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ABSTRACT

Honey bees (*Apis mellifera*) are essential pollinators whose colonies are increasingly compromised by nutritional and oxidative stress. Calcium supports muscle function and the regulation of mitochondrial reactive oxygen species, but conventional calcium salts are poorly absorbed, and reducing calcium carbonate to the nanometer scale may overcome this limitation. This study evaluated whether dietary nano-sized calcium carbonate improves flight-muscle mass and antioxidant defense in honey bees and identified the most effective inclusion level. Calcium carbonate nanoparticles were synthesized by a precipitated-carbonate route and characterized by X-ray diffraction, field-emission scanning electron microscopy and X-ray fluorescence; the product was phase-pure rhombohedral calcite with a mean size of about 94 nm and a composition of $(Ca_{0.987}Mg_{0.013})CO_3$. The nanoparticles were incorporated into pollen patties at 0% (CON), 0.01% (T1) and 0.02% (T2, w/w) and fed to colonies for 43 days. Patty consumption did not differ among groups, indicating that the mineral did not alter palatability. Thorax mass, a structural index of flight-muscle mass because the honey bee thorax consists largely of flight muscle, was greatest in the 0.02% group at weeks 4 and 6 ($p \leq 0.05$), whereas head and abdomen mass were unaffected. The cytosolic antioxidant genes *Sod1* and *Trxr-1* were up-regulated in the supplemented groups, most clearly at week 6, whereas *Sod2* and *Catalase* showed no consistent change. Total antioxidant capacity increased with inclusion level and was significantly higher in the 0.02% group at both time points. Dietary nano-sized calcium carbonate therefore supported flight-muscle mass over the feeding period and increased antioxidant capacity without depressing feed intake, with 0.02% (w/w) the most effective level. These findings identify nano-sized calcium carbonate as a candidate functional-mineral additive that warrants further evaluation for supporting the physiological robustness and antioxidant status of managed colonies.

Keywords: *Apis mellifera*; nano-calcium carbonate; antioxidant capacity; flight muscle

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INTRODUCTION

The western honey bee (*Apis mellifera*) is the most economically important managed pollinator and underpins both crop production and the stability of natural plant communities. About one-third of global crop production depends on animal-mediated pollination, much of which is carried out by bees [1,2]. Over the past two decades, however, the combined action of climate change, pesticide exposure, pathogens and habitat loss has driven widespread pollinator declines, including recurrent honey bee colony losses across temperate regions [2,3]. Because pollination services cannot easily be substituted, these losses translate directly into reduced agricultural output and ecological disruption, and practical measures that improve colony resilience are therefore of

considerable interest.

Among the physiological challenges that bees face, oxidative stress is particularly relevant to colony health. Foraging flight is highly energy-demanding, and the flight-muscle mitochondria that power it generate large quantities of reactive oxygen species (ROS) when metabolic flux is high [4]. Excess superoxide, hydroxyl radicals and hydrogen peroxide oxidize cellular proteins, lipids and DNA; the resulting protein oxidation is a well-characterized route to accelerated cellular aging [5], and such oxidative damage impairs immune competence and ultimately weakens both individual survival and colony stability. The honey bee genome encodes a conserved enzymatic antioxidant system whose expression responds sensitively to environmental and physiological stressors, making antioxidant gene expression a useful read-out of physiological state [6–8].

Cellular calcium (Ca^{2+}) homeostasis has emerged as an important modulator of this oxidative balance. Beyond its classical roles in muscle contraction, neurotransmission and signal transduction, calcium modulates mitochondrial function and cellular redox balance, so that calcium handling and ROS generation are tightly interdependent [9–11]. Adequate calcium supply might therefore support both the contractile machinery of flight muscle and the antioxidant defenses of the bee. Conventional calcium salts, however, have low solubility and limited intestinal absorption, which restricts their bioavailability.

Reducing calcium compounds to the nanometer scale is a well-established strategy for overcoming this limitation. Smaller particles present a much larger surface area and are thought to dissolve more readily, a mechanism proposed to improve intestinal uptake and systemic bioavailability relative to micron-sized material [12]; the particular nano-salt form further influences the resulting bioavailability [13]. Nano-sized calcium sources have been developed from biogenic materials such as eggshell, a calcium carbonate whose nanopowder has been physicochemically characterized [14], and fish bone, a calcium phosphate whose nanoparticles show greater calcium bioavailability than conventional calcium carbonate [15], and nanotechnology more broadly has been proposed for targeted delivery and disease management in apiculture [16]. Despite this, the effect of dietary nano-calcium carbonate on the body composition, antioxidant gene expression and total antioxidant capacity of honey bees has not been examined.

We therefore synthesized calcium carbonate nanoparticles, characterized their crystalline phase, morphology and elemental composition, incorporated the characterized material into pollen patties and fed it to colonies. We then measured patty consumption, body-segment mass, the expression of the antioxidant genes *Sod1*, *Sod2*, *Trxr-1* and *Catalase*, and total antioxidant capacity. We hypothesized that dietary nano-sized calcium carbonate would

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66 enhance flight-muscle development and antioxidant defense in *A. mellifera* in a dose-dependent manner, and we
67 sought to identify the most effective inclusion level.
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MATERIALS AND METHODS

Synthesis of calcium carbonate nanoparticles

Calcium carbonate nanoparticles were produced by a precipitated calcium carbonate (PCC) carbonation route and supplied by the Department of Earth and Environmental Sciences, Chungbuk National University (CBNU). Calcium oxide was hydrated with deionized water to form a calcium hydroxide slurry ($CaO + H_2O \rightarrow Ca(OH)_2$), and sodium glutamate was added at 1% (w/w of $Ca(OH)_2$) as a crystal-growth regulator to promote uniform colloidal seed formation and to control particle morphology at the nanoscale. The conditioned slurry was carbonated with CO_2 gas (≥ 99.5% purity) at 30–50°C and pH 8.5–9.5 for 4 h ($Ca(OH)_2 + CO_2 \rightarrow CaCO_3 + H_2O$), with continuous monitoring of pH and temperature until conversion was complete. These conditions yielded rhombohedral colloidal particles with a mean size of about 100 nm. The slurry was dewatered by filtration and dried, and the dried material was milled to obtain the final nano-calcium carbonate powder, whose crystalline phase, morphology and elemental composition were verified before use as described below.

Characterization of the nanoparticles

The crystalline phase was determined by powder X-ray diffraction (XRD) on a MiniFlex 600 diffractometer (Rigaku, Tokyo, Japan) equipped with a Cu Kα source (λ = 1.5406 Å) operated at 40 kV and 15 mA. Particle morphology and size were examined by field-emission scanning electron microscopy (FE-SEM) on a GeminiSEM 560 (ZEISS, Oberkochen, Germany) at an accelerating voltage of 5.00 kV using an InLens detector, and the mean particle size was estimated from the micrographs. The elemental composition was measured by X-ray fluorescence (XRF) spectrometry on a ZSX Primus-II (Rigaku, Tokyo, Japan). Carbon was not measured directly by XRF but was calculated by stoichiometric balance from the $CaCO_3$ formula.

Experimental colonies and diets

The feeding trial was conducted over 43 days (10 July to 22 August 2025) on *A. mellifera* colonies maintained at Chungbuk National University, Cheongju, Republic of Korea. The base pollen patty was prepared from defatted soybean meal, brewer's yeast, rapeseed pollen, sugar and water (Table 1). Three diets were formulated: the base patty without additive (CON), the base patty containing 0.01% nano-calcium carbonate (T1) and the base patty containing 0.02% nano-calcium carbonate (T2); inclusion levels are expressed relative to the total patty mass. Because the carbonate is about 40% calcium by mass, these levels supply only about 0.004% and 0.008% added elemental calcium (≈40 and 80 mg/kg). The calcium content of the base patty was not analyzed directly but was estimated from published ingredient composition data: solvent-extracted soybean meal, 0.33–0.35% Ca as fed

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[17,18]; brewer's dried yeast, 0.10–0.27% [19,20]; and bee-collected rapeseed pollen, about 0.18–0.28% [21,22] (0.06–0.31% in Korean bee pollen [23]); sucrose contains negligible calcium (≈0.001% [24]). Weighted by the patty formulation (Table 1), the base patty contained an estimated 0.08–0.11% Ca as fed (≈780–1,080 mg/kg; central estimate ≈0.10%), so the supplement increased the calcium content of the patty by only about 4% (T1) and 8% (T2). The concentrations were chosen with reference to the optimal dietary calcium range (143.99–243.99 mg/kg, ≈ 0.014–0.024%) reported for *A. mellifera* larvae reared *in vitro* [25]; because that range refers to calcium provided in a chemically defined larval diet rather than to carbonate added to a complete patty, it was used only as an approximate guide. Each patty was placed inside the hive directly on top of the frames and covered with a thin plastic film to prevent desiccation; patties were never exposed outside the hive, so that only the resident colony had access to its assigned diet. Colonies whose remaining patty fell below 300 g were re-supplied with about 500 g of fresh patty. All colonies were kept in the same apiary under identical management; all hives were fitted with entrance guards, and hornets (*Vespa* spp.) were controlled throughout the trial with traps and by manual removal.

Patty consumption

Patty consumption was determined for each colony as the difference in patty mass between consecutive weighings, and the mean daily intake over the 43-day trial was calculated for each treatment.

Body mass

Adult workers were sampled at weeks 0, 2, 4 and 6, dried at 60°C for 48 h, and dissected into head, thorax and abdomen, each of which was weighed separately [26]. At each time point, at least 15 workers were collected from the combs of each colony, each bee was weighed individually, and the colony mean was used for the analysis.

Antioxidant gene expression

Adult workers were collected at weeks 4 and 6, and the head, wings and legs were removed so that thoracic and abdominal tissue could be used. The thoracic and abdominal tissues of three workers per colony were pooled into one sample per colony, giving three biological replicates (colonies) per treatment. Total RNA was extracted with a Total RNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea) and reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' instructions. Quantitative PCR was performed with EzAmp FAST qPCR 2X Master Mix (ELPIS-Biotech, Daejeon, Korea); each 20 µL reaction contained 1 µL cDNA, 10 µL master mix and 1 µL of each primer. Cycling consisted of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and annealing

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for 20 s. *Gapdh* served as the reference gene, and relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method [27]. The primer sequences (Table 2) were those used previously for these antioxidant genes in honey bees [26].

Total antioxidant capacity

Total antioxidant capacity (T-AOC) was measured at weeks 4 and 6. Thoracic and abdominal tissue pooled from three workers per colony was homogenized, and 100 mg of homogenate was suspended in phosphate-buffered saline (pH 7.4) and centrifuged (12,000×g, 4°C, 15 min). The supernatant was assayed with an EZ-Total Antioxidant Capacity Assay Kit (DOGENBIO, Seoul, Korea). Absorbance was read at 660 nm on a UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used to construct a standard curve, and results are expressed as mM Trolox equivalents. All measurements were performed in technical triplicate.

Statistical analysis

Data were analyzed with IBM SPSS Statistics 29.0 (IBM, Armonk, NY, USA). The colony was the experimental unit (n = 3 per treatment), and for each variable the colony mean was used in the analysis. Treatment means were compared by one-way analysis of variance. Tukey's honestly significant difference test was used for pairwise comparisons. Differences were considered significant at $p \leq 0.05$.

RESULTS

Characterization of the synthesized nanoparticles

The XRD pattern of the synthesized powder matched the rhombohedral calcite structure (Fig. 1). The dominant reflection at about 29.4° 2θ corresponded to the calcite (104) plane, and the accompanying reflections ($10\bar{2}$), (006), (110), ($12\bar{3}$), (202), (108), (126) and higher-angle peaks were all indexable to calcite, with no peaks attributable to vaterite, aragonite or other phases. The powder was therefore phase-pure calcite.

FE-SEM showed discrete particles with the rhombohedral habit characteristic of calcite and a relatively uniform size distribution, with a mean particle size of approximately 94 nm (Fig. 2). XRF analysis gave oxygen 50.45 ± 0.14%, calcium 36.56 ± 0.03% and magnesium 0.28 ± 0.17% by mass, with carbon (12.63%) obtained by stoichiometric balance. Minor and trace constituents included Si (451 ppm), Fe (245 ppm), Sr (69 ppm), Mn (47 ppm), Ni (13 ppm), Zn (5.8 ppm) and Se (1.6 ppm) (Table 3). The corresponding formula was $(Ca_{0.987}Mg_{0.013})CO_3$, indicating a low-magnesian calcite in which a small fraction of the calcium sites is substituted by magnesium. The measured calcium (36.56%) was slightly below, and oxygen (50.45%) slightly above, the values expected for

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pure calcium carbonate (40.0% and 48.0%, respectively), as is common for standardless XRF and surface-adsorbed water; the phase assignment rests on the XRD pattern and is unaffected.

Patty consumption

Mean daily patty consumption did not differ among the three groups (CON, 63.44 ± 13.09 g; T1, 62.03 ± 13.81 g; T2, 65.83 ± 6.70 g; $p > 0.05$; Table 4), showing that inclusion of nano-calcium carbonate at 0.01% or 0.02% did not change how much patty the colonies consumed.

Body mass

Head and abdomen mass did not differ among groups at any time point ($p > 0.05$; Table 5). Thorax mass, in contrast, was affected by treatment. Because the honey bee thorax is composed almost entirely of the indirect flight muscles, thorax mass serves as a structural index of flight-muscle mass. At week 2 the overall ANOVA was significant ($p = 0.02$), but the only pairwise difference was between T1 (10.42 mg) and T2 (9.96 mg); neither supplemented group differed significantly from the control (10.04 mg), so no treatment effect relative to the control was evident at this stage. From week 4 onward the pattern became consistent. The 0.02% group (T2) had the heaviest thorax at both week 4 (9.68 vs 8.89 mg in CON; $p = 0.01$) and week 6 (9.54 vs 8.96 mg in CON; $p = 0.05$), with T1 intermediate and not significantly different from either. Absolute thorax mass declined with time in all groups, but the highest inclusion level consistently maintained greater thorax mass, and hence greater flight-muscle mass, than the control in the later half of the trial.

Antioxidant gene expression

Expression of the four antioxidant genes responded to treatment in a manner that was specific to both gene and time (Fig. 3). At week 4, *Sod1* differed significantly among groups and increased with dose, being highest in T2, intermediate in T1 and lowest in the control, indicating rapid activation of the cytosolic superoxide-scavenging pathway. *Sod2* and *Trxr-1* were numerically higher in the supplemented groups but the differences did not reach significance, and *Catalase* showed only a non-significant tendency toward higher expression in T2.

At week 6 the pattern was clearer: *Sod1* was significantly up-regulated in both T1 and T2 ($p < 0.05$), in keeping with sustained activation of the cytosolic pathway. *Trxr-1* was also significantly up-regulated at week 6, with the highest expression in the 0.02% group and the lowest in the control ($p < 0.05$). *Sod2* behaved differently across time; the non-significant rise in T2 at week 4 was not sustained at week 6, where only T1 showed significantly elevated expression while CON and T2 were comparatively low. *Catalase* again did not differ among groups. Overall, the cytosolic early-response genes *Sod1* and *Trxr-1* were the most responsive to nano-calcium carbonate,

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whereas the downstream *Catalase* was unresponsive.

Total antioxidant capacity

T-AOC increased with the inclusion level of nano-calcium carbonate (Table 6). At week 4 the 0.02% group reached 2.25 mM Trolox equivalents, significantly higher than the control (1.20 mM; $p < 0.05$); the 0.01% group (1.83 mM) was numerically higher than the control but not significantly different from it. The same pattern was stronger at week 6, when the 0.02% group reached 2.55 mM versus 1.58 mM in the control ($p < 0.05$), an increase of about 13% over its own week-4 value, while the 0.01% group (1.75 mM) again did not differ significantly from the control. The robust, statistically supported effect was therefore confined to the 0.02% inclusion level, which raised total antioxidant capacity at both sampling points and tended to do so more strongly with continued feeding.

DISCUSSION

This study set out to determine whether dietary calcium carbonate, reduced to the nanometer scale, improves the physiological robustness and antioxidant defense of honey bees. The material was produced by a precipitated-calcium-carbonate carbonation route in which sodium glutamate acted as a crystal-growth regulator. Characterization confirmed that the product fed to the colonies was phase-pure, low-magnesian calcite with a mean particle size of about 94 nm, in close agreement with the target size of the synthesis (about 100 nm). A particle size below 100 nm is the property most often invoked to explain the superior dissolution and intestinal absorption of nano-calcium relative to conventional micron-sized salts [12,13], so the synthesized material had the physical characteristics associated with improved solubility and absorption. These bioavailability advantages were, however, established in mammalian models [12,13]; given the very different physiology of the honey bee midgut, their translation to bees remains to be demonstrated directly. The small magnesium substitution and the trace levels of Sr, Mn and Fe detected by XRF are consistent with their origin in the lime (CaO) feedstock, help to fingerprint the material, and are unlikely to have had independent physiological effects at the inclusion levels used here.

Patty consumption was unaffected by supplementation. This is consistent with the view that bees regulate intake primarily according to the protein-to-carbohydrate ratio and palatability of the diet rather than its mineral content, a pattern documented in both honey bees [28,29] and bumblebees [30]. Because calcium carbonate at 0.01–0.02% changed neither the carbohydrate content nor the flavor of the patty, the colonies ate similar amounts in all groups, and the physiological differences described below cannot be attributed to differences in total food intake.

The clearest morphological effect of supplementation was on thorax mass, because the honey bee thorax is

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213 composed largely (~90%) of the indirect flight muscles and therefore reflects foraging capacity and physiological
214 robustness [31,32]. Calcium is central to the contractile cycle of muscle, and hymenopteran flight muscle is
215 notable for its unusual, specialized calcium handling, including a paradoxically slow calcium reuptake [32], which
216 provides a plausible route by which improved calcium supply could support flight-muscle mass. The response
217 should nonetheless be read carefully across time. At week 2, although the overall ANOVA was significant, neither
218 supplemented group differed from the control (the only difference was between T1 and T2), so there was no
219 evidence of a treatment effect at this early stage. A consistent pattern emerged only from week 4 onward, when
220 the 0.02% group carried the heaviest thorax. Because thorax mass declined over the feeding period in all groups
221 while the 0.02% group retained the heaviest thorax, the effect in the later half of the trial is best described as
222 maintenance of greater thorax mass at the highest inclusion level, rather than net muscle growth. A similar pattern,
223 in which thorax mass rose only at the highest inclusion level, was recently reported in the same species for pollen
224 patties enriched with a curcumin-steviol glycoside complex [26]. The absence of any effect on head or abdomen
225 mass indicates that the response was specific to the flight-muscle-rich thorax rather than a general change in body
226 size, although muscle fiber structure and contractile function were not examined directly.

227 The decline in absolute thorax mass, which occurred in all groups and was steepest between week 2 (24 July) and
228 week 4 (7 August) (Table 5), most likely reflects environmental conditions during the trial that were common to
229 all colonies. The trial coincided with the mid-summer nectar dearth in Korea, which follows the spring honey flow
230 and spans July and August [33]. In addition, hornet (*Vespa* spp.) predation pressure at the apiary began in early-
231 to-mid July, in line with the onset of hornet worker activity in central Korea [34], and was intense during the trial:
232 although entrance guards, traps and manual removal were used, hornets continued to hover around the hives, and
233 workers remained on guard at the hive entrance rather than leaving to forage. Such foraging paralysis reduces the
234 flight activity of colonies under hornet predation [35] and, under high predation pressure, lowers pollen income
235 and results in lighter workers [36], and a late-summer forage dearth has been associated with declines in colony
236 weight and in the fat stores of nurse bees [37]. Together, these conditions would have sharply reduced the colonies'
237 pollen (protein) income. Pollen deprivation of young adult workers reduces the fresh and dry weight of the thorax,
238 which houses the flight muscles, and later pollen intake does not fully compensate [38]; suboptimal pollen diets
239 delay the attainment of maximum thorax mass [39], and the body weight and protein content of emerging workers
240 vary with season and with the availability of food outside the hive [40]. Consistent with a colony-wide nutritional
241 limitation, head and abdomen mass also declined over the feeding period in all groups (Table 5). Because these
242 conditions affected all colonies equally, they do not bias the comparison among treatments; notably, between

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weeks 2 and 4 the thorax mass of the 0.02% group decreased by only 0.28 mg, compared with 1.15 mg in the control, which is consistent with the supplement partly buffering thorax mass under these nutritionally limiting conditions.

The antioxidant gene data point to a temporally structured response. *Sod1* and *Trxr-1* encode cytosolic, early-response components of the antioxidant system whose expression rises quickly under oxidative or thermal challenge and then subsides [7]. Their up-regulation in the supplemented groups, most clearly for *Sod1* at week 6, points to activation of this first line of defense. The behavior of the mitochondrial *Sod2* is more difficult to interpret. It was not significantly altered at week 4, and at week 6 a significant increase appeared only in the 0.01% group (T1), with the 0.02% group close to the control. Because the effect was confined to the lower dose at a single time point, the data do not form a simple, dose-dependent time-course. One possibility is that the mitochondrial superoxide-handling pathway is governed by a distinct, *Sod2*-centred mitochondrial stress-response program [41], and that its early rise is not sustained once the ROS load is buffered, by analogy with the rise-then-decline kinetics reported for honey bee antioxidant genes after a stressor [7]. However, because a different standing population of bees was sampled at each time point, the present cross-sectional design cannot establish such kinetics. The *Sod2* results were nonetheless variable and did not follow a clean dose relationship, so this interpretation remains tentative. *Catalase* did not respond at either time point, which fits its role as a downstream enzyme that acts on the hydrogen peroxide generated by SOD and tends to be regulated less sharply than the upstream superoxide-handling genes [6]. Notably, the same genes, assayed with the identical primer set, were all up-regulated, including *Catalase*, when honey bees received a curcumin-steviol glycoside complex [26], indicating that the *Catalase* response depends on the type of supplement rather than being uniformly unresponsive.

The functional assay reinforced the transcriptional picture. Total antioxidant capacity rose with inclusion level, and the statistically robust effect, significant at both week 4 and week 6, was confined to the 0.02% group. The parallel between higher T-AOC and the up-regulation of *Sod1* and *Trxr-1* in the same group is consistent with a coordinated response in which ROS-driven transcriptional activation of antioxidant genes [42] is, in the present study, accompanied by a measurable gain in net antioxidant capacity. We did not formally test the correlation between the two read-outs, so this concordance is best described as qualitative agreement rather than a quantified relationship. The fact that the 0.01% group raised T-AOC numerically but not significantly, whereas the 0.02% group did so reproducibly, suggests that a threshold inclusion level is needed to produce a functionally meaningful improvement, and that 0.02% (w/w) is the more effective dose under the present conditions. However, the added elemental calcium (about 40–80 mg/kg) was small relative to the estimated background calcium of the base patty

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(≈780–1,080 mg/kg), which by itself exceeds the optimal range reported for *in vitro*-reared larvae [25]. The effects observed therefore cannot be explained by relief of a calcium deficiency and are more plausibly related to the physicochemical form of the added calcium or to non-calcium effects of the supplement, which a matched comparison with micron-sized calcium carbonate would resolve.

These findings extend to a calcium source the growing evidence that dietary nanominerals can strengthen honey bee antioxidant defense. Supplementation with nano-zinc oxide and nano-selenium has been reported to raise antioxidant status and to modulate *Sod1* expression under heat stress [43], while biogenic nano-calcium sources such as eggshell and fish bone have been characterized as more soluble and bioavailable calcium carriers [14,15]. Our results add nano-calcium carbonate to this list and, importantly, link it to both a structural outcome (thorax mass) and a biochemical one (antioxidant capacity).

The present findings should be interpreted in light of several limitations that also define priorities for future work. The trial used three replicate colonies per treatment over a single 43-day summer period, a modest sample that limits the statistical power available at the colony level and the generality of the conclusions across seasons and genotypes; larger trials with more colonies are needed. Critically, the design included no conventional, micron-sized calcium carbonate arm at a matched calcium dose. The effects reported here are therefore those of the nano-calcium carbonate supplement as fed; whether they arise from the nanoscale properties of the material rather than from the additional calcium itself remains to be determined. Tissue calcium content and the actual uptake of the nanoparticles were not measured, and relative gene expression was normalized to a single reference gene (*Gapdh*); direct markers of calcium absorption and additional validated reference genes would strengthen future work. Although foraging was limited by the summer dearth and hornet predation, the colonies also had access to natural forage, so the patty was not their sole dietary source. Thorax mass is a sound proxy for flight-muscle mass, but muscle fiber structure, contractile function and actual flight performance were not examined, so the functional consequences of the greater thorax mass remain to be confirmed. In addition, worker age at sampling was not standardized and a different cohort of bees was sampled at each time point. Because mean thorax mass depends on the age structure of the sampled workers, and the groups already differed slightly at week 0, part of the between-group difference in thorax mass could reflect colony-level or sampling variation rather than a treatment effect. We also measured physiological and molecular markers but not the downstream outcomes that matter most for beekeeping, namely colony growth, brood production, overwintering success, longevity and resistance to pathogens. The *Sod2* response was variable, and the mechanism by which calcium influences antioxidant gene expression in bees was not directly probed. Future studies should therefore use more colonies and longer

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observation windows spanning the main honey flow, include honey yield and quality as well as productivity, survival and pathogen-challenge endpoints, and combine gene-expression and enzyme-activity measurements with markers of oxidative damage to establish the causal pathway from calcium uptake to antioxidant protection.

CONCLUSION

Calcium carbonate synthesized in the nanometer range was confirmed to be phase-pure, low-magnesian calcite with a mean particle size of about 94 nm. When fed to honey bee colonies in pollen patties, it did not change patty consumption but supported flight-muscle mass at the highest inclusion level in the later half of the trial. It also up-regulated the cytosolic antioxidant genes *Sod1* and *Trxr-1*, and raised total antioxidant capacity, with a statistically robust effect confined to 0.02% (w/w). Dietary nano-sized calcium carbonate is thus a promising functional mineral for supporting flight-muscle mass and antioxidant defense in *A. mellifera*, and 0.02% (w/w) appears to be the most effective inclusion level. Because the present design did not include conventional calcium carbonate at the same calcium dose, whether these effects are specific to the nanoscale form remains to be determined. Confirmation in larger and longer trials that include colony-level productivity and survival endpoints, and a direct comparison with conventional micron-sized calcium carbonate, will be needed before the approach can be recommended for practical colony management.

Acknowledgments

Not applicable.

Declaration of generative AI

No AI tools were used in this article.

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TABLES

Table 1. Composition of the base pollen patty and nano-calcium carbonate inclusion levels

Ingredient (g)	CON	T1	T2
Defatted soybean meal	30	30	30
Brewer's yeast	20	20	20
Rapeseed pollen	20	20	20
Sugar	100	100	100
Water	30	30	30
Subtotal	200	200	200
Nano-calcium carbonate	0	0.02	0.04
Inclusion level (%, w/w)	0	0.01	0.02

Nano-calcium carbonate was added to the 200 g base patty; inclusion levels are expressed relative to total patty mass. CON, control; T1, 0.01% nano-calcium carbonate; T2, 0.02% nano-calcium carbonate.

Table 2. Primers used for quantitative PCR

Target		Sequence (5′–3′)
Catalase	Forward	CCACTCATTCCTGTTGGTAA
	Reverse	GCATCACCGTAAGTGAACAT
Trxr-1	Forward	TGTGCTGGATTTTTAAATGG
	Reverse	TCCACCCAATGTACAAGAAG
Sod1	Forward	CGGCTGAAGTATTCATTACG
	Reverse	ACGCACACTGCTTTAGTCAT
Sod2	Forward	GAAAATACCATTGCGATTCA
	Reverse	ATCGGGTCGAACATTTTTAT
Gapdh	Forward	CACATGGAAAATTCAAAGGA
	Reverse	AATGACCAGAAGCTTTTTCC

Sod1, *Sod2*, superoxide dismutase 1 and 2; *Trxr-1*, thioredoxin reductase 1; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase (reference gene).

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Table 3. Elemental composition of the synthesized nanoparticles determined by X-ray fluorescence

Element	Concentration	± Error
Oxygen (O)	50.45%	0.14
Calcium (Ca)	36.56%	0.03
Carbon (C)[†]	12.63%	-
Magnesium (Mg)	0.28%	0.17
Silicon (Si)	451 ppm	88
Iron (Fe)	245 ppm	25
Strontium (Sr)	69 ppm	2.5
Manganese (Mn)	47 ppm	19
Nickel (Ni)	13 ppm	6.2
Zinc (Zn)	5.8 ppm	2.6
Selenium (Se)	1.6 ppm	1.0

[†] Carbon was calculated by stoichiometric balance from the $CaCO_3$ formula, not measured directly. Derived formula: $(Ca_{0.987}Mg_{0.013})CO_3$.

Table 4. Mean daily pollen-patty consumption of colonies fed nano-calcium carbonate

	CON	T1	T2
Daily intake (g)	63.44 ± 13.09	62.03 ± 13.81	65.83 ± 6.70

No significant difference among groups ($p > 0.05$).

Table 5. Effect of nano-calcium carbonate on body-segment mass (mg) during the feeding period

	CON	T1	T2	SEM	*p*-value
Week 0					
Head	4.22	4.33	4.22	0.07	0.75
Thorax	10.40	10.50	10.10	0.09	0.19
Abdomen	12.43	13.70	12.05	0.61	0.51
Week 2					
Head	3.94	4.22	4.12	0.06	0.14
Thorax	10.04^{ab}	10.42^{b}	9.96^{a}	0.07	0.02
Abdomen	9.17	9.20	9.35	0.25	0.96
Week 4					
Head	3.58	3.74	3.77	0.08	0.62
Thorax	8.89^{b}	9.16^{ab}	9.68^{a}	0.10	0.01
Abdomen	7.91	7.87	7.94	0.20	0.99
Week 6					
Head	3.72	4.14	4.14	0.11	0.20
Thorax	8.96^{b}	9.38^{ab}	9.54^{a}	0.10	0.05
Abdomen	7.26	7.47	7.43	0.20	0.91

Means within a row bearing different superscript letters differ significantly ($p \leq 0.05$). SEM, pooled standard error of the mean.

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448 **Table 6.** Total antioxidant capacity (mM Trolox equivalents) of workers fed nano-calcium carbonate

	CON	T1	T2
Week 4	1.20 ± 0.09^{b}	1.83 ± 0.15^{b}	2.25 ± 0.12^{a}
Week 6	1.58 ± 0.13^{b}	1.75 ± 0.14^{b}	2.55 ± 0.15^{a}

449 Means within a row bearing different superscript letters differ significantly ($p \leq 0.05$).

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FIGURE LEGENDS

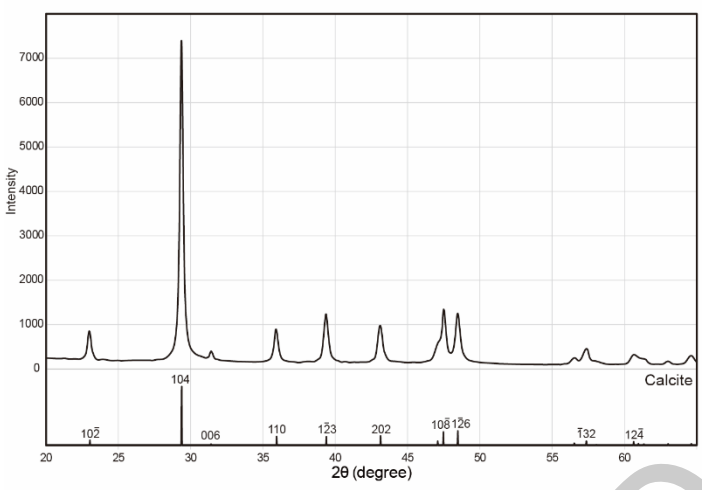


Fig. 1. Powder X-ray diffraction pattern of the synthesized nanoparticles. All reflections index to rhombohedral calcite; the strongest peak near 29.4° 2θ is the calcite (104) reflection. Vertical bars mark the reference calcite positions.

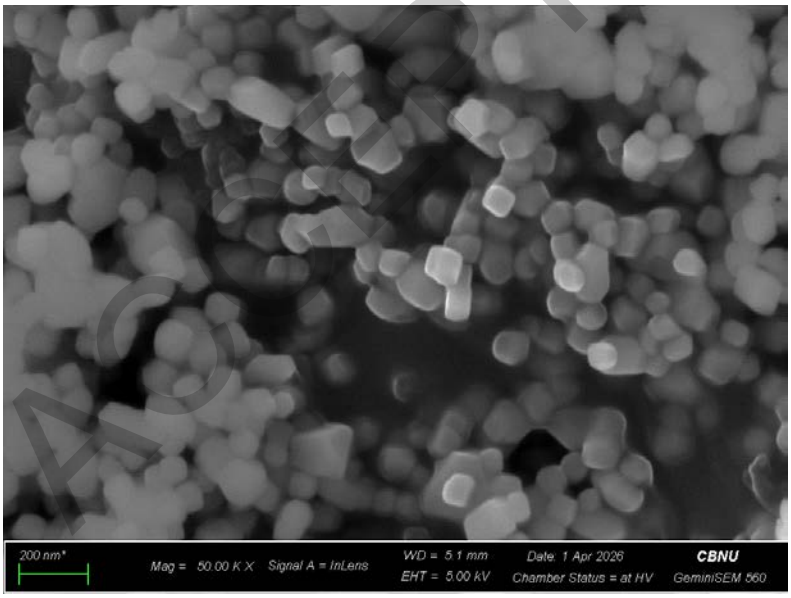
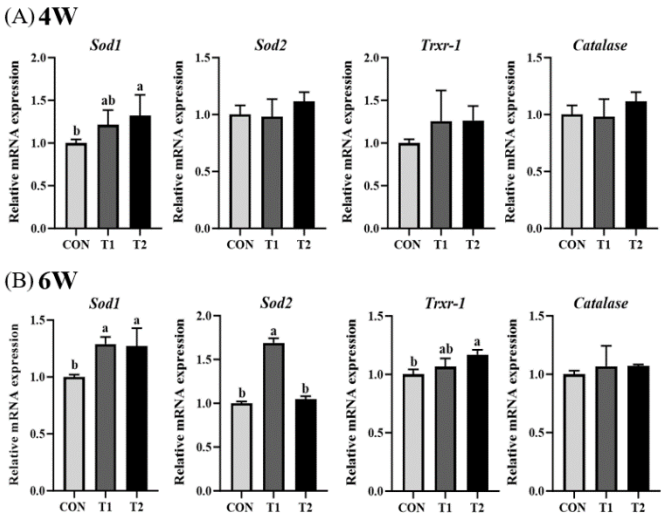


Fig. 2. Field-emission scanning electron micrograph of the synthesized calcite nanoparticles, showing the rhombohedral habit and a mean particle size of about 94 nm (scale bar, 200 nm).

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460 **Fig. 3.** Relative mRNA expression of antioxidant genes (*Sod1*, *Sod2*, *Trxr-1*, *Catalase*) in workers fed nano-

461 calcium carbonate at (A) week 4 and (B) week 6. Bars bearing different letters differ significantly ($p \leq 0.05$).

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