Variability in honey bee cuticular hydrocarbon composition can be understood as a function of gene expression

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Abstract

Most terrestrial insects have a layer of cuticular hydrocarbons (CHCs) that protects them from desiccation and mediates chemical communication. CHC composition is regulated by the expression of genes coding for enzymes in the biosynthetic pathway of hydrocarbons. The diversity and expression of these enzymes determine the abundance and richness of compounds in the CHC profile of an insect. For instance, elongases and desaturases are enzymes that respectively lengthen the hydrocarbon chain and introduce double bonds into it. CHC profiles of honey bees (*Apis mellifera*) vary among castes, social roles, and subspecies. However, little is known about the genetic basis for this variation. Here, we examined the expression of specific elongase- and desaturase-encoding genes and correlated gene expression with CHC composition in nurse and forager bees of two highly divergent *A. mellifera* subspecies: *A. m. carnica* (lineage C) and *A. m. iberiensis* (lineage M). We provide evidence of the difference in specificity among specific desaturases and elongases and demonstrate how such differences shape the CHC profiles of *A. mellifera* workers during their transition from nursing to foraging. Our results expand the current understanding of the genetic basis of CHC biosynthesis and highlight the benefit of further investigating different subspecies of *A. mellifera* to better understand the mechanisms underlying the CHC diversity of honey bees.

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Introduction

Cuticular hydrocarbons (CHCs) protect insects from water loss and mediate inter- and intraspecific communication (Blomquist et al., 1987; Gibbs, 1998; Howard and Blomquist, 2005). The composition of the CHC layer determines its biological function (Blomquist et al., 1987; Howard and Blomquist, 2005). For example, its ability to act as a desiccation barrier depends on the aggregation of its constituent hydrocarbons (Gibbs, 1995; Gibbs, 2002; Menzel et al., 2019). The n-alkanes aggregate denser than methyl-branched or unsaturated hydrocarbons, due to stronger van der Waals forces (Gibbs, 1998; Gibbs and Pomonis, 1995). Furthermore, the aggregation of hydrocarbons increases with their chain length (Gibbs and Pomonis, 1995). The denser the aggregation of the hydrocarbons, the more waterproof the CHC layer is (Gibbs, 1995; Gibbs, 2002; Howard and Blomquist, 2005). However, this also incresases the viscosity of the CHC layer, which limits hydrocarbon diffusion and interindividual CHC exchanges, thereby constraining the function of CHCs as communication cues (Gibbs, 1995; Gibbs, 2002; Gibbs and Pomonis, 1995; Menzel et al., 2019).

CHCs are primarily synthesized by oenocytes, specialized secretory cells associated with the fat bodies of insects (Makki et al., 2014; Moris et al., 2023; Schal et al., 1998; Wigglesworth, 1933). The CHC biosynthetic pathway is closely related to fatty acid metabolism. It is initiated with the synthesis of fatty acyl-coenzyme A (acyl-CoA), from the binding of malonyl-CoA units to acetyl-CoA (Blomquist and Bagnères, 2010; Howard and Blomquist, 2005). Along this pathway, several enzymes modify the fatty acyl-CoA to ultimately produce the different CHCs (Chung and Carroll, 2015; Holze et al., 2021). Some of these enzymes contribute to the elongation of the hydrocarbon chain (elongases) (Cinti et al., 1992; Denic and Weissman, 2007), while others introduce unsaturations (double bonds) into the hydrocarbon chain (desaturases) (Dallerac et al., 2000; Takahashi et al., 2001). The abundance and richness of CHC compounds on the cuticle of an insect are determined by the diversity and expression of the enzymes participating in this biosynthetic pathway (Gu et al., 1997; Juárez et al., 1992; Qiu et al., 2012; Reed et al., 1995). In this sense, the diversity and expression of desaturase-encoding genes influence the diversity and abundance of unsaturated hydrocarbons (e.g., alkenes and alkadienes), while that of elongase-encoding

genes influences the diversity and abundance of compounds with specific chain lengths.

In honey bees (*Apis mellifera*), the CHC profiles of the workers differ according to their age, task-performance, and subspecies (Kather et al., 2011; Rodríguez-León et al., 2024; Vernier et al., 2019). The CHC profiles of nurse bees are characterized by a higher abundance of unsaturated hydrocarbons and longer chain lengths compared to forager bees (Kather et al., 2011; Rodríguez-León et al., 2024). These two groups of honey bee workers are on opposite ends of the age-dependent division of labor in *A. mellifera*. The youngest workers are typically the ones caring for the brood and queen within the hive (nurse bees), while the oldest workers leave the colony to gather pollen and nectar (forager bees) (Huang and Robinson, 1996). Therefore, as the expression of specific elongase- and desaturase-encoding genes varies with age (Vernier et al., 2019), the differences in the CHC composition between honey bee nurses and foragers are assumed to respond to a developmental regulatory process. Moreover, the difference in the CHC profiles of nurse bees and foragers has been shown to be present among *A. mellifera* subspecies (Rodríguez-León et al., 2024).

A. mellifera subspecies have divergent evolutionary histories, leading to both genetic and phenotypic differences (Dogantzis et al., 2021; Garnery et al., 1992; Ruttner, 1988; Ruttner et al., 1978). In consequence, different subspecies have evolved distinct CHC profiles, exhibiting both quantitative and qualitative differences in their composition (Rodríguez-León et al., 2024).

This remarkable variability of CHC profiles makes *A. mellifera* an excellent model organism for studying the molecular mechanisms underlying CHC biosynthesis. A recent study elucidated a clear role of genes encoding for specific elongases and desaturases in the CHC biosynthesis of honey bee workers (Moris et al., 2023). Knocking down these genes resulted in compositional changes in the CHC profile, aligning with the expected function of the respective enzyme type. However, the influence of the expression of specific desaturase- and elongase-encoding genes on CHC profile variability remains unknown.

Here, we aim to fill this knowledge gap by assessing the expression of specific elongase- and desaturase-encoding genes in nurse and forager beesof two highly divergent subspecies, *A. m. carnica* and *A. m. iberiensis*, and correlating it with their CHC profile composition. We hypothesize that the shift in CHC composition during the transition from nursing to foraging is driven by changes in the expression of specific CHC biosynthesis-related genes that occur during the task transition and are preserved among subspecies. In turn, we expect that the CHC composition differences between worker bees of different subspecies correlate with differences in the expression of specific CHC biosynthesis-related genes.

Materials and methods

We performed a common garden experiment, keeping queen-right colonies of *Apis mellifera carnica* and *Apis mellifera iberiensis* in the departmental apiary of the University of Würzburg (Germany). Although these subspecies are both native to Europe, they belong to the most evolutionarily divergent lineages of *A. mellifera* (Wallberg et al., 2014). *A. m. carnica* belongs to the Western European C-lineage, whereas *A. m. iberiensis* belongs to the Eastern European M-lineage (Ruttner, 1988). *A. m. carnica* is native to the Central and Southeastern parts of Europe, but it has been introduced worldwide, and it is now the dominant subspecies in Germany (Maul and Hähnle, 1994). *A. m. iberiensis* is native to the Iberian Peninsula, where it experienced historical secondary contact with the African A lineage (Chávez-Galarza et al., 2015; De la Rúa et al., 2009; Han et al., 2012; Ruttner, 1988).

The colonies were assembled on June 2, 2021, using artificial swarms obtained from *A. m. carnica* colonies and mated queens from both subspecies. *A. m. carnica* queens were obtained from local German beekeepers, and *A. m. iberiensis* queens were obtained from local beekeepers in Portugal.

Honey bee workers were collected between the 22nd and 23rd of September 2021, between 9 a.m. and noon. Ten nurse bees and ten forager bees were collected from two colonies per subspecies, for a total of 80 worker bees. The 40 nurse bees were identified as worker bees that poked their heads into a brood cell for at least 10 seconds. The 40 forager bees were identified as returning worker bees landing at the entrance of the hive with conspicuous pollen loads in their corbiculae. The workers were collected in individual vials and transported to the lab on ice, where they were killed by storing them at -80 °C. The collected bees were preserved at -80 °C until the extraction of their CHCs.

CHC composition analysis

The bees were defrosted and immersed in hexane for only two minutes to extract their CHCs and avoid RNA degradation. Immediately after the extraction with hexane, the bees were dissected to remove their guts and separate the abdomen from the rest of the body. The dissected abdomens were preserved separately in 963 µl of RNA-later, stored at 4 °C for 48 hours, and then at -20 °C until their RNA was extracted. The CHC extracts were stored at -20 °C.

The CHC extracts were analyzed via gas chromatography/mass spectrometry (GC/MS) by injecting 1µl of each extract into an Agilent 7890A Series GC System coupled to an Agilent 5975C Mass Selective Detector (Agilent Technologies, Waldbronn, Germany) operating in electron impact ionization mode at 70 eV and

a source temperature of 230 °C. The split/splitless injector was operated in splitless mode for 1 minute at 300 °C. Separation of compounds was performed on a J&W DB-5 fused silica capillary column (30 m × 0.25 mm ID, df = 0.25 µm, J&W, Folsom, California, USA) with a temperature program starting at 60 °C and increasing by 5 °C per minute to 300 °C, which was held for 10 minutes. Helium served as a carrier gas with a constant flow of 1 ml per minute.

The chromatograms were analyzed using the data analysis software package "MassHunter Workstation Software - Qualitative Analysis Navigator" (version B.08.00; Agilent Technologies, Inc. 2016). The areas of the peaks were determined by integration using the "Agile2" parameter-less integrator, with the peak filters set to 0% of the area of the largest peak. The integration results were aligned regarding the retention time (RT) of the peaks for all the samples of each colony and task-performance (e.g., nurse or forager bees) group. The differences in performance of the total ion counts were corrected based on n-alkane analytical standard solutions (04070-1ML and 04071-5ML, Sigma-Aldrich). The aligned group data sets were merged by the Kováts retention index (RI) and compound identity of the peaks. Compounds that represented less than 0.01% of the total ion count of a sample, and compounds detected in less than 50% of the samples within a group, were excluded from the analysis. Only the compounds identified as alkanes, alkenes, alkadienes, or methyl-branched alkanes were considered in the analysis. The different hydrocarbons in the extracts were identified based on their diagnostic ions and RIs. Double bond positions of monounsaturated hydrocarbons (alkenes) were identified by dimethyl disulfide derivatization (Carlson et al., 1989). The abundances of the compounds were quantified for each sample as the proportion (%) that the area of the corresponding peaks represented from the sum of the area of all the peaks included in the analysis.

Additionally, we calculated the relative abundance of both the alkenes and alkadienes, as well as the mean chain length, in the CHC profile of each bee and compared them between tasks and between subspecies. The relative abundances of the alkenes and alkadienes (mono- and diunsaturated hydrocarbons, respectively) were calculated as the sum of the relative abundances of all the individual alkenes or alkadienes found in the CHC extract of a bee. The mean chain length corresponds to the weighted mean of the chain length of all the compounds found in the CHC profile of each bee, using the relative abundance of the compounds as their weights.

Gene expression analysis

The GenUpTM total RNA kit (Biotechrabbit, Henningsdorf, Germany) was used to extract the total RNA from each of the dissected abdomens, following the manufacturer's instructions. Along the RNA extraction procedure, a DNase I digestion step was performed by adding 30 µl DNase mix containing 30 U RNase-free DNase I (Lucigen Corporation, Middleton, USA) together with the corresponding buffer, after the binding of the RNA to the Mini Filter RNA. The total RNA concentration was determined photometrically, and the extracted RNA was diluted in DEPC-H_2O to a concentration of 100 ng/µl and stored at -80 °C until the synthesis of the cDNA. A total of 400 ng of RNA was used for cDNA synthesis with the cDNA synthesis kit 331475S/L (Biozym, Hessisch Oldendorf, Germany). The resulting cDNA solution was diluted up to a total volume of 180 µl with DEPC-H_2O, and stored at -20 °C.

The expression of two desaturase-encoding genes (*Des1* and *Des2*; Table S1) and two elongase-encoding genes (*Elo1* and *Elo2*; Table S1) was determined via quantitative Polymerase Chain Reaction (qPCR). These four genes have been shown to affect CHC biosynthesis (Moris et al., 2023). Individual cDNAs were analyzed in triplicate for each gene using the SYBR Green BlueMix qPCR kit (Biozym, Hessisch Oldendorf, Germany) and a Rotor-Gene Q (Qiagen, Hilden, Germany). The following cycling conditions were set up for every qPCR run: 2 minutes at 95 °C, 40 cycles with 5 seconds at 95 °C, and 30 seconds at 60 °C. A melting curve was recorded by increasing the temperature from 60 °C to 95 °C in steps of 1 °C every 5 seconds. Four qPCR runs were performed per gene, including the reference genes (*Rpl10* and *Rpl19*; Table S1), for a total of 16 qPCR runs. Each qPCR run was performed with five samples per task and subspecies group. All the samples of both subspecies within a qPCR run corresponded to the same hive, and the same combination of samples per hive per run was used for the four genes. The expression of the four genes was quantified relative to the reference genes, following the qBase algorithm (Hellemans et al., 2007).

Data analysis

We compared the relative abundance of alkenes and alkadienes, as well as the mean chain length, in the CHC profile of the bees between tasks (nurse bees vs. forager bees) and subspecies (*A. m. carnica* vs. *A. m. iberiensis*). We fitted quantile regression models for the 50% quantile, considering both the task and subspecies as independent variables (predictors). The interaction between task and subspecies was only considered for the model cases where it was significant (i.e., the relative abundance of the alkenes).

To evaluate the difference in gene expression of the four assessed genes between tasks and subspecies,

we performed bootstrapped gamma generalized linear model (GLM), with a logarithmic link function, over 10,000 resamples. Inference was made with the bootstrapped marginal means, model coefficients (standardized effect sizes), and their 95% confidence intervals, which were calculated via the percentile intervals method. Due to the use of the logarithmic link function, the model coefficients were transformed by exponentiation. Thus, they represent the proportional difference in gene expression to the reference group (task: nurse bees; subspecies: *A. m. carnica*).

We evaluated the correlation of the relative expression of both desaturase-encoding genes to the relative abundance of both alkenes and alkadienes, as well as to the relative abundance of every unsaturated hydrocarbon in the CHC profiles of the bees. Similarly, for the elongase-encoding genes, we evaluated the correlation of their relative expression to the mean chain length of the CHCs of the bees, as well as to the relative abundance of every compound in the CHC profiles of the bees. All correlations were calculated using Spearman's correlation index. In addition, we performed a bi-dimensional Non-Metric Multidimensional Scaling (NMDS) using the Bray-Curtis dissimilarity index to assess the dissimilarity between samples, regarding the composition of their CHC profiles. Then, for each of the four assessed genes, we plotted on the NMDS the compounds with a positive (> 0.4) or negative (< -0.4) correlation towards the expression of the corresponding gene.

The data analysis was performed using R v4.4.0 (R Core Team, 2024) and RStudio IDE v2024.4.1.748 (Posit Team, 2024). Data wrangling and plotting operations were done using the packages tidyverse v2.0.0 (Wickham et al., 2019), ggtext v0.1.2 (Wilke and Wiernik, 2022), gghalves v0.1.4 (Tiedemann, 2022), ragg v1.3.2 (Pedersen and Shemanarev, 2023), and patchwork v1.2.0 (Pedersen, 2023). The processing of the integrated chromatograms' data (i.e., RT wise alignment, RI calculation, abundance correction, peak filtering, and calculation of the relative abundance of compounds) was done using the packages analyzeGC v0.2.1 (Rodríguez-Leon, 2023a) and GCalignR v1.0.6 (Ottensmann et al., 2018). The relative expression of the genes of interest was calculated using the package easyqpcr2 v0.1.0 (Rodríguez-Leon, 2023b). The bootstrapped GLMs and quantile regression models were fit with the packages tidymodels v1.2.0 (Kuhn and Wickham, 2020) and quantreg v5.98 (Koenker, 2023) respectively. The marginal means were estimated using the package emmeans v1.10.2 (Lenth, 2023). The NMDS was performed using the package vegan v2.6.6.1 (Oksanen et al., 2022). Spearman's correlation indexes and their 95% confidence intervals were calculated using the package confintr v1.0.2 (Mayer, 2023).

Results

CHC composition differs between bees performing different tasks and between subspecies

A. m. carnica nurse bees exhibited a higher abundance of both alkenes and alkadienes, as well as longer chain length hydrocarbons than their forager counterparts (Figures 1 and S1). In *A. m. iberiensis*, the nurse bees elicited a higher abundance of alkadienes and longer chain length hydrocarbons than the forager bees.

Subspecies did not affect the abundance of alkadienes (Figures 1B and S1B). However, the CHC profile of *A. m. carnica* contained a higher abundance of alkenes and longer chain length hydrocarbons than that of *A. m. iberiensis*, in both the nurse and forager bees (Figures 1C and S1C).

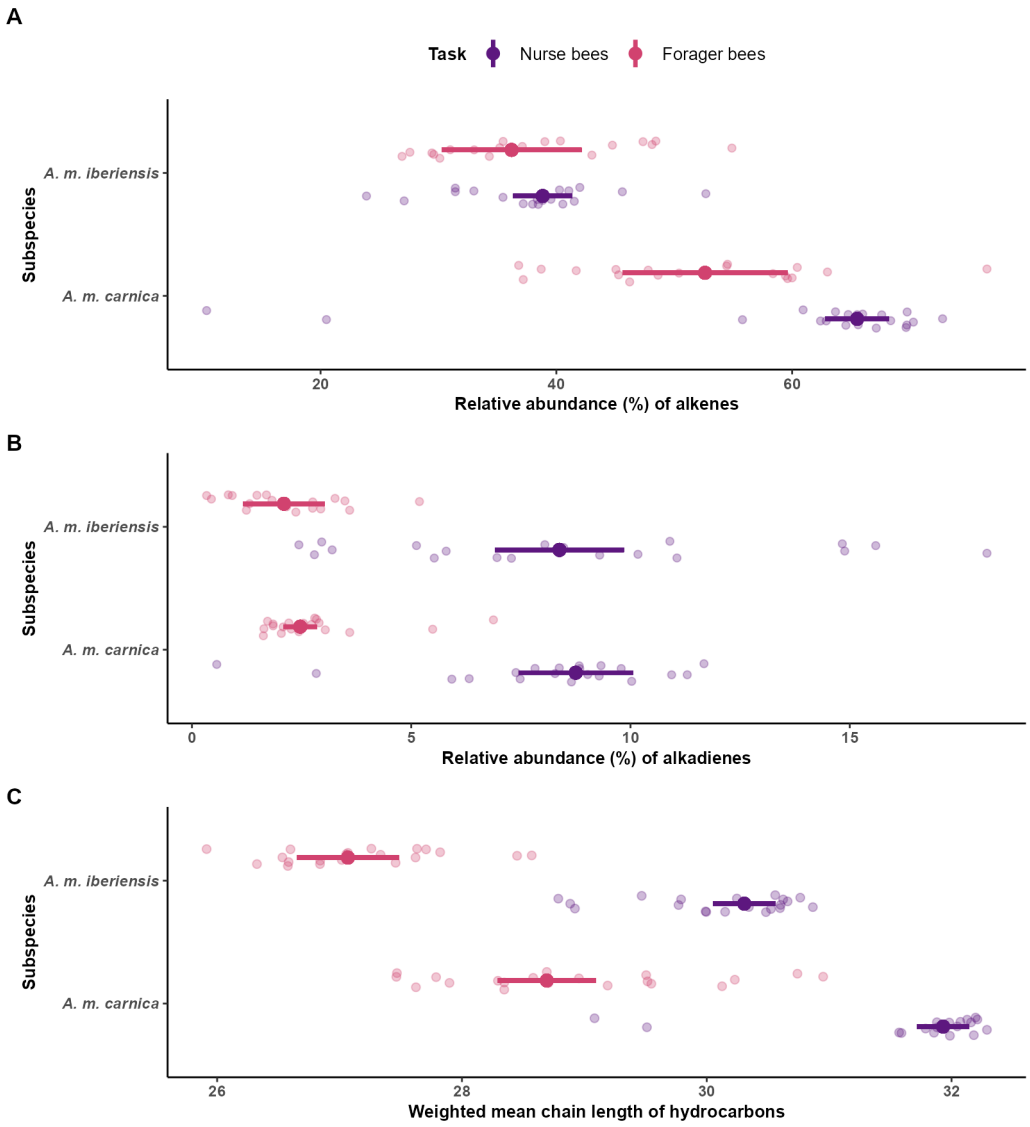

Figure 1: Task and subspecies-related differences in the CHC compositions of honey bee workers (nurse and forager bees) of two subspecies (*A. m. carnica* and *A. m. iberiensis*). The figure is divided into three plots, each corresponding to the results of a quantile regression analysis on the task- and subspecies-related differences in a compositional trait of the CHC profiles of the bees. **A)** Relative abundance of mono-unsaturated hydrocarbons (alkenes). **B)** Relative abundance of di-unsaturated CHCs (alkadienes). **C)** Mean chain length of the hydrocarbons. Point intervals represent the model prediction for the median and its 95% confidence interval. The raw data is visualized with transparent points, which correspond to the measured amount in the CHC profile of every bee.

Nurse and forager bees differ in the expression of both desaturase- and elongase-encoding genes

The expression of both desaturase-encoding genes differed between nurse and forager bees in both subspecies (Figures 2 and S2). Forager bees expressed *Des1* 0.295 times as much as nurse bees (95% CI: 0.240, 0.363). Conversely, the expression of *Des2* in the forager bees was 2.209 times higher than in the nurse

bees (95% CI: 1.722, 2.819).

Nurse and forager bees also differed in the expression of both elongase-encoding genes in both subspecies (Figures 2 and S2). *Elo1* was expressed 5.586 times more by the forager bees than the nurse bees (95% CI: 4.053, 7.774). On the other hand, the expression of *Elo2* in the foragers was 0.145 times that of the nurse bees (95% CI: 0.095, 0.214).

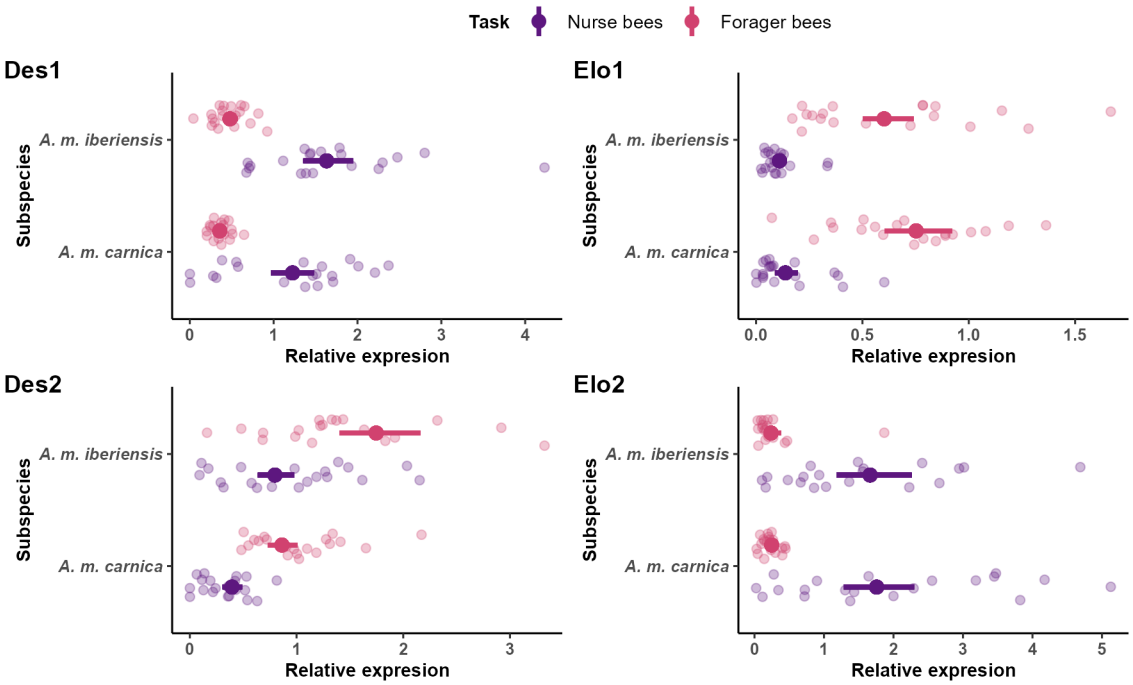

Figure 2: Relative expression of CHC biosynthesis-related genes in honey bee workers (nurse and forager bees) of two subspecies (*A. m. carnica* and *A. m. iberiensis*) genes. The figure is divided into four plots, each corresponding to a gene. Point intervals represent the median of the bootstrapped GLM prediction for the mean relative expression of the genes and its 95% confidence interval. The raw data is visualized with transparent points, which correspond to the relative gene expression value that was measured for every honey bee worker.

Subspecies differ in the expression of desaturase- but not elongase-encoding genes

A. m. iberiensis elicited a higher expression of both desaturase-encoding genes than *A. m. carnica*, for both nurse and forager bees (Figures 2 and S2). The expression of *Des1* was 1.346 times higher in *A. m. iberiensis* than in *A. m. carnica* (95% CI: 1.092, 1.658). Moreover, the expression of *Des2* was 2.039 times higher in *A. m. iberiensis* than in *A. m. carnica* (95% CI: 1.594, 2.593).

In the case of the elongase-encoding genes, the subspecies did not differ in the expression of *Elo1* (95% CI: 0.589, 1.135) or *Elo2* (95% CI: 0.639, 1.426) and this pattern was observed for both nurse and forager bees (Figures 2 and S2).

Correlation between gene expression and CHC composition

Bees with a higher relative expression of *Des1* exhibited a higher abundance of alkadienes in their CHC profiles (Figure 3A). In contrast, the relative expression of *Des2* was negatively correlated with the abundance of both alkenes and alkadienes.

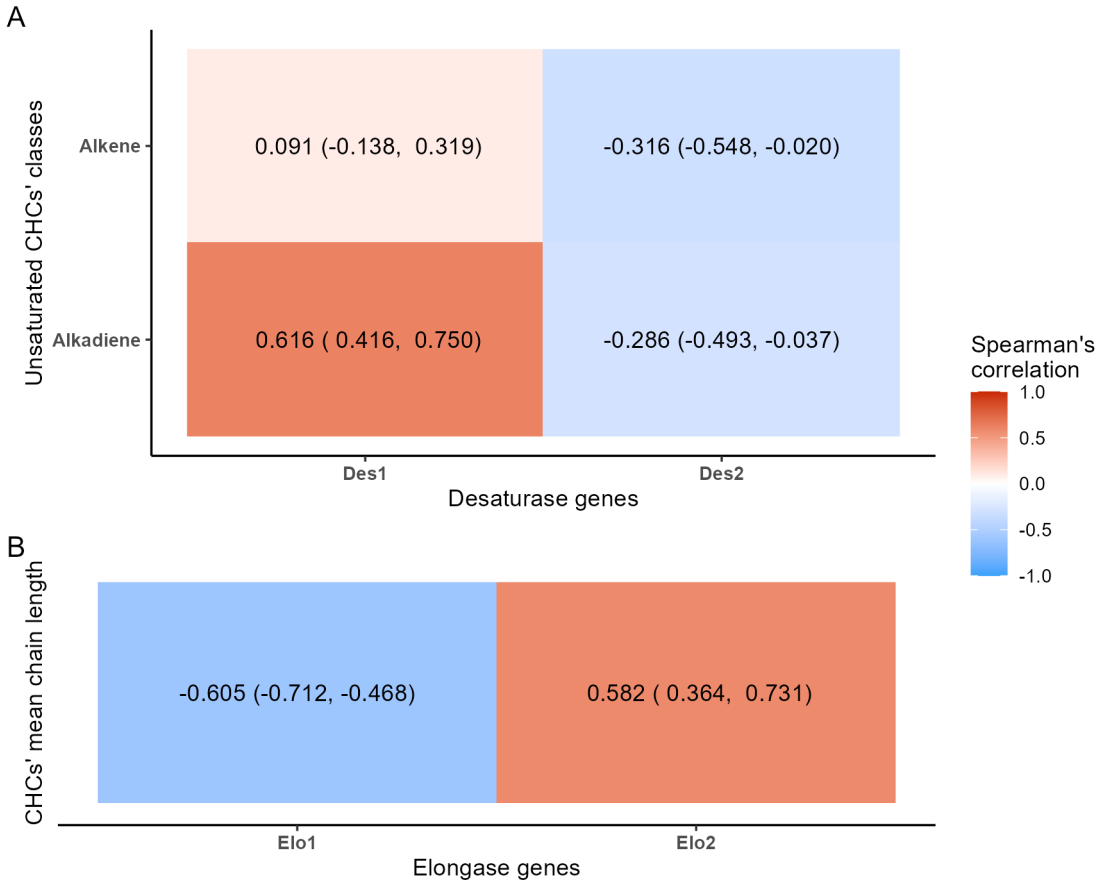

Figure 3: Correlation of the expression of CHC biosynthesis-related genes and CHC composition in honey bee workers (nurse and forager bees) of two subspecies (*A. m. carnica* and *A. m. iberiensis*). **A)** Correlation between the relative expression of both desaturase-encoding genes and the relative abundance of alkenes and alkadienes (%). **B)** Correlation between the relative expression of both elongase-encoding genes and the weighted mean chain length of the CHCs. The estimated Spearman's correlation index is shown besides its 95% confidence interval (lower limit, upper limit).

Bees with a higher expression of *Des1* tended to have a higher abundance of unsaturated hydrocarbons with chain lengths over 29 carbon atoms in their CHC profiles (Figure 4A). On the other hand, bees with a higher expression of *Des2* displayed higher abundances of unsaturated compounds with up to 29 carbon atoms in their CHC profiles.

The compounds that positively correlated with the expression of *Des1* were more abundant in the CHC profile of nurse bees, while those negatively correlated with the expression of *Des1* were more abundant in the CHC profiles of forager bees (Figure 4B). *A. m. iberiensis* nurse bees had a higher abundance of the hydrocarbons that positively correlated with the expression of *Des1* than the nurse bees of *A. m. carnica*. In contrast, forager bees of *A. m. iberiensis* exhibited a higher abundance of the hydrocarbons that negatively correlated with the expression of *Des1* than their *A. m. carnica* counterparts.

In the case of *Des2*, forager bees displayed a higher abundance of the hydrocarbons that were positively correlated with its expression. Nurse bees, in turn, presented a higher abundance of the hydrocarbons that negatively correlated with the expression of *Des2*.

A. m. iberiensis forager bees presented a higher abundance of the hydrocarbons that positively correlated with the expression of *Des2* than the forager bees of *A. m. carnica*. The nurses of *A. m. carnica*, on the contrary, displayed a higher abundance of the hydrocarbons that negatively correlated with the expression of *Des2* than their *A. m. iberiensis* counterparts.

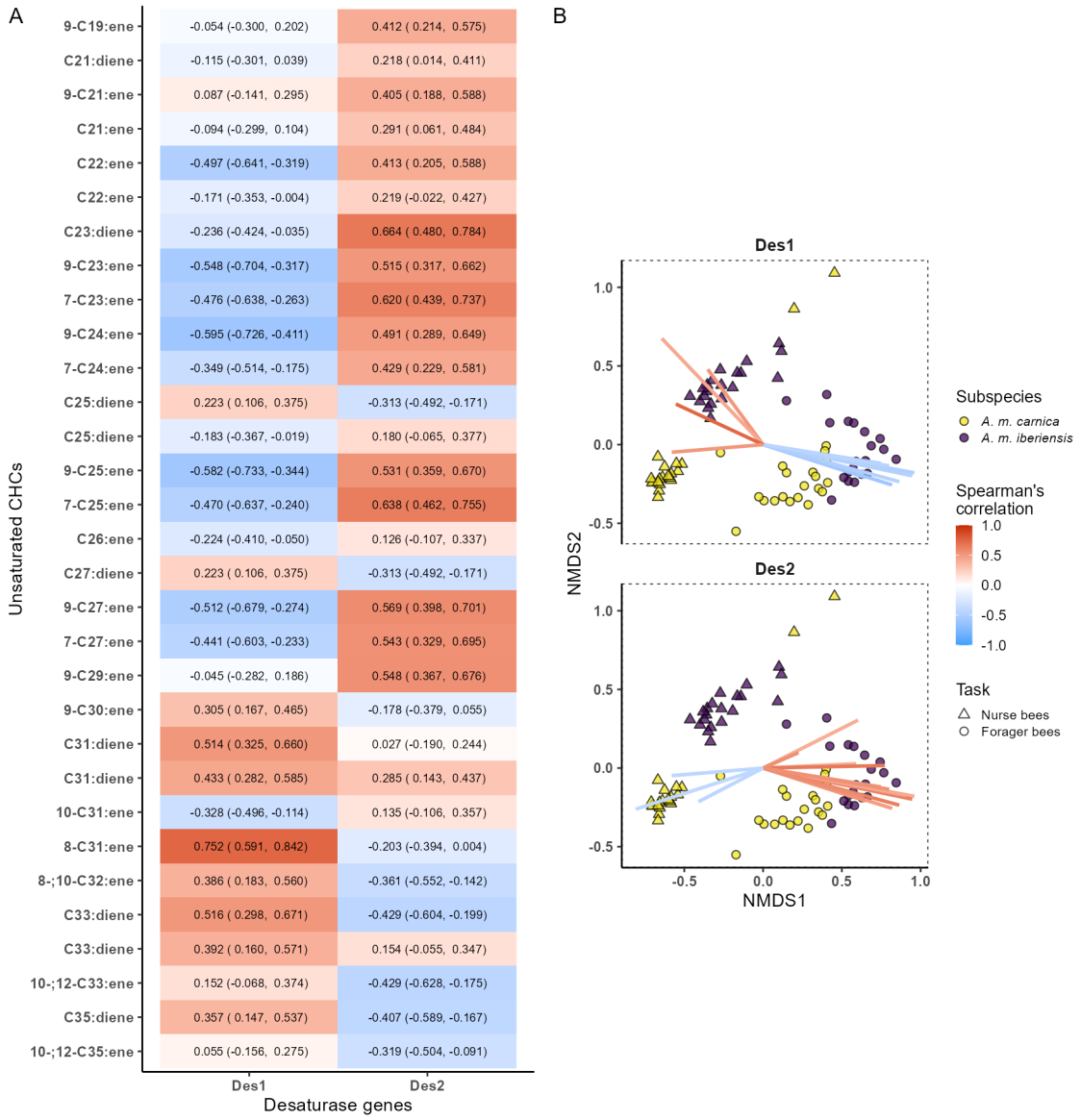

Figure 4: Correlation of the expression of desaturase-encoding genes and the CHC composition of honey bee workers (nurse and forager bees) of two subspecies (*A. m. carnica* and *A. m. iberiensis*). **A)** Correlation between the relative expression of both desaturase-encoding genes and the relative abundance (%) of hydrocarbons. The hydrocarbons are sorted from shorter chain length (top) to longer chain length (bottom). The estimated Spearman's correlation index is shown besides its 95% confidence interval (lower limit, upper limit). **B)** Bi-dimensional Non-metric Multidimensional Scaling (NMDS) analysis on a dissimilarity matrix of the CHC profiles of honey bee workers. Ordination stress: 0.083. The plot is divided into facets, each depicting the same NMDS but only illustrating the coordinates of the CHCs correlated with the expression of a specific desaturase gene. The colored lines illustrate the coordinates of the CHCs. The estimated correlation between hydrocarbons' abundance (%) and the expression of the corresponding gene is indicated by the color of the line (red: positive. blue: negative). Only hydrocarbons with a correlation higher than -0.4 (negative) or 0.4 (positive) are shown.

Bees with a higher expression of *Elo1* tended to have shorter compounds in their CHC profiles (Figure 3B). More specifically, the expression of *Elo1* correlated positively with the abundance of compounds with chain lengths between 22 and 27 carbon atoms and negatively with the abundance of longer compounds (Figure 5A). On the contrary, bees with a higher expression of *Elo2* had the tendency to exhibit CHC profiles with longer compounds (Figure 3B). The higher the expression of *Elo2*, the higher the abundance of the longer compounds ($\geq$ 27 carbon atoms), in particular those with chain lengths over 29 carbon atoms. In turn, compounds with chain lengths shorter than 27 carbon atoms tended to be less abundant in the CHC profiles of the bees with a higher expression of *Elo2*.

The hydrocarbons with the strongest correlation with the expression of both elongase-enconding genes were highly associated with the task performed by the workers (Figure 5B). *Elo1* correlated positively with the hydrocarbons that were more abundant in the CHC profiles of the forager bees and negatively with those that were more abundant in the CHC profiles of nurse bees. *A. m. iberiensis* forager bees displayed a higher abundance of the compounds that were positively correlated with the expression of *Elo1* compared to *A. m. carnica* forager bees. This difference between subspecies was less evident among nurse bees. However, most of the hydrocarbons that correlated negatively with the expression of *Elo1* were more abundant in the *A. m. iberiensis* nurse bees than in their *A. m. carnica* counterparts.

The compounds that correlated positively with the expression of *Elo2* were more abundant in nurse bees compared to forager bees. On the contrary, forager bees displayed a higher abundance of the compounds that correlated negatively with the expression of *Elo2* than nurse bees. Here, the differences between the two subspecies seem to only be evident between the forager bees, with *A. m. iberiensis* displaying a higher abundance of the compounds that were negatively correlated with the expression of *Elo2* than *A. m. carnica*.

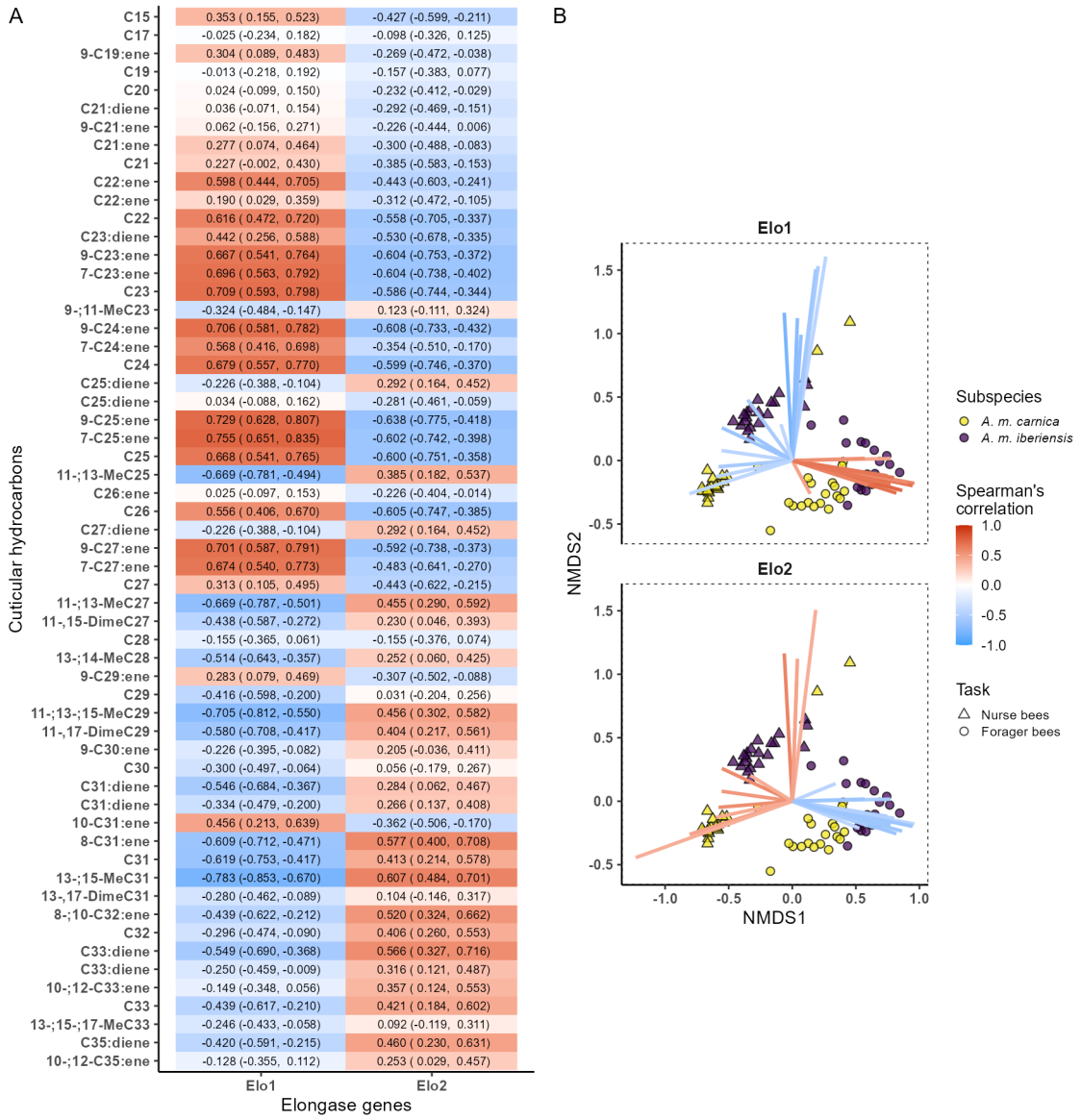

Figure 5: Correlation of the expression of elongase encoding genes and the CHC compositions of honey bee workers (nurse and forager bees) of two subspecies (*A. m. carnica* and *A. m. iberiensis*). **A)** Correlation between the relative expression of both elongase genes and the relative abundance (%) of hydrocarbons. The hydrocarbons are sorted, top-to-bottom, from shorter to longer chain length. The estimated Spearman's correlation index is shown besides its 95% confidence interval (lower limit, upper limit). **B)** Bi-dimensional Non-metric Multidimensional Scaling (NMDS) analysis on a dissimilarity matrix of the CHC profiles of honey bee workers. Ordination stress: 0.083. The plot is divided into facets, each depicting the same NMDS but only illustrating the coordinates of the CHCs correlated with the expression of a specific elongase gene. The colored lines illustrate the coordinates of the CHCs. The estimated correlation between hydrocarbons' abundance (%) and the expression of the corresponding gene is indicated by the color of the line (red: positive. blue: negative). Only hydrocarbons with a correlation higher than -0.4 (negative) or 0.4 (positive) are shown.

Discussion

Nurse and forager bees elicit different CHC compositions

Our data revealed a different CHC composition between nurse and forager bees. Nurse bees typically exhibited compounds with longer chain lengths and a higher abundance of unsaturated compounds in their CHC profile compared to forager bees. This difference was stereotypically consistent for two evolutionary divergent *A. mellifera* subspecies. Furthermore, it suggests that the CHC layer of forager bees protects them better against desiccation compared to nurse bees (Menzel et al., 2018; Florian Menzel et al., 2017; F. Menzel et al., 2017). A similar difference has been described for the workers inside and outside the nest of other social insects (Martin and Drijfhout, 2009; Wagner et al., 2001, 1998). This is thought to respond to the higher desiccation pressure faced by the outside workers (e.g., forager bees) compared to those inside the nest (e.g., nurse bees), due to their higher exposure to weather extremes (e.g., temperature and humidity) (Kather et al., 2011; Martin and Drijfhout, 2009; Rodríguez-León et al., 2024; Wagner et al., 2001, 1998).

Honey bee workers exhibit an age-dependent division of labor, transitioning from nurse to forager bees as they age, which is accompanied by a series of physiological changes (Johnson, 2008; M. Elekonich et al., 2001; Reim and Scheiner, 2014; Scheiner et al., 2017; Scheiner et al., 2014; Schilcher and Scheiner, 2023; Siegel et al., 2013). Therefore, the observed difference in CHC composition between nurse and forager bees could be influenced by shifts in the expression of CHC biosynthesis-related genes that occur along their task transition.

Underlying mechanism of the task-related CHC composition shift in honey bees

The four examined genes (*Des1*, *Des2*, *Elo1*, and *Elo2*) strongly differed in expression between nurse and forager bees. *Des1* and *Elo2* were up-regulated in nurse bees and down-regulated in forager bees, while the opposite pattern was observed for *Des2* and *Elo1*. Honey bees division of labor is flexible and responds to the needs of the colony, allowing individual bees to transition from nursing to foraging at different ages (Huang and Robinson, 1996; Huang and Robinson, 1992; Johnson and Frost, 2012; Winston and Fergusson, 1985). In fact, by artificially manipulating the colony, forager bees can be induced to revert to nurse bees, both behaviorally and physiologically (Robinson et al., 1992). Therefore, it is plausible that the observed differences in the expression of CHC biosynthesis-related genes between nurse and forager bees respond to regulatory changes associated with task transition.

We found that the difference in the expression of CHC biosynthesis-related genes between nurse and forager bees correlates with their different CHC compositions. Specifically, the expression of *Des1* and *Elo2* correlated with the abundance of compounds that were characteristic of nurse bees. The expression of *Des2* and *Elo1*, on the other hand, correlated with the abundance of compounds that were characteristic of forager bees. These findings suggest a yet unknown fine-tuning mechanism involving the up- and down-regulation of these genes, influencing the shift in the CHC composition of honey bee workers during their transition from nursing to foraging. Moreover, this transcriptional mechanism appears to be conserved across evolutionary lineages. As suggested by the consistent correlation between task-related CHC profiles and the expression of these genes in both *A. mellifera* subspecies.

A. m. carnica and *A. m. iberiensis* differ in CHC composition

A. m. carnica displayed longer compounds and a higher abundance of unsaturated compounds in their CHC profiles than *A. m. iberiensis*. These results suggest that *A. m. iberiensis* is better adapted to a higher drought stress than *A. m. carnica* (Menzel et al., 2018; Florian Menzel et al., 2017; F. Menzel et al., 2017). However, differences in CHC composition between *A. mellifera* subspecies have been suggested to respond to their divergent evolutionary histories and genetic drift, as they do not seem to consistently correlate with the climatic differences (i.e., temperature and precipitation) between their native distributions (Rodríguez-León et al., 2024). Therefore, the adaptive difference in CHC composition we observed between *A. m. carnica* and *A. m. iberiensis*, is likely specific to this particular comparison. These two subspecies belong to different *A. mellifera* evolutionary lineages and their natural ranges encompass very different environmental conditions (Chávez-Galarza et al., 2015; De la Rúa et al., 2009; Han et al., 2012; Ruttner, 1988).

As our study was a common garden experiment, we attribute the observed differences in the CHC composition between *A. m. carnica* and *A. m. iberiensis* to innate factors, leading us to expect an innate difference in expression of CHC biosynthesis-related genes between the two subspecies.

A. mellifera subspecies differences highlight CHC biosynthesis complexity

A. m. iberiensis exhibited a higher expression of both desaturase-encoding genes (*Des1* and *Des2*) than *A. m. carnica*. In contrast, the expression of both elongase-encoding genes (*Elo1* and *Elo2*) did not differ between subspecies. These findings suggest that the expression of *Des1* and *Des2* between *A. mellifera* subspecies would respond to their evolutionary divergence, but not the expression of *Elo1* and *Elo2*. Since

we performed a common garden experiment, the similarity in the expression of *Elo1* and *Elo2* by the bees of both subspecies could indicate an influence of the environment on the expression of these two elongase-encoding genes. However, our results do not discard the possibility that the expression these genes responds to intrinsic factors that are not related to the subspecies.

Nevertheless, among the genes assessed here, only the expression of *Des2* suggests a plausible mechanism underlying the differences in the CHC composition between *A. m. carnica* and *A. m. iberiensis*. The expression of this gene is positively correlated with the abundance of hydrocarbons with chain lengths of up to 29 carbon atoms. In consequence, the higher expression of *Des2* in *A. m. iberiensis* compared to *A. m. carnica* could contribute to the shorter mean chain length of hydrocarbons in the CHC profile of *A. m. iberiensis* compared to *A. m. carnica*.

The expression of *Des1*, on the other hand, is correlated with the abundance of alkadienes, a feature not differing between subspecies. Similarly, while the CHC profile of *A. m. carnica* exhibited longer hydrocarbons compared to *A. m. iberiensis*, the expression of both elongase-encoding genes (*Elo1* and *Elo2*) did not differ between subspecies. These discrepancies between the subspecific differences in CHC composition and the expression of the four studied genes evidence the complexity of CHC biosynthesis. Although several genes have been proposed to participate in CHC biosynthesis in honey bees, only a few have been mechanistically confirmed, through targeted knock-down experiments, to directly influence CHC composition in worker bees (Falcón et al., 2014; Moris et al., 2023; Vernier et al., 2019). It is thus plausible that the observed subspecific differences in CHC composition stem from the activity of the enzymes encoded by other CHC biosynthesis-related genes.

Differential roles of specific genes in CHC biosynthesis

Our findings suggest that *Des1* and *Des2* play distinct roles in CHC biosynthesis. Herein, the expression of *Des1* positively correlated with the total relative abundance of alkadienes in the CHC profile of the bees, while the expression of *Des2* negatively correlated with the abundance of both alkenes and alkadienes. We consider that this difference could stem from the specificities of the enzymes encoded by *Des1* and *Des2*. Different desaturases have been found to vary in their substrate specificities and, thereby, in how they influence CHC composition (Dallerac et al., 2000; Holze et al., 2021; Takahashi et al., 2001). Our results support this, as the expression of the two desaturase-encoding genes correlated with the abundance of different unsaturated hydrocarbons in the CHC profiles of honey bee workers. Moreover, the suggested difference in specificities between the enzymes encoded by these two genes explains the negative corre-

lation between the expression of *Des2* and the abundance of both alkenes and alkadienes. Unlike *Des1*, *Des2* expression was positively correlated with the abundance of unsaturated hydrocarbons with chain lengths up to 29 carbon atoms. These compounds were the least abundant in the CHC profile of honey bee workers, considering the mean chain length of the hydrocarbons of every analyzed group (nurse and forager bees of both *A. m. carnica* and *A. m. iberiensis*) (Figure 1C). In consequence, these compounds contributed little to the total abundance of alkenes and alkadienes.

In the case of the elongase-encoding genes, we found that *Elo1* expression negatively correlated with the mean chain length of the CHCs, unlike *Elo2*. While this *Elo1* pattern seems counterintuitive, as elongases catalyze the elongation of the hydrocarbon chain (Chung and Carroll, 2015; Cinti et al., 1992; Jakobsson et al., 2006), this result can be explained by a differential specificity of the elongases encoded by the two genes. Elongases have been found to generally catalyze the biosynthesis of hydrocarbons with chain lengths over 20 carbon atoms. Thus, the difference in specificity among elongases would explain the diversity of chain lengths in the CHC profiles of insects (Blomquist and Bagnères, 2010; Denic and Weissman, 2007; Holze et al., 2021). The expression of *Elo1* and *Elo2* correlated with the abundance of compounds within different chain length ranges, suggesting that both genes influence the synthesis of hydrocarbons with different chain lengths.

The diversity and regulation of the expression of CHC biosynthesis-related genes are thought to influence the richness and abundance of compounds in the CHC profile of an insect (Blomquist and Bagnères, 2010; Chung and Carroll, 2015; Gu et al., 1997; Holze et al., 2021; Howard and Blomquist, 2005; Juárez et al., 1992; Qiu et al., 2012; Reed et al., 1995). However, current understanding of the differential role of specific genes encoding for different enzymes of the same type (e.g., specific desaturases) and how the variation in their expression affects CHC composition remains limited. Here we shed light on this matter by revealing correlations between the expression of specific desaturase- and elongase-encoding genes and CHC compositions in *A. mellifera* workers.

Key Insights and Future Directions

By analyzing both the CHC composition and expression of specific CHC biosynthesis-related genes and their correlation this study expands current understanding of the genetic basis of CHC biosynthesis. Here, we provide evidence of the difference in specificity among specific desaturases and elongases and how such differences could contribute to shaping the CHC profiles of *A. mellifera* workers during their task transition from nursing to foraging. Furthermore, our results highlight the importance of further investi-

gating the different subspecies of *A. mellifera* to better understand the mechanisms underlying the CHCs diversity of honey bees. We predict that the complexity of the genetic basis for the difference in the CHC composition among *A. mellifera* subspecies provides an interesting case for the study of the molecular basis of CHC biosynthesis.

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Data availability

The data and code used for this study are not included in this manuscript, but will be made publicly available as supplementary material for the corresponding paper at the time of publication.

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Supplementary material

Table S1: Oligonucleotide primers used in this study.

	Gene	Gene ID	Primer direction	Primer sequence (5'-3')
Gene of interest	*Des1*	LOC100576797	Reverse	TGATGATAATTATGCCATCCTTC
			Forward	TGGTTGGTAAATTCTGCTGCT
	Des2	LOC551527	Reverse	GTAAGAGGTTGTTTGAAGAA
			Forward	TTGGATTAACGGAACGAAGG
	Elo1	LOC409638	Reverse	CCAAACCACACTGACATTGG
			Forward	TTCATGGACACGATCTTCTTTG
	Elo2	LOC550828	Reverse	TTATTACAAGGACAAATTGAACC
			Forward	TTGGTTTGGCGTTAAGTTCA
Reference gene	*Rpl10*	LOC409589	Reverse	CGTATCTTTGGATCAGGCACACC
			Forward	CGATAAGAAACGTAAGTCAATATGGGGC
	Rpl19	LOC724186	Reverse	GCTTTGACGTGAGTTTGTATTTGCAA
			Forward	GGGACTTCTAGGCTCCATCATGAG

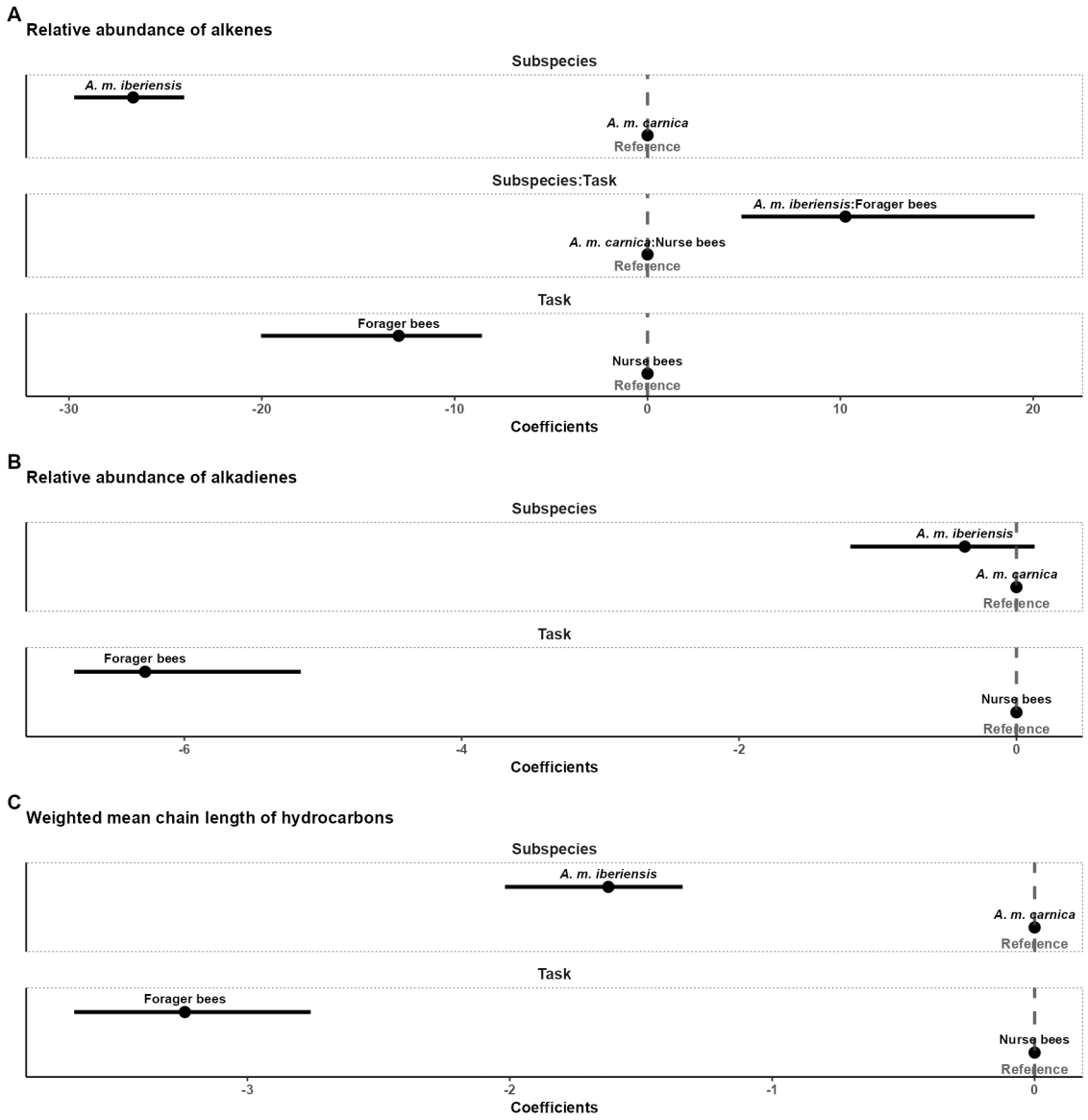

Figure S1: Quantile regression coefficients (effect sizes) for the task and subspecies-related differences in the CHC composition of honey bee workers. The figure is divided into three plots, each corresponding to the results of a quantile regression (50% quantile) analysis on the task- and subspecies-related differences in a compositional trait of the CHC profile of honey bee workers. **A)** Relative abundance of mono-unsaturated hydrocarbons (alkenes). **B)** Relative abundance of di-unsaturated hydrocarbons (alkadienes). **C)** Mean chain length of the CHCs. Each plot is divided into facets, which correspond to the categorical variables used as predictors for the model (task and subspecies) and their interaction (task:subspecies), if included. Point intervals represent the estimated difference to the reference group (task: nurse bees; subspecies: *A. m. carnica*; subspecies:task: *A. m. carnica*:nurse bees) and its 95% confidence intervals. The reference is visualized with a dashed grey line and an overlapping point for the corresponding group.

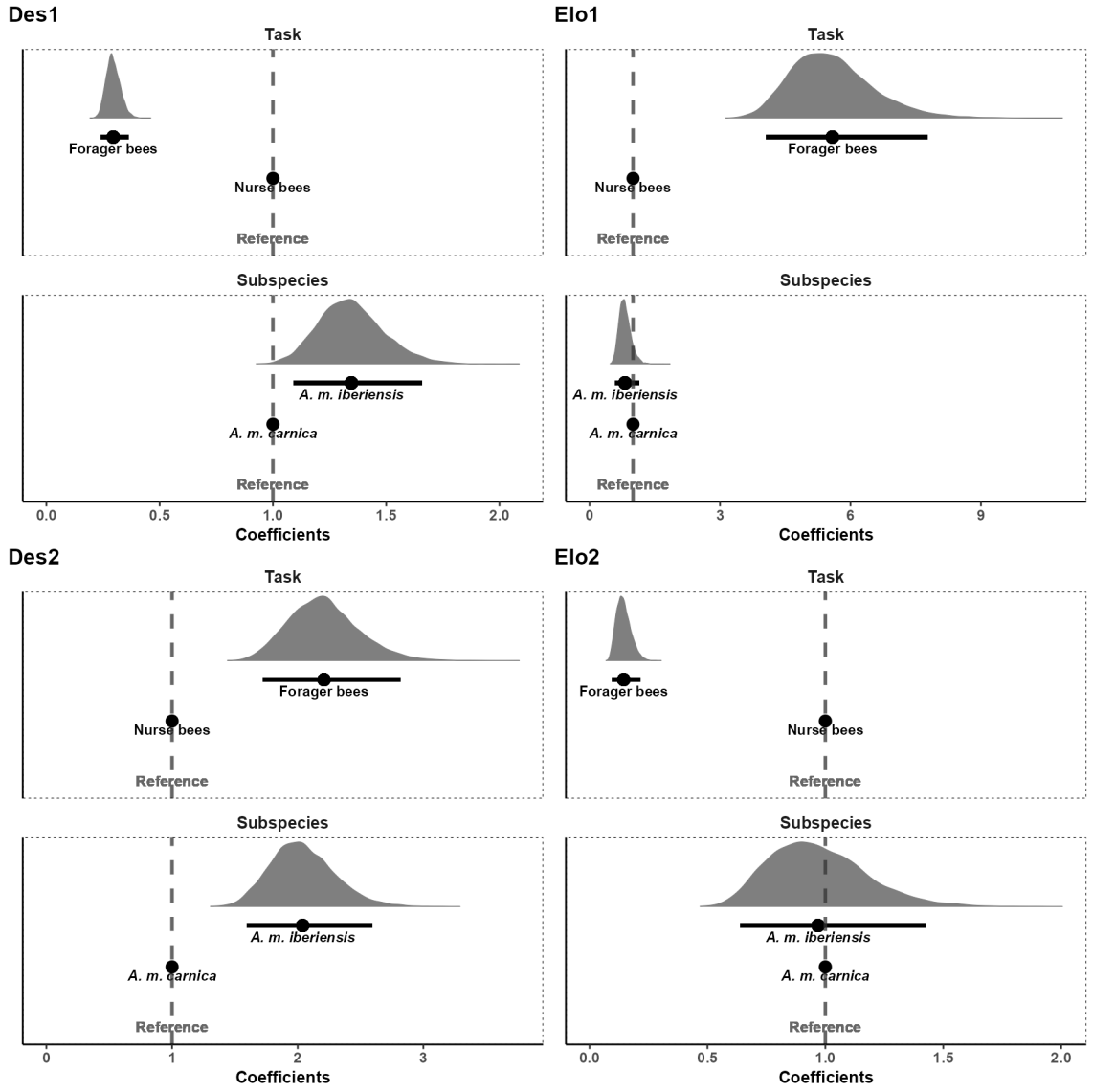

Figure S2: Bootstrapped GLM coefficients (effect sizes) for the difference in the relative expression of the CHCs biosynthetic genes between tasks and subspecies. The figure is divided into four plots, each corresponding to a gene. Each plot is divided into two facets, which correspond to the two categorical variables used as predictors for the model (task and subspecies). Shaded curves depict the distribution of the estimated GLM coefficients across the 10,000 bootstrap simulations. Point intervals represent the median of the bootstrapped GLM mean proportional difference in the gene expression to the reference group (task: nurse bees; subspecies: *A. m. carnica*) and its 95% confidence intervals. The reference is visualized with a dashed grey line and an overlapping point for the corresponding group.