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# Assessing lethal and sublethal effects of pesticides on honey bees in a multifactorial context

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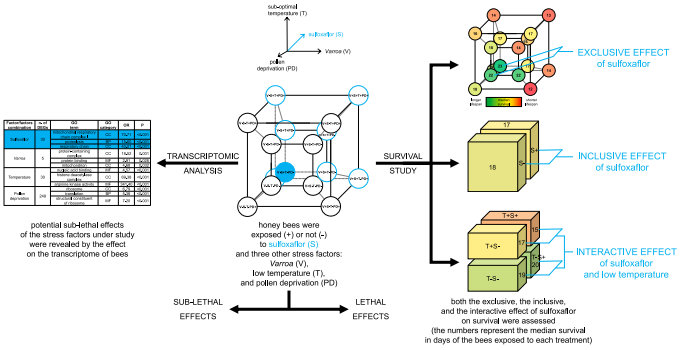
## HIGHLIGHTS

- The inclusive effect is the effect of a stress factor in a multifactorial context.
- Varroa, cold and lack of pollen have a significant inclusive effect on honey bees.
- The inclusive effect of a field realistic concentration of sulfoxaflor in sugar is negligible.
- Sulfoxaflor can influence the gene expression of treated honey bees.
- Transcriptomic analysis can reveal potential sublethal effects of pesticides.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The registration of novel pesticides that are subsequently banned because of their unexpected negative effects on non-target species can have a huge environmental impact. Therefore, the pre-emptive evaluation of the potential effects of new compounds is essential. To this aim both lethal and sublethal effects should be assessed in a realistic scenario including the other stressors that can interact with pesticides. However, laboratory studies addressing such interactive effects are rare, while standardized laboratory-based protocols focus on lethal effects and not on sub-lethal effects.

We propose to assess both lethal and sublethal effects in a multifactorial context including the other stressors affecting the non-target species. We tested this approach by studying the impact on honey bees of the insecticide sulfoxaflor in combination with a common parasite, a sub-optimal temperature and food deprivation. We studied the survival and the transcriptome of honey bees, to assess both the lethal and the potential sublethal effects of the insecticide, respectively. With this method we show that a field realistic concentration of sulfoxaflor in food does not affect the survival of honey bees; however, the significant impact on some key genes indicates that sublethal effects are possible in a realistically complex scenario.

Moreover, our results demonstrate the feasibility and reliability of a novel approach to hazard assessment considering the interactive effects of pesticides. We anticipate our approach to be a starting point for a paradigm shift in toxicology: from an unifactorial, mortality-centered assessment to a multifactorial, comprehensive

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approach. This is something of the utmost importance to preserve pollination, thus contributing to the sustainability of our food production system.

## 1. Introduction

Honey bees play a crucial role both in natural and agricultural environments contributing to the ecosystem service of pollination (Klein et al., 2007; van der Sluijs and Vaage, 2016; Hung et al., 2018). Therefore, any negative impact on honey bees can produce cascading effects on the environment, the economy and the whole society (Klein et al., 2007). Landscape transformation and agriculture intensification expose honey bees to an ever increasing number of pesticides that threaten their survival or affect their performance and consequently pollination (van der Sluijs and Vaage, 2016). For this reason, the evaluation of the effects of pesticides on non-target insect species, particularly those providing important ecosystem services like the honey bee *Apis mellifera* L., prior to their registration, is essential to avoid the impact of cycles of novel pesticide release and banning (Sgolastra et al., 2020).

Current risk assessment procedures require the combined evaluation of the exposure generated by the use of a pesticide in the field and its ecotoxicological effects (EFSA, 2022). The latter involves a tier-based approach, starting with toxicity lab tests normally followed by semi-field and field studies that should complement the first by assessing the possible negative effects of novel pesticides under more realistic conditions (Siviter et al., 2023). However, under field conditions, several stress factors can interfere with agrochemicals, modulating their effect on honey bees (Alaux et al., 2010; Pettis et al., 2012; Di Prisco et al., 2013; Retschnig et al., 2014; Blanken et al., 2015; Doublet et al., 2015). Additional stressors are: parasites and pathogens, adverse environmental conditions and lack of nutrients resulting from landscape deterioration (Goulson et al., 2015). Those factors have the potential to interact with each other and pesticides, which can exacerbate the individual effect and result in synergism (Doublet et al., 2015). Under field conditions both the quality and quantity of confounding factors is extremely variable and hardly controlled; therefore, field studies often produce contradictory results (Breda et al., 2022) and are less informative for the purpose of risk assessment, the results being strongly affected by the context (Woodcock et al., 2017). Furthermore, insecticides do not only affect the survival of bees but can cause sublethal effects, including alterations of homing and foraging, reproductive problems, immune related dysfunctions (Desneux et al., 2007) which in turn can affect the pollination activity. Unfortunately, a pre-emptive direct assessment of all possible sublethal effects is practically unfeasible because pesticides may impact several functions thus requiring a potentially endless number of convenient bioassays.

Better protocols for the pre-emptive evaluation of pesticides are therefore urgently needed to assess both lethal and potential sublethal effects of pesticides in a multifactorial context (Topping et al., 2020).

Convenient methods to address interactive effects through factorial experiments are available (Montgomery, 2013) but they have been rarely applied in entomological studies (Kaunisto et al., 2016). For example, despite the many stressors normally affecting bees under realistic field conditions (Goulson et al., 2015), to our knowledge nobody has ever tested the lethal effect on honey bees of any pesticide as applied in combination with more than one further stressor.

In regard to sublethal effects, the incorporation of 'omic data in risk assessment is more recently taken into consideration (Sauer et al., 2017) since the differential expression of critical genes can be regarded as an early-warning signal of possible physiological alterations that may result in detrimental effects at the organismal level. In fact, studies on the sublethal effects are often supported by data regarding the expression of some critical genes involved in the biological function under study, such as immunity (Annoscia et al., 2020), behavior (Morfin et al., 2019), and orientation (Zhang et al., 2022). Given their multiple roles, major royal jelly proteins are often used as a read-out of sublethal effects involving immunity or behavior (Fent et al., 2020; Wu et al., 2017). Methods are now available to assess the impact of any treatment on the expression of all the genes of the honey bee (i.e. the transcriptome, (Grozinger and Zayed, 2020)); such methods have already been used to test the response of bees to several stressors but, again, only one (Zanni et al., 2017; Ye et al., 2020; Zhang et al., 2023) or at most two stressors (Aufauvre et al., 2014; Schmehl et al., 2014) at a time; only recently a study involving three stressors was published (Kang et al., 2024).

We therefore developed a novel approach for assessing the hazard posed by a pesticide that involves testing both the lethal and sublethal effect of a field realistic dose of the compound on honey bees exposed at the same time to the influence of the other most important stress factors affecting this species. We tested this approach with the insecticide sulfoxaflor because it is widespread and has been the subject of numerous studies that can now be used for an "a posteriori" validation of the method we propose (for a synthetic review of those studies see Table S1). Sulfoxaflor is a sulfoximine insecticide targeting the nicotinic acetylcholine receptor (Zhu et al., 2011) and is regarded as a good replacement candidate of the neonicotinoid insecticides recently banned in the EU (Brown et al., 2016). Similar to other insecticides, a few years after its registration, sulfoxaflor was banned for the use in the open field in Europe because of the potential negative effects on pollinators but it is still in use in the US and elsewhere.

We tested under laboratory conditions the effect of a field realistic dose of sulfoxaflor in combination with three other potential stressors that were selected to cover the most important classes of factors that are deemed responsible for bee decline (Goulson et al., 2015; Potts et al., 2010). In particular, we considered: the infestation with the most important ectoparasite of honey bees, *Varroa destructor* (Nazzi and Le Conte, 2016); the exposure to a sub-optimal temperature (Abou-Shaara et al., 2017) and the deprivation of pollen: an essential nutrient for honey bees whose availability is becoming uncertain because of landscape transformation (Naug, 2009). To include in our analysis the widespread deformed wing virus, we carried out our study late in the season when the pathogen's prevalence reaches 100 % (Nazzi et al., 2012). To assess all the possible relevant interactions among the studied stressors, we adopted a fully factorial experimental design in which honey bees were exposed to all factors both in isolation and in combination with the others (Montgomery, 2013). To gain insight into the possible sublethal effects caused by sulfoxaflor on honey bees, we also assessed the impact of the treatments on metabolism and physiology by means of a transcriptomic analysis of the bees used in the experiment.

With this experimental approach, further than collecting more data on the effect of sulfoxaflor on honey bees, we wanted to assess the potential of a novel ecotoxicological approach to risk assessment that could be applied to test agrochemicals prior to their registration and use, in order to estimate their lethal and sublethal effects under a realistic multifactorial scenario.

## 2. Materials and methods

### 2.1. Biological material

Honey bees and *Varroa* mites used in the experiments were collected from the experimental apiary of the Dipartimento di Scienze Agro-Alimentari, Ambientali e Animali of the University of Udine (Udine, Italy 46°04'54.2" N, 13°12'34.2" E). Previous studies indicated that local colonies are hybrids between *A. mellifera ligustica* Spinola and *A. mellifera carnica* Pollmann (Comparini and Biasiolo, 1991). No

acaricidal treatments were carried out during Summer in the hives of the experimental apiary, so that mite infestation and the associated DWV virus infection, could naturally increase along the season.

### 2.2. Stress factors considered in this study

The effect of four different stress factors was studied: the infestation with an ectoparasite, a sub-optimal temperature, the deprivation of pollen and the chronic contamination with an insecticide as detailed below.

Insecticide: analytical grade sulfoxaflor (Ehrenstorfer GMBH, cod: C17015000) was added to the syrup that was fed to the caged honey bees during the experiment. The pesticide was dissolved in acetone at a concentration of 1 μg/μL. The feeding solutions were prepared by diluting the stock solution in aqueous 2.4 mol/L of glucose and fructose (61% and 31%, respectively) solution (Thom et al., 2003). The same amount of an uncontaminated acetone solution was added to the sugar syrup fed to control bees. Sulfoxaflor concentration in the feeding solution was 0.07 ppm. This concentration is in line with available data on sulfoxaflor contamination of nectar and pollen (Al Naggar and Paxton, 2021).

Pollen deprivation: in order to simulate pollen deprivation, honey bees were fed with the sole sugar solution. Instead, pollen was provided through an open Petri dish (Ø = 35 mm) to the rest of the honey bees. The pollen used in this study was collected near Udine, Italy (46°00′39″ N, 13°20′00″ E) during an extensive flowering of *Amorpha fruticosa* (Fabaceae). A palynological analysis previously carried out revealed that besides *A. fruticosa*, other pollens belonging to plants of the genus *Fagopyrum*, *Lirodendron*, *Lonicera*, *Papaver*, *Taraxacum*, *Vitis* and the family Urticaceae are commonly gathered during this period.

Sub-optimal temperature: to test the effect of a sub-optimal temperature, honey bees were maintained in a thermostatic cabinet whose temperature was set at two degrees and a half lower than the normal hive temperature (i.e. 32 °C) (Tautz et al., 2003).

Parasite: *V. destructor* is the most important ectoparasite of the honey bee mostly exerting its detrimental effects during the reproductive phase (Zanni et al., 2023). For this reason, in the experiment we employed adult honey bees that had been infested during the pupal stage. For that purpose, L5 honey bee larvae were obtained from brood cells capped in the preceding 15 h and artificially infested with one mite or maintained uninfested as control, inside 6.5 mm Ø i.d. gelatin capsules (Agar Scientific Ltd., UK) (Nazzi and Milani, 1994). Mite-infested and uninfested bees were maintained for 11 days (up to 24 h prior to the presumed emergence time) at 34.5 °C, 75% R.H., dark. Then, *Varroa*-infested bees were separated from the mite and transferred as well as control bees into the plastic cages (185 × 105 × 85 mm) for the survival study.

### 2.3. Four factors fully factorial experiment

To evaluate the effect of the stress factors described above on honey bee survival, we adopted a fully factorial experiment which implies the establishment of sixteen experimental groups, half of which were treated with each of the four factors. Practically, 11 days after setting up the experiment (i.e. <24 h before eclosion, so that bees could be exposed to the factors under study immediately after eclosion), groups of 25 uninfested or mite infested bees, that were maintained during pupation under artificial conditions inside gelatin capsules, were placed into plastic cages (185 × 105 × 85 mm) (eighth cages with mite infested (V+) and eight with uninfested bees (V-)). The following day, all cages were inspected to remove the bees that died before reaching the adult stage. The honey bees from half of the sixteen cages were fed with sugar syrup contaminated with 0.07 ppm of sulfoxaflor (S+) and half with the control solution (S-). The sugar solution was supplied through 20 mL syringes. A Petri dish containing pollen was put into the cages hosting bees not exposed to pollen deprivation (PD-) whereas the other half of cages was left without pollen (PD+). Finally, half of the cages were maintained in a climatic chamber with standard conditions (34.5 °C, 75% R.H., dark) (T-) and the other half in a climatic chamber whose temperature was set at 32 °C (75% R.H., dark) (T+). Water was provided to the bees of all cages through a 20 mL syringe. Sugar solution, pollen, and water were provided ad libitum and replaced every 7 days; daily consumption was not measured, to reduce to the minimum the time spent by honey bees outside the climatic chambers.

To sum up, the following experimental groups were established (Fig. 1):

1. uninfested honey bees fed with sugar syrup and pollen maintained at 34.5 °C (control group: V − S − T − PD−);
2. uninfested honey bees fed with sugar syrup only at 34.5 °C (V − S − T − PD+);
3. uninfested honey bees fed with sugar syrup contaminated with 0.07 ppm of sulfoxaflor and pollen at 34.5 °C (V − S + T − PD−);
4. uninfested honey bees fed with sugar syrup contaminated with 0.07 ppm of sulfoxaflor at 34.5 °C (V − S + T − PD+);
5. mite infested honey bees fed with sugar syrup and pollen at 34.5 °C (V + S − T − PD−);
6. mite infested honey bees fed with sugar syrup only at 34.5 °C (V + S − T − PD+);
7. mite infested honey bees fed with sugar syrup contaminated with 0.07 ppm of sulfoxaflor and pollen at 34.5 °C (V + S + T − PD−);
8. mite infested honey bees fed with sugar syrup contaminated with 0.07 ppm of sulfoxaflor at 34.5 °C (V + S + T − PD+);
9. uninfested honey bees fed with sugar syrup and pollen maintained at 32 °C (V − S − T + PD−);
10. uninfested honey bees fed with sugar syrup only at 32 °C (V − S − T + PD+);
11. uninfested honey bees fed with sugar syrup contaminated with 0.07 ppm of sulfoxaflor and pollen at 32 °C (V − S + T + PD−);
12. uninfested honey bees fed with sugar syrup contaminated with 0.07 ppm of sulfoxaflor at 32 °C (V − S + T + PD+);
13. mite infested honey bees fed with sugar syrup and pollen at 32 °C (V + S − T + PD−);
14. mite infested honey bees fed with sugar syrup only at 32 °C (V + S − T + PD+);
15. mite infested honey bees fed with sugar syrup contaminated with 0.07 ppm of sulfoxaflor and pollen at 32 °C (V + S + T + PD−);
16. mite infested honey bees fed with sugar syrup contaminated with 0.07 ppm of sulfoxaflor at 32 °C (V + S + T + PD+).

The cages were checked daily to count and remove dead bees. The experiment was terminated at day 45, when honey bees that were still alive were censored. The experiment was replicated three times late in the season (August – September 2020). Ideally, 25 bees per cage were used for each experimental group in each replication; however, in some cases artificially reared bees did not reach the adult stage and were thus discarded at the beginning of the experiment. In conclusion, the numbers of adult bees reported in table S2 were used per experimental group per replicate and on total. It should be noted that the differential number of bees per cage is the unavoidable consequence of using bees that developed in the laboratory from the larval stage onwards; this in turn is dictated by the use of artificially mite infested bees, instead of naturally infested bees collected from the hive. Remarkably, in this way also the confounding effect of any possible thermal shock to which honey bees may be exposed during pupation in the hive, was prevented.

### 2.4. Sulfoxaflor contamination and thermoregulation

To test the thermoregulation of honey bees exposed to sulfoxaflor via oral route through the sucrose solution, we performed two additional ad hoc experiments; one using the pesticide as a single stress factor and another where we included pollen deprivation as an additional stress factor. Experiments were performed from June to July 2022.

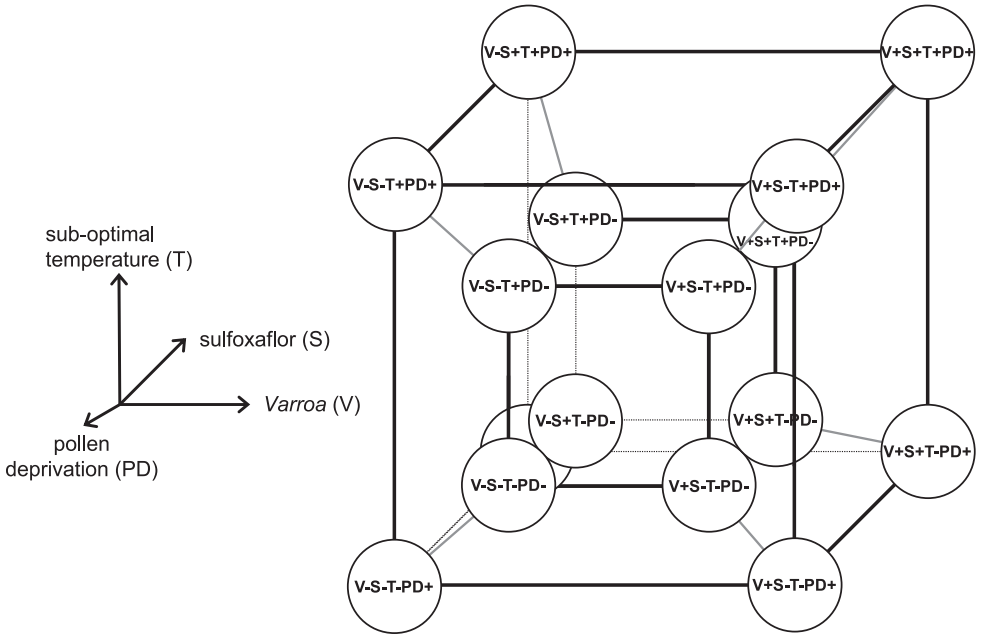


**Fig. 1.** The fully factorial experiment described in the text. Caged honey bees were exposed (+) or not (−) to the four different stress factors illustrated on the four axis depicted on the left (infestation with *Varroa* at the pupal stage (V), 0.07 ppm of sulfoxaflor in sucrose solution (S), a sub-optimal temperature of 32 °C (T) and the deprivation of pollen (PD)). Each circle in the hypercube represents an experimental group; the factors are denoted in the circles with a plus or minus sign when present or absent, respectively.

For this purpose, the day before the experiments, several combs from different hives containing emerging bees were randomly collected from the apiary and stored overnight in a climatic chamber (34.5 °C, 75% R. H., dark). The day after, groups of 25 newly emerged honey bees were transferred into 6 plastic cages (185 × 105 × 85 mm) and maintained under the same controlled conditions (each cage was set up with the same number of bees per frame). In experiment 1 three cages were provided with water, pollen and a sucrose solution (61% glucose, 39% fructose) (Thom et al., 2003), while the sugar solution provided to the other three cages was treated with sulfoxaflor at 0.07 ppm. In experiment 2, pollen was not delivered to the bees inducing a pollen deprivation in both sulfoxaflor treated and untreated cages.

From day 7 to 14, two bees per cage were collected randomly with a total of six bees per treatment per day. Then, we evaluated the body temperature of each single honey bee after exposure at room temperature (Tosi et al., 2016). To this purpose, bees were placed individually in a polystyrene box, transferred to room temperature (25 °C) and then photographed with an infrared thermographic camera (brand: FLIR; model: i5; thermal resolution = ±0.1 °C) with emissivity set at 0.97 (Stabentheiner et al., 2010). Pictures were taken through a hole in the polystyrene lid to reduce the possible interference of light radiation. Three pictures per honey bee were taken as technical replicates and the temperature of each single bee was calculated using the average of the three technical replicates. Images were analyzed with FLIR Tools® software; the recorded temperature was the average value of the warmest part of the bee body which always corresponded to the thorax. The area used to calculate the mean temperature was equal for each bee. Because of the adopted measurement method, we did not assume our data were normally distributed and therefore the recorded temperatures were compared using the Mann Whitney *U* test. Experiment 1 was replicated once, using a total of 75 and 77 bees per treatment. Experiment 2 was replicated twice, using a total of 148 and 147 bees per treatment.

### 2.5. Transcriptomic analysis of honey bees by means of RNAseq

The whole body of three bees per experimental group (for a total of 48 bees) was homogenized by means of mortar and pestle in liquid nitrogen. Total RNA was extracted and purified according to the procedure provided with the RNeasy Plus mini kit (Qiagen®, Germany). The amount and the integrity of the RNA in each sample were quantified by means of a Lab chip GX touch nucleic acid analyzer (Perkin Elmer™ UK). Libraries preparation and RNA sequencing in paired reads of length 150 bp were performed by IGA technology services s.r.l. of Udine (Italy) using a NovaSeq™ platform (Illumina, US).

Reads were aligned using hisat2 with default parameters (Kim et al., 2019) on the genome of *A. mellifera* (GCF_003254395.2). Using the hisat2 option –no-unal, unaligned reads were discarded. Aligned reads were then assigned to transcripts and expressed as FPKM using cufflinks (Roberts et al., 2011). This approach allowed normalizing by the total number of non viral reads; this is a desirable outcome, since the number of viral reads was the major factor of variation in the number of reads aligning on *Apis* genome in our experiments.

For the analysis of these data see the paragraph "Statistical analysis of data" below.

Gene expression heatmap was plotted using the R library ComplexHeatmap (https://doi.org/10.1093/bioinformatics/btw313).

The gene ontology (GO) annotation of *A. mellifera* transcriptome was performed as follows.

1. Gene Ontology annotation file was downloaded from UniProt.
2. The full transcriptome of *A. mellifera* was matched against the Swissprot section of the UniProt database annotation using blastx (https://pubmed.ncbi.nlm.nih.gov/2231712/) setting an evalue threshold of $10^{-10}$ and allowing a maximum of two hits per gene.
3. *A. mellifera* transcripts mapping against Uniprot entries were associated to the corresponding GO terms.

The final results is a list of *A. mellifera* genes associated to the corresponding GO terms (if any).

For each comparison, enrichment of GO associated to differentially expressed genes was performed using Fisher's exact test of independence.

Scripts and R functions used in this work are available at: https://gi

thub.com/genomeud/sulfoxaflor.

### 2.6. qRT-PCR validation of RNAseq results and further analysis of possible interactions

A sample of 500 ng of RNA previously extracted for the RNAseq analysis were used to synthesize cDNA following the manufacturer specifications (M-MLV reverse transcriptase, Invitrogen, US). Ten ng of cDNA from each sample were analyzed using qRT-PCR with the primers reported in Table S3 using TB Green® Premix Ex Taq™ II, according to the manufacturer specifications (Takara Bio, Japan) on a BioRad CFX96 Touch™ Real time PCR Detector. In order to ensure that primers efficiency was included in the desirable range of 99–100 %, this was calculated according to the formula E = 10^(−1/slope)−1) * 100. Relative quantification of four genes encoding for major royal jelly proteins 1–4 was performed adopting the Livak & Schmittgen method (Livak and Schmittgen, 2001) using actin and GAPDH as housekeeping genes.

### 2.7. Statistical analysis of the four factors fully factorial experiment

Medians were calculated on pooled data, including censored individuals (Dataset 1, sheet "medians"). The effect of each single factor as compared to the control was analyzed by means of a log-rank test on bee survival data using the pooled data from the three replicates (Dataset 1, sheet "log-rank comp. between exp. gr."). To test the effect of each factor in combination with all the others as well as all the interactions, we carried out an analysis of variance on the longevity data, after excluding censored individuals, and checking data for normality. This was performed with package "car", function "Anova" (type = II) of RStudio (Dataset 1, sheet "survival and stat.").

### 2.8. Transcriptomic analysis of honey bees by means of RNAseq

First FPKM data were checked for the presence of possible outliers by testing if any of the 48 samples had >20 % of genes with transcription values exceeding two standard deviation intervals from the average expression value (Dataset 3, sheet "data+outlier selection"). In this way one sample out of 48 was highlighted and the expression value of each gene in that sample was replaced with the average expression of that experimental group as calculated using the remaining two samples (Dataset 3, sheet "data after outlier treatment"). To limit the impact of this treatment and adopt the most conservative approach, we reduced by one the number of degrees of freedom. F statistics and *P* values were calculated according to Montgomery (2013) with Excel in a data file that accompanies this submission (Dataset 3, sheet "data after outlier treatment"). Only genes with an average expression value higher than 10 were considered as expressed and included in this analysis. Out of the expressed genes, only those for which a P value smaller than 0.005 were considered as differentially expressed. We opted for this approach rather than a more conservative correction for multiple comparisons, to reduce to the minimum the number of false negative, in view of the purpose of the analysis that was aimed at gaining any possible early warning signal of potential sub-lethal effects.

## 3. Results

### 3.1. Lethal effect of sulfoxaflor and the other stress factors

To assess the lethal effect of sulfoxaflor and the other factors either alone and in combination with the other stressors, we carried out a fully factorial experiment in which we exposed or not caged bees, fed ad libitum with sugar syrup and pollen to the following factors and their combinations: an infestation at the pupal stage with the parasitic mite *V. destructor* (V+), a sub-optimal environmental temperature (T+), the deprivation of pollen (PD+) and the contamination of the sugar diet with a field realist dose of sulfoxaflor (S+) (Fig. 1).

#### 3.1.1. Assessing the "exclusive effect" of each stress factor

The described experiment allowed us to estimate the median survival of honey bees exposed to various stressors (Dataset 1); these estimates were reported on the vertexes of an hypercube with the edges aligned along the direction of change of the factors under consideration (Fig. 2A). In this way, starting from the bottom left internal vertex of the hypercube, representing the median survival of control bees, and moving along the three edges departing from that vertex, the effect of each single factor in isolation from the others can be recognized (Fig. 2A). We may call this the "exclusive effect" of each factor because it is the effect of that factor when no other stressors are present; this corresponds to the effect that would be obtained with a simple unifactorial analysis. In this perspective, in presence of pollen, neither *Varroa* nor sulfoxaflor had a negative effect on honey bee survival (control vs. V+: Log-rank test, $P = 0.693$; control vs. S+: Log-rank test, $P = 0.874$), the effect of a sub-optimal temperature only approached significance (control vs. T+: Log-rank test, $P = 0.088$), whereas a significant impact of pollen deprivation was observed (control vs. PD+: Log-rank test, $P = 0.014$).

#### 3.1.2. Assessing the "inclusive effect" of each stress factor

We then considered the effect of each stressor when applied together with any of the other factors and their combinations; in this way we wanted to assess if each stressor is harmful or not under most circumstances (i.e. with or without three other concurring stressors). We call this the "inclusive effect" of the factor under study because it includes both the effect of that factor and all the possible interactions with the other stressors. Graphically, the inclusive effect of a stressor can be appreciated by comparing the two halves of the hypercube obtained by cutting the solid with a plane perpendicular to the direction along which the stressor under study varies (Fig. 2B, C, D, E). A difference between the two half cubes denotes the inclusive effect of that stressor, because shorter/longer survival is observed in bees exposed to that stressor both in presence or not of three other stressors of different quality; to check the statistical significance of the observed differences a four ways ANOVA was applied (Table S4).

The survival values (in days) in the right half of the cube (where experimental groups including mite infested bees are located) were always smaller than the values in the left half cube regardless of the identity of the other concurring stressors (Fig. 2A). In fact, the median survival of all bees exposed to *Varroa* was 26 % lower than that of uninfested bees (Fig. 2B; median survival of uninfested bees = 19, median survival of mite infested bees = 14; $F = 17.935$, $Df = 1$, $P < 0.001$; Table S4). This denotes a significant inclusive effect of *Varroa* parasitism, indicating that the mite exerts an effect which is negative under most conditions.

Similarly, by comparing the lower half cube with the upper one, a significant reduction in survival (i.e. −16 %) could be noted (Fig. 2C; median survival of bees exposed to a normal temperature = 19.0, median survival of bees exposed to a low temperature = 16.0; $F = 25.583$, $Df = 1$, $P < 0.001$ Table S4). In fact, in all cases but one (i.e. *Varroa* infested, pollen deprived bees) smaller values of median survival were found moving upwards along the edges of the hypercube (Fig. 2A). Therefore, a temperature lower by only two degrees and a half with respect to the hive temperature can exert an effect that is negative under most circumstances, irrespective of the quality/quantity of the other stressors.

To assess the inclusive effect of pollen deprivation on bees, the internal cube, displaying the median survival of bees from all the experimental groups which were fed pollen ad libitum was compared to the external cube, where the median survival of pollen deprived bees is represented (Fig. 2D). In this case a 16 % difference in median survival was noted (median survival of pollen fed bees = 19.0, median survival of pollen deprived bees = 16.0; $F = 10.566$, $Df = 1$, $P = 0.001$; Table S4). In this case effects were more variable; for example, pollen deprivation

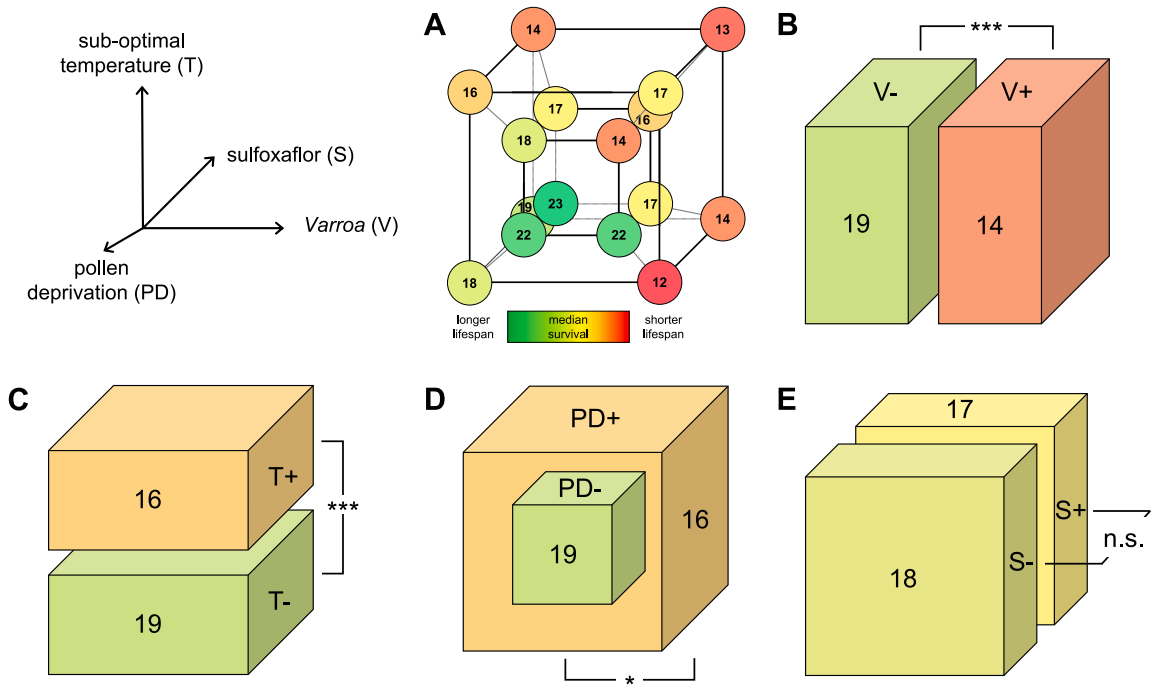


**Fig. 2.** Graphical representation of the results of the four-factors fully factorial experiment. A) median survival (numbers in the colored circles) in days of honey bees exposed to the factors displayed along the four axes represented on the left; different values are denoted with different colors (dark green for the longest survival and red for the shortest). Moving along the four edges departing from the bottom-left, internal vertex of the hypercube, the "exclusive effect" of each stressor can be recognized by the changing color. B, C, D, E) Graphical representation of the "inclusive effect" of B) *Varroa*, C) temperature, D) pollen deprivation, E) sulfoxaflor. The figures in the half cubes represent the median survival in days of the bees belonging to all the experimental groups treated or not with the factor under consideration; different values are denoted with different colors. Three, two and one asterisk mark significant differences between treatments at $P < 0.001$, 0.01 and 0.05, respectively.

appeared to be always negative in case of an optimal temperature (moving from the internal square to the external one in the lower half of the hypercube), whereas the same was not always true at a lower temperature (Fig. 2A).

Finally, the back half of the cube, where sulfoxaflor treated experimental groups are represented, was only little different from the front half (i.e. −6 %), indicating that this compound had only a small, not significant, general impact on bees (Fig. 2E; median survival of untreated bees = 18.0, median survival of sulfoxaflor treated bees = 17.0; F = 0.305, Df = 1, $P = 0.581$ Table S4). This situation is the result of a variable effect of sulfoxaflor on bees exposed to different stressors; for example, while sulfoxaflor does not seem to reduce the survival of pollen fed uninfested bees maintained at normal temperature, it appears to decrease the survival of pollen fed, mite infested bees at the same temperature (Fig. 2A).

### 3.1.3. *Assessing the "interactive effect" of each stress factor*

To study how each stressor affects the response of honey bees to the others, and in particular to test if sulfoxaflor can influence the response of bees to the other stress factors, aggravating or mitigating their effect, we studied the binary interactions between factors and in particular those involving sulfoxaflor; we may call this the "interactive effect" of sulfoxaflor. In the hypercube such effects can be noted graphically by comparing the quarter cubes obtained by further dividing by two the half cubes illustrating the effect of a certain factor (i.e. *Varroa*, temperature and pollen deprivation) with a plane separating the front and the back of the hypercube, so as to separate the effect of sulfoxaflor on the bees exposed or not to the factor under study (Fig. 3).

By doing so it is possible to note that, sulfoxaflor does not seem to influence the response of bees to mite infestation, since the reduced survival of mite infested bees is not further modified by sulfoxaflor contamination (Fig. 3A; sulfoxaflor x *Varroa*: F = 2.779, Df = 1, $P = 0.096$; Table S4).

Instead, while the effect of sulfoxaflor on bees exposed to an optimal temperature is limited and the two lower quarter cubes are similar (Fig. 3B), that on thermally stressed bees seems to be higher, in that the survival of bees exposed to a low temperature is further decreased by sulfoxaflor contamination (Fig. 3B; sulfoxaflor x temperature: F = 3.894, Df = 1, $P = 0.049$; Table S4). Finally, when a similar approach was applied to assess the possible interaction between sulfoxaflor and pollen deprivation (Fig. 3C), no significant effect was found (sulfoxaflor x pollen deprivation: F = 0.008, Df = 1, $P = 0.927$; Table S4); in other words, pollen deprivation doesn't seem to aggravate the effect of the tested dose of sulfoxaflor.

To gain insight into the interactive effect of sulfoxaflor and temperature, we carried out a complementary study on the thermoregulation capacity of sulfoxaflor exposed worker bees. We found that both pollen fed and pollen deprived honey bees, that had been exposed to a sublethal dose of sulfoxaflor were less capable of maintaining a body temperature above 34 °C when exposed to room temperature, as compared to untreated control bees (Dataset 2; Fig. 3D, E; pollen fed bees: Mann Whitney *U* test, n1 = 48, n2 = 48, U = 550, $P < 0.001$; pollen deprived bees: n1 = 96, n2 = 96, U = 2870, $P < 0.001$).

The study of all the remaining combinations of factors revealed a significant interaction between temperature and pollen deprivation (temperature x pollen deprivation: F = 7.873, Df = 1, $P = 0.005$; Table S4) indicating that the negative effect of pollen deprivation is reduced at low temperature. Finally, no triple or quadruple interaction appeared to be significant.

### 3.2. *Potential sublethal effects of sulfoxaflor and the other stress factors*

To test the effect of the studied stressors and their combinations on honey bee metabolism and physiology so as to preview possible sublethal effects, we sampled 3 individual bees from each group of the experiment previously described (Fig. 1) and measured the transcription

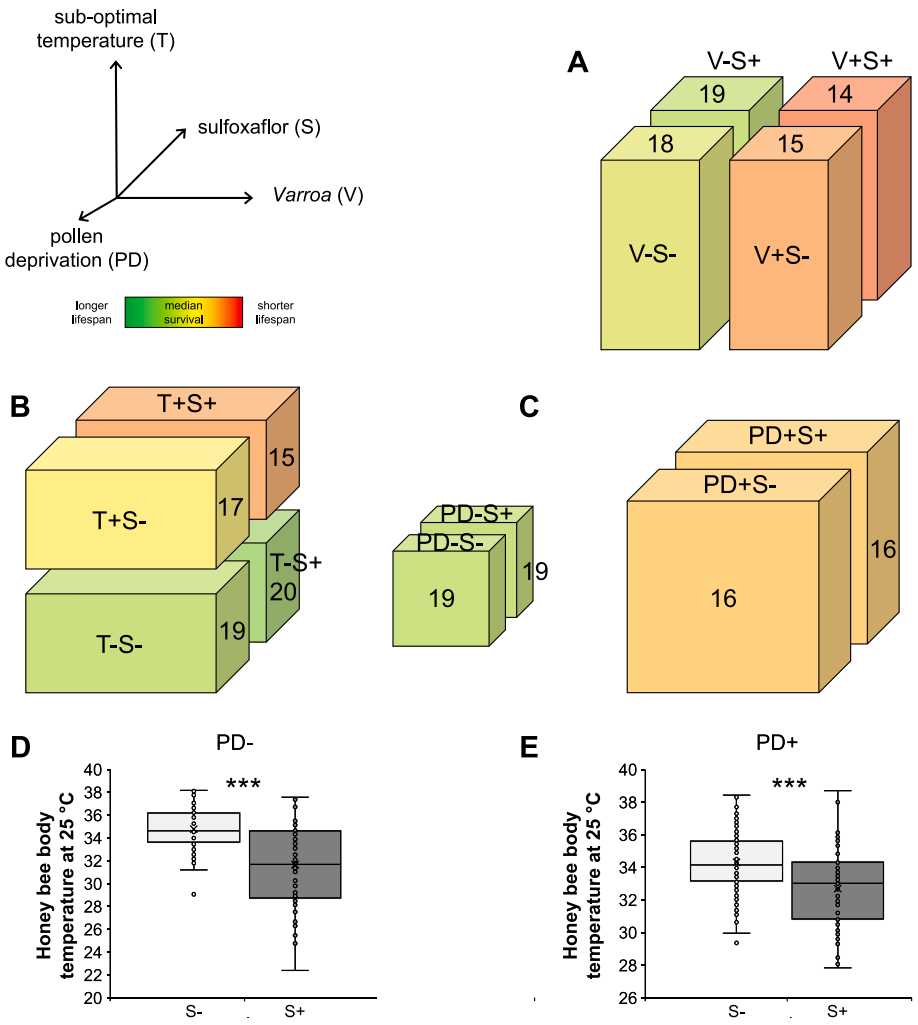


**Fig. 3.** Binary interactions between sulfoxaflor and the other factors considered in this study. A) *Varroa*, B) low temperature, C) pollen deprivation. The figures in the quarter cubes are the median survival in days of the bees belonging to all the experimental groups treated or not with that combination of factors; different values are denoted with different colors (dark green for the longest survival and red for the shortest). D) and E) body temperature of honey bees exposed to room temperature after chronic treatment with 0.07 ppm of sulfoxaflor in sucrose solution (S+) as compared to control bees (S-). Bees with free access to pollen during the experiment (PD-, panel D) or not (PD+, panel E) were used in the experiment. Three asterisks mark significant differences at $P < 0.001$.

level of each gene by means of RNAseq (Dataset 3).

The clustering of samples according to the similarity of the global pattern of gene expression highlighted a major impact of pollen deprivation, in that samples formed two major groups, mostly including either pollen fed or pollen deprived bees, respectively (Fig. 4). A factorial analysis confirmed that pollen deprivation is a major driver of gene expression, affecting the expression of 249 genes (Table 1). Among the genes differentially expressed by pollen deprivation, three major royal jelly proteins and vitellogenin were found (Dataset 3).

The other factors, and all the possible combinations, influenced the transcriptome of honey bees to different extents (Table 1; Dataset 3). In particular, sulfoxaflor caused a differential expression of 30 genes. A gene ontology study of the genes differentially expressed after sulfoxaflor contamination revealed several terms related to mitochondrial activity (Table 1; Table S5; Dataset 3), suggesting that sulfoxaflor may impair energy production in exposed bees.

Similar to sulfoxaflor, a sub-optimal temperature, significantly affected the response of 30 genes (Table 1), while a mite infestation suffered at the pupal stage did not cause a notable impact on the transcriptome of adult bees (i.e. only 5 genes appeared to be differentially expressed; Table 1).

The proposed analytical approach also allowed to clarify the interactive effect of sulfoxaflor on the transcriptome of bees, i.e. how the pesticide can influence the response of honey bees to the other stressors in terms of gene expression. It appeared that sulfoxaflor can interact with all the other factors and their combinations (Table 1, Dataset 3); interestingly, one major royal jelly protein was regulated because of the triple interaction between sulfoxaflor, temperature and pollen deprivation (Dataset 3).

To gain a deeper insight into the interactive effect of sulfoxaflor at the molecular level (i.e. its capacity to influence the response of the bees' transcriptome to other stress factors) we used qRT-PCR to further investigate the expression of the genes encoding four major royal jelly proteins and assessed how sulfoxaflor interacts with either *Varroa* or a low temperature or pollen deprivation (Dataset 4). We concentrated on royal jelly proteins because, in this study, three major royal jelly proteins were down-regulated in pollen deprived bees and MRJP1 was also regulated by the interaction between sulfoxaflor, temperature and pollen deprivation. Furthermore, these proteins play multiple roles in honey bees and may be influenced by pesticides (Fent et al., 2020). Our analysis revealed that, despite the limited effect of sulfoxaflor on the expression of these genes (Fig. 5, top row of panels), the insecticide interacted with both *Varroa* and low temperature, significantly altering the response of bees to those stressors, as highlighted by the crossing lines in the second and third rows of panels in Fig. 5.

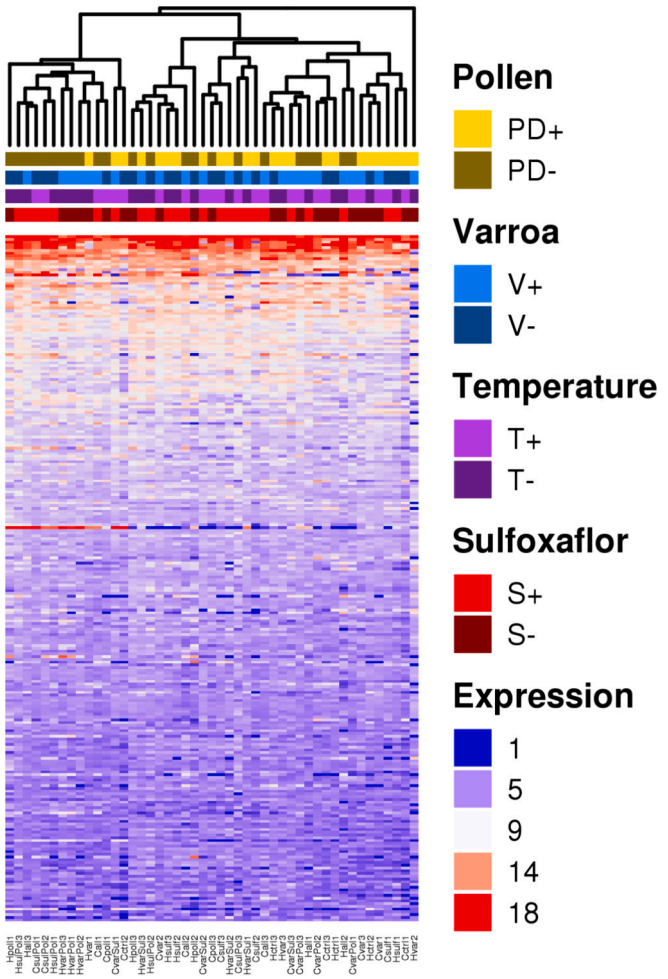


**Fig. 4.** Clustering of bee samples exposed or not to pollen deprivation, *Varroa* mite infestation, low temperature or sulfoxaflor contamination, according to the pattern of gene expression. Each column is a sample and the most expressed 250 genes are the rows in the heatmap.

## 4. Discussion

The study of the effect of each single factor in isolation from the others (i.e. the "exclusive effect" of the factors), revealed that only pollen deprivation had a significant impact on honey bee survival, whereas the effect of low temperature only approached significance. Instead, neither *Varroa* nor sulfoxaflor had a negative impact on honey bee survival.

The fact that, in presence of pollen, 0.07 ppm of sulfoxflor in sucrose solution did not cause a significant reduction in the survival of adult bees is in line with previous findings (Barascou et al., 2021; Tamburini et al., 2021). The lack of effect of the main ectoparasite of honey bees, in presence of pollen was expected in view of the beneficial effect of pollen in mite infested bees (Annoscia et al., 2017; Frizzera et al., 2022). A cooler temperature didn't significantly reduce bee survival contrary to previous results (Frizzera et al., 2023) while the negative effect of pollen deprivation was confirmed (Brodschneider and Crailsheim, 2010).

Overall, these results show that an unifactorial approach to testing, highlighting the exclusive effect of each stressor, would indicate that at the tested dose, sulfoxaflor via nectar route have little or no effect, consistent with a No Observed Effect Concentration (NOEC) of 0.5 mg/Kg previously proposed (EFSA (European Food Safety Authority), 2019). On the other hand, such kind of analysis would indicate a negligible effect of another stress factor (i.e. the *Varroa* mite) whose negative effect, instead, is well known; this clearly highlights the limits of the unifactorial approach, encouraging a more comprehensive evaluation of the pesticide's effect under a more realistic scenario, incorporating in the analysis the effect of the other stressors.

When the effect of each factor in combination with the others was studied, a different scenario emerged, in that both *Varroa* parasitism, low temperature and pollen deprivation, in a mutifactorial context, appeared to significantly impact honey bee survival, consistent with current knowledge (Nazzi and Le Conte, 2016; Frizzera et al., 2023; Brodschneider and Crailsheim, 2010). Instead, sulfoxaflor had no significant effect on honey bee survival. According to the nomenclature we proposed, we can state that both *Varroa* parasitism, a temperature lower by two degrees to the in-hive conditions and the lack of pollen have a negative inclusive effect on honey bee survival, whereas that of a field realistic dose of sulfoxaflor is negligible. It may be argued that the negative results we obtained with sulfoxaflor may be related to the tested dose and a dose-response study would have been more informative. However, it should be noted that the dose used here was selected based on previous studies regarding the chemical's concentration in nectar and pollen (Al Naggar and Paxton, 2021) and can therefore be regarded as a likely exposure. Under this point of view, the approach we propose underlines the importance of parallel exposure assessment studies, already implemented in pesticide risk assessment procedures (EFSA (European Food Safety Authority), 2019).

The study of the binary interactions between stressors, to highlight how each stressor can modify the response of honey bees to the other stress factors, revealed some interesting cases. On one hand, sulfoxaflor did not alter the response of bees to mite infestation or pollen deprivation, contrary to what observed by Barascou and coworkers, whom, however, tested this interaction at a single temperature of 30 °C (Barascou et al., 2021). On the other hand, the insecticide appeared to influence the survival of honey bees concurrently exposed to low temperature. This result is consistent with that of our complementary experiment which showed that sulfoxaflor exposed worker bees have a reduced thermoregulation capacity. This result suggests that indeed sulfoxaflor may influence the response of honey bees to low temperatures as already shown for other insecticides (Alburaki et al., 2023).

Regarding the other binary interactions, the significant one between temperature and pollen deprivation is certainly worth of further study, also in relation to the dynamics of pollen collection along the season (Danner et al., 2017). Instead, the lack of a significant interaction between *Varroa* and pollen deprivation was rather unexpected, because it apparently contradicts previous observations regarding the beneficial effect of pollen on mite infested bees (Annoscia et al., 2017; Frizzera et al., 2022).

According to our analysis, both the four triple interactions and the quadruple interaction between stress factors were not significant (Table S4). This was largely expected because most systems are dominated by some of the main effects and low order interactions, while most high order interactions are normally negligible (Montgomery, 2013). For this reason, we would suggest not to consider the lack of significance of high order interactions as an indirect indication of a limited effect of the factors under study, but rather concentrate the attention on inclusive effects and binary interactions.

In conclusion, our multifactorial approach to the study of the effect of sulfoxaflor on honey bee survival, indicates that this pesticide at the tested concentration, does not significantly reduce honey bee lifespan, even in presence of three other common stressors. However, sulfoxaflor seems to aggravate the negative effect of a low temperature.

The study of the transcriptome of the honey bee samples collected from the four factors, fully factorial experiment allowed to investigate at the molecular level the effect of each factor in combination with the others on honey bees. This work was carried out to highlight any interesting pattern that could reveal early signals of potential sub-lethal effects of the studied factors on bees.

Pollen deprivation appeared to be responsible of the major impact on gene expression in honey bees as it was largely expected since pollen is

**Table 1**
Number of genes that were differentially expressed (DEGs) in response to each factor and all their possible combinations and significantly over-represented gene ontology terms. The three GO terms with highest *P*-value are reported along with their category (CC: cellular component, BP: biological process, MF: molecular function), odd-ratio and P-value). Sulfoxaflor treated samples are highlighted in grey. For a more detailed list of differentially expressed genes see Dataset 3; for the genes regulated by sulfoxaflor see also Table S5.

| Factor/factors combination | n. of DEGs | GO term | GO category | OR | *P* |
|---|---|---|---|---|---|
| Sulfoxaflor | 30 | mitochondrial respiratory chain complex I | CC | 70.71 | <0.001 |
| | | proteolysis | BP | 15.60 | <0.001 |
| | | respiratory chain | CC | 40.01 | <0.001 |
| *Varroa* | 5 | protein-containing complex | CC | 18.52 | 0.001 |
| | | protein binding | MF | 2.91 | 0.026 |
| | | mitochondrion | CC | 4.68 | 0.032 |
| Temperature | 30 | nucleic acid binding | MF | 4.57 | <0.001 |
| | | histone deacetylase complex | CC | 69.38 | <0.001 |
| | | arginine kinase activity | MF | 341.40 | <0.001 |
| Pollen deprivation | 249 | ribosome | CC | 6.79 | <0.001 |
| | | translation | BP | 5.38 | <0.001 |
| | | structural constituent of ribosome | MF | 7.20 | <0.001 |
| Sulfoxaflor x *Varroa* | 2 | | | | |
| Sulfoxaflor x Temperature | 11 | chromocenter | CC | 655.55 | <0.001 |
| | | nucleic acid binding | MF | 12.47 | <0.001 |
| | | RNA binding | MF | 10.61 | <0.001 |
| Sulfoxaflor x Pollen deprivation | 3 | | | | |
| *Varroa* x Temperature | 4 | mRNA splice site selection | BP | 732.59 | <0.001 |
| | | U2-type prespliceosome | CC | 446.11 | <0.001 |
| | | U1 snRNP | CC | 389.49 | <0.001 |
| *Varroa* x Pollen deprivation | 31 | caudate nucleus development | BP | Inf | <0.001 |
| | | putamen development | BP | Inf | <0.001 |
| | | mitochondrial ATP synthesis coupled proton transport | BP | 272.46 | <0.001 |
| Temperature x Pollen deprivation | 4 | transcription by RNA polymerase II | BP | 73.79 | <0.001 |
| | | nucleus | CC | 3.50 | 0.042 |
| Sulfoxaflor x *Varroa* x Temperature | 11 | transepithelial L-ascorbic acid transport | BP | Inf | 0.042 |
| | | L-ascorbate: sodium symporter activity | MF | Inf | <0.001 |
| | | sodium-dependent L-ascorbate transmembrane transporter activity | MF | Inf | <0.001 |
| Sulfoxaflor x *Varroa* x Pollen deprivation | 12 | translation | BP | 17.16 | <0.001 |
| | | translational elongation | BP | 125.53 | <0.001 |
| | | translation elongation factor activity | MF | 111.04 | <0.001 |
| Sulfoxaflor x Temperature x Pollen deprivation | 10 | mitochondrion | CC | 6.24 | 0.005 |
| *Varroa* x Temperature x Pollen deprivation | 12 | histone pre-mRNA 3'end processing complex | CC | 1151.63 | <0.001 |
| | | negative regulation of translation | BP | 58.71 | <0.001 |
| | | DNA binding | MF | 5.70 | <0.001 |
| Sulfoxaflor x *Varroa* x Temperature x Pollen deprivation | 5 | mitochondrial respiratory chain complex II assembly | BP | 3789.94 | <0.001 |
| | | succinate metabolic process | BP | 986.25 | <0.001 |
| | | mitochondrial intermembrane space | CC | 86.85 | <0.001 |

the major source of aminoacids for protein synthesis in honey bees (Frias et al., 2016). On the other hand, regardless of the limited impact on survival, sulfoxaflor influenced the expression of thirty genes, indicating that this compound may affect the metabolism and/or the physiology of honey bees; in particular, a significant impact on genes involved in energy production was noted. It has been previously shown that neonicotinoid insecticides can affect mitochondrial bioenergetics in honey bees (Nicodemo et al., 2014) and bumblebees (Powner et al., 2016). More recently, Liu and coworkers, using the midge *Chironomus kiinensis* as a model species, found that sulfoxaflor can display a similar inhibitory activity on mitochondrial energy production (Liu et al., 2021). In view of the increased oxygen consumption when flight muscles are activated during heating (Goller and Esch, 1991), we speculate that the reduced thermoregulatory capacity of sulfoxaflor exposed bees, that we demonstrated with our complementary experiment, may depend on the effect of sulfoxaflor on mitochondrial activity highlighted through the transcriptomic analysis. If practical constraints had not precluded the measurement of sugar consumption in our experimental bees, we could have assessed if any compensation mechanism (e.g. increased sugar consumption to produce more energy) is exploited by sulfoxaflor treated bees to counteract the effect we noted.

Overall, it appears that a transcriptomic approach has the potential of revealing critical functions that could be affected by a pesticide in a multifactorial context. We suggest that, once identified through this approach, such functions should be better studied using the most convenient bioassays under appropriate conditions. For example, in this case, given the importance of the energetic metabolism to support the contraction of flight muscles, both thermoregulation and flight activity should be addressed. Our own results about thermoregulation (Fig. 3D, E) and those reported elsewhere regarding the flight activity of sulfoxaflor exposed bees (Table S1) further corroborate the robustness of our approach.

The little number of genes regulated by *Varroa* parasitism may appear surprising in view of previous transcriptomic studies of mite infested bees which highlighted a larger number of differentially expressed genes (Zanni et al., 2017; Annoscia et al., 2019) and the strong impact of the mite on bee survival. On the other hand, the limited number of differentially expressed genes observed here is consistent with the long time elapsed after parasitization (i.e. bees were sampled 7 days after emergence, when mite infestation ceased) and the fact that, in this case, the bees suffering the most severe effect of mite infestation had likely died before sampling (as shown by the reduced median survival of mite infested bees).

Finally, the factorial analysis of gene expression data evidenced

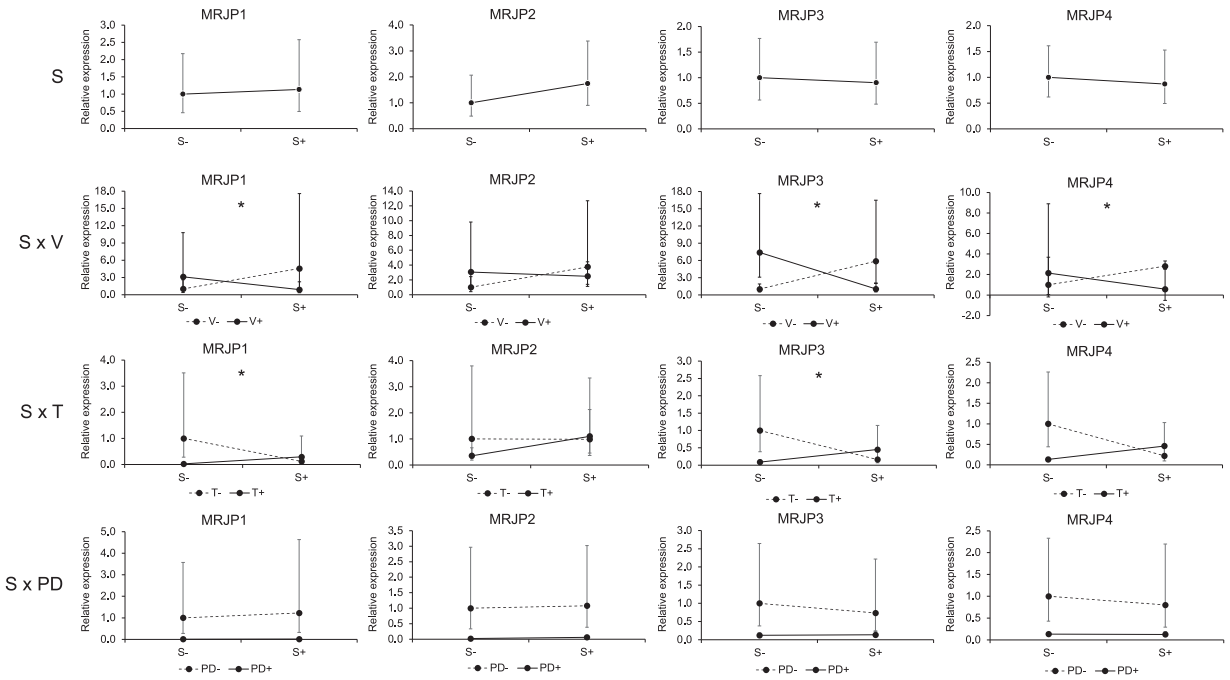


**Fig. 5.** Impact of sulfoxaflor (S) and the interactions between sulfoxaflor and *Varroa* (S × V), sulfoxaflor and temperature (S × T), sulfoxaflor and pollen deprivation (S × PD) on the expression of four major royal jelly proteins of the honey bee. An asterisk marks significant effects at $P < 0.05$.

other little groups of genes regulated by the triple and quadruple interactions between factors. This limited number of regulated genes is consistent with the relatively scarce number of higher order interactions that are normally found through multifactorial experiments (Montgomery, 2013). For this reason, the fact that a major royal jelly protein (i.e. MRJP1) was found to be regulated by the triple interaction between sulfoxaflor, temperature and pollen deprivation was regarded as worth of interest. Royal jelly proteins represent the main protein components of royal jelly that is used to feed honey bee larvae but likely play multiple roles (Buttstedt et al., 2013). MRJP1 has a fundamental role in honey bee development (Kamakura, 2011) but also possesses some remarkable antibiotic properties (Park et al., 2019). Furthermore, this protein plays important roles in learning and memory (Hojo et al., 2010). Recently, it was suggested that MRJP1 may also attenuate the harmful effects of sulfoxaflor on honey bees (Shi et al., 2022); furthermore, a recent study investigating the interactive effect of Acetamiprid, *Nosema ceranae* and *V. destructor* found that four major royal jelly proteins were down-regulated in all treated groups (Kang et al., 2024).

Our gene expression study revealed a significant influence of sulfoxaflor on the expression of MRJPs in bees exposed to *Varroa* and low temperature. Given the fundamental role of MRJP1 for honey bee development and activity, the observed effect is indicative of important interactive effects of sulfoxafor. They are worth to be investigated more in detail with convenient bioassays addressing all the potentially affected functions, including development, caste differentiation, immunity and bee brain functions (Kamakura, 2011; Park et al., 2019; Hojo et al., 2010; Shi et al., 2022). A similar approach could also be applied to the other genes highlighted by our transcriptomic analysis (Table 1 and Dataset 3).

## 5. Conclusions

Our results suggest that sulfoxaflor has a limited effect on honey bee survival at the tested dose even when applied in combination with the other most common stress factors. On the other hand, it was found that sulfoxaflor may induce alterations that can affect individual metabolism and physiology; these, in turn, could induce significant sublethal effects on bees.

Moreover, our work demonstrates how a multifactorial approach to hazard assessment allows to study, in a realistic context, possible lethal effects and reveal unexpected sublethal effects that are worth of further investigation prior to authorization, so as to reduce the environmental risks related to the release and subsequent ban of novel pesticides (Sgolastra et al., 2020; Siviter et al., 2023). We suggest that the concept of inclusive and interactive effect of pesticides that we introduced here should be incorporated into risk assessment procedures of insecticides, further than the already largely used concept of exclusive effect. In this way, it will be possible to account for the interactions among different stress factors that could never be clarified through traditional field experiments. We also propose to exploit the power of omic techniques and in particular transcriptomics to collect early warning signals of potential sublethal effects that can guide further investigations by facilitating the selection of the most convenient bioassays to be carried out for the purpose.

With this work, we wish to contribute to a paradigm shift in toxicology: from an unifactorial, mortality-centered assessment to a multifactorial, comprehensive approach. This progress is of the utmost importance to preserve pollination, thus contributing to biodiversity maintenance and food production (Potts et al., 2016).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.174892.

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## Credit authorship contribution statement

**Davide Frizzera:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Virginia Zanni:** Writing – review & editing, Investigation, Data curation. **Elisa Seffin:** Writing – review & editing, Investigation, Data curation. **Joachim Rodrigues de Miranda:** Writing – review & editing, Formal analysis, Data curation. **Fabio Marroni:** Writing – review & editing, Formal analysis, Data

curation. **Desiderato Annoscia:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francesco Nazzi:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Francesco Nazzi reports financial support was provided by European Union. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Datasets 1, 2 and 4 are available in the Supplementary material section as Excel files, while dataset 3 is available at https://figshare.com/articles/dataset/dataset3_xlsx/24658518

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