Molecular Ecology

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MOLECULAR ECOLOGY

ORIGINAL ARTICLE OPEN ACCESS

Susceptibility to the Neonicotinoid Pesticide Imidacloprid Is Linked to Life History Regulation in Honey Bees (*Apis mellifera* L.)

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Received: 29 July 2025 | **Revised:** 29 January 2026 | **Accepted:** 3 March 2026

Keywords: age of first foraging | division of labor | foraging | genetic variation | insecticide | life history | pollinators | social evolution

ABSTRACT

The Western honey bee (*Apis mellifera*) is a globally important pollinator. Its health in natural and managed populations is compromised by numerous factors, including pesticides. Neonicotinoid pesticides are widely used even though they can cause a variety of detrimental effects. Exposure can disrupt the complex life history regulation of worker honey bees and induce precocious foraging and premature death. However, honey bee variation in neonicotinoid susceptibility and underlying mechanisms are poorly understood. We hypothesised that neonicotinoid susceptibility and life history of worker honey bees are innately linked through constitutive patterns of gene expression. We confirmed the relation between worker survival of an acute exposure to the neonicotinoid imidacloprid and the inherent rate of the workers' most fundamental life history transition from in-hive tasks to initiate foraging in a colony-level experiment. To investigate the underlying mechanisms, we also compared the whole-body transcriptomes of young nurses, young foragers, old nurses, and old foragers between bees from colony sources with low versus high imidacloprid susceptibility that were raised in a common single cohort colony environment. These comparisons identified age- and behaviour-related transcriptome changes that are consistent with current models of the life history regulation of honey bee workers but also indicated that age itself has a profound effect on the whole-body transcriptome. Importantly, hundreds of gene expression changes distinguished honey bees of low and high imidacloprid susceptibility, largely in an age- and caste-specific manner with the most significant biological functions ranging from stress responses and protein homeostasis to apoptosis. General age and behavioural effects on the overall transcriptome correlated with each other, but the transcriptome differences according to imidacloprid susceptibility correlated with the age- and behaviour-related gene expression changes in an age-specific manner. However, significant directional overlap in differentially expressed genes from low and high imidacloprid susceptible bees existed among age and behavioural groups. Notably, three genes of known function differed consistently between bees of low and high imidacloprid susceptibility: A chitinase potentially providing structural resilience to pesticides, a potentially detoxifying cytochrome P450, and the vitellogenin receptor with putative functions in life history regulation and stress resistance in worker honey bees. These genes provide prime candidates for studying imidacloprid resistance in honey bees and other insects, as well as providing potential mechanistic links between pesticide effects and life history regulation.

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1 | Introduction

Western honey bees (*Apis mellifera* L.) are eusocial insects inhabiting colonies in which most tasks are performed by non-reproductive, female workers (Cervoni and Hartfelder 2021). Worker bees specialise on certain tasks, largely in an age-dependent manner (Lindauer 1952; Winston 1987; Hamilton et al. 2019; Robinson 1992; Ulgezen et al. 2024). The most pronounced transition in the life history of workers occurs when they shift from in-hive tasks, such as nursing behaviour, to outside foraging, typically during the second or third week of their adult life (Johnson 2010; Robinson 1992; Rueppell, Bachelier, et al. 2007), although profound genetic variation for this complex trait exists (Robinson et al. 1989; Rueppell et al. 2004). This transition requires profound physiological changes and is primarily driven by the mutual regulation of juvenile hormone (JH) and vitellogenin (Vg): The typically young and well-nourished nurses are characterised by low JH and high Vg titers, while the opposite is true for the later foraging stage (Amdam and Omholt 2003). Experimental ablation of the JH-synthesising corpora allata delays the onset of foraging (Sullivan et al. 2000), while injection of JH-analogue induces foraging initiation at a younger age (Robinson 1987). Vg-downregulation also increases JH titers and correspondingly leads to the onset of foraging (Nelson et al. 2007). The combined Vg-JH regulatory axis thus combines nutrient sensing through the central storage and transport protein Vg with a hormonal response to stress in the form of JH (Amdam and Omholt 2003; Ament et al. 2008; Gruntenko and Rauschenbach 2008; Mutti et al. 2011). Other molecular influences on the worker's transition from in-hive to foraging status have been identified, and numerous genes also change expression in response to the transition (Zayed and Robinson 2012) to accommodate the differing demands on in-hive and foraging workers (Fahrbach et al. 1998; Harrison 1986; Toth and Robinson 2005; Withers et al. 1993, 1995).

Honey bee workers face numerous external threats impacting their health and survival such as pathogens and pesticides (Goulson et al. 2015; Hristov et al. 2020; Tsvetkov et al. 2017; Wang et al. 2016). The workers' exposure and corresponding defences depend on their life history stage. For example, in-hive honey bees can be exposed to a greater diversity of viruses than foragers (Kraberger et al. 2019). In parallel, nurses are more resilient against bacterial stressors compared to foragers despite similar overall immune-gene expression (Byhrø et al. 2019; Lourenço et al. 2019). Foraging requires metabolically intensive flight that can generate reactive oxygen species in flight muscles (Bordier et al. 2017; Margotta et al. 2018; Williams et al. 2008). While the expression of many anti-oxidant genes does not differ between young and old workers (Corona et al. 2005), the depletion of vitellogenin reduces the foragers' ability to protect against oxidative stress compared to nurses (Bordier et al. 2017; Seehuus et al. 2006). Correspondingly, foraging but not chronological age may affect cognitive functioning and other performance measures (Behrends et al. 2007; Rueppell, et al. 2007). Typically, foragers are also more exposed to chemical stress from agrochemicals or plant produced phytochemicals (Liao et al. 2017; Tosi et al. 2017; Vannette et al. 2015; Vidkjær et al. 2024). Even though foragers express various cytochrome P450s and other detoxification enzymes constitutively (Mao et al. 2011; Rand et al. 2015; Vannette et al. 2015; Zeng et al. 2024) and can respond via induced increases in stress tolerance metabolites and enzymes (Chen et al. 2021; Colin et al. 2019; Rand et al. 2015; Vannette et al. 2015), their susceptibility to chemical stressors is similar or even higher than that of nurses (Barascou et al. 2022; Bordier et al. 2017).

Lost foragers are replaced by increased recruitment of young bees as foragers, and exceptionally high forager mortality leads to workers transitioning prematurely to foraging (Leoncini et al. 2004; Perry et al. 2015). Such precocious workers display poor spatial memory, low foraging performance, and short life expectancy, which can lead to a positive feedback loop via increased recruitment of even younger workers and ultimately lead to colony failure (Chang et al. 2015; Perry et al. 2015; Ushitani et al. 2016). Precocious foragers are generally identified as foragers that have transitioned to the role prior to 15 days of age (Perry et al. 2015; Ushitani et al. 2016; Vance et al. 2009), and the regulation of their transition may be determined by fewer factors than that at older ages (Jassim et al. 2000; Marco Antonio et al. 2008). Premature transitioning can be caused by single stressors, such as disease (Benaets et al. 2017; Ellis and Rangel 2024; Traniello et al. 2020) and pesticide exposure (Chen et al. 2021; Colin et al. 2019; Morfin et al. 2019; Tokach et al. 2024).

Induction of precocious foraging leads to profound changes in the brain transcriptome compared to the in-hive nursing state and these changes are similar to those experienced by older foragers (Ament et al. 2012; Chen et al. 2021; Whitfield et al. 2003). Comparatively little is known about transcriptome profile changes in other tissues that accompany the nurse-to-forager transition (Ament et al. 2012; Chen et al. 2021; Toma et al. 2000; Whitfield et al. 2003, 2006). Additionally, it is unclear whether precocious foraging induced by stressors (Benaets et al. 2017; Chen et al. 2021; Colin et al. 2019; Ellis and Rangel 2024; Morfin et al. 2019; Tokach et al. 2024; Traniello et al. 2020) is related to the bees' susceptibility to succumb to these stressors (Herman et al. 2026). Here, we hypothesised that inherent stress susceptibility of honey bee workers is linked to their life-history transition from in-hive to foraging tasks. We predicted that worker survival of an acute stress is positively correlated to the age of first foraging and tested this prediction by comparing worker cohorts across colonies with regards to their survival of imidacloprid exposure and their tendency to initiate foraging in a common social environment. The neonicotinoid insecticide imidacloprid targets primarily the nicotinic acetylcholine receptor with behavioural effects (Decourtye et al. 2004), has other systemic consequences (He et al. 2021), and can induce precocious foraging even at low doses (Colin et al. 2019). The mechanisms underlying variability in imidacloprid susceptibility in honey bees are unknown. Therefore, we compared the whole-body transcriptomes of the previously determined most and least susceptible worker cohorts in nurses and foragers at two different ages to test our second, mechanistic prediction that the transcriptome patterns correspond to the predicted phenotypic relation: Transcriptomes of workers from imidacloprid-susceptible colonies were predicted to resemble older and/or forager-like transcriptomes and transcriptomes from less susceptible sources were predicted to be more like the transcriptomes of young workers and/or nurses.

2 | Materials and Methods

2.1 | Honey Bees

Honey bees were obtained from 17 pre-established colonies in Edmonton (Crop Diversification Centre North, trial 1) and 16 in Lethbridge (University of Lethbridge, trial 2). These colonies were established 1 month before the start of the experiment with queens from three different commercial sources. For both trials, the experiment was carried out using identical methods unless otherwise specified. One brood frame with emerging brood was taken from each colony with at least 1000 remaining capped brood cells, and these frames were transported to the University of Alberta, where they were then incubated at 37°C and 60% humidity for 24 h in one incubator (Forma Model 3940 Stability Chamber, Thermo Scientific).

2.2 | Exposure to Imidacloprid

After a 24 h incubation of the colonies' brood frames (one frame per colony, 16 colonies in trial 1 and 17 colonies in trial 2), we brushed off all newly emerged honey bee workers into separate plastic bins (33 × 46 × 12 cm) based on their source colonies with honey dispersed throughout all bins. Workers capable of flying were excluded as they might represent older bees. In order to test their survival when exposed to a pesticide, 45 newly emerged bees were taken from each colony and separated into 3 plastic cups (disposable beverage cups with flat lids and 472 mL volume), with each cup containing 15 honey bees. One of the three cups per colony was used as a solvent control and fed a sucrose solution containing 0.1% DMSO through a 2 mL microcentrifuge tube (Fisherbrand). The two other cups were fed ad lib with a sucrose solution containing 10 ppm of the neonicotinoid imidacloprid. As an additional negative control, 45 bees were randomly selected from a mix of bees from all source colonies. They were split into three cups of 15 bees each to estimate bee mortality when fed with only a plain 30% sucrose solution. Prior to feeding, all tubes were weighed and their mass was recorded to account for differences in feeding.

Honey bee survival was recorded every 3 h, over a period of 48 h. Honey bees were not removed from cups to avoid disturbance, but microcentrifuge tubes containing sucrose were removed once empty and replaced immediately. Total food consumption of each experimental group was determined by weighing tubes before and after providing them to the bees. However, the correlation between feeding amount and survival was not significant in either trial (Data S1) and consequently feeding amounts were ignored in all subsequent analyses. After 48-h, final survival was measured, and honey bees were frozen and discarded. Since there was no mortality in the negative controls and no significant differences among colonies in the solvent control groups, cox proportional hazards analyses in 'R' were performed on the treatment group data without correction to determine and compare imidacloprid survival of these bees based on their source colony.

2.3 | Colony Set-Up

For source colonies with at least 150 additional newly emerged workers ($n = 14$ for trial 1 and $n = 13$ for trial 2), all remaining workers were paint-marked using different colours (Testors acrylic paint) to indicate their source colony. After marking each bee, they were pooled and randomly distributed into three nucleus hives containing two honey frames, two drawn-out empty frames and a young queen. Thus, we did not specifically determine how many workers from each source were introduced into each of the experimental colonies at the start of the experiment but approximately 1500 marked workers were included in each colony overall. Approximately 1000 unmarked, newly-emerged honey bees from unrelated colonies were added until each colony contained approximately 2500 workers. The resulting single cohort colonies were located next to one another at the Laird W McElroy Environmental & Metabolism Research Centre (53°30′10.4″N 113°32′10.3″W) located on the South Campus of the University of Alberta. Workers that died or left the colony prior to the onset of the experiment on day 5 were excluded from the analysis. At the end of the foraging observations (see below), all remaining bees were frozen to determine the exact number of bees from each source colony that was involved in the foraging observations (trial 1: 298.4 ± 100.2 (SD); trial 2: 331.6 ± 151.5). This procedure was repeated for both trials.

2.4 | Precocious Foraging Capture & Observation

Honey bee foraging was recorded from 5 to 15 days of age (post-emergence). Workers that left or were carried from the hive prior to day 5 were considered damaged and day 15 was selected based on prior definitions of precocious foraging. Workers returning from foraging flights were recorded daily from approximately 8 AM to 4 PM or until the onset of orientation flights (to avoid counting orienting workers that had not started foraging) by blocking hive entrances periodically with a wire mesh. Returning foragers accumulated on this mesh and were identified to source colony based on their paint colour and marked with black paint to prevent double-counting individuals. The age of this first foraging activity was recorded as the age of the first foraging for each worker.

Based on the lowest and highest imidacloprid survival in the lab experiments, three source colonies were selected for a group of low (colonies #107, #122, and #127) and a group of high imidacloprid susceptibility (colonies #109, #115, and #137). Seven foragers and seven nurses were captured from each selected source colony on days 7 and 26, sampled in equal proportion from the three replicate nucleus host hives as much as possible (total sample size = 168). Foragers were captured after being identified by their paint colour from outside the entrance of the hive after returning from a foraging flight. Nurses were captured from a brood frame from the centre of the colony after we observed them attending a brood cell. All bees were captured in individual 1.5 mL microcentrifuge tubes and immediately placed on dry ice. Captured bees were then moved to a −80°C freezer until further processing.

2.5 | RNA Extraction

Briefly, we prepared tubes with 10 μL of 2M DTT and 500 μL of lysis buffer, and added metallic beads to each tube. Frozen bees were added to individual 1.5 mL microcentrifuge tubes and homogenised using a FastPrep24-5G Tissue Disruptor (MP Biomedicals). Following homogenisation, RNA was extracted from our samples using the Invitrogen PureLink RNA mini kit following the manufacturer's instructions and stored at −80°C until library preparation.

2.6 | TagSeq Library Preparation

Libraries for poly-A selective Illumina sequencing were made using an in-house library preparation protocol following our established procedures (Kennedy et al. 2021) for 3–4 of the 7 samples of each of the 24 experimental groups (6 colonies, 2 ages, 2 behavioural states) with the best RNA quality. Initially, 96-well index plates were prepared with unique combinations of diluted ILL-BC and TruSeq_UN Oligos barcoding primers. We then performed RNA fragmentation of isolated total RNA by incubating samples at 70°C in a preheated thermocycler for 10 min, followed by first-strand cDNA synthesis, using one poly-T tailed primer. Cleanup of the cDNA was performed with sparQ PureMag magnetic beads (Qiagen). Cleaned cDNA was then amplified using PCR for 18 cycles, which was verified by 2% (w/v) agarose gel electrophoresis of an aliquot, while the main product was again cleaned using magnetic beads. Subsequently, each sample was barcoded by adding 20 μL of 5 ng/μL cDNA from each sample to separate wells in our prepared index plate followed by PCR. The size of PCR products was then confirmed using a 2% (w/v) agarose gel, before pooling 5 μL of each sample into a 1.5 mL tube, followed by another bead cleanup. This cleaned, pooled sample was then sent off for sequencing at the Centre for Applied Genomics at the Hospital for Sick Children in Toronto (Ontario) for one lane of NovaSeq X (PE 150 bp) Illumina sequencing.

2.7 | Bioinformatics

Untrimmed reads were generated using the NovaSeq X onboard DRAGEN software (v.4.1.7) and demultiplexed with bcl2fastq. We additionally quality-tested our sequencing samples using FastQC (Andrews 2010). Four individuals with < 3 million reads were excluded due to low data availability. The resulting 90 individuals consisted of 15 individuals from colony 107 (4 young nurses, 3 young foragers, 4 old nurses, 4 old foragers), 15 individuals from colony 109 (4 young nurses, 4 young foragers, 4 old nurses, 3 old foragers), 16 from colony 115 (4 for each subgroup), 15 from colony 122 (4 young nurses, 3 young foragers, 4 old nurses, 4 old foragers), 13 from colony 127 (2 young nurses, 3 young foragers, 4 old nurses, 4 old foragers), and 16 from colony 137 (4 for each subgroup). Following quality control analysis, we uploaded our sequencing data onto the Narval Compute Canada cluster (narval.computecanada.ca). We removed adapters and PCR duplicates using Eli Meyer's TagSeq Perl scripts (TagTrimmer.pl. and RemovePCRDups.pl.), available at https://github.com/Eli-Meyer/TagSeq. The cleaned reads were then aligned with the latest version of the indexed honey bee reference genome GCF_003254395.2 (Wallberg et al. 2019) using HiSat2 (Kim et al. 2019). Alignments were sorted into BAM files using Samtools (Li et al. 2009). Lastly, we used HT-Seq to count the assigned reads of represented genes to create a count table for each of our samples (Anders et al. 2015).

We created annotation files to specify for each sample its source colony, behavioural role (nurse vs. forager), age (7 vs. 26 days), adult colony environment, and susceptibility to imidacloprid. Annotation file and counts table file from HiSat2 were combined in R using the *BiocManager* (v.1.30.26), and *Deseq2* (v. 1.49.3) R packages (Love et al. 2014; Morgan and Ramos 2025). We filtered out low gene counts, normalised expression values and identified differentially expressed in genes in various pairwise comparisons with DESeq analyses using a 0.05 Benjamini-Hochberg FDR cut-off. We performed Enrichment analysis with the topGO package (v. 2.61.1) to test which gene ontologies were significantly over- or under-represented among up- or down-regulated genes (Alexa and Rahnenfuhrer 2025). Other secondary analyses were either performed directly in R or Excel after exporting normalised log2-fold changes and corresponding *p*-values of each gene.

2.8 | Statistical Analysis

Unless otherwise specified, data analysis was conducted in Microsoft Excel and R Studio. Survival data and foraging data were counted for each observed honey bee in order to perform a cox proportional hazards analysis and create survival curves. Survival data was analysed in R Studio using the 'survival' package on mortality data collected in the field, which was compiled in Excel. In order to create survival curves, we used the 'ggplot2' R package with our pesticide survival and foraging datasets. A log-rank statistical test was performed using the 'ggplot2' and 'survminer' packages. Chi-square tests of independence of the proportion of foragers after 15 days and their source colony were performed with the online calculator www.standarddeviationcalculator.io/chi-square-calculator.

Pearson's correlation and *p*-values were created manually using Microsoft Excel. Briefly, we correlated the average age of each colony to the average survival when exposed to imidacloprid. Correlation values and significance estimates were created manually through Excel for each of our two replicate experiments ($n = 13$ for trial 1 and $n = 12$ in trial 2 after removal of one outlier in each trial). The subsequent scatterplot was also rendered in Microsoft Excel by combining the data from the two datasets into one plot and adding significance and correlation values.

To assess overall transcriptome patterns across all samples with sufficient coverage ($N = 91$), a principal component analysis (PCA) with the R package *ggplot* (v. 3.5.2) was performed based on all genes with sufficient data ($N = 9935$) and sample grouping was visualised in a PCA plot. Additionally, a heatmap of 2261 significantly expressed genes (p-value < 0.05) was generated with the *pheatmap* package (v. 1.0.13) in R using normalised counts (Kolde 2025). After one sample proved to be an outlier in

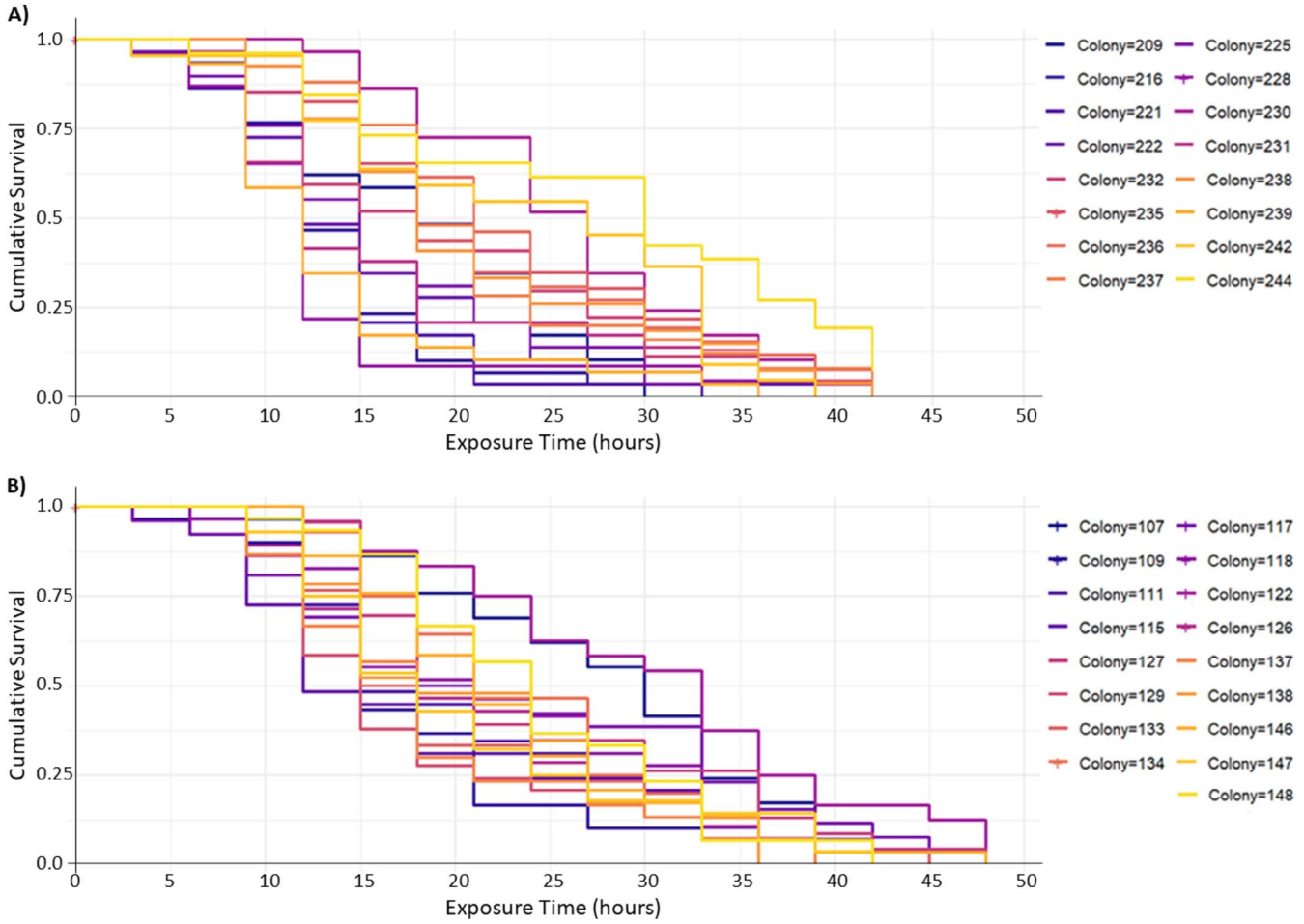

FIGURE 1 | Variation of imidacloprid survival among honey bee colonies. Cumulative survival of honey bee workers from different colonies while feeding ad libitum on a sucrose solution with 10 ppm imidacloprid. Two independent experiments with colonies from different apiary locations (A: Edmonton, B: Lethbridge) included 33 colonies overall. Each colony's mortality curve represents 30 workers distributed over two cages kept in a temperature and humidity controlled incubator for 48 h. Results of the corresponding solvent controls (one cage of 15 workers per colony) are not shown because negligible mortality was observed (Data S1). Log-rank test-based *p*-values indicate significant differences in survival among colonies from the Edmonton (A) and Lethbridge (B).

both analyses, this individual was excluded from all subsequent analyses and the PCA and heatmap regenerated. *pheatmap* was also used to cluster all remaining samples to visualise how clustering aligned with age (7 days vs. 26 days), task group (nurse vs. forager), imidacloprid susceptibility (low vs. high), source colony (six source colonies), and colony environment (across the three replicate nucs).

To assess similarity between transcriptome comparisons between different experimental groups, we correlated log2FoldChange of all genes in pairwise comparisons between two comparisons, generating a Pearson correlation matrix with the *Hmisc* (v. 5.2-3) and *corrplot* (v. 0.95) R packages (Harrell 2024; Wei and Simko 2024). As one example, the relative expression of all genes in old individuals relative to young individuals was correlated to the gene expression of individuals that came from imidacloprid susceptible colonies relative to that of individuals from less-susceptible colonies. Furthermore, we analysed directional overlap (Lawhorn et al. 2018) among the sets of significantly DEGs for each pairwise DeSeq2 comparison of the bees from colonies of low and high imidacloprid susceptibility (7-day-old nurses, 7-day-old foragers, 26-day-old nurses, and 26-day-old foragers) and visualised the results in an upset plot using the *UpSetR* package (Conway et al. 2017). In order to test for significance of overlap, we used a custom Microsoft Excel script (Data S2) to randomly permute the data and generate 1000 bootstrapped overlap lists to compare the actual numbers of overlapping genes with this empirically determined random distribution.

3 | Results

3.1 | Imidacloprid Survival and Onset of Foraging Covary Among Colonies

In order to investigate differences in susceptibility to imidacloprid among colonies, we fed 1-day-old worker honey bees from distinct source colonies with a sucrose solution containing 10 ppm imidacloprid and monitored survival in a common incubator environment. In both experiments, worker survival was significantly different among colonies (Log-rank tests: Edmonton—$N = 480$, $p < 0.001$, Lethbridge—$N = 510$, $p = 0.004$, Figure 1, Data S1).

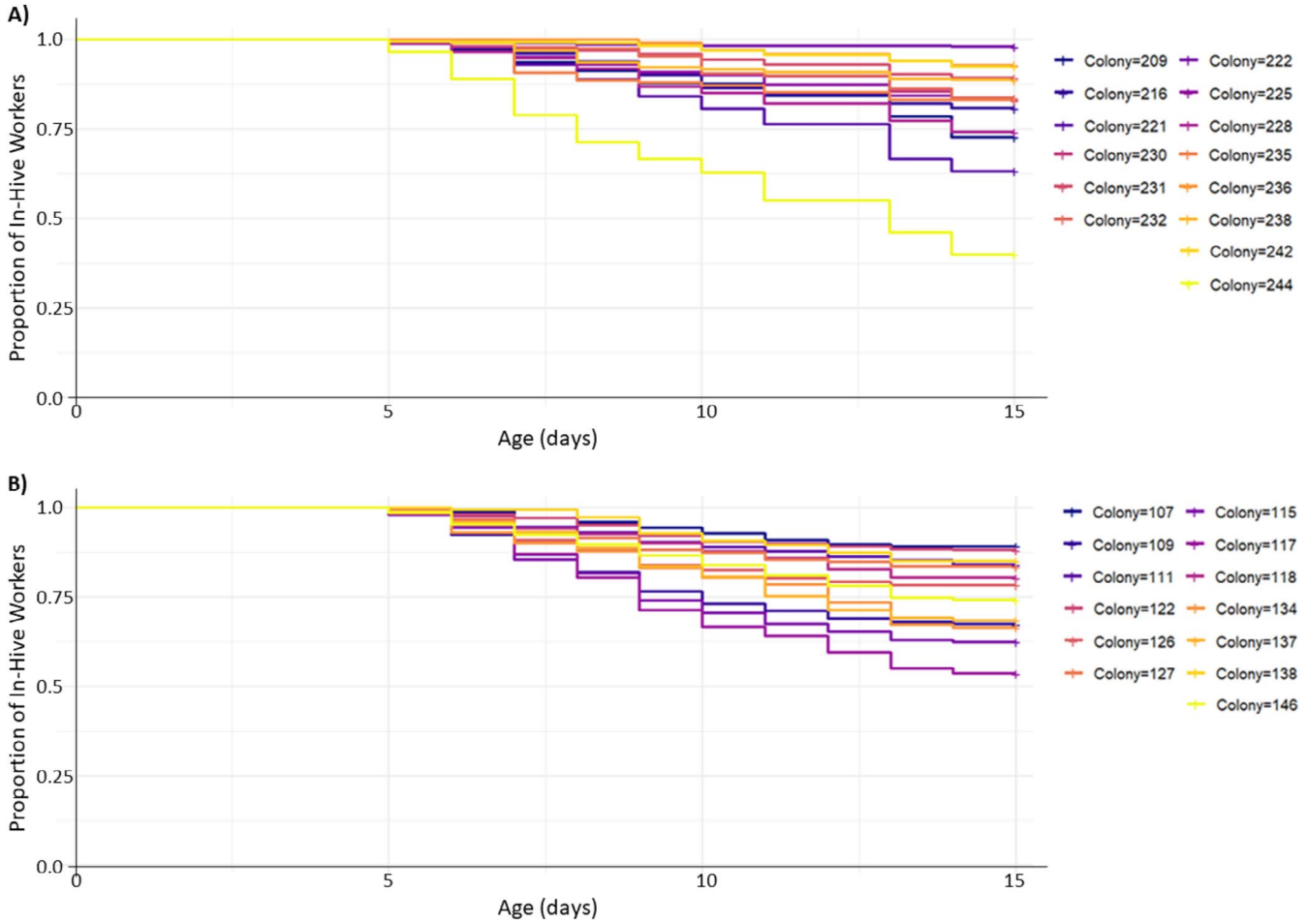

FIGURE 2 | Variation of the onset of worker foraging among honey bee colonies. Newly emerged workers from 14 colonies of the Edmonton (A) and 13 colonies of the Lethbridge (B) apiary were colour-marked and introduced into single-cohort colonies to record their onset of foraging from five to 15 days of age. Log-rank test indicated significant differences among colony sources in the probability of transition to foraging ($p < 0.001$ for both experiments).

To test for concomitant differences in the age of first foraging among these colonies, remaining newly emerged workers were colour-marked, introduced to single cohort colonies, and monitored for foraging between the age of 5 and 15 days. For each experiment, data from the three replicate single cohort colonies were combined to increase statistical power. The age of first foraging differed significantly among workers from the different source colonies in both experiments (Log-rank tests: Edmonton—$N = 4629$, $p < 0.001$, Lethbridge—$N = 3750$, $p < 0.001$, Figure 2). The proportion of workers from each source colony that was not observed foraging before 15 days of age also varied significantly in the Edmonton ($\chi^2 = 503.2$, df = 13, $p < 0.001$) and Lethbridge ($\chi^2 = 215.1$, df = 12, $p < 0.001$) experiments, ranging from 2.0% to 60.1% and 10.8% to 46.4%, respectively (Data S3).

To investigate whether imidacloprid survival relates to the onset of foraging behaviour, we correlated the source colonies' average imidacloprid survival estimates to their estimated age of first foraging and the proportion of workers that had initiated foraging after 15 days. While the age of first foraging trended positively with imidacloprid survival, the correlation was not significant (Pearson's $R = 0.21$, $p = 0.507$, $n = 13$ for Edmonton and $R = 0.50$, $p = 0.102$, $n = 12$ for Lethbridge). The proportion of workers that were observed initiating foraging was significantly negatively correlated to imidacloprid survival (Pearson's $R = -0.46$, $p = 0.003$, $n = 13$ for Edmonton and $R = -0.76$, $p = 0.004$, $n = 12$ for Lethbridge; Figure 3).

3.2 | Transcriptome Differences Between Workers From Colonies With Low and High Imidacloprid Susceptibility Varies Across Life History

Workers from the three least and three most imidacloprid-susceptible colonies in the Lethbridge trial (colonies #107, #122, and #127 vs. #109, #115, and #137, respectively) were sampled from two age groups (7 vs. 26 days) and two behavioural groups (nurses vs. foragers) to compare their whole-body transcriptomes. Based on 90 individuals with at least 10.5M reads, expression profiles of 9935 genes were analysed. Samples most prominently grouped according to age, followed by behavioural status and imidacloprid resistance, while clustering by colony within these categories was not apparent (Figure 4), and workers from the three susceptible and three non-susceptible source colonies were combined in the respective treatment groups. Likewise, potential effects of the host colony environment were ignored based on our balanced sampling design to focus the

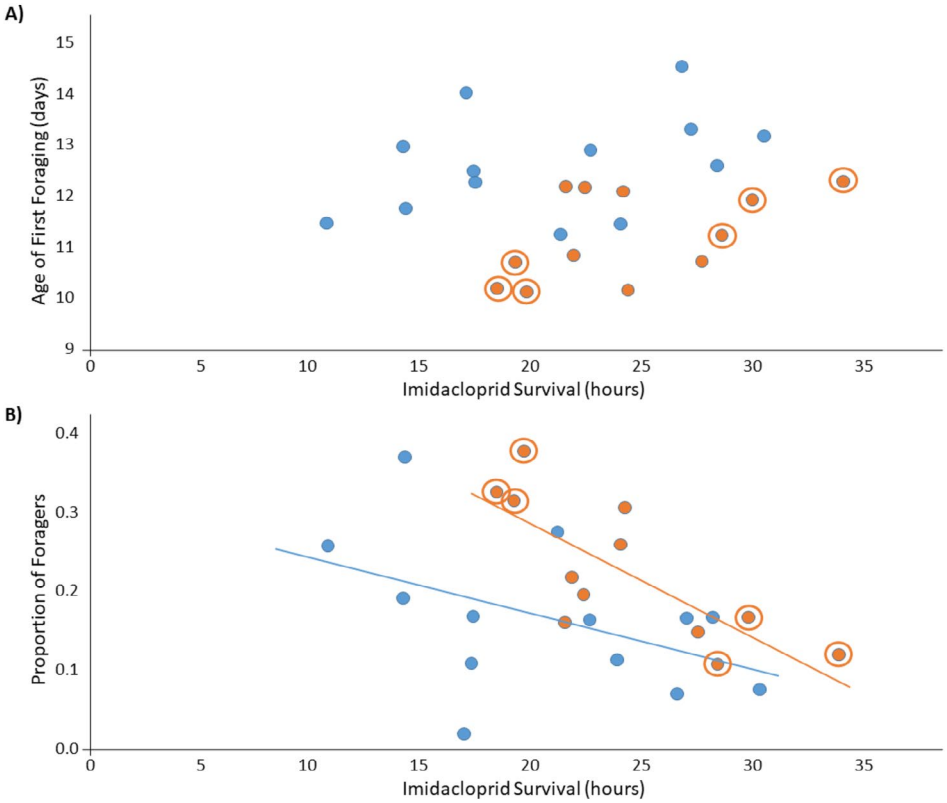

FIGURE 3 | Relation between imidacloprid survival and foraging in honey bee workers. The average age at which individuals from a particular colony source start initiate foraging in a common hive environment shows a positive, but non-significant correlation (Edmonton trial in orange: $R = 0.21$, $p = 0.507$, $n = 13$; Lethbridge trial in blue: $R = 0.50$, $p = 0.102$, $n = 12$) with the average survival duration of workers from the same colony sources to survive an acute imidacloprid exposure in the laboratory (A). Correspondingly, the proportion of individuals from the same colony sources starting to forage at all during the observation window of 5 to 15 days exhibited significantly negative correlation with the imidacloprid survival of workers across colonies in both trials (Edmonton in orange: $R = -0.46$, $p = 0.003$, $n = 13$; Lethbridge in blue: $R = -0.76$, $p = 0.004$, $n = 12$). Solid lines indicate lines of best fit (B).

analyses on our main influences: age, behavioural status, and imidacloprid susceptibility (Figure 4).

Overall, we identified significant expression differences in 2797 genes based on age (1447 up- and 1350 down-regulated in 26-day vs. 7-day old workers), 3015 genes based on behavioural status (1609 up- and 1406 down-regulated in foragers compared to nurses), and 76 genes based on imidacloprid susceptibility (35 up- and 41 down-regulated in workers from less susceptible compared to more susceptible colonies). We also compared specifically the transcriptome differences according to age in both behavioural groups and according to behaviour in both age groups with similar results (Data S4). Most central to our hypothesis, we compared the transcriptomes between the low and high imidacloprid-susceptible groupings within each behavioural age group: in young nurses 576 genes were differentially expressed (310 up- and 266 down-regulated genes in the low- compared to the high-susceptibility group). We identified 519 differentially expressed genes in young foragers (249 up and 270 down), 526 in old nurses (245 up and 281 down), and 727 in old foragers (340 up and 387 down). While interesting candidate genes were identified by each pairwise comparison (Data S4), gene ontology analyses found numerous biological functions that were enriched in significant differentially expressed genes (Figure 5; Data S5 and S6). Particularly notable biological functions were neuronal processes that were up-regulated in the overall comparisons of foragers to nurses and 26- to 7-day olds, and the up-regulated response to oxidative stress in 26-day olds compared to 7-day olds. Both gene ontology terms were also enriched in more specific comparisons of age and task groups. Bees of low- compared to high imidacloprid susceptibility were distinguished by numerous processes, most notably upregulation of protein stabilisation and heat response in young nurses (Figure 5), apoptosis in young foragers, protein repair in old nurses, and TOR signalling and stress responses in old foragers.

Significant directional overlap between these lists of differentially expressed genes was observed (Figure 6, Data S7). Pairwise overlap was strongest between old nurses and old foragers with 58 genes (16 up, 28 down [both $p < 0.001$], and 14 inconsistent). The overlap between young and old foragers (41 genes: 17 up and 15 down, 9 inconsistent) was also significant in both directions ($p < 0.001$). Between young and old nurses, the overlap of 12 upregulated genes was significant ($p < 0.001$), while their overlap in terms of 10 downregulated genes was not ($p = 0.092$). Similarly, 15 shared upregulated genes between young nurses and young foragers were significant ($p < 0.001$) but not 11 downregulated genes ($p = 0.452$). Likewise, overlap between young foragers and old nurses was significant for downregulated genes (15, $p < 0.001$) but not for upregulated genes (13, $p = 0.091$), while overlap between young nurses and old foragers was significant for downregulated genes (20, $p < 0.001$)

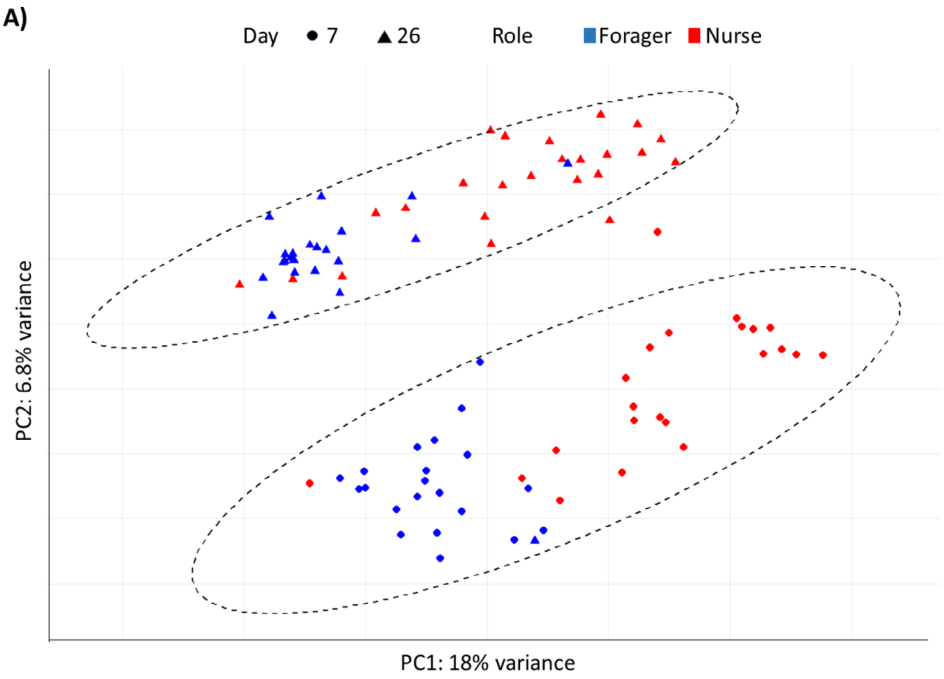

FIGURE 4 | Transcriptome profiles of worker honey bees distinguish primarily age and task groups. In a principal component analysis based on expression of all genes (A), individuals of different ages are primarily separated along PC1 (explaining 18% of total variance), while task-based expression differences accounted for a lesser proportion of variation along PC1 but also relate to PC2 (explaining 6% of total variance). Hierarchical clustering of all samples based on differentially expressed genes (B) similarly indicated the strongest division by age, followed by behavioural status (task). Within the four general clusters of 7-day old nurses, 7-day old foragers, 26-day old nurses, and 26-day old foragers, some separation was also apparent based on whether workers came from colonies with low or high imidacloprid susceptibility (Susceptibility), but source colony (Colony) or colony environment (Replicate) appeared to have little impact. The colour code of sample is indicated in the figure legend and colours of the heatmap indicate relative gene expression levels.

but not for upregulated genes (11, $p = 0.189$). Furthermore, the three-way overlap for young foragers, old nurses, and old foragers showed significant downregulated genes (2, $p < 0.001$), but the same was not seen for upregulated genes (2, $p = 0.092$). The remaining three- and the four-way comparisons of differentially expressed genes showed significant directional overlap in all cases (all $p < 0.001$). 'Cytochrome P450 9E2' was consistently upregulated in bees with low versus high imidacloprid susceptibility across all four demographic groups and four genes (uncharacterised LOC102655953 and LOC727649, 'acidic mammalian chitinase', and 'putative vitellogenin receptor yl') were consistently downregulated.

To assess overall similarities between transcriptome profile changes with regards to our three factors (age, behaviour, and imidacloprid susceptibility), we correlated gene expression differences from different comparisons (Figure 7). Thus, correlation coefficients indicate the similarity of gene expression differences due to two different splits of the dataset. Gene expression changes due to older age were positively correlated with behavioural maturation from nursing to foraging when all samples were combined ($R = 0.28$, $n = 9518$ $p < 0.001$). Overall behavioural gene expression changes exhibit high correlations with behavioural changes at 7 and 26 days of age, and overall age-related changes also show similarly high correlations with age-related changes in nurses and foragers (all $R > 0.8$, $n > 9254$, $p < 0.001$). Age-related changes were similar in nurses and foragers ($R = 0.51$, $n = 9206$, $p < 0.001$) and behaviour-related changes were similar in both age groups ($R = 0.55$, $n = 9179$, $p < 0.001$). Behavioural differences in 7 day-old worker were positively correlated to age-related changes in nurses but not foragers, while the opposite was true for behavioural differences in 26 day-old workers (Figure 7). Low but significant positive correlations were found between the results of all comparisons of workers from sources of low and high imidacloprid susceptibility ($R = 0.08$ to 0.14, $n = 8846$ to 9040, $p < 0.001$), but comparisons of the imidacloprid-related differences to age- and behaviour-related differences (highlighted rectangle in Figure 7) revealed important differences. While the differences of low versus high susceptibility in young nurses and foragers mostly showed negative correlations with gene expression changes due to increased age or behavioural maturation (9 of 12 correlation significantly negative), the opposite was true for older nurses and foragers (9 of 12 correlations significantly positive, Figure 7). Overall gene expression differences between workers from low and high imidacloprid susceptible sources were negatively correlated with expression changes with behavioural ontogeny (overall: $R = -0.07$, $n = 9453$, $p < 0.001$; in 7 day-olds: $R = -0.03$, $n = 9189$, $p = 0.006$; in 26 day-olds: $R = -0.09$, $n = 9247$, $p < 0.001$) and increasing age (overall: $R = -0.04$, $n = 9453$, $p < 0.001$; not significant in nurses: $R = -0.01$, $n = 9189$, $p = 0.432$; in foragers: $R = -0.07$, $n = 9267$, $p < 0.001$).

4 | Discussion

Our study demonstrates a novel link between life history and stress susceptibility in honey bees. Specifically, we found that

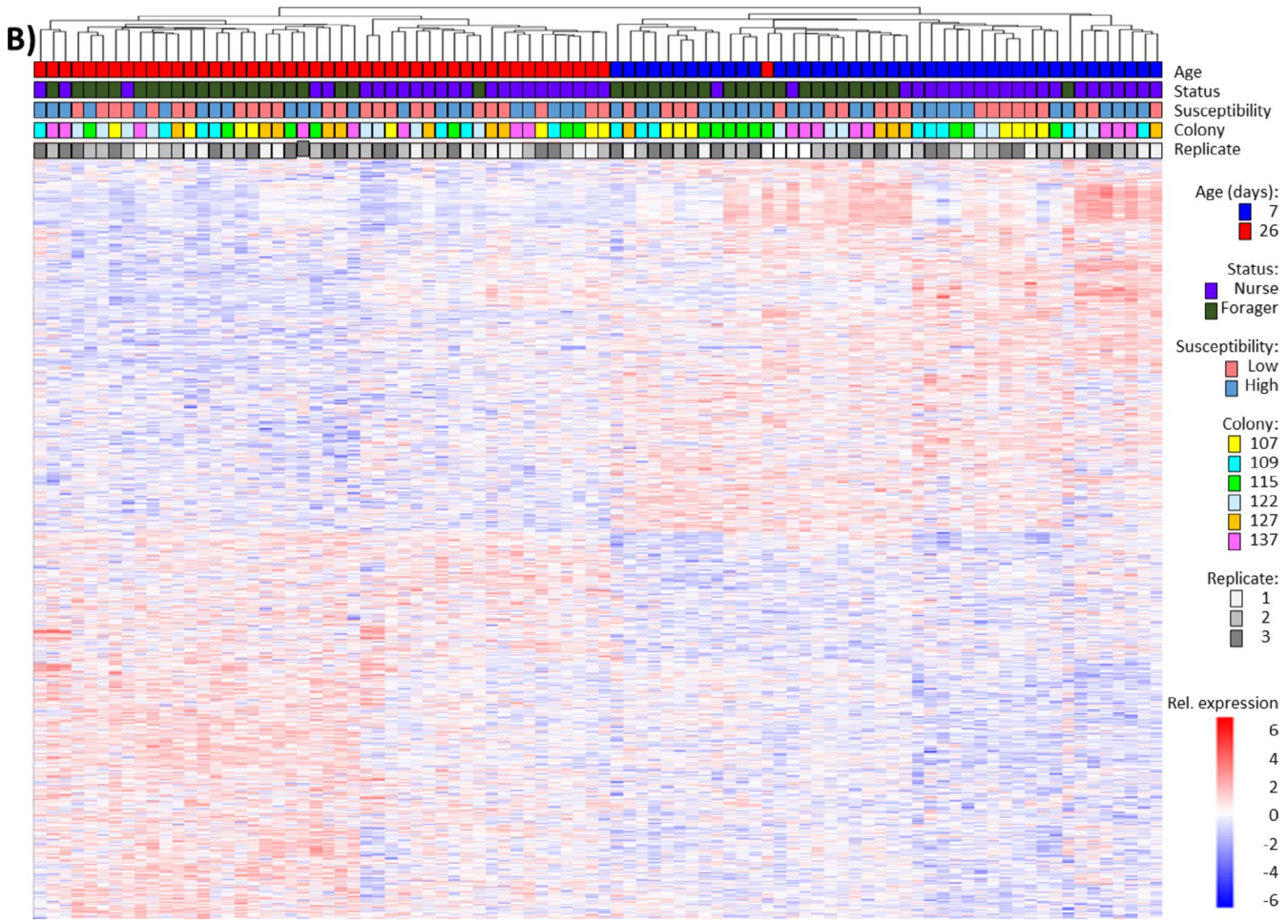

FIGURE 4 | (Continued)

workers that exhibit higher imidacloprid-induced mortality are more likely to transition from in-hive tasks to foraging in the first 2 weeks of life. We tested this connection by comparing populations of workers from different colony sources because it is impossible to independently measure these two variables from the same individuals: Exposure to chemical stressors, such as the neonicotinoid imidacloprid, influences the probability of honey bee workers to transition to foraging (Chen et al. 2021; Colin et al. 2019; Shi et al. 2020), and some stressors induce precocious foraging (Colin et al. 2019). A mortality assay also precludes subsequent behavioural studies for the obvious reason that dead bees cannot initiate foraging. Conversely, honey bee foragers are more susceptible to chemical stressors than in-hive workers (Barascou et al. 2022), and therefore a mortality assay after behavioural analysis would also yield confounded results. However, the comparison of the group phenotypes 'average imidacloprid survival' with 'proportion of foragers by age 15 days' allowed us to establish this link. However, the number of colonies was modest and such links depend on the standing genetic variation. Consequently, we cannot be certain that the identified relation is universal in honey bees.

Although our hypothesis was motivated by the induction of foraging through stress (Benaets et al. 2017; Chen et al. 2021; Colin et al. 2019; Ellis and Rangel 2024; Morfin et al. 2019; Tokach et al. 2024; Traniello et al. 2020), our demonstration that innate imidacloprid susceptibility of newly emerged workers correlates with the intrinsic propensity of non-exposed workers from the same cohort to initiate foraging at a young age provides evidence for a more fundamental link between stress susceptibility and life history regulation in honey bees. Despite our common garden experiment with three replicate colonies in two experimental trials, we cannot distinguish whether the observed relation is due to genetic covariance or some influence of the source colony environment during the development of our experimental workers. Both explanations are possible: Genetic covariation is the hallmark of the pollen hoarding syndrome, which links genetic variation in nutritional and reproductive physiology to various behavioural phenotypes, including the age of first foraging (Nelson et al. 2007; Page et al. 2012; Rueppell 2014). Nutritional differences during development are equally plausible because poor nutrition has been linked to precocious foraging (Toth et al. 2005) and lower pesticide resistance (Tosi et al. 2017). Ultimately, it might not be important whether genetic or environmental causes lead to the physiological and transcriptomic differences responsible for this correlation (Robinson 2004).

Differences among source colonies were most pronounced with regards to the proportion of foragers by day 15 (between the colony with the lowest and highest values 1742.1% and 247.4% difference existed in the Edmonton and Lethbridge trials, respectively), followed by imidacloprid survival (up to 181.9% and

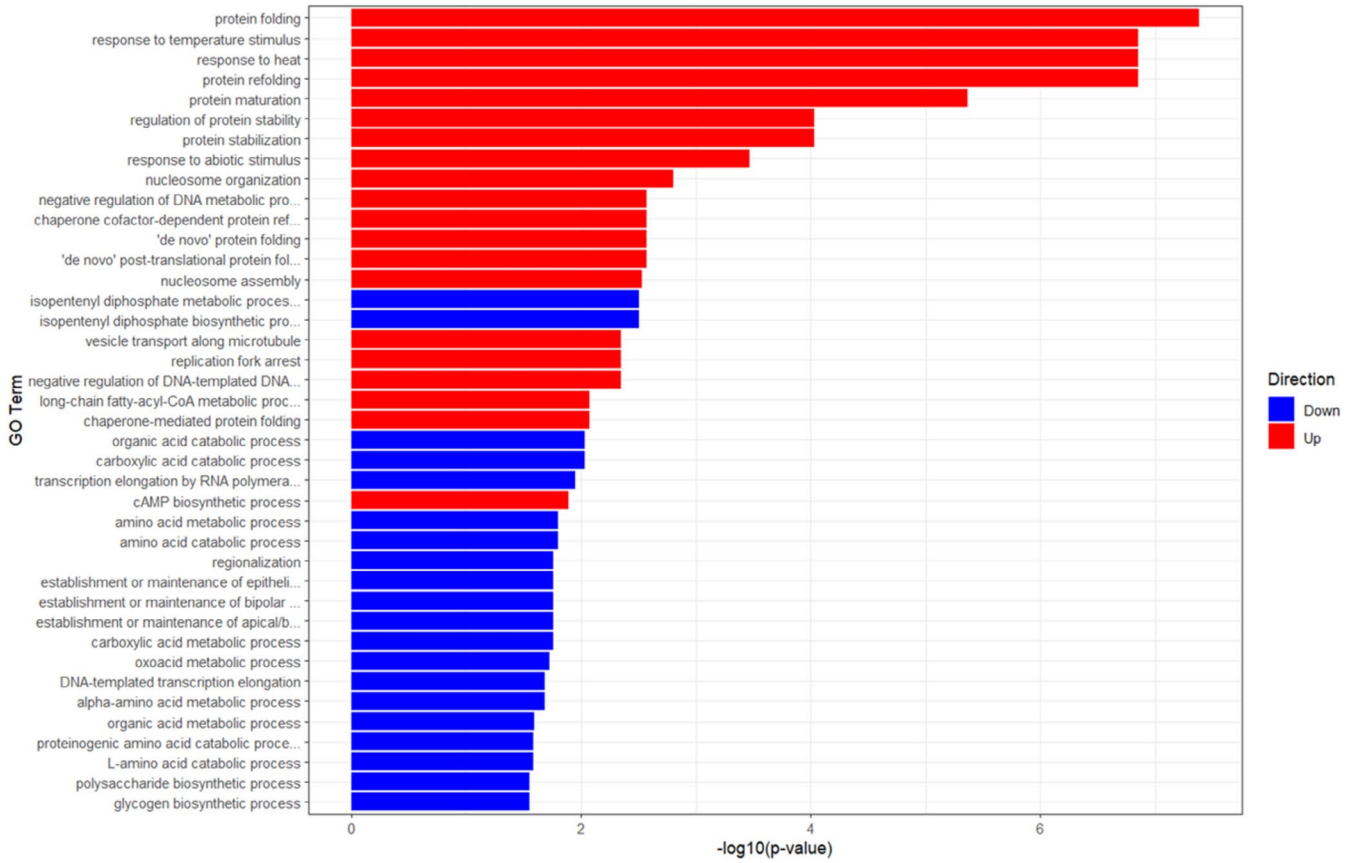

FIGURE 5 | Enriched gene ontology terms in differentially expressed genes in young nurse bees from colonies that show low imidacloprid susceptibility compared to their equivalents from colonies with higher susceptibility. Top 20 enriched GO terms for significantly up-regulated (red) and down-regulated (blue) genes in 7-day old nurses from source colonies that survived an acute imidacloprid exposure better than 7-day old nurses (sampled from the same foster colonies) from source colonies that were more susceptible to imidacloprid. GO terms on the left were ordered by significance of enrichment, indicated by the length of the bars.

84.3% differences among colony values), and the age of first foraging (colonies varied only up to a maximum of 29.1% and 21.5%). While the proportion of foragers accounted for all individuals in our colony samples, our measure of the age of first foraging compared only a select group of precocious foragers that initiated foraging before the 15th day of age. Variation in precocious foraging ages might be smaller than in the proportion of foragers because the foraging transition is less complex at precocious ages (Marco Antonio et al. 2008). Our data also show that imidacloprid susceptibility is less correlated to the age of first foraging than to the proportion of foragers by day 15, which may be due to less precision in its measurement but could also reflect biological differences: Even though stress is commonly seen triggering precocious foraging, inherent stress susceptibility may be more related to the normal regulation of the transition from in-hive tasks to foraging.

Our transcriptome comparison between nurses and foragers at 7 and 26 days of age support the notion that the behavioural transition at young ages shares some features but is distinct from that same transition at older ages: Important events shared between the age group comparisons were the downregulation of six MRJPs, hexamerin 110 and 70a, vhdl, and vitellogenin, several of which may be involved in the actual regulation of the behavioural transition (Fang et al. 2023; Martins et al. 2023; Nelson et al. 2007). Shared upregulated genes were less common and top genes were one uncharacterised protein, the odorant receptor 1, and digestive enzymes (e.g., a lipase and the alpha-glucosidase Hbg3). However, the expression changes from nursing to foraging in 7 day-olds only explained about 30% of the corresponding changes in 26 day-olds and vice versa, as approximated by squaring the correlation coefficient between the two transcriptomic comparisons (Figure 7). Moreover, the correlation of the changes associated with behaviour of 7 day-olds was positively correlated with the age-related ages in nurses but not in foragers, while the opposite was true for the behavioural changes at 26 days. Finally, age was more important for grouping overall worker transcriptome profiles than behavioural status (Figure 4).

Our general result of age outweighing behavioural status for the overall whole-body transcriptome is in stark contrast to previous studies of the brain transcriptome that report brain gene expression to be primarily associated with behaviour instead of age (Chen et al. 2021; Hamilton et al. 2019; Traniello et al. 2020; Whitfield et al. 2003, 2006). Usually, age and behavioural status are correlated in worker honey bees (Robinson 1992), but the differences between brain and body indicate that gene expression in the body may be influenced more strongly by chronological age than gene expression in the brain. This interpretation may also explain the uncoupling of functional senescence in cognitive functions and the age-dependent mortality increase

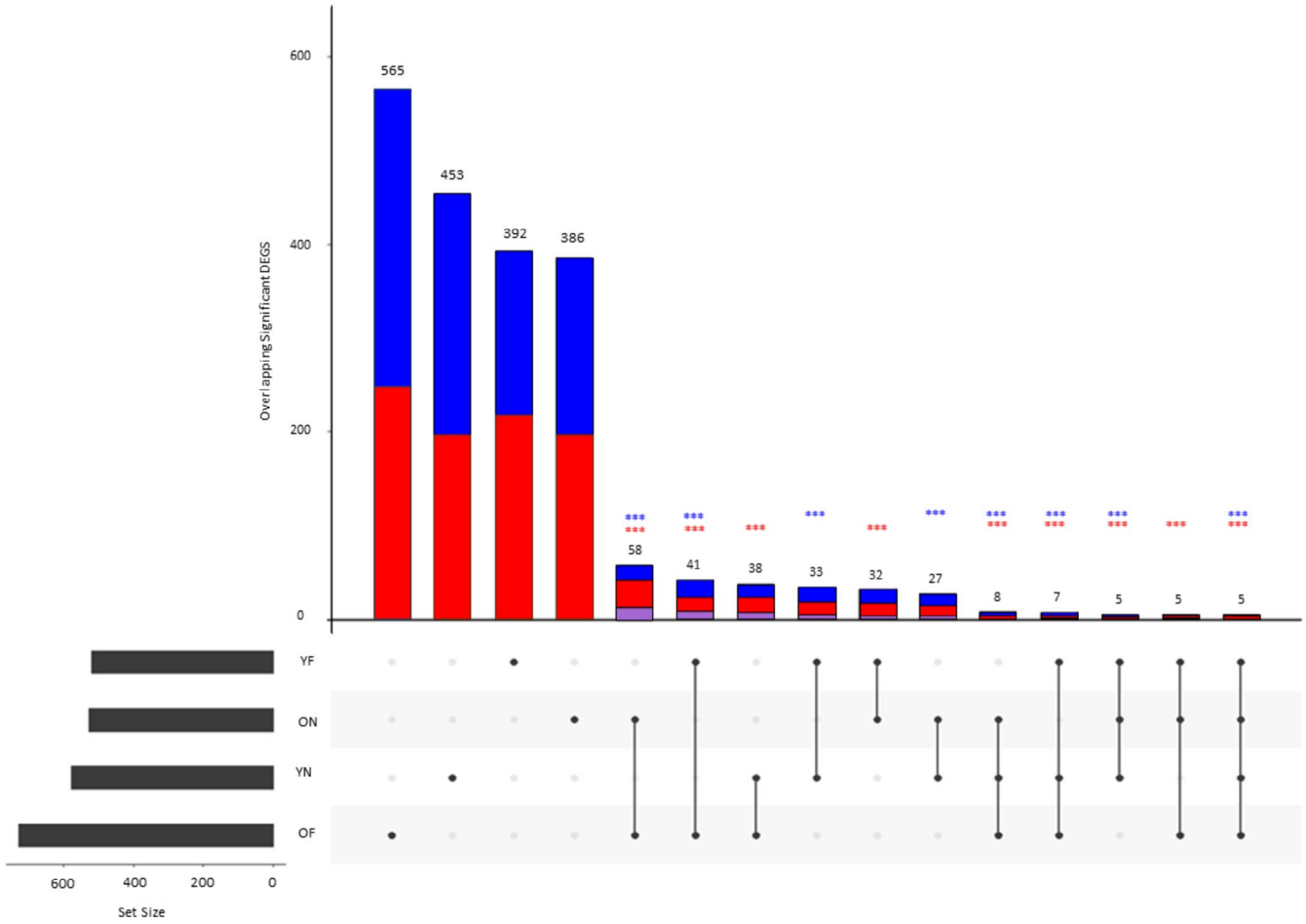

FIGURE 6 | Overlap in constitutive gene expression differences between imidacloprid-susceptible and non-susceptible honey bees across different worker demographic groups. As indicated in grey horizontal bars ('Set Size'), transcriptome comparisons between workers from sources that displayed low versus high imidacloprid susceptibility revealed 392 differentially expressed genes (DEGs) in young foragers (YF), 526 in old nurses (ON), 576 in young nurses (YN), and 727 in old foragers (OF). The majority of these DEGs were unique to each comparison (4 left-most vertical bars) with relatively similar numbers of up- (in green) and down-regulated (in red) genes. Overlap analyses among these up- and down-regulated DEG lists as denoted in the intersection matrix below the bar graphs revealed significant directional overlap in most cases, indicated by *** ($p < 0.001$) in the respective colour. Purple segments of the overlap bars depict shared DEGs with inconsistent patterns of up- and downregulation between bees of low and high imidacloprid susceptibility.

(Rueppell, Christine, et al. 2007). Nevertheless, our findings also support the notion that age-related and behaviour-related genes have connections: Changes from 7-day to 26 day olds regardless of behaviour are correlated to general changes from nurse to forager status (Figure 7), supporting the notion that physiological changes that usually occur with time increasingly predispose workers to transition to foraging tasks (Ament et al. 2011).

We focused on the imidacloprid susceptibility of the worker source colonies as our third factor for evaluating the gene expression patterns in our samples, while ignoring the host colony environment and individual source colony identity based on the overall clustering of our transcriptome profiles (Figure 4). We ignored the latter factors due to their small effect in this particular experiment but cannot rule out that they play important roles in other circumstances, as the natural colony demography certainly would affect the transition to foraging behaviour (Leoncini et al. 2004; Perry et al. 2015). Overall, the factor 'imidacloprid susceptibility' led to the identification of only 76 DEGs compared to almost 3000 DEGs in the overall comparison of age groups or behavioural status groups. However, 7.3 to 10.2 times more DEGs in relation to imidacloprid susceptibility were identified when gene expression was evaluated in each of the four behavioural/age groups separately, thus reducing residual variation within the low and high imidacloprid groupings. These hundreds of DEGs based on imidacloprid susceptibility of the source colonies of our samples were unanticipated, suggesting that numerous genetic differences contribute to the observed difference in imidacloprid susceptibility. Our pooling of three colonies in each susceptibility type should reduce other spurious differences that could arise when comparing single colony sources. Approximately equal numbers of genes were up- and downregulated in workers of low versus high imidacloprid susceptibility. Gene ontology enrichment analyses of these DEG lists indicated that low imidacloprid susceptibility in young nurses was linked to upregulated protein stabilisation and downregulation of several metabolic functions (Figure 5). In other groups, low susceptibility was associated with an upregulation of apoptotic processes and lipid metabolism (in young foragers), upregulation of protein repair, purine biosynthesis,

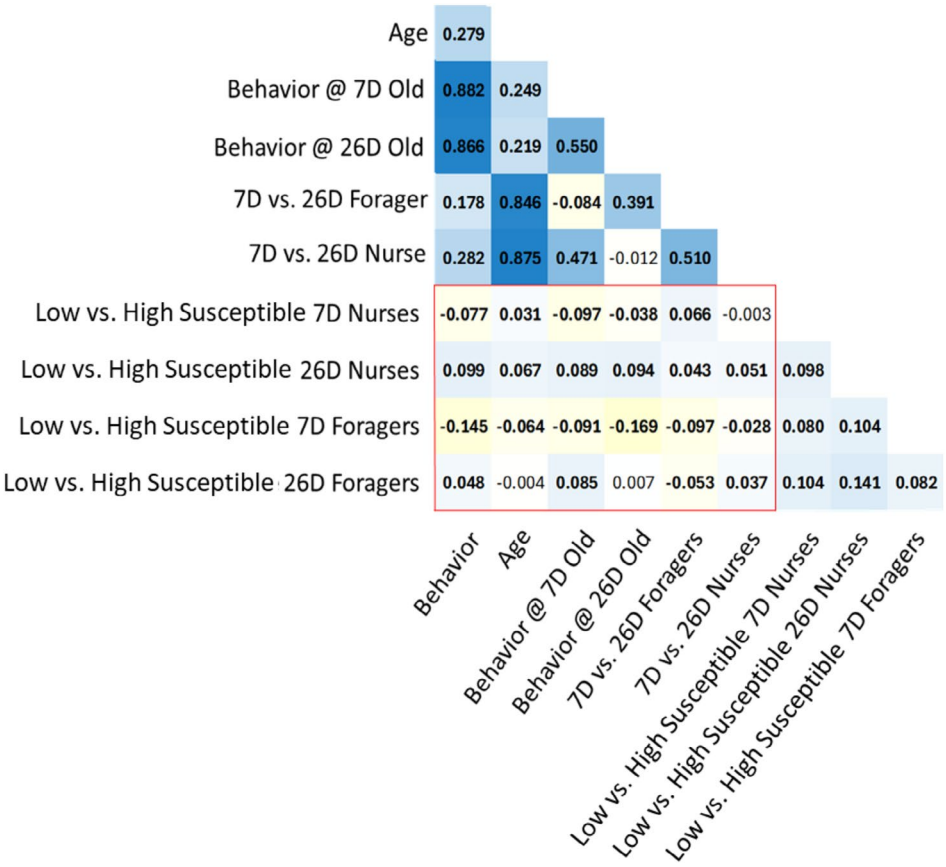

FIGURE 7 | Transcriptome differences based on imidacloprid susceptibility are related to age- and behaviour-related gene expression changes. The transcriptome profiling of workers from source colonies with low and high imidacloprid susceptibility across two behavioural states (nurses and foragers) and two ages (7 and 26 day old) allowed for systemic comparisons of these three factors. The transcriptome dataset or subsets of it were subdivided in different ways and the expression differences between these divisions were correlated to differences of another comparison across all genes. In accordance to previous results, gene expression changes were largely related to behavioural maturation from nurse to forager state (upper five rows). The relation between constitutive gene expression differences due to imidacloprid susceptibility and age-based and behavioural gene expression variation differed between young and old individuals (red-outlined square): In young nurses and foragers, more susceptible bees were distinguished from less susceptible ones by gene expression differences that were correlated to differences that overall distinguished young from old workers and nurses from foragers. The opposite was true in old nurses and foragers, although the correlations in both cases were not very strong or universal. Correlations among the differences between bees of low versus high imidacloprid susceptibility were consistent yet also not very strong across age and behavioural groups (right-most three columns). Pairwise Pearson's coefficients are shown against a colour-coded background and bold-faced when significant (based on expression comparisons of between 8846 and 9518 genes with sufficient data for pairwise comparisons).

and quinone metabolism, and with a downregulation of chromosome organisation (in old nurses), and diverse changes in cellular transport and cytoskeletal organisation, and an upregulation of TOR signalling and responses to radiation and metal ions (in old foragers). Some of these processes are directly related to imidacloprid effects on honey bees, such as apoptosis (Carneiro et al. 2022). These processes also involve several other plausible mechanisms that could either resist or mitigate imidacloprid-induced stress, but they are fundamentally different from canonical pesticide resistance mechanisms (Bass et al. 2015). Despite characterisation of transcriptome responses to imidacloprid (Wu et al. 2017) and considerable comparative work within and between species, imidacloprid susceptibility is poorly understood (Nagloo et al. 2024). Our findings provide an initial list of potential mechanisms that need to be further investigated.

Among the differentially expressed genes (DEGs) according to imidacloprid susceptibility, the majority was unique to a particular behavioural/age group with most DEGs in old foragers, followed by young nurses, old nurses, and young foragers. These unique differences could indicate that genetic differences in pesticide susceptibility among colonies are age-specific. However, no direct evidence to support this hypothesis exists to our knowledge. Directional overlap of DEGs was significantly higher than expected by chance for most pairwise and 3-way comparisons among groups, confirming some commonalities among the sampled groups despite their broad differences described above. Five DEGs were shared among all four comparisons, which included the two uncharacterised proteins (LOC727649 and LOC102655953) and an acidic mammalian chitinase homologue. The consistent association of a high expression of this chitinase with high imidacloprid susceptibility could be due to its potential role in influencing the permeability of the peritrophic membrane or other chitinous barriers of imidacloprid entry into the body (Oliveira et al. 2019). The fourth consistent DEG was the vitellogenin receptor gene (yl), which was consistently higher expressed in bees of high imidacloprid

susceptibility. In honey bee workers, this receptor enables cellular uptake of vitellogenin into the hypopharyngeal glands (Dohanik et al. 2024), which could remove circulating vitellogenin levels in the haemolymph and thus lead to a decreased potential for vitellogenin to act as a systemic protectant against reactive oxygen damage and other stressors (Havukainen et al. 2013; Seehuus et al. 2006). The fifth shared DEG was a cytochrome P450 9E2, which was upregulated in less susceptible workers. This finding is consistent with results from planthoppers where overexpression of this gene is associated with resistance (Bass et al. 2011) and may be due to the generally detoxifying functions of cytochrome P450s (Ye et al. 2022). These five genes are linked with imidacloprid susceptibility across the lifespan of worker honey bees and thus top candidates for follow-up studies to understand the genetic basis of imidacloprid susceptibility in honey bees.

Corresponding to the small but significant overlap in DEGs between the different comparisons of low and high susceptibility workers, the pairwise correlations between the entire transcriptome comparisons (Figure 7) were also weak but significant, confirming a mix of similarities and idiosyncrasies in the distinction between low and high imidacloprid susceptibility among young nurses, young foragers, old nurses, and old foragers. The differences were also correlated to the general age- and behaviour-dependent transcriptome changes, albeit in a distinctly age-dependent manner: Gene expression changes associated with increasing age and the nurse to forager transition correlated predominantly positively with the gene expression differences between low and high imidacloprid susceptible 26-day old nurses and foragers. In contrast, mostly negative correlations were found between the age- and behaviour-related changes and the gene expression patterns of 7-day old bees (nurses and foragers) of low relative to high imidacloprid susceptibility. These findings suggest that there is not one optimal physiological state for surviving imidacloprid across all life history stages. Instead, workers with a gene expression profile that was more typical for their respective age group were less susceptible to imidacloprid: Although this effect is weak, as indicated by the small correlation coefficients, more nurse-like and 'typical 7-day old' transcriptome patterns are associated with better imidacloprid survival in 7-day olds, while more forager-like and 'typical 26-day old' transcriptome patterns are associated with better survival in 26-day olds. Thus, the steady states of 'young nurse' and 'old forager' may be more stress-resistant than intermediate or transitory states that are less stabilised (Page and Amdam 2007), although this idea needs to be tested more directly.

Instead of adding to the numerous studies to document negative impacts of imidacloprid on honey bees (Cresswell 2011), we have opted to link colony-level differences in imidacloprid survival to the timing and transcriptome profiles of fundamental life-history states of worker honey bees. This allowed us to deduce biological processes and candidate genes that may be responsible for the considerable variation in imidacloprid survival. From a practical point of view, these mechanisms could be used to mitigate imidacloprid-induced mortality or precocious foraging (Colin et al. 2019). Our results indicate that selective breeding for a delayed life history transition to foraging in honey bee workers could simultaneously increase their resistance to neonicotinoid stress. Since precocious foraging leads to low foraging performance and premature death (Chang et al. 2015; Perry et al. 2015; Ushitani et al. 2016), such measures could benefit honey bee health in multiple ways. However, we have also demonstrated that differences between imidacloprid survival and death differ substantially among young nurses, young foragers, old nurses, and old foragers; thus, defence and mitigation strategies may have to be life stage specific.

Author Contributions

Gursimran Toor: conceptualisation, methodology, validation, formal analysis, investigation, data curation, visualisation, writing – original draft. **Robert X. Lu:** formal analysis. **Olav Rueppell:** conceptualisation, methodology, formal analysis, resources, writing – review and editing, supervision, project administration, funding acquisition.

Acknowledgements

We appreciate the support and collegiality of all members of the Social Insect lab group at the University of Alberta. We thank the Alberta Tech Transfer team and particularly Nicole McCormick for maintaining experimental colonies and their help in collecting emerging brood frames from their colonies at the Crop Diversification Center North in Edmonton. Likewise, we thank Shelley Hoover and her research group at the University of Lethbridge for maintaining experimental colonies and their help in gathering emerging brood frames from their colonies for our study.

Funding

This work was supported by the Natural Sciences and Engineering Research Council of Canada (Grant RGPIN-2022-03629), the Results Driven Agricultural Research and Alberta Beekeepers Commission. Infrastructure supporting this research was funded by the Canada Foundation for Innovation's John R. Evans Leaders Fund (JELF), with matching support from the Government of Alberta, Technology and Innovation, through the Research Capacity Program - Small Equipment Grant (RCP-SEG).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw sequences as well as the count table output from Deseq2 can be found online in the Geo dataase (GSE300082) at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE300082.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** Imidacloprid survival and consumption raw data, separated according to our two trials (with colonies from Edmonton and colonies from Lethbridge, respectively). Each row in each data matrix represents an individual worker honey bee. **Data S2:** Visual basics for applications code (implemented in Microsoft Excel) for resampling tests to empirically determine statistical significance of our gene overlap analyses. **Data S3:** Raw data of the foraging age measurements. Each row represents data from an individual worker, with the day it was first observed foraging, its colony origin, and the single cohort nucleus hive (=replicate) indicated. Workers that were not recorded as foraging in the observation period were censored on day 15. **Data S4:** Lists of gene expression differences (fold changes) and associated *p*-values for each transcriptomic contrast performed in this study. In each list, transcripts (primarily idenfied by LOC IDs) are sorted by ascending *p*-values and significance is indicated in red. **Data S5:** Graphs depicting the 20 gene ontology terms that were most significantly overrepresented in the up- (red) and downregulated (blue) genes

for each transcriptomic contrast performed. **Data S6:** All gene ontology terms that were significantly enriched in each of the transcriptomic contrasts that were analysed in this study. TopGO output indicating *p*-values, actual and expected number of DEGs for each GO term based on the total number of genes annotated for this term, and direction of DEGs (up or down). **Data S7:** Lists of differentially expressed genes included in the overlap analysis illustrated in the upset plot (Figure 6). Accordingly, upregulated transcripts are indicated in green, downregulated transcripts in red, and transcripts that exhibited opposing differences between low and high imidacloprid susceptibility comparisons for different age/behaviour groups are shaded in purple.