularly interested in immune proteins (due to their importance for withstanding pathogenic infections), HSPs (due to their combined function as protein chaperones that mitigate protein aggregation during temperature stress (Shan et al. 2020) as well as potential antiviral activity (McMenamin, Daughenbaugh, and Flenniken 2020; Merkling et al. 2015)), detoxification enzymes (due to their role in breaking down endogenous and xenobiotic toxic compounds), antioxidant enzymes (as protectors from cellular damage due to oxidative stress), and vitellogenin and related proteins (due to their importance in oogenesis, longevity and lipid trafficking). We therefore extracted all quantified proteins belonging to these groups to identify patterns in reproductive stage transitions (Figures 4 and 5). Accession numbers associated with these proteins are in Data S2.

We found a strong tendency for reproductive *A. mellifera* and *B. impatiens* queens to express higher levels of immune proteins and lower levels of HSPs relative to virgins, with the exception of a subset of small HSPs (within the protein-lethal 2 essential for life family, or pl.(2)el) (Figure 4A,B). Reproductive *L. niger* and *B. terrestris* queens, however, did not have conserved patterns of immune protein expression. Although several immune proteins were differentially expressed in these species, some increased while others decreased with nest establishment in *L. niger*, and only two of fourteen immune proteins were differentially expressed in *B. terrestris*. Patterns of HSP expression were also protein-specific in *L. niger* (some

TABLE 1 | GO terms significantly enriched in reproductive transitions in two or more species.

ID	Name	Direction[a]	p[b]	MF score[c]	Species	Transition
Carbohydrate metabolism						
GO:0005975	Carbohydrate metabolic process	Down	1.3E-03	0.52	*B. terrestris*	Mated to established
		Down	3.8E-03	0.68	*L. niger*	Mated to established
		Up	3.2E-03	0.01	*A. mellifera*	Virgin to mated
GO:0006082	Organic acid metabolic process	Down	1.3E-03	0.94	*B. impatiens*	Virgin to established
		Down	1.0E-12	0.94	*B. impatiens*	Virgin to mated
		Down	2.5E-03	0.94	*L. niger*	Virgin to established
		Down	6.5E-03	0.94	*L. niger*	Mated to established
		Up	2.6E-03	0.95	*A. mellifera*	Virgin to established
		Up	1.0E-04	0.95	*B. impatiens*	Mated to established
GO:0015980	Energy derivation by oxidation of organic compounds	Down	6.0E-04	0.76	*B. terrestris*	Mated to established
		Down	1.0E-04	0.67	*L. niger*	Virgin to established
		Down	2.0E-04	0.72	*L. niger*	Mated to established
GO:1901135	Carbohydrate derivative metabolic process	Down	4.0E-04	0.95	*B. impatiens*	Virgin to mated
		Down	3.7E-03	0.91	*L. niger*	Mated to established
		Up	5.4E-03	0.11	*A. mellifera*	Virgin to mated
GO:0051287	NAD binding	Down	4.0E-03	0.06	*B. impatiens*	Virgin to established
		Down	8.7E-03	0.08	*L. niger*	Mated to established
		Up	1.2E-03	0.09	*L. niger*	Virgin to established
GO:0032787	Monocarboxylic acid metabolic process	Down	2.0E-04	0.85	*B. impatiens*	Virgin to mated
		Down	4.3E-03	0.92	*L. niger*	Mated to established
Nucleotide metabolism						
GO:0006163	Purine nucleotide metabolic process	Down	6.2E-03	0.96	*B. impatiens*	Virgin to mated
		Down	3.3E-03	0.96	*L. niger*	Mated to established
GO:0006753	Nucleoside phosphate metabolic process	Down	1.5E-02	0.98	*B. impatiens*	Virgin to mated
		Down	2.8E-03	0.97	*L. niger*	Mated to established
		Up	3.3E-03	0.09	*A. mellifera*	Virgin to mated
GO:0009150	Purine ribonucleotide metabolic process	Down	3.9E-03	0.96	*B. impatiens*	Virgin to mated
		Down	2.4E-03	0.96	*L. niger*	Mated to established
GO:0009259	Ribonucleotide metabolic process	Down	9.8E-03	0.97	*B. impatiens*	Virgin to mated
		Down	5.0E-03	0.97	*L. niger*	Mated to established
GO:0055086	Nucleobase-containing small molecule metabolic process	Down	6.7E-03	0.97	*B. impatiens*	Virgin to mated
		Up	2.2E-03	0.07	*A. mellifera*	Virgin to mated
Amino acid metabolism						
GO:0006520	Amino acid metabolic process	Down	4.0E-04	0.94	*B. impatiens*	Virgin to mated
		Up	2.7E-03	0.95	*A. mellifera*	Virgin to established

(Continues)

TABLE 1 | (Continued)

ID	Name	Direction[a]	p[b]	MF score[c]	Species	Transition
GO:0009063	Amino acid catabolic process	Down	2.3E-03	0.74	*B. impatiens*	Virgin to established
		Down	1.0E-04	0.74	*B. impatiens*	Virgin to mated
		Up	3.1E-03	0.75	*A. mellifera*	Virgin to established
GO:1901605	Alpha-amino acid metabolic process	Down	1.4E-03	0.93	*B. impatiens*	Virgin to established
		Down	2.0E-04	0.94	*B. impatiens*	Virgin to mated
		Up	1.5E-03	0.94	*A. mellifera*	Virgin to established
GO:1901564	Organonitrogen compound metabolic process	Down	1.0E-04	1.00	*B. impatiens*	Virgin to mated
		Up	2.6E-03	0.97	*L. niger*	Virgin to established
		Up	3.0E-04	0.98	*L. niger*	Virgin to established
Lipid metabolism						
GO:0006629	Lipid metabolic process	Down	6.0E-04	0.65	*B. impatiens*	Virgin to mated
		Up	7.0E-03	0.03	*A. mellifera*	Virgin to mated
GO:0044255	Cellular lipid metabolic process	Down	5.0E-04	0.73	*B. impatiens*	Virgin to mated
		Up	5.9E-03	0.03	*A. mellifera*	Virgin to mated
Other metabolism						
GO:0016491	Oxidoreductase activity	Down	1.0E-12	0.01	*B. impatiens*	Virgin to established
		Down	1.0E-12	0.01	*B. impatiens*	Virgin to mated
		Down	1.2E-03	0.03	*B. terrestris*	Virgin to mated
		Up	1.0E-04	0.02	*A. mellifera*	Mated to established
		Up	1.0E-03	0.02	*A. mellifera*	Virgin to established
		Up	1.0E-04	0.05	*L. niger*	Virgin to established
GO:0016614	Oxidoreductase activity, acting on CH-OH group of donors	Down	1.0E-12	0.06	*B. impatiens*	Virgin to established
		Down	1.0E-12	0.04	*B. impatiens*	Virgin to mated
		Up	5.0E-03	0.03	*A. mellifera*	Virgin to mated
GO:0016616	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	Down	1.1E-03	0.07	*B. impatiens*	Virgin to established
		Down	1.0E-12	0.05	*B. impatiens*	Virgin to mated
		Up	3.1E-03	0.03	*A. mellifera*	Virgin to mated
GO:0044281	Small molecule metabolic process	Down	1.0E-04	0.98	*B. impatiens*	Virgin to established
		Down	1.0E-12	0.99	*B. impatiens*	Virgin to mated
		Down	3.4E-03	0.98	*L. niger*	Virgin to established
		Up	1.0E-12	0.05	*A. mellifera*	Virgin to mated
		Up	8.0E-04	0.99	*B. impatiens*	Mated to established
GO:0016829	Lyase activity	Down	2.0E-04	0.11	*B. impatiens*	Virgin to mated
		Up	8.0E-04	0.10	*A. mellifera*	Virgin to established
		Up	2.6E-03	0.02	*A. mellifera*	Virgin to mated
GO:0016835	Carbon–oxygen lyase activity	Down	5.0E-03	0.14	*B. impatiens*	Virgin to mated
		Up	1.0E-12	0.18	*A. mellifera*	Virgin to established

(Continues)

TABLE 1 | (Continued)

ID	Name	Direction[a]	p[b]	MF score[c]	Species	Transition
GO:0006793	Phosphorus metabolic process	Down	9.8E-03	0.35	*B. impatiens*	Virgin to mated
		Up	1.0E-12	0.00	*A. mellifera*	Virgin to mated
GO:0019637	Organophosphate metabolic process	Down	2.5E-03	0.97	*B. impatiens*	Virgin to mated
		Up	4.0E-04	0.03	*A. mellifera*	Virgin to mated
Biosynthesis						
GO:0009058	Biosynthetic process	Down	5.0E-04	0.98	*B. impatiens*	Virgin to mated
		Up	1.0E-04	0.00	*A. mellifera*	Virgin to mated
GO:0044249	Cellular biosynthetic process	Down	3.8E-03	0.99	*B. impatiens*	Virgin to mated
		Up	1.0E-12	0.02	*A. mellifera*	Virgin to mated
GO:0044271	Cellular nitrogen compound biosynthetic process	Down	7.8E-03	0.97	*L. niger*	Virgin to established
		Up	1.9E-03	0.04	*A. mellifera*	Virgin to mated
GO:1901576	Organic substance biosynthetic process	Down	1.9E-03	0.99	*B. impatiens*	Virgin to mated
		Up	1.0E-04	0.01	*A. mellifera*	Virgin to mated
GO:0044283	Small molecule biosynthetic process	Down	3.0E-04	0.94	*B. impatiens*	Virgin to mated
		Up	2.8E-03	0.94	*L. niger*	Virgin to established
		Up	8.0E-04	0.92	*L. niger*	Virgin to established
GO:0044391	Ribosomal subunit	Down	1.0E-04	0.04	*B. terrestris*	Virgin to established
		Down	6.0E-04	0.17	*L. niger*	Virgin to established
Development						
GO:0048731	System development	Up	2.0E-04	0.05	*A. mellifera*	Virgin to established
		Up	1.0E-04	0.08	*B. impatiens*	Mated to established
		Up	1.0E-12	0.05	*B. impatiens*	Virgin to established
		Up	4.0E-04	0.08	*L. niger*	Virgin to established
		Up	2.0E-04	0.10	*L. niger*	Mated to established
GO:0048856	Anatomical structure development	Up	4.0E-04	0.04	*A. mellifera*	Virgin to established
		Up	1.0E-12	0.06	*B. impatiens*	Virgin to established
		Up	2.0E-04	0.45	*L. niger*	Virgin to established
		Up	1.0E-12	0.28	*L. niger*	Mated to established
GO:0032502	Developmental process	Up	3.0E-04	0.03	*A. mellifera*	Mated to established
		Up	8.0E-04	0.06	*A. mellifera*	Virgin to established
		Up	1.0E-12	0.06	*B. impatiens*	Virgin to established
		Up	1.0E-12	0.79	*L. niger*	Virgin to established
		Up	1.0E-12	0.73	*L. niger*	Mated to established

[a]Direction refers to up- or down-regulation of proteins within each GO term.
[b]All terms in this table were significant at 10% FDR (Benjamini–Hochberg method).
[c]MF score refers to multifunctionality score (scores closer to 1 indicate that the proteins belonging to the GO term are more highly multifunctional, and thus more likely to be enriched by chance).

HSPs transitioned from low to high and others from high to low abundance during reproductive development), whereas HSP levels in *B. terrestris* were generally highest in the established queen group (opposite to the patterns in other species). However, since *L. niger* samples originated from whole body extracts while the others originated from haemolymph, the impact of sample type versus species differences, for HSPs as well as other proteins described below, is uncertain. Although

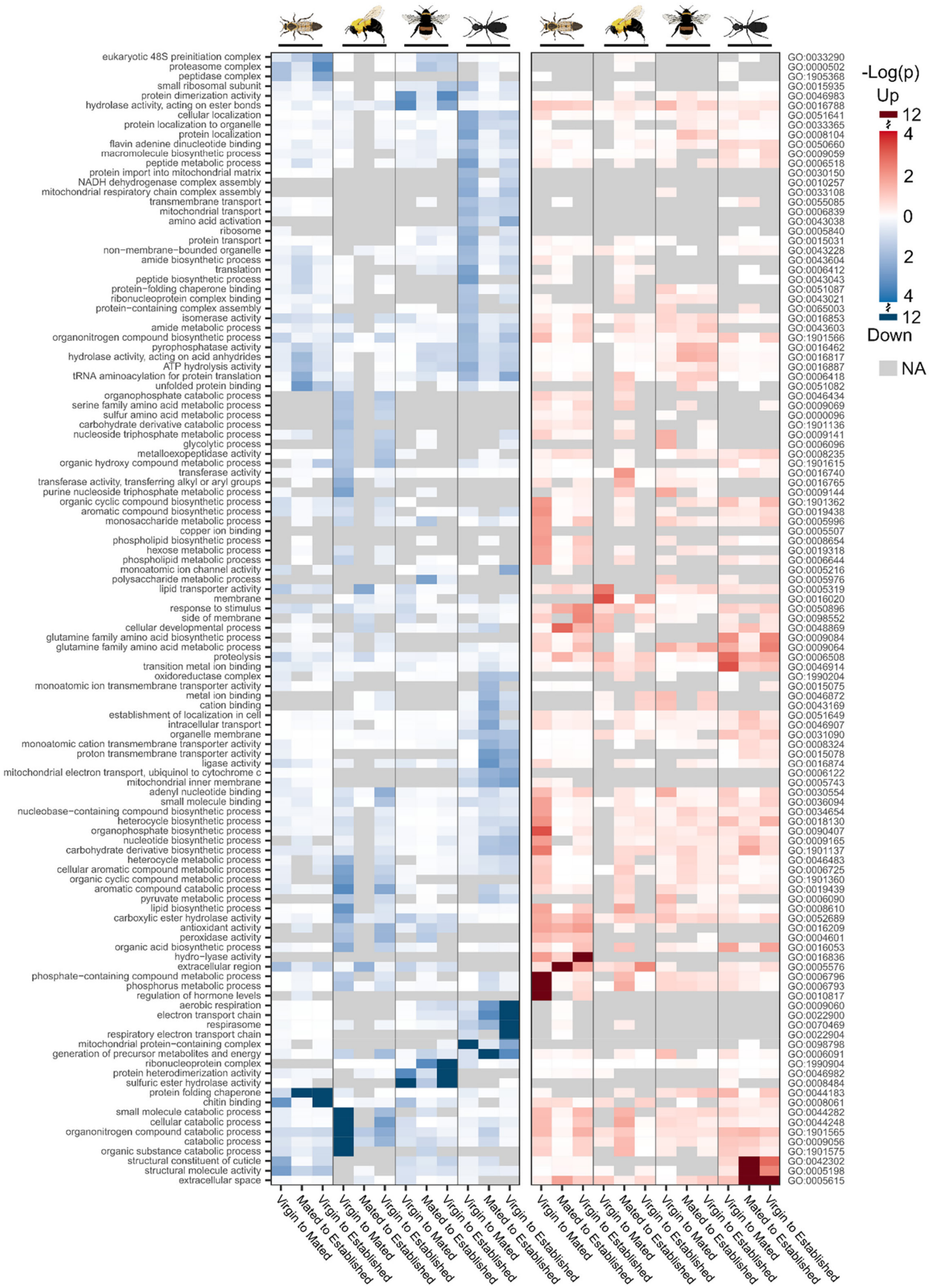

FIGURE 3 | Enriched GO terms across reproductive stages, uniquely significant in one species. Each GO term depicted is significant at 10% FDR (Benjamini–Hochberg) in at least one contrast. Each GO term displayed is significantly enriched in at least one reproductive stage transition, but not in more than one species. All statistics associated with GO term enrichments are available in Data S4.

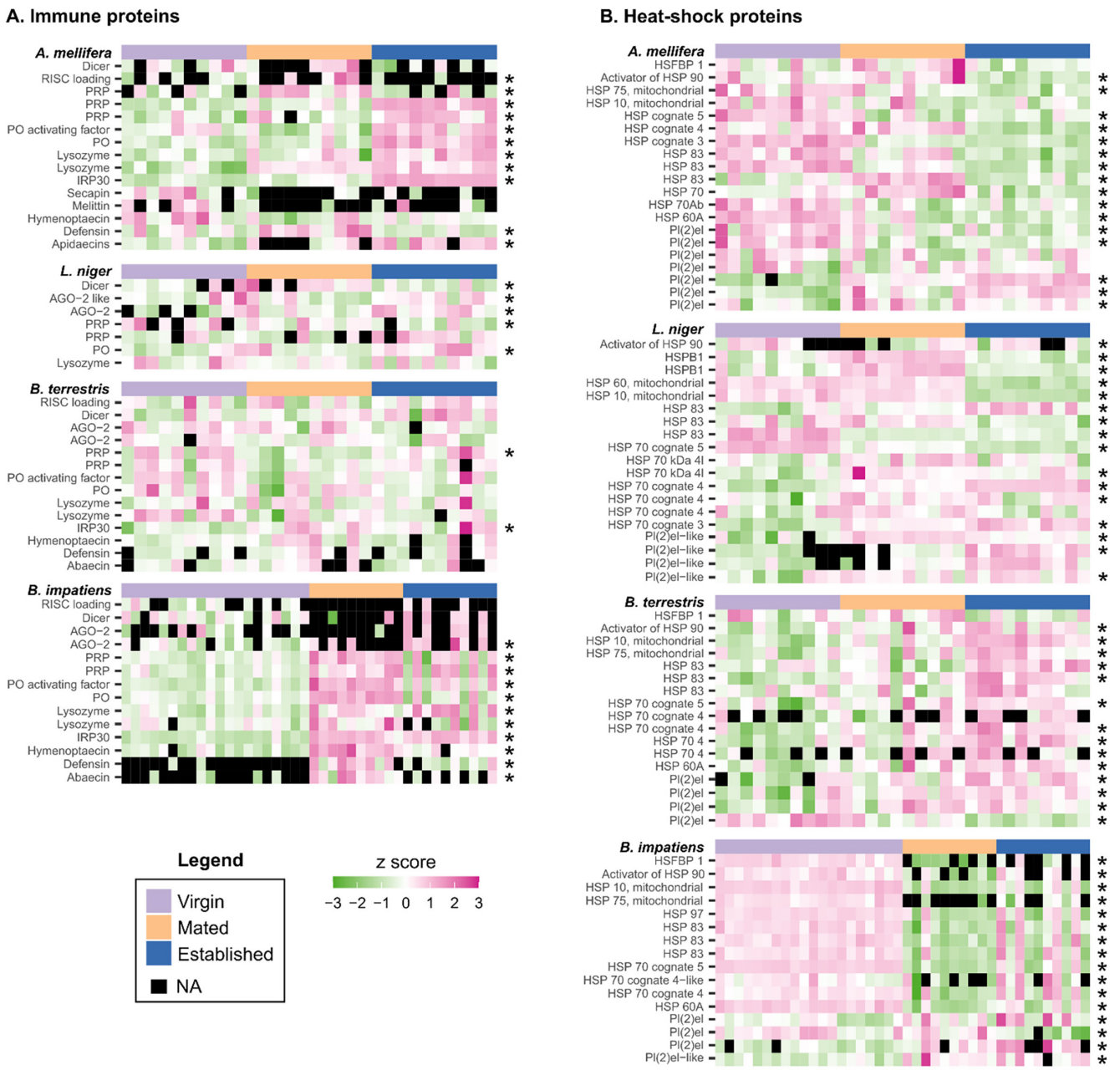

FIGURE 4 | Expression patterns for immune proteins and heat-shock proteins (HSPs). Each row represents a protein, and each column represents a sample (colour bars indicate the developmental stage of that sample). NA (black tiles) refers to instances where proteins were not quantified. Asterisks indicate that the protein was significant in at least one contrast (5% FDR, Benjamini–Hochberg). AGO = argonaut; HSBP = heat-shock protein beta; HSFBP = heat-shock factor binding protein; HSP = heat-shock protein; IRP = immune response protein; PO = phenoloxidase; PRP = peptidoglycan recognition protein; RISC = RNA-induced silencing complex.

not evaluated statistically, we also note that our ability to detect some proteins appears biased towards reproductive stages (Figures 4 and 5, black tiles within heatmaps), indicating that there is structure in the likelihood of peptides belonging to some proteins falling below our limit of detection. Interestingly, immune-responsive protein (IRP)30, a protein involved in immunity and ostensibly reproduction in many hymenopteran species (Dong et al. 2020), followed similar expression trajectories in all species which have an IRP30 homologue (*A. mellifera*, *B. impatiens* and *B. terrestris*) with lower levels in virgins and higher levels in reproductive individuals.

Regarding detoxification enzymes, *A. mellifera* and *L. niger* exhibited similar patterns of expression, with levels tending to be lower in virgins and higher in reproductive individuals (Figure 5A). Notably, *L. niger* appears to express a highly expanded repertoire of cytochrome p450 enzymes. Cytochrome p450 enzymes were identified in the other species, but were fewer in number and not considered quantified as coverage across samples was < 30%. While some of the differences in numbers of cytochrome p450 enzymes identified may be due to the whole-body nature of *L. niger* samples (which we would expect to yield greater coverage of tissue-specific proteins), the data reflect the general

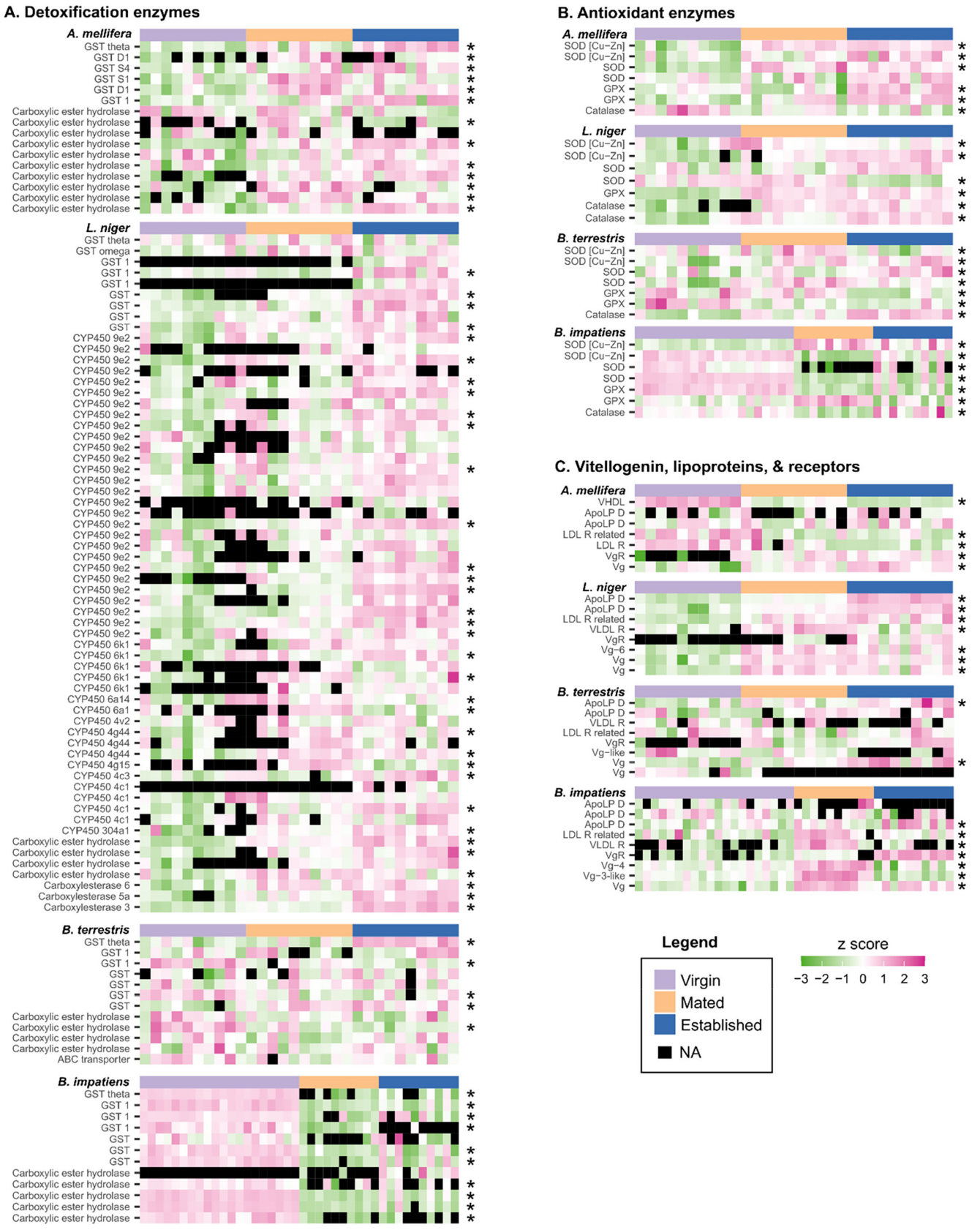

FIGURE 5 | Expression patterns for detoxification enzymes, lipoproteins and antioxidant enzymes. Each row represents a protein, and each column represents a sample (colour bars indicate the developmental stage of that sample). NA = data not available. Asterisks indicate significance in at least one contrast (5% FDR, Benjamini–Hochberg). ABC = ATP-binding cassette; CYP450 = cytochrome p450; GPX = glutathione peroxidase; GST = glutathione S-transferase; SOD = superoxide dismutase; Vg = vitellogenin; VgR = vitellogenin receptor.

proliferation of detoxification enzymes in the *L. niger* genome (Konorov et al. 2017). Indeed, 131 cytochrome p450 enzymes are annotated in *L. niger*, compared to 61, 52 and 57 in *A. mellifera*, *B. impatiens* and *B. terrestris*, respectively, as determined by inspecting the Uniprot protein databases. In contrast to *L. niger*, reproductive *B. impatiens* queens expressed lower levels of detoxification enzymes compared to virgins, and patterns in *B. terrestris* were again variable. Patterns of antioxidant enzymes mirrored those of detoxification enzymes for all species (Figure 5B), possibly pointing to coregulation of these systems.

In all four species, vitellogenin and vitellogenin receptor proteins exhibited similar expression trajectories, with low levels in virgins followed by higher levels upon mating, as one might expect. While only one vitellogenin isoform was identified in *A. mellifera*, three isoforms were identified in each of the other species. While the isoforms covaried in *L. niger*, in *B. impatiens* and *B. terrestris* they did not. Additional lipoproteins and lipoprotein receptors were quantified in all species and those which were differentially expressed tended to have higher levels in reproductive queens, except in *A. mellifera*, where the opposite trend is apparent (Figure 5C).

3.3 | Analysis of Additional Proteins of Interest

We next investigated expression patterns of hexamerins, odorant-binding proteins (OBPs) and proteins linked to insulin-like signalling. We expected that, similar to vitellogenins, hexamerins (conserved proteins with roles in amino acid storage, immunity and hormone transport (Burmester 2013)) would have largely conserved patterns across species; however, while levels were generally high in virgins and decreased upon initiation of reproduction, patterns in *L. niger* did not follow this trend (Figure 6); rather, they were lower in virgin queens and higher post-mating. In contrast to the other species, in honey bees a large number (nine) of OBPs were differentially expressed and most, but not all, increased in established queens. Accessions of these proteins are reported in Table 2.

Rewiring of relationships between vitellogenin, JH and insulin-like/IGF-1 signalling has been previously implicated as a mechanism underlying the paradoxical high longevity and high fecundity of eusocial queens (Rodrigues and Flatt 2016). We therefore wished to compare expression patterns of proteins involved in insulin signalling (insulin-like growth factor 1, insulin degrading enzymes, insulin receptor substrates, insulin-like growth factor-binding proteins) and JH metabolism (JH acid o-methyltransferase, JH esterase binding protein and JH epoxide hydrolase). Unfortunately, the only two types of proteins identified in more than one species were insulin degrading enzymes and insulin-like growth factor-binding proteins (Figure 6). Insulin degrading enzyme expression generally decreased after mating, as did most isoforms of insulin-like growth factor binding protein. The notable exception was in *L. niger*, where expression markedly increased after nest establishment.

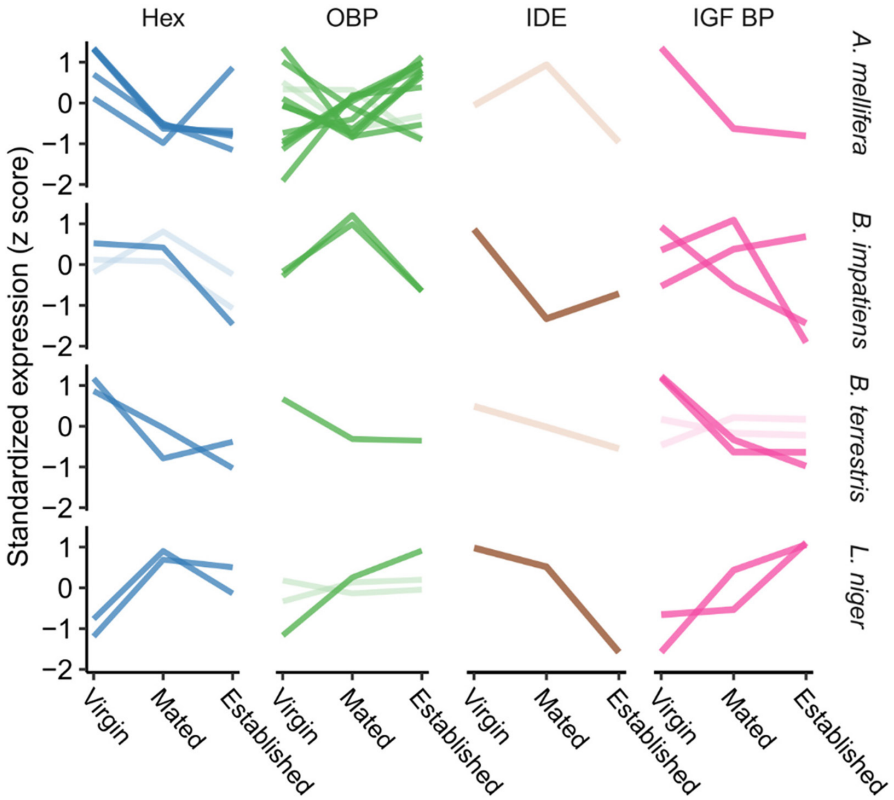

FIGURE 6 | Expression patterns of hexamerins, odorant-binding proteins, prostaglandin enzymes, insulin degrading enzymes and IGF-binding proteins. Each line represents a single protein (accessions found in Table 2). Darker colours indicate that the protein was differentially expressed among reproductive stages (Benjamini–Hochberg correction, 5% FDR). Lighter colours indicate that the protein was quantified but not differentially expressed. Hex = hexamerins; IDE = insulin-degrading enzyme; IGF BP = insulin-like growth factor-binding protein; OBP = odorant-binding proteins.

TABLE 2 | Hexamerins, odorant-binding proteins, prostaglandin enzymes, insulin degrading enzymes and IGF-binding proteins.

Accession	Description	Species	Significant
A0A7M6UMJ2	OBP13	*A. mellifera*	*
A0A7M6UUL3	OBP17	*A. mellifera*	*
Q1W645	OBP9	*A. mellifera*	*
A0A7M6UBW1	OBP18	*A. mellifera*	*
A0A8U0WQC3	OBP14	*A. mellifera*	*
Q1W638	OBP16	*A. mellifera*	*
A0A7M6UQF8	OBP4	*A. mellifera*	*
Q1W633	OBP21	*A. mellifera*	*
Q1W647	OBP3	*A. mellifera*	*
Q1W635	OBP19	*A. mellifera*	*
Q1W639	OBP15	*A. mellifera*	
A0A7M7GT67	OBP10	*A. mellifera*	
A0A7M7IH71	Hexamerin 110	*A. mellifera*	*
A5YV87	Hexamerin 70c	*A. mellifera*	*
Q6J4Q1	Hexamerin	*A. mellifera*	*
A5YVK7	Hexamerin 70a	*A. mellifera*	*
A0A7M6UMR7	Hexamerin 110 precursor	*A. mellifera*	*
A0A7M7IHY3	Insulin degrading enzyme	*A. mellifera*	
A0A7M7M5Q4	Insulin-like growth factor binding protein	*A. mellifera*	*
A0A6P3UU36	OBP19d	*B. impatiens*	*
A0A6P3E3E5	OBP56d	*B. impatiens*	*
A0A6P3V2I2	Hexamerin	*B. impatiens*	*
A0A6P3E3A5	Hexamerin	*B. impatiens*	
A0A6P3DW69	Hexamerin	*B. impatiens*	
A0A6P3UVV3	Insulin-like growth factor binding protein	*B. impatiens*	*
A0A6P3DXW5	Insulin-like growth factor binding protein	*B. impatiens*	*
A0A6P6FH38	Insulin-like growth factor binding protein	*B. impatiens*	*
A0A6P3V4Y4	Insulin degrading enzyme	*B. impatiens*	*
A0A9B0F6X3	OBP56d	*B. terrestris*	*
A0A9B0C594	PGF synthase 1	*B. terrestris*	*
A0A9B0C025	PGE synthase 3	*B. terrestris*	*
A0A6P3DGX7	Hexamerin	*B. terrestris*	*
A0A6P3DHB8	Hexamerin	*B. terrestris*	*
A0A9B0F2Q1	Insulin-like growth factor binding protein	*B. terrestris*	*
A0A9B0F6M5	Insulin-like growth factor binding protein	*B. terrestris*	*
A0A9B2JV75	Insulin-like growth factor binding protein	*B. terrestris*	
A0A9C6SJ11	Insulin-like growth factor binding protein	*B. terrestris*	

(Continues)

TABLE 2 | (Continued)

Accession	Description	Species	Significant
A0A9B2JV90	Insulin degrading enzyme	*B. terrestris*	
A0A0J7K786	OBPa10	*L. niger*	*
A0A0J7KP79	OBP	*L. niger*	
A0A0J7MRR3	OBP56d	*L. niger*	
A0A0J7LBP8	Hexamerin 2	*L. niger*	*
A0A0J7LBG8	Hexamerin	*L. niger*	*
A0A0J7L774	Insulin-like growth factor binding protein	*L. niger*	*
A0A0J7L9B8	Insulin-like growth factor binding protein	*L. niger*	*
A0A0J7K367	Insulin-degrading enzyme	*L. niger*	*

*Indicates significance at 5% FDR (Benjamini–Hochberg) in at least one contrast.

4 | Discussion

Our goal in this study was to take a broad view of functional shifts in protein expression profiles using gene set enrichment analyses and to map patterns of how specific groups of proteins change with reproductive stage transitions across species. We were particularly interested in assessing constitutive levels of proteins putatively involved in responses to pathogens (immune effectors), temperature stress (HSPs), toxins (detoxification enzymes), oxidative stress (antioxidant enzymes) and allocation of lipids (vitellogenin and other lipid transporters), as these proteins are linked to common stressors and ovary or oocyte investment. We expected to identify conserved patterns of proteins linked to immunity and reproduction, and that proteins associated with abiotic stress and longevity would vary according to the demands of each species' nest-founding strategy. By extension, we anticipated that profiles in the two *Bombus* species (both with non-claustral, independent nest founding) would be similar, whereas profiles in *A. mellifera* and *L. niger* (with fission and claustral, independent founding, respectively) would diverge.

We found that, on the contrary, patterns did not consistently reflect the queen's social context, phylogeny, or colony founding strategy, with many patterns of immune and stress-response proteins appearing to be species- or protein-specific within functional groups. We found that far more GO terms were uniquely enriched in transitions within a single species than overlapped with one or more species (Figures 2 and 3), and enriched GO terms were not disproportionately shared among the two *Bombus* species. Particularly evident patterns include the vast expansion of cytochrome p450 enzyme expression in *L. niger*, the upregulation of numerous OBPs in established *A. mellifera* queens, the uniquely high levels of detoxification and antioxidant enzyme expression in *B. impatiens*, and weak correlation of immune proteins and reproductive stage in *B. terrestris*. These data show that the protein expression profiles in queens are complex and often divergent between species, even those sharing similar nesting strategies (*Bombus*), with comparatively few consistent patterns. However, and importantly, some of this lack of consistency is likely due to differences in sampling methodology (see Study Limitations below), including the ages of sampled queens (affecting *B. impatiens*) and differences in the tissues analysed (affecting *L. niger*), which impedes our ability to draw strong conclusions. With this caveat in mind, addressing our specific hypotheses, we found that (1) immune effectors were not upregulated after mating in all species (strong patterns were only observed in *A. mellifera* and *B. impatiens*), (2) only *A. mellifera* and *B. impatiens* exhibited consistent HSP upregulation in virgins, (3) changes in proteins linked to xenobiotic exposure appear not to be linked to claustral versus non-claustral nest founding and (4) antioxidant enzyme expression was only partially linked to queen longevity, with upregulation apparent in mated queens of long-lived *L. niger* and *A. mellifera*, as predicted, but inconsistent patterns in the two *Bombus* species. Regarding point 3, although *L. niger* was the only fully claustral species examined (which confounds with differences in the tissues analysed), the overall conclusion that nest founding strategy appears unlinked to detoxification enzyme abundance is also supported by the observation that the *L. niger* genome contains far more detoxification enzyme genes than other Hymenoptera (Konorov et al. 2017).

Nevertheless, some proteins linked to egg-laying and anatomical structure/system development were consistently differentially expressed across reproductive stages of different species, which is noteworthy especially because these trends survive the methodological differences in sampling between species. Specifically, *B. impatiens* queens were older than *B. terrestris* queens at each reproductive stage, and *L. niger* queen samples consisted of whole-body extracts, rather than haemolymph. These sampling differences likely impacted the protein expression patterns observed—most strongly for the *L. niger* samples, for which the data represent a proportional average of the whole body with no tissue-level granularity—but clearly this did not preclude measuring consistent patterns for the core proteins involved in oocyte development (vitellogenin orthologues, vitellogenin receptors, and IRP30, discussed further below) and those sharing the GO term for anatomical structure (GO:0048856) and system (GO:0048731) development. Expression of genes related to system development have been previously shown to be overrepresented in queen honey bee ovaries after mating (Kocher et al. 2008),

and the development-related proteins we identified in haemolymph and body extracts here are likely similarly linked to the dramatic changes that occur in the ovaries post-mating. Several researchers have independently found genes involved in biosynthetic processes and metabolism enriched in queen reproductive transitions (Kocher, Tarpy, and Grozinger 2010; Kocher et al. 2008; Chen et al. 2021; Liu et al. 2022; Lucas, Romiguier, and Keller 2017), which is reflected in our data as well. Most specifically, Kocher et al (Kocher, Tarpy, and Grozinger 2010). previously found that virgin honey bees had elevated expression levels of HSPs relative to mated queens, which is the same pattern we found here.

Patterns of vitellogenin and vitellogenin receptor expression were also overall consistent across species, despite *L. niger* samples being the only ones containing fat bodies and ovaries (the sites of vitellogenin synthesis and deposition respectively). Vitellogenin is a conserved protein that is strongly associated with not only reproduction (as a yolk protein) but also transgenerational immunity and longevity, likely through its antioxidant functions and ability to carry pathogen fragments into oocytes (Salmela, Amdam, and Freitak 2015; Salmela et al. 2016; Morandin et al. 2014; Amdam et al. 2003; Zhao et al. 2021). While canonical vitellogenin (Vg) expression patterns were conserved, with generally low levels in virgins and higher levels occurring after mating, patterns of non-canonical vitellogenin isoforms (Vg-like or numbered isoforms, e.g., Vg-6) were not consistent across species (Figure 5). This agrees with data showing that vitellogenin isoforms have distinct expression profiles and functions which diverge from yolk deposition, though these functions are still poorly defined (Salmela et al. 2016; Morandin et al. 2014; Zhao et al. 2021). Interestingly, vitellogenin receptor, which mediates oocyte incorporation of Vg, was often not identified in virgin or mated queen samples, but was always quantified in established queens, which are also those with the highest reproductive output; therefore, receptor expression may offer another layer of control over directing vitellogenin's multifunctional roles.

IRP30 is another protein which we expected to observe in higher amounts after queen mating, and indeed this was the case. IRP30 has only been identified in Hymenoptera and appears to be a secreted immune protein (Albert et al. 2011) that also positively regulates vitellogenin expression (Tian et al. 2021) and is associated with initiating oviposition (Dong et al. 2020; Tian et al. 2021). In our study, IRP30 expression patterns mirror that of canonical vitellogenin, which further substantiates the importance of IRP30 for reproduction and its relationship with vitellogenin (Tian et al. 2021). IRP30 is not merely correlated with reproductive stage; it is also elevated in *A. mellifera*, *B. lantschouensis*, *B. terrestris* and *B. impatiens* reproductive workers compared to non-reproductive workers (McAfee, Chapman, et al. 2024; Dong et al. 2020; Tian et al. 2021), which share genetics and a common colony environment. Therefore, the association with reproduction is strong and not likely an artefact of potentially confounding factors. The dual role of IRP30 in both reproduction and immunity (the context in which it was originally discovered) (Albert et al. 2011) is just one of several ways that social hymenopteran queens appear to not be bound to typical tradeoffs between fecundity and other capabilities.

It seems like the more data are generated addressing reproduction-based trade-offs in queens, the less clear the trends become. What initially seems like a simple framework—that limited resources combined with the energetically expensive task of reproduction lead to a reduced investment in other functions—has in several instances been upended in queens of eusocial species. The most obvious example is that eusocial queens are both highly fecund and long-lived, possibly due to reorganisation of the insulin/IFG-1, JH, and vitellogenin interactions (Rodrigues and Flatt 2016). In addition, new data in honey bees suggests that queens have at least partially escaped a trade-off between reproduction and immunity, as injecting queens with viral fragments elicited a strong immune response but did not cause a decrease in oocyte output (Chapman et al. 2024). Some researchers have also proposed that, unlike other invertebrates, queen honey bees have escaped the typical trade-off between reproduction and the heat-shock response (Shih et al. 2020). Data that do support reproduction-immunity trade-offs in hymenopteran queens seem to be specific to sperm storage, rather than egg-laying (Baer, Armitage, and Boomsma 2006; Chérasse and Aron 2018; McAfee et al. 2021). This framework may therefore have limited utility outside the topic of sperm storage.

The data on queen honey bee heat-shock responses are particularly intriguing in the context of our study, as this highlights the importance of investigating inducibility in addition to constitutive levels of effectors. Our data and data from Kocher, Tarpy, and Grozinger (2010). show that in honey bees and *B. impatiens*, baseline (unmanipulated) HSP expression levels are generally lower after mating, which would ostensibly support a reproduction-heat-shock trade-off (Figure 4). However, Shih et al. (2020) found that mated honey bee queens still had a strongly inducible heat-shock response, comparable to that of non-reproductive workers. This suggests that, even if constitutive expression is low, in effect there is no trade-off since expression can still surge upon stimulation. Combined, these data suggest that expression patterns of HSPs may be driven less by a trade-off, per se, and more by the advantage of maintaining broadly high (*A. mellifera* and *B. impatiens*) or selectively high (*L. niger*) constitutive expression during the virgin phase in particular, when queens are more likely to experience heat events.

The contrasting low expression of HSPs in *B. terrestris* virgins was initially puzzling, as we expected the two *Bombus* species to display more similar patterns to one another than to the more distantly related species tested here; however, in light of differences in the two species' climatic niches and ages of sampled queens, the results are not unreasonable. Feuerborn et al. (2023) recently reported that *B. impatiens* queens exhibited lower heat tolerance after initiating oviposition compared to non-ovipositing queens, which is consistent with our data (where HSP expression is generally lower in mated *B. impatiens* queens and higher in virgins). The same researchers also showed that rearing environment affected heat tolerance, and the heat tolerance in virgins was highly dependent on their age (with very low tolerance early in life and significantly higher heat tolerance in queens just 3 days older) (Feuerborn et al. 2023). This small difference in age leading to a large difference in heat tolerance is one potential reason why the *B. impatiens* and *B. terrestris* data do not agree, since *B. impatiens* virgins were sampled as they exited the nest (> 7 days old) and

B. terrestris virgins were sampled when they were 2 days old. In addition, *B. terrestris* is a Euro-Mediterranean (hot, dry summers and cool to mild winters) species with generally high heat tolerance compared to other bumble bee species (Martinet et al. 2021). *B. impatiens*, although also considered a relatively heat-tolerant species (Feuerborn et al. 2023), is abundant in Eastern United States, extending northward into Eastern Canada, where temperatures fluctuate dramatically between seasons with humid, hot summers and cold winters. Since niche climatic conditions are linked to bumble bee species thermal limits (Feuerborn et al. 2023) (with those adapted to warmer conditions exhibiting intuitively higher thermal tolerance) (Feuerborn et al. 2023; Martinet et al. 2021, 2015) and humidity influences the effectual temperatures (McAfee et al. 2020), some differences in protein expression between these species should not be surprising. However, this does not fully explain the differences observed, as we would still expect virgin queens in both species to be more vulnerable, relative to ovipositing queens, to temperature fluctuations as they exit the nest and search for mates in the summer. Some combination of climate niche adaptation, rearing environment and age differences of the sampled queens may be affecting the results.

4.1 | Study Limitations

Our work offers a rich view into constitutive protein expression in queens, but we caution that these data do have several limitations. First, especially in light of the work of Shih et al., challenge experiments are necessary to test effector inducibility on top of constitutive expression. Second, as mentioned previously, the *L. niger* samples were composed of whole-body extracts rather than haemolymph, and the expression data were therefore heavily influenced by the most abundant tissues, such as the muscles, ovaries, brain, and digestive tract. These data therefore represent a proportional average across all tissues. While expression patterns of proteins associated with reproductive system development were conserved across species, it is unfortunately impossible to determine the extent to which proteins that differed between *L. niger* and the other species were influenced by life histories versus tissue differences. Third, the *B. impatiens* dataset (published previously (McAfee, Chapman, et al. 2024)) was unlike the others in that it included post-diapause (but pre-nesting) queens as the 'mated' stage, and queens sampled from larger nests (>150 individuals) as the 'established' stage (roughly 3 months after nest establishment). Conversely, in the other species, mated queens were sampled days after copulation and established queens were sampled days after emergence of the first workers. This means that the social context of *B. impatiens* and *A. mellifera* established queens were most similar to one another, which could be in part why they tend to express immune and HSPs similarly; however, patterns of detoxification and antioxidant enzymes diverge, so it is not clear if this is the case. Fourth, the post-diapause *B. impatiens* queens were collected from the wild, whereas the other reproductive stages of *B. impatiens* and all *B. terrestris* samples were derived from laboratory-propagated queens, which may change their genetic diversity or phenotype (as has been recently documented for heat tolerance (Feuerborn et al. 2023)). Fifth, we were disappointed in our inability to quantify more proteins in the insulin-like/IGF-1 and JH signalling pathways. This may be linked to our choice of tissue (haemolymph for bees and whole-body extracts for ants) which not only complicated species comparisons involving *L. niger* but also may be why proteins involved in these specific pathways were difficult to quantify. We and others have obtained high coverage of these proteins in whole-body extracts (Chen et al. 2021); thus, while haemolymph is a suitable and functionally relevant tissue for studying patterns of stress response proteins and some proteins involved in reproduction, it may not be a suitable tissue for assessing proteins involved in insulin-like/IGF-1 and JH signalling, specifically. Finally, it is possible that other variables not accounted for in this study may also be influencing protein expression patterns, such as pre-existing infections, weather conditions, genotype, or nutrient availability. Future work may benefit from including additional covariates, particularly pathogens, into such analyses, and sampling more tightly controlled reproductive stages. All of these limitations detract from our ability to draw seamless comparisons between species, but the data do still highlight some conserved patterns and provide a rich view of potential differences between species.

5 | Conclusion

We initially postulated that the molecular underpinnings of the major life stage transitions in hymenopteran queens would be strongly linked to exposure risks associated with their nest-founding strategy. Instead, our data demonstrate that while some processes do seem to be common, there are generally vast differences in protein expression patterns in four species of hymenopteran queens. Only the most conserved processes linked to oogenesis and anatomical/system development were consistently reflected across species, and the majority of patterns were unique or shared in only some species. These data are derived from haemolymph (*A. mellifera*, *B. impatiens* and *B. terrestris*) and whole body (*L. niger*) extracts, and it is possible that we may have found more conserved patterns if more specialised reproductive tissues (such as the ovaries or spermathecae) were analysed. While the factors underlying these observed differences remain unclear, we expect that these deep, complex data will assist in further hypothesis generation for investigating queen biology.

Author Contributions

A.M., B.M., and S.A. conceptualised the work. B.M. and K.P. produced the *B. terrestris* samples. F.D. produced the *L. niger* samples. S.E.H. produced the *A. mellifera* samples. A.M. produced the *B. impatiens* samples, wrote the first draft of the manuscript, prepared the mass spectrometry samples, produced the figures, and analysed the data. Grants to L.J.F., S.E.H., and S.A. supported the infrastructure needed to complete this work. All authors edited the manuscript and assisted with data interpretation and presentation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Proteomic data: All raw proteomics data are available via the MassIVE proteomics data archive (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp). The *B. impatiens* data are previously published and available under accession MSV000091414 (Foster 2023a). The *A. mellifera* data are available under accession MSV000094277 (Foster 2024). The *L. niger* and *B. terrestris* data are located under accession MSV000092460 (Foster 2023b). Please see the cited references for the relevant digital object identifier (doi). In addition, all data underlying Figures 1 through 6 are available in Data S2–S4. Sample metadata: Sample metadata are available via the above cited accessions on the MassIVE proteomics data archive.

Benefit-Sharing Statement

Benefits from this research include establishing a collaboration between researchers in different countries and allowing for coordinated research efforts on species distributed on different continents. All contributing researchers are included as authors and these data are now publicly available to the scientific community, as described above.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.