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Chronic laboratory exposure to environmentally relevant concentrations of Mospilan 20 SP (acetamiprid): effects and molecular responses in honeybees (*Apis mellifera* L.)

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Abstract: Bees were exposed for 10 days to a sucrose solution containing acetamiprid at concentrations of 48, 4.8, and 0.48 mg a.i./kg of solution, corresponding to the maximum recommended field application rate and its 1/10 and 1/100 dilutions. An increase in mortality was observed at the highest tested concentration, with a cumulative mortality of 30.4% by day 10, whereas lower concentrations showed effects comparable to the control. In addition, the expression of selected genes related to detoxification (glutathione S-transferase, thioredoxin reductase), oxidative stress (superoxide dismutase 1, superoxide dismutase 2), immunity (abaecin, hymenoptaecin, apidaecin), and neural regulation (acetylcholinesterase 1) was analysed. No significant changes were detected in most genes, except for *sod2*, which showed increased expression at the lowest concentration. Overall, the results suggest that acetamiprid exhibits relatively low chronic toxicity at environmentally relevant concentrations but may induce significant effects at higher or continuous exposure levels. These findings highlight the importance of considering both lethal and sublethal endpoints, including molecular responses, in assessing pesticide risks to honeybees.

Keywords: agrochemical; bee colony; pollinator; plant protection; neonicotinoid

Honeybees (*Apis mellifera* L.) inhabit a wide range of environments, from wild to intensively farmed and urban landscapes, where they are exposed to various stress factors. The most significant of these include pathogens, limited food availability, and exposure to agrochemicals. These factors have been shown to affect bee immunocompetence, viral replication, and the course of pathogenesis (Goulson et al. 2015). The health of bee colonies is closely associated with the condition of the landscape. Habitat degradation and reduced diversity of flowering plants diminish nutritional quality, increasing bee susceptibility to infections. Forage sources thus play a dual role in this regard: on the one hand, they provide essential nutrients; on the other, they act as potential vectors for pathogens and agrochemicals (McMenamin et al. 2016, Lin 2024). The consequences of these stressors are

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reflected in the globally observed losses of bee colonies (Steinhauer et al. 2018, Hristov et al. 2020).

Given their fundamental importance to the pollination of both agricultural and wild plants, bees are a key ecological and economic resource. A decline in their populations can have significant negative impacts on crop production and ecosystem stability, which is why identifying the factors causing population losses is extremely important. In addition to pathogens, the most commonly cited stressors include exposure to plant protection products (PPPs), low food nutritional quality and habitat degradation (Lin et al. 2024).

The global use of PPPs in agriculture is increasing, a trend associated with population growth and the intensification of agricultural production (Popp et al. 2013). While these products contribute to the economic efficiency of growers, they also pose a risk to humans and non-target organisms (Geiger et al. 2011). A salient disadvantage of this approach is its non-selective nature (Carlile 2006), which can harm beneficial species, including honeybees (Genersch 2010, Calatayud-Vernich et al. 2018). Research has demonstrated that diverse classes of pesticides have the capacity to modify the detoxification and antioxidant systems of bees, thereby affecting enzyme activity, ATP levels, proteins, and glutathione (Renzi et al. 2016, Almasri et al. 2020, Paleolog et al. 2020, 2021). The most vulnerable are worker bees, which come into the most frequent contact with contaminated food. Furthermore, numerous *in vitro* studies have utilised short-term exposure to a singular substance at elevated doses (Medrzycki et al. 2003, Yang et al. 2020, Battisti et al. 2021), a condition that does not align with the real-world scenario in which bees are exposed to combinations of various PPPs at low concentrations (Tang et al. 2021).

One of the most significant groups of PPPs, whose impact on bees is widely discussed, is that of neonicotinoids. These substances target the nicotinic acetylcholine receptors of insects and disrupt the functioning of their nervous system (Elbert et al. 2008, Simon-Delso et al. 2015). Neonicotinoids can be categorised into two distinct groups: the nitro group, which includes compounds such as imidacloprid, clothianidin and thiamethoxam, and the cyano group, which comprises acetamiprid and thiacloprid (Iwasa et al. 2004, Blacquière et al. 2012). While nitro-group neonicotinoids are recognised for their high toxicity to pollinators and have been prohibited in the EU (EU 485/2013, Suchail et al. 2000), cyano-neonicotinoids, including acetamiprid, demonstrate lower acute toxicity (Iwasa et al. 2004) and consequently have received less attention regarding their chronic effects.

Acetamiprid is a widely active substance of plant protection products in the agricultural sector, particularly in the context of fruit and vegetable crops. It is predominantly administered through foliar spraying (USEPA 2002, Elbert et al. 2008). After application, it can contaminate nectar and pollen (Mullin et al. 2010, David et al. 2016, Böhme et al. 2018, Sabo et al. 2024), thereby posing a genuine exposure risk to pollinators. Despite the perceived minimal risk of acute exposure, there remains a paucity of data on the effects of chronic exposure to low doses, which are more characteristic of prolonged exposure than of short-term contact with high concentrations.

The objective of this study was to test the hypothesis that chronic exposure to Mospilan 20 SP (acetamiprid) increases mortality and affects gene expression related to detoxification, oxidative stress, immunity, and neural regulation in honeybees (*Apis mellifera*) under standardised OECD 245 (2017) conditions. Test concentrations were based on the recommended field application rate and included the maximum concentration and its 1/10 and 1/100 dilutions to ensure environmental relevance.

MATERIAL AND METHODS

Chemicals and concentrations used. In this study, rather than utilising the active substance acetamiprid in isolation, we tested the commercial product Mospilan 20SP 0.6 g®, purchased from Floraservis, spol. s r. o. (NIPPON SODA, Tokyo, Japan), to emulate agricultural conditions.

The test substance that was the subject of the study was the registered plant protection product Mospilan 20 SP, which contains acetamiprid as the active substance (200 g/kg). Test concentrations were prepared by dissolving the product in a 50% (*w*/*v*) sucrose solution, followed by dilution of the stock solution to obtain a series of three concentrations (1/1 M, 1/10 M, and 1/100 M; M = Mospilan 20 SP solution). The highest concentration represented the maximum recommended field application rate, while the lower concentrations were prepared as serial dilutions to reflect decreasing exposure levels. The selection of concentrations was based on the product's recommended application dosage in Slovakia and aligned with the requirements of OECD guideline 245 (2017).

The reference substance employed in the present experiment was dimethoate (nominal concentration

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of 1.0 mg a.i./kg diet) in accordance with OECD 245 (2017).

Bee collection and preparation. The experimental honeybee workers were collected from the university apiary. A comprehensive series of checks and assessments was conducted to ensure that the colonies had not been exposed to chemicals that could affect the research objectives. The experiment utilised young bees, with a maximum age of two days, reared from brood combs obtained from colonies containing a healthy queen with no signs of bee disease. These brood combs were selected based on their ability to maintain the queen's health and physiological status. The brood combs contained stores, allowing newly emerged bees access to pollen during the first hours after emergence. The combs were transferred to an incubator simulating hive conditions and stored until worker bees hatched. The incubator was maintained at a temperature of 34 °C (± 0.5) and a relative humidity of 70% (± 5), with the environment kept in darkness. The newly emerged worker bees were collected and placed in stainless steel boxes or cages, with approximately 50 bees allocated to each box. The dimensions of the boxes are as follows: 10 × 10 × 5 cm.

Experimental design. Each test group was replicated five times, with the total number of bees utilised in each group as follows: control group (301 individuals), group 1/1 M (260 individuals), group 1/10 M (265 individuals), and group 1/100 M (278 individuals). The study utilised approximately 1 100 test bees.

Before the commencement of the exposure phase, the bees were provided with a 50% (*w*/*v*) sucrose solution *ad libitum* during a 24-hour acclimatisation period (OECD 245 2017). The bees were then exposed to feeding solutions containing Mospilan 20 SP at the maximum tested concentration and its dilutions of 1/10 M and 1/100 M (hereinafter referred to as 1/1 M, 1/10 M, and 1/100 M). The control group was administered an untreated 50% (*w*/*v*) sucrose solution.

During the 10-day exposure phase, the subjects were provided with feed solutions *ad libitum*, which were replaced daily. Diet consumption was meticulously monitored by weighing the feeding microtubes (Ependorf) before and after a 24-hour feeding interval. The expected daily sucrose solution consumption of 40.0 mg per bee was based on results from Schmitzer and Kling (2014). The quantity of diet consumed was expressed. The mean daily syrup intake for each treatment was corrected for evaporation losses throughout the exposure period, in accordance with OECD 245 (2017).

As Mospilan 20 SP is characterised by high water solubility, the utilisation of organic solvents was deemed unnecessary in the experimental procedure. Aqueous sucrose solutions with the test product were prepared one day before use, stored at +4 °C in the dark, and prepared fresh daily during the exposure phase (OECD 245 2017).

To verify the sensitivity and reliability of the biological test system, the reference substance, dimethoate, was utilised in the bioassay at a nominal concentration of 1.0 mg a.i./kg diet, as recommended by OECD method 245 (2017).

The mortality and any behavioural abnormalities were recorded daily throughout the experiment. On the tenth day, the test bees were anaesthetised by short-term cooling. This was achieved by placing the cages in a refrigerator for 30 min. The bees from each treatment were then pooled and transferred to labelled plastic containers for further analysis.

RNA isolation and cDNA synthesis. At the end of the experiment, the surviving bees from each group were dissected to obtain the intestine. A total of 10 intestines were collected for one cage. Each intestine was washed in phosphate-buffered saline (Sigma-Aldrich, Burlington, USA) to remove any remaining intestinal content and was stored in RNAlater (Thermo Fisher, Vilnius, Lithuania) at –20 °C until further processing.

Pooled intestines were first homogenised using a hand pestle. The RNA was isolated using TRIzol (Invitrogen, Carlsbad, USA) reagent according to the manufacturer's protocol. The concentration and purity of the obtained RNA were assessed using a spectrophotometer, Nanodrop 8000 (Thermo Scientific, Waltham, USA) at 260/280 nm. For the cDNA synthesis, the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) was used. The same amount of RNA (1 000 ng) was transcribed from each sample. The resulting cDNA was used as a template for qPCR.

Gene expression analysis (qPCR). Relative gene expression of selected genes (Table 1) was quantified using the thermocycler croGENE Real-Time PCR System (GeneProof, Brno, Czech Republic). PCR was then done in 10 µL reactions, each containing 5 µL Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, USA), 0.3 µL forward and 0.3 µL reverse primer (c = 10 µmol/µL) for selected genes (Table 1), 4 µL of cDNA with a concentration of

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Table 1. Primers used in the present study

Primer	Sequence	References
rp49 (ribosomal protein 49)	F: AAGTTCATTCGTCACCAGAG R: CTTCGAGTTCCTTGACATTATG	de Miranda and Fries (2008)
gapdh (glyceraldehyde-3-phosphate dehydrogenase)	F: GATGCACCCATGTTTGTTTG R: TTTGCAGAAGGTGCATCAAC	Scharlaken et al. (2008)
gst (glutathione S-transferase)	F: GGAGAGGTGTGGAGAGATAGTG R: CGCAAATGGTCGTGTGGATG	Li et al. (2014)
sod1 (superoxide dismutase 1)	F: AGCAGATGCAAGTGGTGTTG R: GAGCACCAGCATTTCCTGTAG	Collins et al. (2004)
sod2 (superoxide dismutase 2)	F: TCGCCAAAGGTGATGTCAATAC R: CGTCTGGTTTACCGCCATTTG	Li et al. (2014)
trxr (thioredoxin reductase)	F: CAGTGAATTTTGGTGCAAAAGT R: CACCTAGACCCCAAGTGCTACC	Corona et al. (2005)
apid (apidaecin)	F: TTTTGCCTTAGCAATTCTTGTTG R: GTAGGTCGAGTAGGCGGATCT	Li et al. (2016)
hym (hymenoptaecin)	F: CTCTTCTGTGCCGTTGCATA R: GCGTCTCCTGTCATTCCATT	Evans et al. (2016)
aba (abaecin)	F:CGCACTACTCGCCACGATAT R:TCGGATTGAATGGTCCCTGAC	Li et al. (2022)
ace1 (acetylcholinesterase 1)	F: GATCGACGGCGCTTTCCTCG R: GACCCGTCGATGTGGAACAAC	Kim et al. (2023)

10 ng/µL, and 0.4 µL PCR-grade water. The PCR protocol consisted of initial denaturation at 95 °C for 60 s, followed by 40 cycles of denaturation at 95 °C for 15 s and extension at 60 °C for 30 s. Melt curve analysis was performed after the PCR to confirm amplification of the specific product. Each reaction was run in duplicate, and each assay contained a negative control without a cDNA template. The efficiency of reactions was calculated using a serial dilution and a standard curve. The reaction efficiency was 100% ± 10%. Ribosomal protein 49 (*rp49*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) were used as reference genes. Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ (± SD) method.

Statistical analysis. The toxicity data obtained were analysed using ToxRat Professional® software (ToxRat Solutions GmbH, Alsdorf, Germany).

Statistical analysis was performed using JASP (ver. 0.96.0, JASP Team, Amsterdam, the Netherlands). Due to a small sample size, the non-parametric Kruskal-Wallis test was used with a post-hoc Dunn test for multiple comparisons.

RESULTS AND DISCUSSION

In the present *in vivo* laboratory test, the effects of Mospilan 20 SP on laboratory-kept bees were determined in accordance with OECD protocol 245 (2017).

Table 2. Cumulative mortality of honeybees during a 10-day exposure period

Test item	Treatment nominal (mg a.i./kg diet)	Cumulative mortality (%)									
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Control	n.a.	0.0	0.7	1.7	1.7	1.7	2.0	2.3	3.0	5.3	6.0
Mospilan 1/1	48	0.0	0.0	3.5	3.8	3.8	5.4	6.9	7.3	20.8	30.4
Mospilan 1/10	4.8	0.0	1.1	1.9	1.9	1.9	2.3	2.3	2.3	2.3	2.3
Mospilan 1/100	0.48	0.7	1.4	3.6	3.6	3.6	5.0	5.4	6.8	7.2	7.2
Reference dimethoate	1	0.0	0.0	0.0	0.0	0.0	27.3	45.5	100.0	100.0	100.0

D – day of assessment; a.i. – active ingredient; n.a. – not applicable

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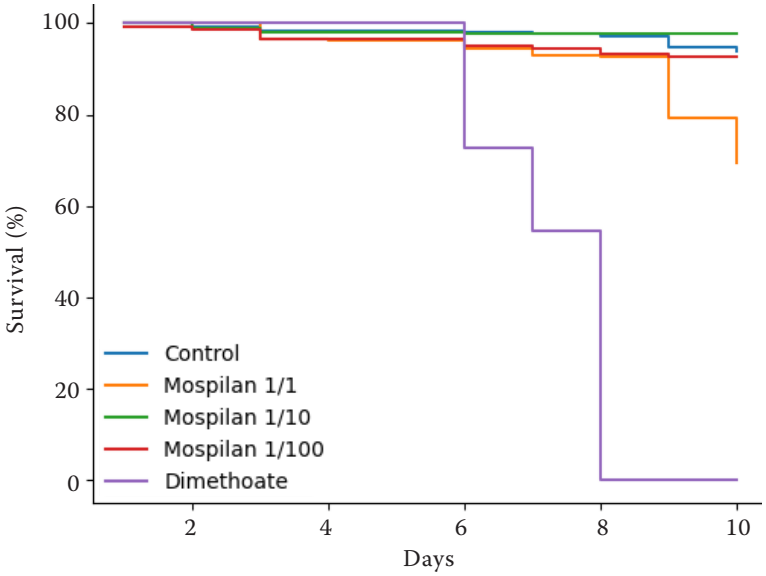

Figure 1. Kaplan-Meier survival curves *Apis mellifera*

Adult bees in cages were exposed to doses of 48 mg a.i./kg feed (1/1 M), 4.8 mg a.i./kg feed (1/10 M) and 0.48 mg a.i./kg feed (1/100 M). The observed cumulative mortality during the 10-day exposure period is presented in Table 2, while the survival dynamics are shown in Figure 1. The applied and consumed doses (nominal and recalculated) are summarised in Table 3.

The anticipated daily consumption of 40 mg of syrup per food bee was not attained in any of the groups examined. The maximum value of the total average daily syrup intake was observed in the group 0.48 mg a.i./kg feed (1/100 M) (38.12 mg/bee/day), and the minimum value 23 18 mg/bee/day in the positive control group treated with 1.0 mg dimethoate/kg die (Table 3).

In the course of 10 days of chronic exposure to honeybees, no clear linear relationship between the dose of Mospilan 20SP (acetamiprid) and mortality was observed at the lower concentrations that were tested. The cumulative mortality rate in the untreated control group was 6.0% at the end of the experiment, below the acceptance limit set by OECD Method 245 (2017). The highest mortality was observed in the group exposed to the highest Mospilan 20 SP concentration (1/1 M), indicating a concentration-dependent effect of acetamiprid on honeybee survival.

A pronounced decline in survival was observed at the highest concentration and in the reference treatment. In contrast, lower concentrations showed survival comparable to the control at the end of the experiment.

The validity criteria defined by OECD 245 (2017) were fulfilled, as mortality in the control group remained below 15% and reached 6.0%, whereas 100% mortality was observed in the reference control group at the end of the experiment. These results confirm the reliability of the experimental design and the validity of the obtained data.

The tested concentrations of acetamiprid were selected based on the maximum recommended field

Table 3. Doses applied and consumed during the 10-day exposure period

Treatment group	Treatment nominal (mg a.i./kg diet)	Treatment nominal (µg a.i./bee/day)*	Overall mean daily syrup intake (mg/bee/day)	Consumed dosage (µg a.i./bee/day)*
Control	n.a.	n.a.	31.78	n.a.
Mospilan 1/1	48	1.92	30.25	1.45
Mospilan 1/10	4.8	0.192	32.52	0.156
Mospilan 1/100	0.48	0.0192	38.12	0.0183
Reference dimethoate	1	0.04	23.18	0.023

*Based on calculations performed with non-rounded values and on theoretical daily food consumption of 40.0 mg diet/bee/day. a.i. – active ingredient; n.a. – not applicable

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application rate of Mospilan 20 SP in Slovakia. The highest tested concentration of 48 mg a.i./kg corresponded to the upper limit of the active substance in the field application solution, while the lower concentrations (4.8 and 0.48 mg a.i./kg) represented its 1/10 and 1/100 dilutions, respectively. This approach enabled the inclusion of both a worst-case exposure scenario and environmentally realistic concentrations reflecting exposure *via* contaminated nectar and pollen. As bees are not directly exposed to spray liquids but rather to residues in food, these lower concentrations are considered relevant for chronic exposure conditions.

In the present study, the maximum daily intake of acetamiprid (1.45 µg/bee/day) remained well below the reported oral median lethal dose (LD_{50} value) of (~7 µg/bee) (Iwasa et al. 2004), confirming that the observed effects were associated with chronic rather than acute toxicity. Despite this, the highest concentration tested (48 mg a.i./kg) resulted in increased cumulative mortality, reaching 30.4% by day 10. In contrast, lower concentrations caused only minor effects (2.3% at 4.8 mg a.i./kg and 7.2% at 0.48 mg a.i./kg), comparable to the control group (6.0%). However, a longer exposure period could provide additional information on potential delayed effects at lower concentrations, as suggested in studies with boscalid exposure by Simon-Delso et al. (2018).

These findings are consistent with those of previous toxicological studies. In the study conducted by Mohamadzade et al. (2025), a concentration-dependent increase in mortality was reported. The findings indicated that chronic exposure to concentrations ranging from 10 to 50 ppm resulted in an approximate 20% increase in mortality compared to the control group. Furthermore, concentrations of 100 to 150 ppm led to a substantially higher mortality rate, ranging from 70% to 90%. The median lethal concentration (LC_{50}) for this concentration range was 45.7 ppm. Furthermore, the reported LD_{50} values (1.039 µg/bee for newly emerged bees and 15.3 µg/bee for older individuals) (Mohamadzade et al. 2025) indicate that the maximum intake observed in our present study (1.45 µg/bee/day) falls within the lower range of toxicologically relevant doses, which corresponds with the moderate mortality observed at the highest concentration.

Comparable results were reported by Yang et al. (2020), who identified an NOAEC value of 5 mg/L under chronic exposure conditions. In the present study, the lower concentrations (4.8 and 0.48 mg a.i./kg) fall within or below this range and resulted in negligible mortality, supporting their classification as environmentally relevant and sublethal exposure levels.

The results are further supported by a study focusing on sublethal and chronic effects. Sublethal doses of acetamiprid (0.5–2 µg/bee) (Shi et al. 2019) have been demonstrated to exert a substantial impact on lifespan, with a concomitant impairment of behavioural traits. The most pronounced effects were observed at doses ≥ 1 µg/bee. This finding is in close alignment with our result, wherein the highest consumed dose (1.45 µg/bee/day) was associated with increased mortality. In contrast, lower doses resulted in only minor lethal effects. Collectively, these findings imply that the biologically relevant effects of acetamiprid may occur at exposure levels of approximately 1 µg/bee under chronic conditions.

From a mechanistic perspective, the observed mortality pattern can be explained by acetamiprid's mode of action as a neonicotinoid, which targets nicotinic acetylcholine receptors. At lower concentrations, bees are likely able to compensate for neurotoxic stress through detoxification mechanisms, resulting in minimal mortality. However, at higher concentrations or under prolonged exposure, these mechanisms may become overwhelmed, leading to increased physiological stress and mortality (Iwasa et al. 2004).

Furthermore, exposure to pesticides can influence social interactions within the colony. As demonstrated in an earlier study, exposure to pesticides has been shown to reduce the frequency of trophallaxis, which may reduce the transfer of contaminated food items among individuals (Yousuf et al. 2026). This disruption may further influence the distribution of pesticide residues within the colony and contribute to variability in individual exposure.

In contrast to the results obtained in laboratory studies, field research has indicated a comparatively low risk of acetamiprid to honeybee colonies, particularly when alternative uncontaminated forage resources are present (Capela et al. 2022). This discrepancy can be explained by differences in exposure scenarios, as field conditions typically involve only partial contamination of foraging resources. In contrast, in the present study, bees were continuously exposed to a treated diet without access to alternative food sources.

Taken together, these findings confirm that acetamiprid exhibits relatively low acute toxicity compared

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to other neonicotinoids (Iwasa et al. 2004) but can induce significant mortality under chronic exposure conditions at higher concentrations. The results further demonstrate that NOEC/NOAEC thresholds reported in the literature (Yang et al. 2020) serve as a reliable benchmark for environmentally relevant exposure levels.

The relative gene expression analysis (Figure 2) revealed no statistically significant differences between the control group and any of the treated groups

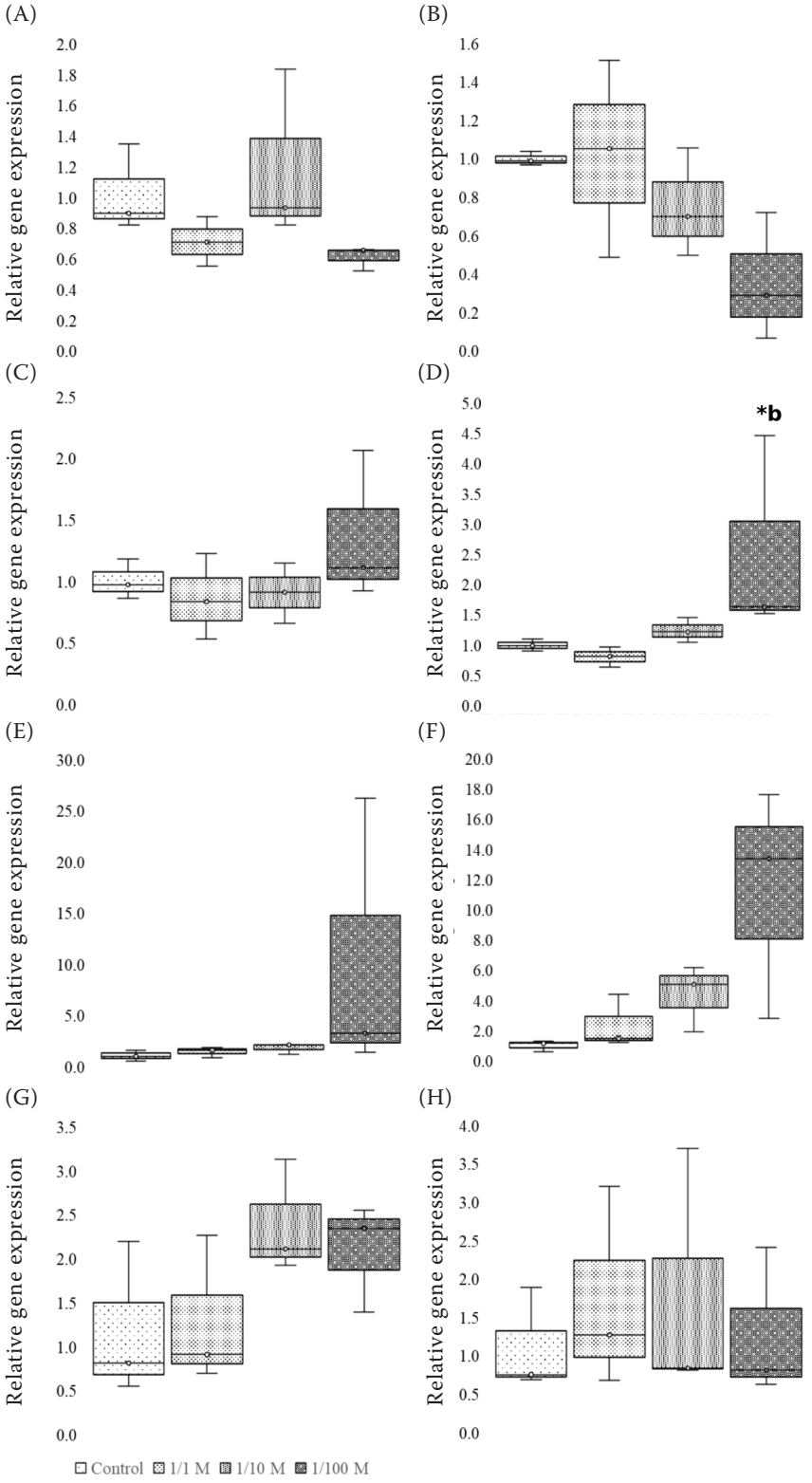

Figure 2. Relative gene expression of (A) *gst*; (B) *trxr*; (C) *sod1*; (D) *sod2*; (E) *aba*; (F) *hym*, (G) *apid* and (H) *ace1* between the experimental groups. Group differences were evaluated using the Kruskal-Wallis test. Boxes represent the first and third quartiles, the centre line indicates the median, and whiskers extend to the minimum and maximum values. Significant differences are indicated by *($P < 0.05$); the letter b denotes a significant difference compared with the 1/1 M group

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in the case of *gst*, *trxr*, or *sod1*. In the case of *trxr*, no significant trend toward lower expression with increasing acetamiprid concentration was observed. *Sod2* gene expression was significantly higher in the 1/100 M group than in the 1/1 M group. GST is a xenobiotic detoxification enzyme, while TRXR, SOD1, and SOD2 function primarily as antioxidant defence enzymes, neutralising reactive oxygen species generated during oxidative stress (Gong and Diao 2017). However, in the present study, no transcriptional upregulation of the analysed detoxification- and oxidative stress-related genes was detected. Similar results were recorded by Han et al. (2023). One reason for this could be the sampling time point, as transcriptional upregulation of these defence genes may have been transient and had already returned to baseline by the time of sampling. A further study focusing on protein concentration and enzymatic activity in honeybees would be suitable for elucidating whether these changes are reflected in the proteins produced, as it was previously established that acetamiprid affects antioxidant activity in bee brains (Mackei et al. 2024). In bee larvae, acetamiprid was also observed not to affect gene expression (Lu et al. 2023). Another possible explanation is survival bias, whereby the sampled individuals may represent a subset of bees with greater physiological tolerance to acetamiprid exposure. The only statistically significant difference was observed for *sod2* between the 1/1 M and 1/100 M groups, with the group receiving the lower concentration showing the highest gene expression. Interestingly, published evidence suggests that honeybees cannot detect neonicotinoid pesticides through taste or smell and will consume neonicotinoid-laced food without avoidance (Kessler et al. 2015). This argues against olfactory aversion as an explanation for differences in consumption among concentration groups.

The next group of genes analysed in this study comprised genes encoding antimicrobial peptides (*aba*, *hym*, and *apid*), which are important components of the honeybee innate immune response. No significant changes were observed; only a non-significant trend toward higher expression at lower concentrations. Chronic exposure does not usually affect gene expression (Tahir et al. 2023) in bees, and only high concentrations, such as 2 ug/mL of feed, were able to stimulate this response in larvae (Lu et al. 2023). It is positive that acetamiprid does not impair immune defence mechanisms.

Lastly, *ace1* was evaluated because neonicotinoids (such as acetamiprid) act as agonists of nicotinic acetylcholine receptors, thereby interfering with signal transmission (Matsuda et al. 2001). We hypothesised that sustained acetylcholine receptor activation could trigger a compensatory transcriptional upregulation of acetylcholinesterase (*ace1*), the enzyme responsible for degrading acetylcholine at the synapse, as a homeostatic response to excess stimulation. Despite this, no significant differences were observed among the experimental groups. In other works, no effect was observed on the enzymatic activity of acetylcholinesterase (Gao et al. 2022), which is consistent with our finding of unchanged *ace1* transcript levels, though it should be noted that enzymatic activity and gene expression represent distinct regulatory levels and do not necessarily co-vary. In fact, a much higher dose (0.6 mg/L) than used in our work was capable of suppressing the ACE1 enzymatic activity (Badawy et al. 2015). However, it should be noted that while the concentration of acetamiprid in this work did not alter *ace1* gene expression, the situation in other tissues, such as the brain, may be different.

In this study, the chronic effects of the neonicotinoid insecticide Mospilan 20 SP (acetamiprid) on adult honeybees (*Apis mellifera*) were evaluated under OECD 245 (2017) laboratory conditions. The results showed a clear concentration-dependent decrease in survival, with the highest exposure (48 mg a.i./kg) causing a significant increase in mortality, while lower concentrations were comparable to the control.

Overall, acetamiprid exhibited relatively low chronic toxicity at environmentally relevant concentrations, but induced pronounced lethal effects at higher doses. In contrast, molecular analyses revealed no consistent changes in detoxification (*gst*, *trxr*, *sod1*), immune-related (*aba*, *hym*, *apid*), or neural (*ace1*) gene expression, suggesting limited transcriptional responses under the laboratory conditions tested.

These findings indicate that acetamiprid primarily affects honeybee survival at elevated exposure levels, while sublethal molecular responses remain largely unchanged. Therefore, risk assessment should prioritise lethal endpoints while still considering potential long-term exposure effects.

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