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## Nano-La<sub>2</sub>O<sub>3</sub> undermines honeybee cognition by invading the brain and accelerating neuronal apoptosis

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The widespread application of nanoparticles in agriculture poses threats to pollinators like honeybees, but the mechanisms of nanoparticle effects on honeybee cognitive behaviours remain poorly understood. To begin to address this knowledge gap, we examined the cognitive and physiological implications of lanthanum oxide nanoparticles (nano-La<sub>2</sub>O<sub>3</sub>) on honeybees (*Apis mellifera* L.). Our findings revealed that exposure to nano-La<sub>2</sub>O<sub>3</sub> (e.g., >10 mg L<sup>-1</sup>) significantly impaired olfactory associative learning and memory of honeybees ( $p < 0.05$ ) in a dose-dependent fashion, attributable to the increased apoptosis of neural cells and the downregulation of genes related to cognitive functions (*cAMP-dependent protein kinase (pka)*, *cAMP-responsive element binding protein (creb)*, *n-methyl-D-aspartate receptor 1 (nmdar1)*) due to the invasion of nano-La<sub>2</sub>O<sub>3</sub> into brains. This further led to decreased sucrose consumption and reduced survival rates among exposed honeybees. Our research documents the first evidence of nano-La<sub>2</sub>O<sub>3</sub> accumulation in honeybee brains and provides insights into the mechanisms through which nanoparticles negatively affect the cognitive behaviour of honeybees, highlighting new potential ecological risks posed by nanomaterials.

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### Environmental significance

Lanthanum oxide nanoparticles (nano-La<sub>2</sub>O<sub>3</sub>) are rare earth oxide nanoparticles that are extensively employed in the agrochemical industry. Honeybees (*Apis mellifera* L.) will be adversely influenced upon ingesting nectar, pollen, and water contaminated with nano-La<sub>2</sub>O<sub>3</sub>. Our findings revealed that the impairment of cognitive behavior and physiological wellness in honeybees can be attributed to the invasion of nano-La<sub>2</sub>O<sub>3</sub> into the brain, potentially affecting the honeybee population. These results provide valuable evidence for the environmental hazards and health threats of nanoparticles on habitats in the ecosystem.

## 1. Introduction

Honeybees are integral to global ecosystems as vital pollinators, playing crucial roles in maintaining terrestrial biodiversity and agricultural productivity.<sup>1</sup> However, because of their intensive foraging activities and food storage nature, honeybees are susceptible to contact with a wide range of environ-

mental pollutants including manufactured nanoparticles.<sup>2,3</sup> Honeybee populations declined by approximately 25% in Europe from 1985 to 2005, and by 59% in North America from 1947 to 2005.<sup>4</sup> Moreover, the United States reported a 44% honeybee colony loss during the period 2019 to 2020.<sup>5</sup> While there are many hypotheses for such declines, the roles of nanomaterials in impacting honeybee populations are relatively understudied.<sup>6</sup>

The rapid advancement of nanotechnology has led to the widespread incorporation of manufactured nanoparticles in various agrochemicals, for example, as vectors in pesticides to conferring antibacterial functions for plant protection.<sup>7,8</sup> The increasing application of manufactured nanoparticles is increasing their environmental release and accumulation, raising concerns about their adverse environmental consequences. For example, rare earth elements and their oxide nanoparticles are widely used in the agrochemical

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industry for making fertilizers, pesticides, and additives,<sup>9–11</sup> among which lanthanum oxide nanoparticles (nano-La<sub>2</sub>O<sub>3</sub>) account for around 30% of rare earth oxide nanoparticulate additives.<sup>12</sup> Due to their wide use, nano-La<sub>2</sub>O<sub>3</sub> can transfer and accumulate within soils, water, and plants in the agricultural system, with subsequent bioaccumulation through the food chain and exposure risk to higher trophic organisms including honeybees—a keystone species in ecosystems.<sup>1,12–14</sup> Honeybees could be contaminated by nano-La<sub>2</sub>O<sub>3</sub> through body surface adhesion, respiratory uptake, and the ingestion of contaminated pollen, nectar, and water during foraging,<sup>15,16</sup> potentially eliciting unintentional toxicological effects.

Our previous research revealed that the exposure of honeybees to nano-La<sub>2</sub>O<sub>3</sub> negatively affects their physiological health by disrupting the composition and abundance of gut microbiota.<sup>14</sup> A similar report was made on TiO<sub>2</sub> nanoparticle effects on honeybee gut microbiota and health.<sup>17</sup> Additionally, studies have found that exposure to metal oxide nanoparticles (*i.e.*, nano-CdO, nano-PbO, and nano-ZnO) results in neurotoxic effects on honeybees, resulting in brain maldevelopment and increased acetylcholinesterase (AChE, a cholinergic enzyme hydrolysing the neurotransmitter of acetylcholine) levels.<sup>16,18</sup> Recently, honeybee learning and memory were shown to be impaired by sublethal concentrations of a neonicotinoid pesticide.<sup>19</sup> Further, the co-administration of TiO<sub>2</sub> nanoparticles with a pyrethroid pesticide induced brain damage in honeybees.<sup>20</sup> However, it remains unknown whether rare earth nanoparticles such as nano-La<sub>2</sub>O<sub>3</sub> affect honeybee cognition and associated functions. Such understanding could be important, given the significant role of cognitive abilities in maintaining honeybee foraging behaviours, social interactions, and pollinating services.<sup>21</sup> The intentional use of nanoparticles in the honeybee industry has been discussed,<sup>22</sup> in addition to the ancillary exposure of honeybees to a wide range of nanosized particles.<sup>23</sup> The exposure of honeybees to manufactured or incidental nanoparticles is thus inevitable, making understanding the hazards of nanomaterials to honeybee populations a vital step toward assessing potential ecosystem-level risks.

This study aimed to evaluate the impacts of nano-La<sub>2</sub>O<sub>3</sub> on the learning and memory abilities of honeybees (*Apis mellifera* L.) following a 2-week oral exposure, then to test whether nano-La<sub>2</sub>O<sub>3</sub> can penetrate the haemolymph–brain barrier of these insects and accumulate in the brain, and thus lead to neurotoxic effects. Moreover, neural apoptosis and gene expression related to learning and memory formation were measured to explore the neurobiological mechanisms underlying honeybee cognitive alterations due to nano-La<sub>2</sub>O<sub>3</sub> exposure. To the best of our knowledge, this is the first study to reveal the dose-dependent cascading adverse effects of nano-La<sub>2</sub>O<sub>3</sub> on honeybee learning and memory by invading the brain, accelerating neuronal apoptosis, and downregulating genes related to cognitive functions. These findings serve as new benchmarks for evaluating the ecological implications of manufactured nanomaterials and

deepening the understanding of the decline of honeybee populations.

## 2. Methods

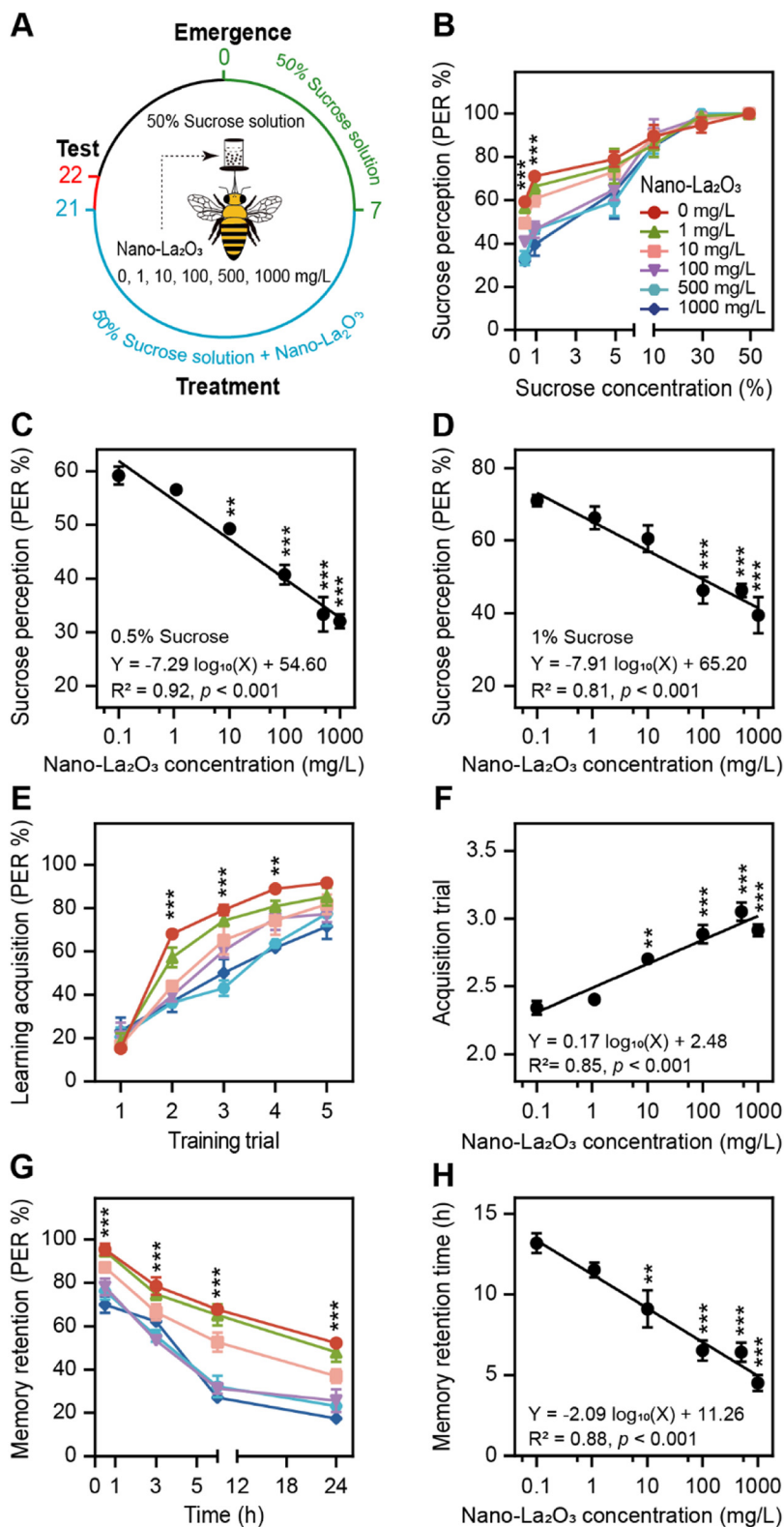
### 2.1 Experimental design

La<sub>2</sub>O<sub>3</sub> nanoparticles (25 ± 5 nm, 99.99% purity) were provided by the University of California Center for Environmental Implications of Nanotechnology (UC CEIN).<sup>24</sup> As reported previously, the hydrodynamic size of nano-La<sub>2</sub>O<sub>3</sub> in deionized water (pH 6.8) was from 573 to 605 nm, with the zeta potential ranging from 8 to 10 mV.<sup>24</sup> To maintain a stable and uniform distribution of nanoparticles, nano-La<sub>2</sub>O<sub>3</sub> was initially mixed with a sterilized sucrose solution (50%, w/w) and dispersed using a vortex oscillator for 5 min; subsequently, the mixture was ultrasonicated for 1 h using a KQ5200 ultrasonic machine (Kunshan Ultrasonic Instrument, Kunshan, China). The concentrations of nano-La<sub>2</sub>O<sub>3</sub> solution were 1, 10, 100, 500, and 1000 mg L<sup>-1</sup>, denoted as T1, T10, T100, T500, and T1000, respectively. The low exposure dose (1 mg L<sup>-1</sup>) in our study approximately matches the upper range of La residues in bee products (*e.g.*, 0.15–3.6 µg kg<sup>-1</sup> in honey and 7.64–164.46 µg kg<sup>-1</sup> in pollen),<sup>25</sup> and the middle doses (10 and 100 mg L<sup>-1</sup>) are used to reflect La amounts in plants (*e.g.*, 0.004–40 mg kg<sup>-1</sup>).<sup>26</sup> The highest dose, 1000 mg L<sup>-1</sup>, is within the reported ranges of metal oxide nanoparticle concentrations in soil environments<sup>27</sup> and simulates an extreme exposure concentration of nano-La<sub>2</sub>O<sub>3</sub>, allowing for the examination of potential effects of nanoparticle environmental accumulation as previously considered.<sup>12,28</sup> For the control group (T0), honeybees were provided with the sterilized sucrose solution (50%, w/w) without nanoparticles. All treatment solutions were freshly prepared daily.

Honeybees (*A. mellifera* L.) were raised at the Beijing experimental apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences. The capped brood frames were collected from three distinct colonies. Upon collection, newly emerged honeybees from the same frame were transferred into a wooden wire-mesh cage, which was maintained in a honeybee incubator at 30 °C with a relative humidity of 45% ± 5%. Before nano-La<sub>2</sub>O<sub>3</sub> exposure, the honeybees received a daily diet of sterilized sucrose solution (50%, w/w) for 1 week to allow their normal growth. Subsequently, during the 2-week exposure, honeybees were treated with a sterilized sucrose solution (50%, w/w) containing different concentrations of nano-La<sub>2</sub>O<sub>3</sub> (1, 10, 100, 500, and 1000 mg L<sup>-1</sup>, Fig. 1A). For each concentration treatment, three independent cages, each containing 70 honeybees, were employed.

Throughout the 2-week exposure, the honeybee feeding rate as measured by the consumption of feeding solution, and the survival rate as measured by live honeybee counts across time, were measured daily. Afterward, the honeybees were evaluated for their sucrose perception and olfactory associative learning and memory capabilities. Furthermore, analyses were conducted to detect nano-La<sub>2</sub>O<sub>3</sub> residues in





**Fig. 1** Nano-La<sub>2</sub>O<sub>3</sub> treatment affects sucrose perception, olfactory associative learning and memory in honeybees. (A) A schematic diagram of the experiment. (B) Sucrose perception in honeybees treated with different concentrations of nano-La<sub>2</sub>O<sub>3</sub>, as shown by the proboscis extension reflex (PER) to increasing concentrations of sucrose solution (0.5%, 1%, 5%, 10%, 30%, and 50%). Linear regression between different concentrations of nano-La<sub>2</sub>O<sub>3</sub> and sucrose perception to (C) 0.5% and (D) 1% sucrose solution. (E) Olfactory associative learning in honeybees exposed to varying concentrations of nano-La<sub>2</sub>O<sub>3</sub>, where honeybees were trained to associate hexanol with a sucrose reward. (F) Linear regression between different concentrations of nano-La<sub>2</sub>O<sub>3</sub> and the trial numbers of honeybees that successfully learned. (G) Memory retention tests for olfactory learning in nano-La<sub>2</sub>O<sub>3</sub>-treated honeybees were conducted at 0.5, 3, 6, and 24 h after learning training. (H) Linear regression between nano-La<sub>2</sub>O<sub>3</sub> concentration and memory retention time.



honeybee brains and bodies, assess apoptosis in brain cells, and measure gene expression related to learning and memory. These indicators were used to assess the neurotoxicity of nano-La<sub>2</sub>O<sub>3</sub> to honeybees.

## 2.2 Sucrose perception test

To test whether nano-La<sub>2</sub>O<sub>3</sub> could undermine the sucrose perception ability of honeybees after the 2-week exposure, we examined the proboscis extension reflex (PER) of honeybees to different concentrations of sucrose solutions (0.5%, 1%, 5%, 10%, 30%, and 50%, w/w).<sup>29</sup> A high perception ability was implied if the honeybee exhibited PER to a low concentration of sucrose solution. Specifically, the body of the honeybee was restrained in a custom-designed plastic tube but allowing for the free movement of the head, proboscis, and antennae. Each honeybee was supplemented with 30 μL sterilized sucrose solution (50%, w/w) and then rested for 2 h to ensure a uniform hunger level. The PER of each honeybee was recorded after touching the antennae with 5 μL of different concentrations of sucrose solution (0.5%, 1%, 5%, 10%, 30%, and 50%, w/w) in ascending sequential order at 5-minute intervals to prevent any potential habituation effects.<sup>30</sup> If the proboscis was extended, a score of 1 was assigned; otherwise, the score was 0. For each nano-La<sub>2</sub>O<sub>3</sub> treatment, three independent cages were employed, and >17 honeybees per cage were used for the sucrose perception test (Table S1).

## 2.3 Learning test

The olfactory associative learning protocol was employed to test whether nano-La<sub>2</sub>O<sub>3</sub> could undermine the learning ability of honeybees.<sup>31</sup> Learning ability was quantified by examining the PER of honeybees trained with a sucrose-associated odour, with a high learning ability indicated if honeybees exhibited PER in response. Before conditioning, each honeybee was gently harnessed and allowed to recover for 1 h. Only individuals that were healthy, responsive, and showed a full PER to antennal stimulation with 50% (w/w) sucrose solution were selected for the learning test. Non-responsive honeybees were excluded. Specifically, honeybees were exposed to a hexanol-laden gas stream for 6 s (flow rate, 1 mL s<sup>-1</sup>) using a custom-made air flow controller, with the last 2 s coinciding with a reward of a 1 μL sucrose solution (50%, w/w). The PER was recorded during the first 4 s immediately following the hexanol presentation to the honeybees. If the proboscis was extended, a score of 1 was assigned; otherwise, the score was 0. For each honeybee, learning trials were repeated five times in succession at 10-minute intervals. For each nano-La<sub>2</sub>O<sub>3</sub> treatment, three independent cages were employed, and >14 honeybees per cage were used for the learning test (Table S1).

## 2.4 Memory test

After the learning test, honeybees that had successfully learned—as evidenced by the extension of their proboscis to hexanol at the fifth learning session—were used for memory

retention assessments.<sup>31</sup> Similar to the learning test, the memory ability was quantified by examining the PER of honeybees to hexanol at 0.5, 3, 6, and 24 h post-learning test. Different retention times were used to reflect the short-term memory (0.5 h), intermediate memory (3 h and 6 h), and long-term memory (24 h) effects. A high memory ability was suggested if the honeybee exhibited PER to the sucrose-associated odour (hexanol). Following each memory test (e.g., at 0.5 h), honeybees were fed with a 10 μL sucrose solution (50%, w/w) and returned to the incubator to maintain their normal physiological state. For each nano-La<sub>2</sub>O<sub>3</sub> treatment, three independent cages were employed, and >10 honeybees per cage were used for the memory test (Table S1).

## 2.5 Quantification of nano-La<sub>2</sub>O<sub>3</sub> residues in honeybees

To explore the presence of nano-La<sub>2</sub>O<sub>3</sub> within the brains of honeybees, we employed scanning electron microscopy (SEM).<sup>32</sup> Because the mushroom bodies (MBs) of the brain mainly account for learning and memory behaviours in honeybees, SEM analysis focused on the MBs. Five honeybee brains were tested in each treatment group. Brain samples were fixed using glutaraldehyde (2.5%, v/v) and osmium tetroxide (1%, v/v) followed by dehydration with progressively increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%, w/w). After dehydration, the samples were sputter-coated with a thin layer of gold metal. Afterward, the prepared samples were examined using a Sigma 300 SEM (ZEISS, Oberkochen, Germany) to visualize nano-La<sub>2</sub>O<sub>3</sub> particles.

To quantify residual La<sub>2</sub>O<sub>3</sub> in the honeybees, an inductively coupled plasma optical emission spectrometer (ICP-OES) (PerkinElmer, Palo Alto, USA) was employed for the measurement of La concentrations, serving as an additional indicator of the La<sub>2</sub>O<sub>3</sub> residues present.<sup>14</sup> Quantification of La residues was conducted separately for the body (thorax and abdomen) and the head of honeybees. For each nano-La<sub>2</sub>O<sub>3</sub> treatment, there were three biological replicates, with five honeybee bodies or twenty honeybee heads pooled together as test replicates, respectively. Honeybees were initially rinsed five times with sterile 1× phosphate-buffered saline (PBS) (Solarbio, Beijing, China) to eliminate the La residues from the honeybee surface, followed by drying at 60 °C in a constant oven and flash-freezing in liquid nitrogen.<sup>33</sup> Afterward, these honeybee samples were finely ground and homogenized in a solution containing 4 mL of concentrated nitric acid and 1 mL of hydrogen peroxide (30%, v/v) and digested for 30 min with a microwave digestion system (Anton Paar GmbH, Graz, Austria). Subsequently, the La residues were quantified by ICP-OES.

## 2.6 Neuronal apoptosis in honeybee brains

To evaluate neuronal apoptosis in honeybee brain cells, the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) method was employed, utilizing an *in situ* cell death detection kit (Roche, Indianapolis, IN,



USA).<sup>34</sup> Honeybee brains were peeled out carefully and placed in paraformaldehyde solution (4%, v/v) for 2 h, then the brains were sectioned into 30  $\mu\text{m}$  slices using a CM1900 microtome (Leica, Wetzlar, Germany). Brain sections were incubated with immunohistochemical blocking solution (Beyotime Biotech, Zhejiang, China) for 1 h at room temperature. Subsequently, they were incubated with freshly prepared TUNEL reaction solution in the dark at room temperature for 2 h. The sections were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Darmstadt, Germany) for 10 min and covered with mounting medium (Beyotime, Shanghai, China).

Stained sections were visualized using an SP8 confocal microscope (Leica, Wetzlar, Germany). Apoptosis in specific brain regions (Fig. S1), including whole mushroom bodies (MBs), antennal lobes (ALs), and optic lobes (OLs) (*i.e.*, lobula (LO), medulla (ME), and lamina (LA)), was quantified using ImageJ 1.52 software (National Institutes of Health, Bethesda, MD, USA). The proportion of apoptotic cells was calculated by dividing the number of TUNEL-positive neurons (stained green) by the total number of neurons (stained blue). For each nano-La<sub>2</sub>O<sub>3</sub> treatment, five honeybee brains were used as biological replicates.

## 2.7 Gene expression assays in the honeybee brains

Real-time quantitative reverse transcription PCR (RT-qPCR) was used to examine the relative expression of eight genes, including two genes linked to brain apoptosis (*caspase-1* and *caspase-3*), three genes related to learning and memory (*cAMP-dependent protein kinase (pka)*, *cAMP-responsive element binding protein (creb)*, *n-methyl-D-aspartate receptor 1 (nmdar1)*), and three genes involved in physiological health (*vitellogenin (vg)*, *catalase (cat)*, and *apidaecin 1 (apid1)*). For each nano-La<sub>2</sub>O<sub>3</sub> treatment, there were three biological replicates, with five honeybee brains pooled together as a test replicate. Total RNAs of honeybee brains were isolated using the SG High Purity Total RNA Extraction Kit (SinoGene, Beijing, China) according to the manufacturer's manual. Subsequently, cDNA was constructed by transcribing the RNAs using the Thermo First cDNA Synthesis Kit (SinGene, Beijing, China) in accordance with the manufacturer's protocol. The ribosomal protein L32 (*rpl32*) was used as the reference gene. Primer sequences are listed in Table S2.<sup>34</sup> RT-qPCR was conducted using 2  $\times$  SG Green qPCR Mix (with ROX) (SinoGene, Beijing, China) on a StepOnePlus Real-Time PCR Systems instrument (Applied Biosystems, Carlsbad, CA, USA), employing a total reaction volume of 15  $\mu\text{L}$ : 7.5  $\mu\text{L}$  of 2  $\times$  SG Green qPCR Mix, 1  $\mu\text{L}$  of cDNA, 0.25  $\mu\text{L}$  of forward primer, 0.25  $\mu\text{L}$  of reverse primer, and 6  $\mu\text{L}$  of nuclease-free water.<sup>34</sup> The relative expression of genes was calculated using the comparative C<sub>T</sub> method.<sup>35</sup>

## 2.8 Statistical analyses

The normality of the data was analysed by the Shapiro–Wilk test. Honeybee sucrose perception, learning, and memory

tests were analysed using one-way ANOVA with Dunnett's multiple comparisons. Linear regressions were conducted to examine the relationship between nano-La<sub>2</sub>O<sub>3</sub> exposure concentrations and behaviour test parameters. The quantification of La residues in honeybee bodies and heads as well as the results of neuronal apoptosis and gene expression were compared *via* one-way ANOVA with Dunnett's multiple comparisons. For honeybee survival rates, the log-rank (Mantel–Cox) test was applied.<sup>14</sup> Food consumption was analysed using the Kruskal–Wallis test followed by Dunnett's multiple comparisons. All statistical analyses in this study were performed with GraphPad Prism 9 software (GraphPad Software, Boston, MA, USA). Significance levels were defined as \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

## 3. Results and discussion

### 3.1 Nano-La<sub>2</sub>O<sub>3</sub> undermines sucrose perception, learning, and memory in honeybees

Nano-La<sub>2</sub>O<sub>3</sub> undermined the sucrose perception ability of honeybees, as reflected by the diminished PER proportion to sucrose solution due to nano-La<sub>2</sub>O<sub>3</sub> exposure, notably with the relatively mild stimuli (0.5% sucrose,  $p < 0.001$ ; 1% sucrose,  $p < 0.001$ ; Fig. 1B). There was a significant negative correlation between different concentrations of nano-La<sub>2</sub>O<sub>3</sub> and PER proportion to 0.5% and 1% sucrose (linear regression,  $p < 0.001$ ; Fig. 1C and D). However, when applying relatively strong stimuli, *i.e.*, 5%, 10%, 30%, and 50% sucrose, there was no significant difference in the PER proportion among nano-La<sub>2</sub>O<sub>3</sub> treatments ( $p > 0.36$ ; Fig. 1B). The reduction in sucrose perception could be attributed to physiological disorders in honeybees, such as gut disorders,<sup>14</sup> brain damage,<sup>36</sup> and impaired gustatory receptor function on the antennae or proboscis, which may also lead to a diminished appetitive response. Thus, chronic exposure to nano-La<sub>2</sub>O<sub>3</sub> might result in decreased perception of honeybees to lower concentrations of nectar, affecting food storage in the hive and possibly leading to colony death during famine.

Nano-La<sub>2</sub>O<sub>3</sub> also impaired the learning ability of honeybees, as reflected by the decreased learning acquisition (the PER percentage of acquiring the ability of associating odour with food;  $p < 0.01$ ; Fig. 1E) with increasing nano-La<sub>2</sub>O<sub>3</sub> concentrations. Also, there was a significant positive correlation between nano-La<sub>2</sub>O<sub>3</sub> concentration and the needed learning trials by which honeybees successfully learned the ability of associating odour with food (linear regression,  $p < 0.001$ ; Fig. 1F), with a significant increase for concentrations of  $>10 \text{ mg L}^{-1}$  nano-La<sub>2</sub>O<sub>3</sub> ( $p < 0.01$ ; Fig. 1F). These results suggest that honeybees need a longer time to acquire a skill after exposure to nano-La<sub>2</sub>O<sub>3</sub>. Moreover, nano-La<sub>2</sub>O<sub>3</sub> could potentially interfere with peripheral odorant detection in the antennal sensilla or with the initial neural processing of olfactory signals, making the odour a weaker or less salient stimulus. A weaker perceived conditioned stimulus can indeed require more paired trials to form a robust association with the sucrose reward. Therefore, the observed results may



reflect a combination of impaired olfactory perception and impaired learning and memory formation. Future studies employing electrophysiological techniques such as electroantennography (EAG) would be necessary to directly assess the impact of nano-La<sub>2</sub>O<sub>3</sub> on peripheral olfactory function and to decouple perceptual effects from central cognitive deficits.

Meanwhile, nano-La<sub>2</sub>O<sub>3</sub> significantly impaired the memory of honeybees, as reflected by the decreased memory retention (the PER percentage of keeping the ability of associating odour with food;  $p < 0.001$ ; Fig. 1G) with increasing nano-La<sub>2</sub>O<sub>3</sub> concentrations. Also, there was a significantly negative correlation between nano-La<sub>2</sub>O<sub>3</sub> concentration and the mean memory retention time over which honeybees retained the ability of associating odour with food (linear regression,  $p < 0.001$ ; Fig. 1H). Consistently, the memory retention time was significantly shorter at concentrations of  $>10$  mg L<sup>-1</sup> nano-La<sub>2</sub>O<sub>3</sub> ( $p < 0.01$ ; Fig. 1H).

Perception, learning, and memory are essential for honeybee survival, foraging, and communication with their mates.<sup>21</sup> Our results demonstrated that the sucrose perception and olfactory associative learning and memory of honeybees could be adversely influenced by chronic nano-La<sub>2</sub>O<sub>3</sub> exposure. As a result, in natural settings, the ability of honeybees to associate the odour, colour, and shape of flowers with a food source may deteriorate due to environmental nano-La<sub>2</sub>O<sub>3</sub> exposure.

### 3.2 Penetration and accumulation of nano-La<sub>2</sub>O<sub>3</sub> in honeybee brains

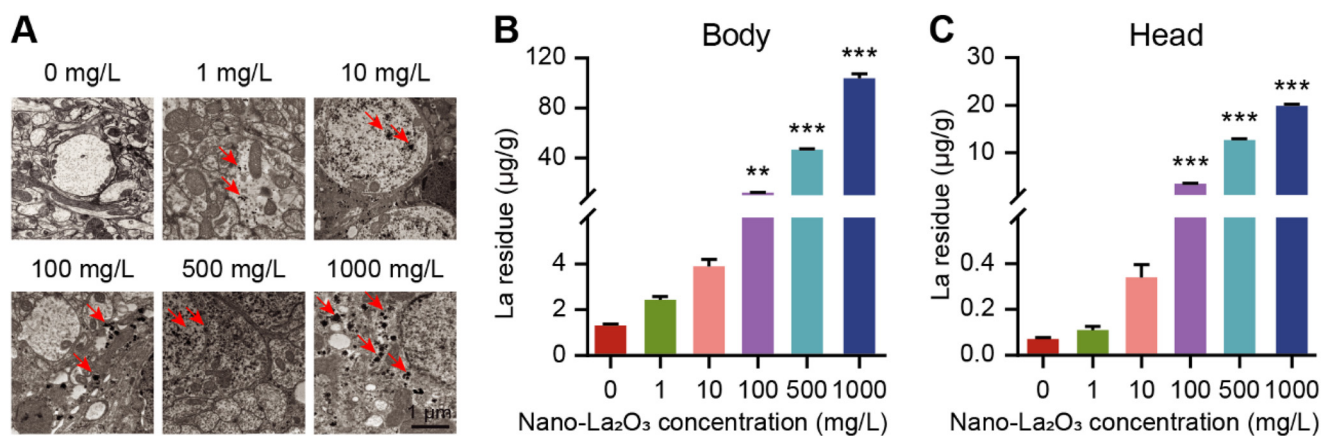
Given the impact of nano-La<sub>2</sub>O<sub>3</sub> on the cognitive behaviours of honeybees, there is a possibility that the nanomaterial has either accumulated in the brain or damaged neurons. To determine whether nanoparticles penetrate and accumulate in honeybee brains, SEM was used to identify nano-La<sub>2</sub>O<sub>3</sub> within the brain tissue of honeybees (Fig. 2A). An increase in

the dosage of nano-La<sub>2</sub>O<sub>3</sub> treatment led to a visible accumulation of electron-dense appearances in the brains, which manifested as clustered clumps. Furthermore, a marked increase in La deposition in honeybee bodies and heads was observed; in particular, T100, T500, and T1000 resulted in significantly elevated La residues in bodies ( $p < 0.01$ , Fig. 2B) and heads ( $p < 0.001$ , Fig. 2C) compared to the control.

The dose-dependent accumulation of La residue in honeybee bodies aligns with our previous findings,<sup>14</sup> but in this study, we provide the first evidence that La residue accumulated in the heads of honeybees with nano-La<sub>2</sub>O<sub>3</sub> exposure, which may disrupt synaptic plasticity and neural processes, and contribute to the observed cognitive deficits. Previous studies have indicated that nano-La<sub>2</sub>O<sub>3</sub> and other nanoparticles can bypass the blood–brain barrier and reach the brain in mammals.<sup>37,38</sup> Similar to mammals, honeybees have a haemolymph–brain barrier.<sup>39,40</sup> However, it remains unclear if nano-La<sub>2</sub>O<sub>3</sub> can cross similar barriers in insects. Our research has directly detected nano-La<sub>2</sub>O<sub>3</sub> within honeybee brain tissue, suggesting that nano-La<sub>2</sub>O<sub>3</sub> can be transported in the alimentary tract, cross the haemolymph–brain barrier, and accumulate in the honeybee brains. Once nano-La<sub>2</sub>O<sub>3</sub> invades the brain, it may lead to potential neurotoxic effects such as neuronal apoptosis by inducing oxidative stress in brain cells.<sup>41</sup>

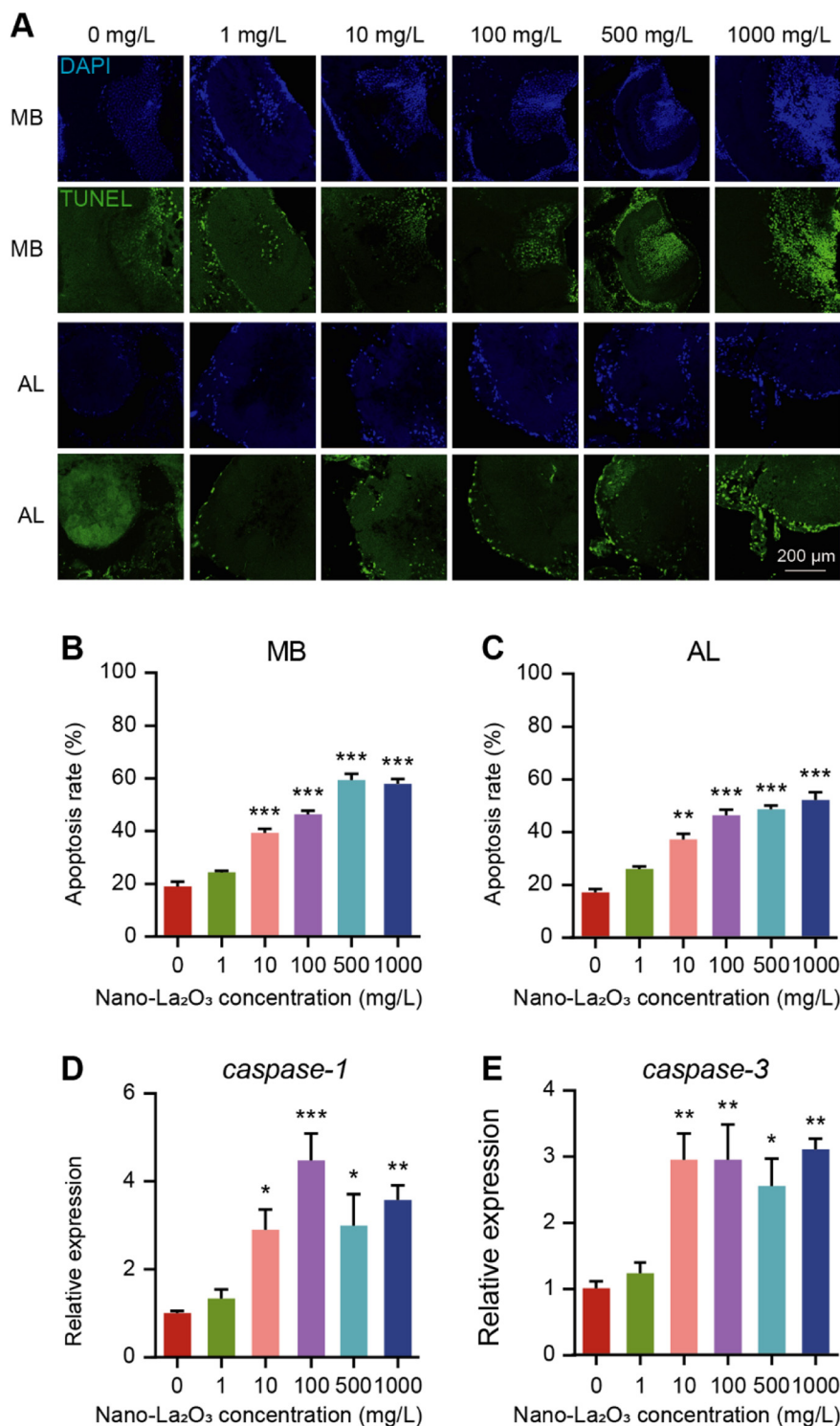
### 3.3 Nano-La<sub>2</sub>O<sub>3</sub> induces apoptosis in honeybee brain cells

After discovering that nanoparticles accumulate in honeybee brains, we proceeded to evaluate the risk of nano-La<sub>2</sub>O<sub>3</sub> on neuronal apoptosis by TUNEL assays. Because nano-La<sub>2</sub>O<sub>3</sub> impaired honeybee learning and memory abilities, and the brain regions of MBs and ALs are crucial in information processing related to olfactory learning and memory in honeybees, the apoptosis of brain cells in these two areas was analysed. The results showed that honeybee brain neuronal cells of the MB and AL regions



**Fig. 2** Nano-La<sub>2</sub>O<sub>3</sub> residue accumulation in honeybee brains. (A) Scanning electron microscope (SEM) images showing nano-La<sub>2</sub>O<sub>3</sub> residue in the brains of honeybees treated with different concentrations of nano-La<sub>2</sub>O<sub>3</sub>. The content of La residue in the (B) body and (C) head of honeybees following nano-La<sub>2</sub>O<sub>3</sub> treatment.





**Fig. 3** Nano-La<sub>2</sub>O<sub>3</sub> treatment induces apoptosis in honeybee brain neurons. (A) Examples of apoptosis in regions of mushroom bodies (MBs) and antennal lobes (ALs) in honeybees treated with different concentrations of nano-La<sub>2</sub>O<sub>3</sub>. Apoptotic cells were stained by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL, green) and counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). (B) and (C) display the apoptosis rates in MBs and ALs, respectively, at different concentrations of nano-La<sub>2</sub>O<sub>3</sub>. (D) and (E) show the relative expression of genes related to apoptosis (*caspase-1* and *caspase-3*) in the brains of honeybees treated with different concentrations of nano-La<sub>2</sub>O<sub>3</sub>. Scale bar, 200  $\mu$ m.

that had been chronically treated with nano-La<sub>2</sub>O<sub>3</sub> showed increased apoptosis (Fig. 3A). Specifically, the apoptotic cell number of MBs and ALs in the T10, T100, T500, and T1000 groups

was significantly higher than that of the control group (MBs,  $p < 0.001$ , Fig. 3B; ALs,  $p < 0.01$ , Fig. 3C). This observation of neuronal apoptosis, especially in the olfactory information



coding and memory formation-related areas of MBs and ALs, indicates that nano-La<sub>2</sub>O<sub>3</sub> exposure induces the deterioration of brain function, thereby affecting olfactory learning and memory. Additionally, apoptosis in the OLs (LO, ME, and LA), which are important for visual processing, was also increased in the nano-La<sub>2</sub>O<sub>3</sub> treatment groups, indicating a broader neurotoxic impact (LO,  $p < 0.01$ , Fig. S2A; ME,  $p < 0.01$ , Fig. S2B; LA,  $p < 0.05$ , Fig. S2C). The accelerated neuronal apoptosis could be induced by the increased production of reactive oxygen species (ROS) owing to nanoparticle exposure, since ROS play a crucial role in the process of cell death cascade as signalling molecules.<sup>42,43</sup>

Previous studies have found that the apoptotic pathway in both mammals and insects can be mediated by caspase-1 and caspase-3, which act as pro-cell death factors, ultimately leading to apoptosis.<sup>44,45</sup> Given the prominent role of these caspases in apoptosis, the expression of caspase-1 and caspase-3 in honeybee brains was assessed. As shown in Fig. 3, except for the T1 group, nano-La<sub>2</sub>O<sub>3</sub> exposure significantly increased the expression of genes related to apoptosis, including *caspase-1* and *caspase-3*, in comparison to the control group (*caspase-1*,  $p < 0.05$ , Fig. 3D; *caspase-3*,  $p < 0.05$ , Fig. 3E). The results confirmed from the gene expression that

nanoparticle exposure accelerated the neuronal apoptosis as observed in TUNEL assays (Fig. 3A–C), suggesting that nano-La<sub>2</sub>O<sub>3</sub> treatment potentially deteriorates honeybee cognitive abilities by inducing neuronal apoptosis. Taken together, nano-La<sub>2</sub>O<sub>3</sub> appears to cause neuron apoptosis through increased ROS and apoptosis-related gene expression, which further accounts for the deficient performance of olfactory associative learning and memory in honeybees treated with nano-La<sub>2</sub>O<sub>3</sub>.

### 3.4 Nano-La<sub>2</sub>O<sub>3</sub> affects memory-related gene expression in honeybee brains

Nano-La<sub>2</sub>O<sub>3</sub> was found to affect honeybee behaviour, possibly by altering the expression of genes associated with learning and memory. We subsequently examined the transcription of these genes in honeybee brains to understand the molecular impact. Compared to the control (T0), nano-La<sub>2</sub>O<sub>3</sub> treatment resulted in the downregulation of *pka* in T100, T500, and T1000 groups, and of *creb* and *nmdar1* in T10, T100, T500, and T1000 groups (*pka*,  $p < 0.05$ , Fig. 4A; *creb*,  $p < 0.01$ , Fig. 4B; *nmdar1*,  $p < 0.05$ , Fig. 4C). The cAMP-dependent protein kinase (PKA) and its

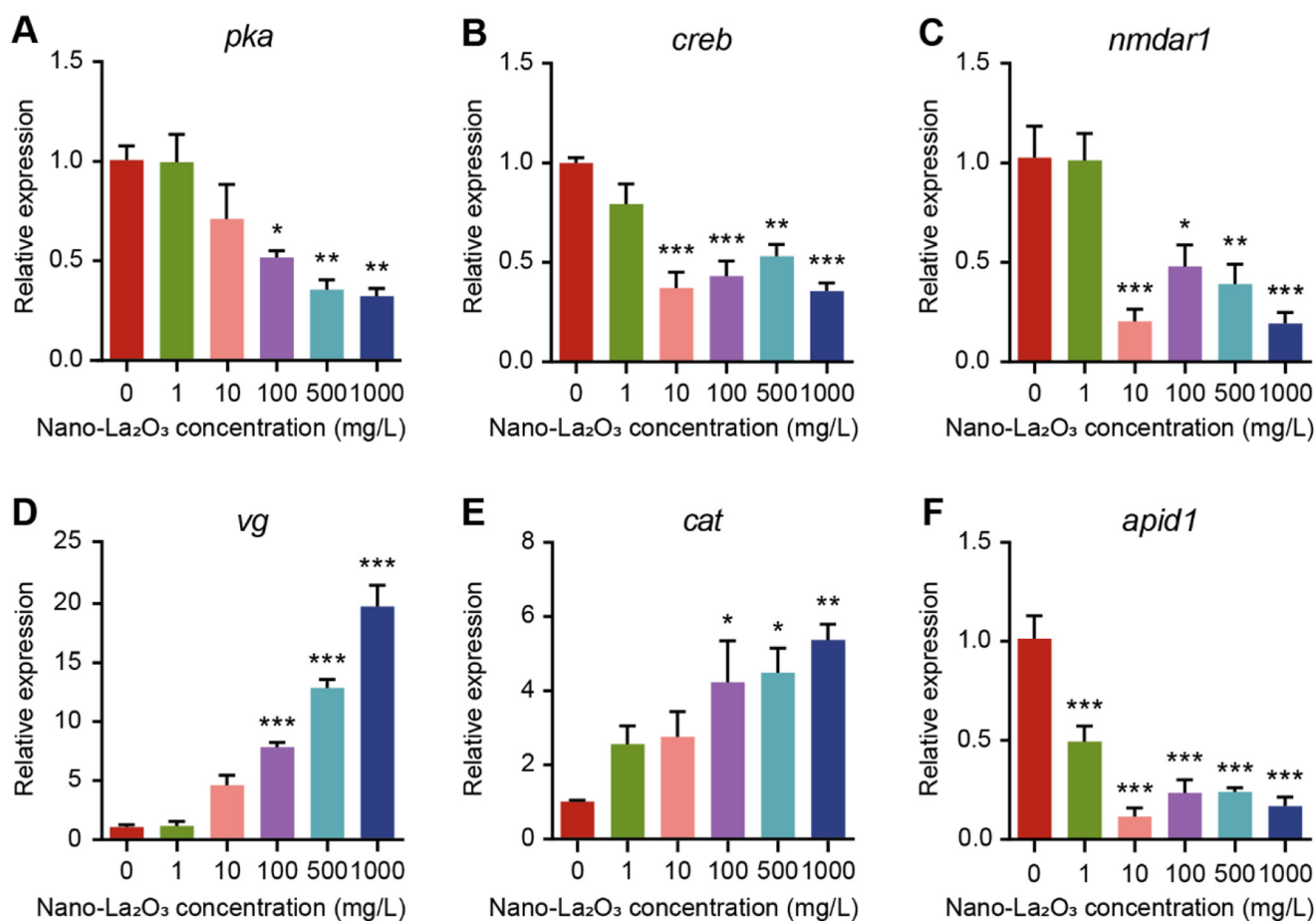


Fig. 4 Nano-La<sub>2</sub>O<sub>3</sub> treatment affects brain gene expression in honeybees. Relative expression of brain genes, including (A) cAMP-dependent protein kinase (*pka*), (B) cAMP-responsive element binding protein (*creb*), (C) *n*-methyl-D-aspartate receptor 1 (*nmdar1*), (D) vitellogenin (*vg*), (E) catalase (*cat*), and (F) *apidaecin 1* (*apid1*), in honeybees treated with various concentrations of nano-La<sub>2</sub>O<sub>3</sub>.



downstream target, cAMP-responsive element binding protein (CREB), which are crucial in the molecular process underlying memory formation in honeybees, play key roles in synaptic plasticity.<sup>46,47</sup> Previous studies suggested that reduced PKA activity impairs long-term memory formation.<sup>48</sup> Moreover, the *N*-methyl-D-aspartate receptor (NMDAR) is widely expressed throughout the brain, and *nmdar1* is the primary receptor in the MBs of honeybees. This receptor mediates glutamate activity which is critical for memory formation and recall.<sup>49,50</sup> Disruption of NMDAR with receptor antagonists or RNA interference (RNAi) leads to impaired long-term memory of honeybees.<sup>51,52</sup> As such, considering the results of olfactory associative learning and memory in this study, the marked decrease in the expression of *pka*, *creb*, and *nmdar1* also implies that the learning and memory abilities of honeybees are negatively influenced by the chronic higher-concentration treatment of nano-La<sub>2</sub>O<sub>3</sub>.

Additionally, the expression of several genes associated with physiological health was quantified in honeybee brains. Vitellogenin is an egg yolk protein associated with queen bee egg production and is believed to regulate honeybee behaviour.<sup>53</sup> As a nutrient synthesized by the fat body, vitellogenin contributes to immunity against xenobiotics and protects DNA from ROS damage.<sup>54</sup> In this study, the potential toxicity of nano-La<sub>2</sub>O<sub>3</sub> might induce oxidative stress in honeybees, partially explaining the observed increase in *vg* expression with rising concentrations of nano-La<sub>2</sub>O<sub>3</sub> ( $p < 0.001$ , Fig. 4D). Catalase, a crucial antioxidant enzyme, catalyses the decomposition of hydrogen peroxide into H<sub>2</sub>O and O<sub>2</sub>, thereby reducing ROS damage in organisms.<sup>55</sup> A study has found that sublethal levels of metal pollution, such as copper and cadmium, result in a significant increase in *cat* expression in honeybees after 48 h.<sup>56</sup> Similarly, in a survey of aquatic organisms *Daphnia magna*, the ROS produced by oxidative stress was also observed after 48 h exposure to LaCoO<sub>3</sub> perovskite nanoparticles.<sup>57</sup> As expected, we detected significantly higher expression of *cat* in honeybee brains in T100, T500, and T1000 compared to T0 ( $p < 0.05$ , Fig. 4E), implying that catalase actively works to prevent cellular damage caused by oxidative stress following nano-La<sub>2</sub>O<sub>3</sub> exposure. Apidaecin is an

immunity-related antimicrobial peptide, which acts as an immune effector to defend against external pathogen attacks.<sup>58</sup> Yet, in this study, we observed that the expression of *apid1* was suppressed by long-term exposure to nano-La<sub>2</sub>O<sub>3</sub> ( $p < 0.001$ , Fig. 4F), suggesting that such treatment induces immune system inhibition. Thus, the significantly higher expression of *vg* and *cat* in the high concentrations of nano-La<sub>2</sub>O<sub>3</sub> treatment groups, coupled with the significant decrease of *apid1* across all nano-La<sub>2</sub>O<sub>3</sub> treatment groups, appears to account for the increase in brain apoptotic cells, which also implies an increase in abnormal death of honeybees.

### 3.5 Nano-La<sub>2</sub>O<sub>3</sub> decreases sucrose consumption and survival of honeybees

Results herein showed that nano-La<sub>2</sub>O<sub>3</sub> can accumulate in the brain, leading to neuronal apoptosis and gene expression disorders and impairing the learning and memory behaviour of honeybees. This raises the question of whether these adverse effects directly affect the physiological health and overall survival of honeybees. To further investigate this issue, we analysed the daily sucrose consumption and survival rates of honeybees. The results revealed a decline in sucrose consumption with increasing concentrations of nano-La<sub>2</sub>O<sub>3</sub>. There was no significant difference in sucrose consumption between the lower concentrations of nano-La<sub>2</sub>O<sub>3</sub> treatment groups (T1 and T10) and the control group (T0) ( $p > 0.05$ , Fig. 5A). However, compared to T0, a significant reduction in sucrose consumption was observed in T100, T500, and T1000 within the 2-week exposure period ( $p < 0.05$ , Fig. 5A). A similar study demonstrated that the feeding rate of honeybees decreased after 9 days of oral exposure to CdO and PbO nanoparticles.<sup>16</sup> Furthermore, the accumulation of CdO or PbO nanoparticles in the honeybee midgut, which is in charge of nutrient absorption, can trigger apoptosis and necrosis in the epithelial cells of the peritrophic membrane.<sup>16,59</sup> Therefore, the observed reduction in food consumption could also stem from direct physical damage to the honeybee digestive system by nano-La<sub>2</sub>O<sub>3</sub> ingestion.

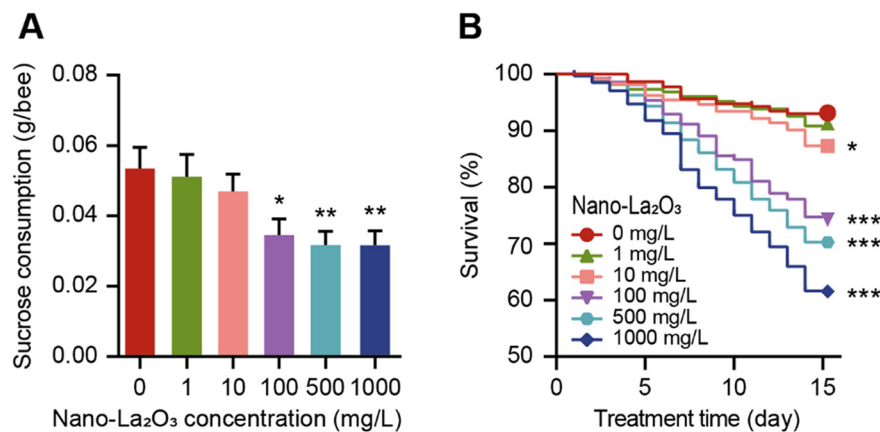


Fig. 5 Nano-La<sub>2</sub>O<sub>3</sub> treatment decreases sucrose consumption and survival in honeybees. (A) Average daily sucrose intake per honeybee during 14 days of nano-La<sub>2</sub>O<sub>3</sub> exposure. (B) Survival curves of honeybees treated with different concentrations of nano-La<sub>2</sub>O<sub>3</sub>.



Survival rate serves as an essential indicator for evaluating the physiological state of honeybees. Throughout 14 days of nano-La<sub>2</sub>O<sub>3</sub> treatment, the survival rate in the treatment groups progressively decreased as compared to the control group. Except for the T1 group, the survival proportions in the other treatment groups (T10, T100, T500, and T1000) were significantly lower than that in the control ( $p < 0.05$ ; Fig. 5B). Among them, the groups T100, T500, and T1000 showed higher mortality, with survival percentages dropping to 75%, 70%, and 62%, respectively, after 14-day exposure. The accumulation of nano-La<sub>2</sub>O<sub>3</sub>, indicated by measured La within the honeybees (Fig. 2), appears to cause abnormal death in these organisms, likely due to decreased sucrose consumption stemming from the disorder of the digestive tract.<sup>60</sup> Meanwhile, impaired cognitive behaviour might influence individual foraging activities, potentially threatening honeybee populations by decreasing the food storage by colonies.<sup>18,36,61</sup>

## 4. Conclusions

This study revealed anew that nano-La<sub>2</sub>O<sub>3</sub> impairs honeybee learning and memory by invading the brain and then inducing increased neuronal apoptosis and downregulating the expression of genes related to cognitive functions. Our findings address existing knowledge gaps regarding the negative effects of nano-La<sub>2</sub>O<sub>3</sub> on cognitive behaviours and brain neural cells in honeybees. We speculate that the decline of honeybee populations may be partly due to the neurotoxicological and histopathological influence of rare earth nanoparticles such as nano-La<sub>2</sub>O<sub>3</sub>. These findings could also apply to other ecologically and economically important pollinators, highlighting the potential ecological risks posed by manufactured nanomaterials entering the environment.

## Author contributions

Wangjiang Feng: formal analysis, data curation, methodology, writing – original draft, writing – review & editing. Yuan Ge: conceptualization, resources, supervision, project administration, funding acquisition, writing – review & editing. Patricia A. Holden: writing – review & editing. Yongxiang Zhang: investigation, methodology. Pingli Dai: writing – review & editing. Yong-Jun Liu: conceptualization, investigation, methodology, resources, formal analysis, supervision, funding acquisition, validation, project administration, writing – review & editing.

## Conflicts of interest

The authors declare that there are no competing financial interests.

## Data availability

Supplementary information: Tables S1 and S2 and supplementary figures: Fig. S1 and S2. See DOI: <https://doi.org/10.1039/D5EN00105F>.

The data supporting this article have been included as part of the supplementary information (SI).

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