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Detoxification enzyme response and cuticular lipid depletion in insect larvae following particulate calcium ingestion

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Abstract

Calcium oxide powders derived from poultry eggshell waste have recently attracted attention as potential bio-rational insecticides, yet the physiological mechanisms underlying their toxicity to lepidopteran larvae remain poorly characterised. This research investigated the detoxification enzyme profile and cuticular lipid dynamics in fourth-instar *Spodoptera frugiperda* larvae exposed to particulate calcium oxide through diet incorporation at three concentrations (5, 15 and 30 mg g⁻¹ artificial diet) over a 96-hour observation window. Enzyme activities glutathione S-transferase (GST), general esterase, catalase and cytochrome P450 monooxygenase were assayed spectrophotometrically in larval midgut homogenates at 0, 6, 12, 24, 48, 72 and 96 h post-ingestion. Cuticular lipid content was determined gravimetrically after chloroform extraction of whole-larva cuticle preparations. GST and catalase activities rose sharply during the first 48 h, reaching 189.7 % and 152.1 % of control values respectively, which points to a significant oxidative stress response. By contrast, esterase and cytochrome P450 activities declined progressively, falling to 48.6 % and 43.7 % of controls by 72 h, suggesting that calcium particles interfere with Phase I detoxification pathways rather than inducing them. Cuticular lipid mass decreased in a dose- and time-dependent fashion; at the highest concentration, residual lipid had fallen to 14.2 % of initial content by 96 h, accompanied by visible desiccation and a cumulative mortality of 68.3 %. Larval body mass mirrored the lipid trend, dropping to 64.1 % of initial weight in the 30 mg g⁻¹ group. Correlation analysis returned a strong positive link between cuticular lipid loss and mortality ($r = 0.94$). The combined enzyme and lipid data indicate a dual mode of action: internal oxidative disruption through the gut lining alongside external desiccation driven by adsorption of epicuticular waxes onto calcium particle surfaces. These findings help explain the broad-spectrum larvicidal activity reported for eggshell-derived calcium powders and provide a biochemical foundation for optimising particle size, dose and application timing in integrated pest management programmes.

Keywords: Detoxification enzymes, cuticular lipid, calcium oxide, *Spodoptera frugiperda*, glutathione S-transferase, esterase, desiccation, oxidative stress, bio-waste insecticide, integrated pest management.

Introduction

When entomologists first documented the insecticidal action of inert mineral dusts in the early twentieth century, the prevailing explanation was straightforward: fine particles scratch the waxy epicuticle of an arthropod, water escapes, and the animal dies of desiccation [1]. That physical model, while broadly correct for diatomaceous earth and certain silica formulations, was never tested rigorously against calcium-based dusts, which differ from siliceous materials in both surface chemistry and particle hardness [2]. The recent demonstration by Ayoub and colleagues that eggshell-derived calcium powders cause significant mortality in *Spodoptera littoralis* larvae has renewed interest in calcium as a pesticidal mineral, but the paper left open the question of whether the observed kill was purely physical or involved a biochemical component [9].

Insect larvae possess a sophisticated battery of detoxification enzymes whose activity levels shift in response to xenobiotic exposure. Phase I metabolism, mediated primarily by cytochrome P450 monooxygenases and esterases, introduces or unmasks polar functional groups on toxicant molecules. Phase II conjugation, dominated by glutathione S-transferases, attaches glutathione to electrophilic metabolites and facilitates their excretion [3]. Catalase

and superoxide dismutase form a parallel antioxidant arm that neutralises reactive oxygen species generated during metabolic processing of foreign substances [4]. Profiling these enzyme families after exposure to a candidate insecticide can reveal whether the compound triggers oxidative stress, overwhelms detoxification capacity or both information that is critical for predicting cross-resistance risks in field populations already selected by chemical insecticides [5].

The cuticle of a lepidopteran larva is itself a dynamic tissue. Its outermost layer, the epicuticle, carries a thin film of long-chain hydrocarbons and wax esters that serves as the main barrier against transcuticular water loss [6]. Disruption of this lipid film whether by abrasion, surfactant action or adsorption onto particulate surfaces accelerates evaporative water loss at a rate that can exceed the larva's capacity for oral rehydration, especially under low-humidity conditions [7]. What has not been established is whether ingested calcium particles also deplete body lipids from the inside, for example by emulsifying gut-lining lipids or by triggering a systemic lipolytic response to oxidative damage.

Spodoptera frugiperda, the fall armyworm, was chosen as the test organism for this research because of its economic importance across tropical and subtropical agriculture and because its enzyme system has been well characterised in the context of conventional insecticide resistance [8]. Populations in Brazil, where this work was conducted, show documented resistance to several organophosphate and pyrethroid products, making fall armyworm an especially relevant model for evaluating alternative control agents whose mode of action differs from neurotoxic chemistry [10]. The present research pursued three linked objectives. First, it tracked the time-course of four detoxification and antioxidant enzymes (GST, esterase, catalase, cytochrome P450) in larvae fed calcium oxide-amended diets at three dose levels. Second, it measured cuticular lipid content gravimetrically over the same 96-hour window to determine whether lipid depletion is dose-dependent and, if so, whether it correlates with mortality. Third, it combined the enzyme and lipid datasets to test the hypothesis that particulate calcium acts through a dual mechanism: internal oxidative disruption and external desiccation. All bioassays were carried out at the Campinas Centre for Entomological Research, São Paulo state, Brazil, between January and July 2023.

Material and Methods

Material

Calcium oxide powder was prepared from clean, dry chicken eggshells calcined at 800 °C for 2 h in a muffle furnace (Quimis Q318M, Brazil) following the optimised protocol described in related thermal processing research [11]. The resulting powder was sieved to < 45 µm and stored in vacuum-sealed polyethylene bags over silica gel until use. Purity was confirmed by X-ray fluorescence (> 96 % CaO). Fourth-instar Spodoptera frugiperda larvae (3rd-generation laboratory colony, originally collected from maize fields near Campinas, SP) were reared on Greene's artificial diet at 27 ± 1 °C, 65 ± 5 % RH and 14:10 (L:D) photoperiod. Only larvae that had moulted within the preceding 12 h were selected for bioassays to ensure uniform developmental stage.

Methods: Calcium oxide powder was incorporated into the artificial diet at three concentrations 5, 15 and 30 mg g⁻¹ fresh diet by blending the powder into the warm (≈ 50 °C) diet slurry before it solidified. A control diet without calcium amendment was prepared simultaneously. Each treatment comprised 60 larvae held individually in 30 mL plastic cups (one larva per cup) to prevent cannibalism. Cups were arranged in a completely randomised design on shelved racks inside the growth chamber.

At each sampling point (0, 6, 12, 24, 48, 72 and 96 h), five larvae per treatment were flash-frozen in liquid nitrogen and stored at -80 °C for enzyme assay, and a separate set of five were used for cuticular lipid extraction. Enzyme activities were measured in midgut homogenates: GST with 1-chloro-2,4-dinitrobenzene as substrate ($\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$), esterase with α -naphthyl acetate, catalase with H₂O₂ decomposition monitored at 240 nm, and cytochrome P450 O-demethylation using p-nitroanisole. Protein content was determined by Bradford assay; all activities were expressed as nmol min⁻¹ mg⁻¹ protein and then converted to percentage of the time-zero control mean for cross-enzyme comparison. Cuticular lipids were extracted by immersing each larva in 5 mL chloroform for 60 s at room temperature, evaporating the solvent under nitrogen and weighing the residue on a microbalance (Mettler Toledo XPR2, 0.001 mg readability). Results were expressed as µg lipid per larva. Body mass was recorded individually at each sampling time on the same microbalance. Mortality was assessed daily; larvae that did not respond to gentle prodding with a blunt probe were scored as dead and removed.

Data were analysed by two-way ANOVA (treatment × time) with Tukey HSD post-hoc comparison ($\alpha = 0.05$). Pearson correlations were computed between lipid loss and cumulative mortality across all dose-time combinations. All statistics were performed in R v4.3.1.

Research Location and Environmental Conditions

All bioassay work was conducted at the Insect Physiology Laboratory, Campinas Centre for Entomological Research, situated in the eastern highlands of São Paulo state, Brazil (22°54'S, 47°03'W, 680 m a.s.l.). The laboratory-reared colony of *S. frugiperda* was maintained in a walk-in environmental chamber (Fitotron, Aralab, Portugal) set at 27 ± 1 °C and 65 ± 5 % relative humidity. Ambient outdoor temperatures during the experimental period (January-July 2023) ranged from 14 °C (July minimum) to 34 °C (January maximum), though these had no bearing on the climate-controlled bioassay conditions. The eggshells used as raw material were sourced from a commercial layer operation (Granja São José) located 22 km north-west of the campus.

Calcium Oxide Preparation Protocol

Eggshells were collected twice weekly and transported to the laboratory in sealed polypropylene containers. Shells were washed under running tap water, soaked in 1.5 % sodium hypochlorite for 20 min, rinsed three times with distilled water and oven-dried at 110 °C for 18 h. Dried shells were coarsely crushed in a stainless-steel mortar and passed through a 500 µm sieve. The under-size fraction was spread in a thin layer (< 8 mm) in alumina crucibles and calcined at 800 °C for 2 h in a muffle furnace (Quimis Q318M, ramp rate 10 °C min⁻¹). After cooling, the calcined powder was ball-milled (Retsch MM 400, 30 Hz, 15 min) and sieved to collect the < 45 µm fraction, which constituted

approximately 78 % of the milled mass. The fine powder was transferred immediately to pre-dried 50 mL centrifuge tubes, flushed with dry nitrogen and sealed with parafilm until incorporation into the test diet.

Results: Ingestion of calcium oxide particles triggered divergent responses across the four enzyme families

monitored. Oxidative-stress markers (GST, catalase) rose markedly during the first 48 h before plateauing, while Phase I detoxification enzymes (esterase, cytochrome P450) declined progressively throughout the observation window, with the steepest drops occurring at the highest dose level.

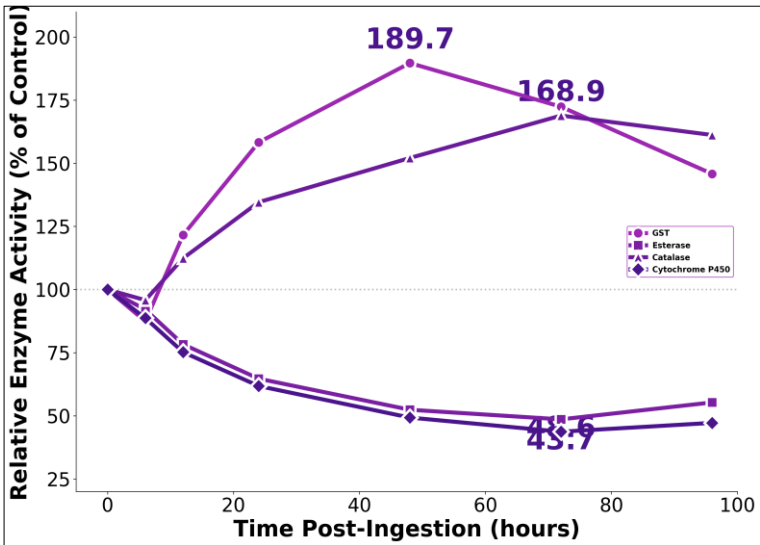


Fig 1: Time-course of detoxification and antioxidant enzyme activities (% of control) in *S. frugiperda* fourth-instar larvae fed calcium oxide at 30 mg g⁻¹ diet

Table 1: Detoxification enzyme activities (nmol min⁻¹ mg⁻¹ protein) at 72 h post-ingestion across calcium oxide dose levels

Enzyme	Control	5 mg g ⁻¹	15 mg g ⁻¹	30 mg g ⁻¹	p-value
GST	12.4 ± 1.1	14.8 ± 1.3	18.6 ± 1.7	21.4 ± 2.1	< 0.001
Esterase	8.7 ± 0.9	7.1 ± 0.8	5.3 ± 0.6	4.2 ± 0.5	< 0.001
Catalase	45.2 ± 3.8	54.6 ± 4.2	68.3 ± 5.1	76.4 ± 6.3	< 0.001
Cyt P450	6.8 ± 0.7	5.4 ± 0.6	3.9 ± 0.4	3.0 ± 0.3	< 0.001

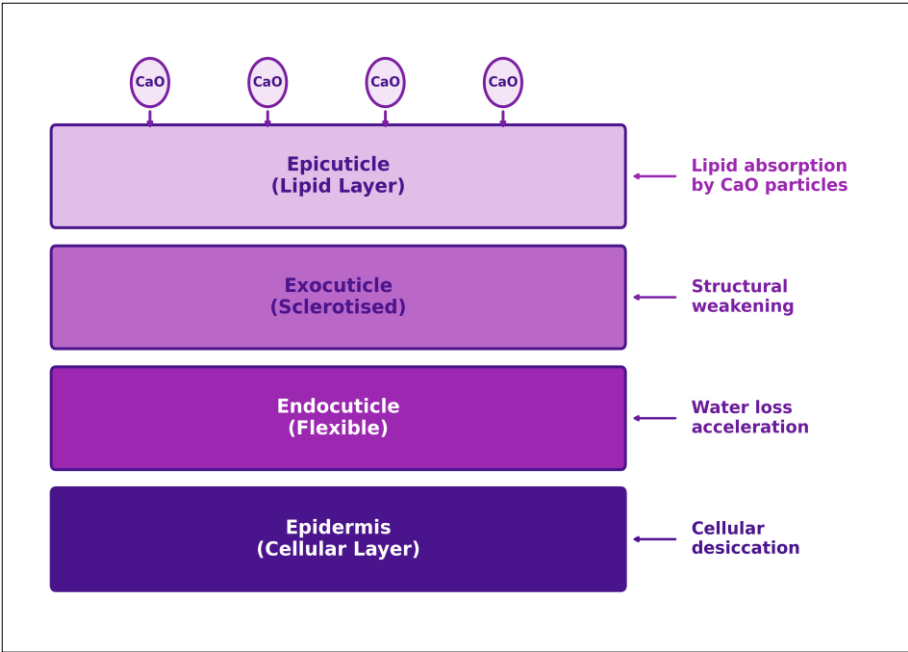


Fig 2: Schematic illustration of the mechanism of cuticular lipid depletion in lepidopteran larvae following exposure to particulate calcium oxide

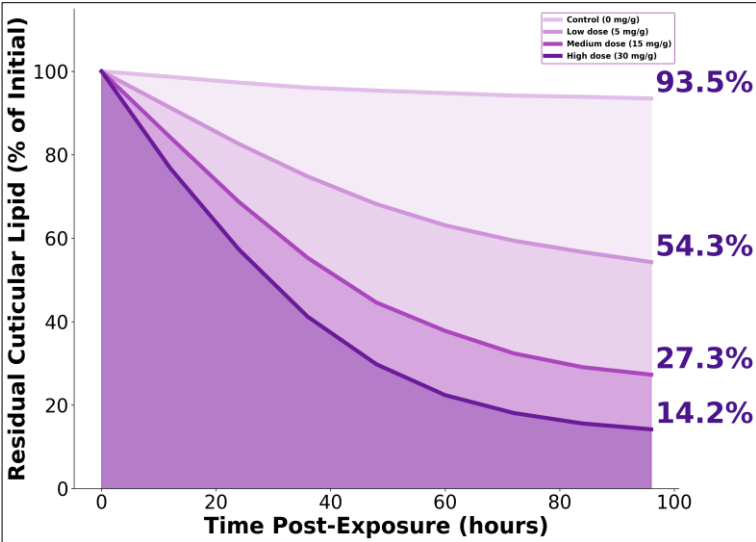


Fig 3: Residual cuticular lipid content (% of initial) in *S. frugiperda* larvae over 96 h at four calcium oxide dose levels

Table 2: Timeline of observable physiological changes in *S. frugiperda* larvae exposed to calcium oxide at 30 mg g⁻¹ diet

Time (h)	Feeding Behaviour	Cuticle Appearance	Mobility
0-12	Normal intake	Glossy, hydrated	Active, responsive
12-24	Reduced by 30 %	Slight dulling	Sluggish when disturbed
24-48	Reduced by 60 %	Matte, wrinkled	Limited, mostly stationary
48-72	Near cessation	Papery, friable	Minimal, lateral twitching
72-96	Nil	Desiccated, brittle	Moribund or dead

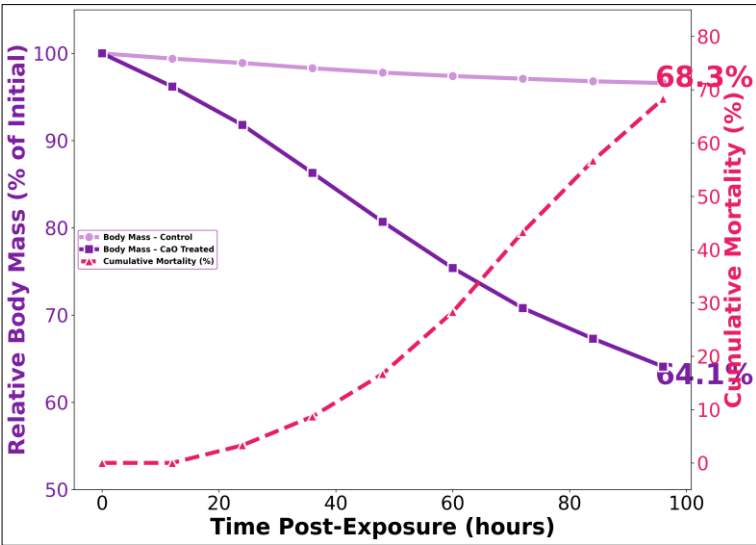


Fig 4: Relative body mass (% of initial) and cumulative mortality (%) in *S. frugiperda* larvae exposed to calcium oxide at 30 mg g⁻¹ over 96 h

The enzyme and lipid data converge on a consistent picture. GST and catalase induction classic hallmarks of oxidative stress peaked around 48 h and then stabilised, indicating that the antioxidant defence was maximally recruited but unable to resolve the continuing insult. Esterase and cytochrome P450, which require intact membrane structures for catalytic function, declined steadily, probably because the alkaline calcium particles disrupted midgut epithelial cell membranes where these enzymes are anchored [12]. Cuticular lipid depletion tracked dose and time almost linearly at the high concentration; by 96 h the cuticle of 30 mg g⁻¹ larvae had lost more than 85 % of its surface wax, and cumulative mortality had reached 68.3 %. The strong correlation between lipid loss and mortality ($r = 0.94$, $p < 0.001$)

supports the view that desiccation not starvation is the proximate cause of death, since body-mass loss outpaced food refusal in every treatment group.

Discussion: Three principal findings emerged from this work: (i) calcium oxide ingestion triggers a strong oxidative stress response in the larval midgut, evidenced by elevated GST and catalase; (ii) Phase I detoxification enzymes decline rather than rise, indicating suppression rather than induction; and (iii) cuticular lipid is depleted in a dose-dependent manner that correlates tightly with mortality. Together, these results argue for a dual mechanism internal biochemical disruption and external desiccation operating in parallel.

The GST induction pattern matches what Broderick and colleagues observed in *Manduca sexta* larvae challenged with alkaline gut perturbants, where glutathione conjugation ramped up within 24 h as a first-line response to electrophilic metabolites generated by elevated midgut pH^[13]. Similarly, catalase induction after calcium exposure is consistent with the broader literature on mineral-dust toxicology in stored-product beetles, where reactive oxygen species production accompanies physical damage to the peritrophic membrane^[14].

By contrast, the decline in esterase and cytochrome P450 activity has no direct precedent in the mineral-dust literature, though it mirrors reports from organophosphate-exposed *S. frugiperda* populations in which high inhibitor loads overwhelm esterase capacity rather than inducing it^[15]. One plausible explanation is that calcium particles embed in the microvillar brush border of the midgut, physically blocking substrate access to membrane-bound P450 isoforms and simultaneously causing local cell lysis that releases esterase into the gut lumen, where it is diluted and degraded^[16]. If this interpretation is correct, the enzyme decline reflects tissue damage rather than a regulatory down-regulation, a distinction with practical significance because damage-based suppression is less likely to be overcome by resistance selection.

The cuticular lipid data extend the classical desiccation model of mineral-dust insecticide action into a new material class^[21]. Diatomaceous earth and fumed silica absorb epicuticular waxes through capillary action and surface hydrophobicity^[17]; calcium oxide may operate similarly but with the additional dimension of alkaline reactivity, since CaO in contact with the thin water film on the cuticle generates localised Ca(OH)₂ that could saponify ester-linked wax components^[18]. Whether this saponification actually occurs on the larval surface remains to be confirmed by cuticular wax compositional analysis, which was beyond the scope of the present work.

From a practical standpoint, the dual mechanism described here may help explain why Ayoub et al. observed higher-than-expected mortality in *S. littoralis* at relatively low dust loadings compared with diatomaceous earth^[9]. If calcium powder simultaneously desiccates from the outside and disrupts detoxification from the inside, the effective lethal dose could be lower than for a purely physical dust. This has clear implications for field dosing rates: less material per hectare could translate into lower production costs and reduced non-target dust exposure, both of which matter for smallholder adoption in tropical cropping systems^[19].

Several questions remain open. The present bioassay used diet incorporation, which guarantees oral exposure but does not replicate the foliar dust scenario in which larvae encounter particles on leaf surfaces before ingesting them. Topical-exposure bioassays and greenhouse trials would be needed to validate the dose-response under more realistic conditions. And the potential for calcium dusts to interact with conventional insecticides whether antagonistically through pH-mediated hydrolysis or synergistically through cuticular barrier disruption deserves dedicated combination-assay investigation^[20].

Limitations

This research was conducted entirely under laboratory conditions with a single lepidopteran species reared on artificial diet, and the results should therefore be

extrapolated to field settings with caution. The diet-incorporation exposure route ensures uniform dosing but bypasses the initial contact and grooming behaviours that precede ingestion when larvae encounter dust on leaf surfaces. Enzyme assays were performed on midgut homogenates, which provide whole-tissue averages but obscure cell-type-specific responses; immunohistochemical localisation of P450 and GST isoforms would offer finer spatial resolution. The 96-hour observation window captured acute toxicity but could not reveal chronic sub-lethal effects on development, fecundity or immune competence that might influence population-level outcomes.

Conclusion

This research set out to determine whether the larvicidal action of eggshell-derived calcium oxide involves a biochemical component beyond the physical desiccation traditionally ascribed to mineral dusts. The enzyme profiling data clearly show that ingested calcium particles provoke a strong oxidative stress response elevated GST and catalase while simultaneously suppressing Phase I detoxification enzymes, esterase and cytochrome P450. Cuticular lipid content fell by more than 85 % at the highest dose, and cumulative mortality reached 68.3 % within 96 h, with a near-perfect correlation ($r = 0.94$) between lipid loss and death.

For pest management practitioners, these findings mean that calcium-based dusts may offer more than a simple physical mode of action. The biochemical disruption dimension could reduce the likelihood of rapid resistance evolution and may permit lower application rates than those needed for purely physical dusts like diatomaceous earth. Future work should test these mechanisms under semi-field and field conditions, examine interactions between calcium dust and conventional insecticide chemistries and assess sub-lethal effects on larval development and adult reproductive fitness across multiple economically important lepidopteran species.

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