CALCIUM FORTIFICATION OF *TENEBRIO MOLITOR* (LINNAEUS, 1758) LARVAE: POWDER MIXING VERSUS SPRAY APPLICATION

FORTIFICAÇÃO COM CÁLCIO DE LARVAS DE *TENEBRIO MOLITOR* (LINNAEUS, 1758): MISTURA DE PÓ VERSUS APLICAÇÃO POR PULVERIZAÇÃO

ENRIQUECIMIENTO CON CALCIO DE LARVAS DE *TENEBRIO MOLITOR* (LINNAEUS, 1758): MEZCLA EN POLVO FRENTE A APLICACIÓN POR PULVERIZACIÓN

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ABSTRACT
Low calcium (Ca) content and inverted Ca:P ratios are major nutritional limitations of *Tenebrio molitor* larvae when used as feeder insects or ingredients in compound feeds. This study evaluated two Ca fortification strategies by comparing wheat-bran diets nominally supplemented with calcium carbonate ($CaCO_3$) at 4, 8, and 12% (w/w): dry powder mixing (POW) and spraying an aqueous $CaCO_3$ suspension onto the substrate followed by drying (SPR). Larvae were fed the experimental diets *ad libitum* for 48 h and then fasted for 48 h to promote gut clearance prior to analysis. Calcium concentrations in diets and larvae were quantified by flame atomic absorption spectrophotometry. Dietary Ca increased strongly with $CaCO_3$ inclusion in both series, and all fortified diets exceeded the control. At 4% and 8% $CaCO_3$, SPR diets contained more Ca than the corresponding POW diets, whereas no difference was observed at 12%. Larval Ca increased significantly with inclusion level: relative to control larvae, POW diets produced approximately 1.7–2.7-fold enrichment and SPR diets produced approximately 2.5–3.4-fold enrichment. Two-way ANOVA confirmed significant effects of fortification strategy and inclusion level, with SPR yielding higher larval Ca than POW at matched $CaCO_3$ levels. These results identify spray-based $CaCO_3$ application as an effective approach to improve calcium transfer from substrate to *T. molitor* larvae and produce calcium-fortified larvae with elevated Ca content.
Keywords: *Tenebrio molitor*; Gut-loading; Calcium carbonate; Spray application; Wheat bran; Nutritional plasticity.

RESUMO
O baixo teor de cálcio (Ca) e as razões Ca:P invertidas são limitações nutricionais importantes das larvas de *Tenebrio molitor* quando utilizadas como insetos-alimento ou ingredientes em rações compostas. Este estudo avaliou duas estratégias de fortificação com Ca, comparando dietas à base de farelo de trigo suplementadas nominalmente com carbonato de cálcio ($CaCO_3$) nas proporções de 4%, 8% e 12% (p/p): mistura de pó seco (POW) e pulverização de uma suspensão aquosa de $CaCO_3$ sobre o substrato, seguida de secagem (SPR). As larvas foram alimentadas com as dietas experimentais *ad libitum* por 48 h e, em seguida, submetidas a um jejum de 48 h para promover o esvaziamento do trato digestório antes da análise. As concentrações de cálcio nas dietas e nas larvas foram quantificadas por espectrofotometria de absorção atômica com chama. O teor de Ca nas dietas aumentou acentuadamente com a inclusão de $CaCO_3$ em ambas as séries, e todas as dietas fortificadas apresentaram níveis superiores aos da dieta controle. Nos níveis de 4% e 8% de $CaCO_3$, as dietas SPR apresentaram maior teor de Ca do que as dietas POW correspondentes, enquanto não foi observada diferença no nível de 12%. O teor de Ca nas larvas aumentou significativamente com o nível de inclusão: em relação às larvas do grupo controle, as dietas POW proporcionaram um enriquecimento de aproximadamente 1,7 a 2,7 vezes, e as dietas SPR, um enriquecimento de aproximadamente 2,5 a 3,4 vezes. A análise de variância (ANOVA) de dois fatores confirmou efeitos significativos da estratégia de fortificação e do nível de inclusão, com o método SPR resultando em maiores teores de Ca nas larvas do que o método POW para os mesmos níveis de $CaCO_3$. Estes resultados apontam a aplicação de $CaCO_3$ via pulverização como uma abordagem eficaz para melhorar a transferência de cálcio do substrato para as larvas de *T. molitor* e produzir larvas fortificadas com elevado teor de Ca.
Palavras-chave: *Tenebrio molitor*; Enriquecimento nutricional; Carbonato de cálcio; Aplicação por pulverização; Farelo de trigo; Plasticidade nutricional.

RESUMEN
El bajo contenido de calcio (Ca) y las relaciones Ca:P invertidas son limitaciones nutricionales importantes para las larvas de Tenebrio molitor cuando se utilizan como insectos de consumo o ingredientes en dietas compuestas. Este estudio evaluó dos estrategias de fortificación de Ca, comparando dietas a base de salvado de trigo suplementadas nominalmente con carbonato de calcio ($CaCO_3$) en proporciones de 4%, 8% y 12% (p/p): mezcla de polvo seco (POW) y pulverización de una suspensión acuosa de $CaCO_3$ sobre el sustrato, seguida de secado (SPR). Las larvas fueron alimentadas con las dietas experimentales ad libitum durante 48 h y luego sometidas a un ayuno de 48 h para promover el vaciamiento del tracto digestivo antes del análisis. Las concentraciones de calcio en las dietas y las larvas se cuantificaron mediante espectrofotometría de absorción atómica de llama. El contenido de calcio en las dietas aumentó bruscamente con la inclusión de $CaCO_3$ en ambas series, y todas las dietas fortificadas mostraron niveles más altos que la dieta de control. En los niveles de $CaCO_3$ del 4% y 8%, las dietas SPR mostraron un contenido de calcio mayor que las dietas POW correspondientes, mientras que no se observó diferencia en el nivel del 12%. El contenido de calcio en las larvas aumentó significativamente con el nivel de inclusión: en comparación con las larvas del grupo control, las dietas POW proporcionaron un enriquecimiento de aproximadamente 1,7 a 2,7 veces, y las dietas SPR, un enriquecimiento de aproximadamente 2,5 a 3,4 veces. El análisis de varianza bidireccional (ANOVA) confirmó los efectos significativos de la estrategia de fortificación y el nivel de inclusión, con el método SPR resultando en un mayor contenido de calcio en las larvas que el método POW para los mismos niveles de $CaCO_3$. Estos resultados sugieren que la aplicación de $CaCO_3$ mediante pulverización es un enfoque eficaz para mejorar la transferencia de calcio del sustrato a las larvas de T. molitor y producir larvas fortificadas con un alto contenido de Ca.
Palabras-clave: *Tenebrio molitor*; Enriquecimiento nutricional; Carbonato de calcio; Aplicación por pulverización; Salvado de trigo; Plasticidad nutricional.

INTRODUCTION

The use of edible insects as feed ingredients has gained increasing attention as a sustainable strategy to support animal production, given their efficient feed conversion, comparatively low environmental footprint, and attractive nutritional profiles (RUMPOLD & SCHLÜTER, 2013; OONINCX & DE BOER, 2012; ZHOU et al., 2022; ROSSI et al., 2025; RAGOZZINO-PAULINO et al., 2025). Many species provide high levels of metabolizable energy and protein, as well as essential fatty acids, vitamins, and minerals, positioning insects as promising alternatives or complements to conventional feedstuffs in monogastric and aquaculture systems (HONG et al., 2020; OONINCX & FINKE, 2021; ROSSI et al., 2025). Among the most widely reared species, *Tenebrio molitor* is particularly valued due to its ease of mass production, relatively undemanding husbandry requirements, and broad acceptance in the feed industry (HONG et al., 2020; FIALHO et al., 2021; LANGSTON et al., 2024).

Despite these advantages, *T. molitor* larvae exhibit an imbalanced mineral profile characterized by very low calcium and relatively high phosphorus contents (OONINCX & DIERENFELD, 2012; FIALHO et al., 2021; LATNEY et al., 2017; LANGSTON et al., 2024; NOYENS et al., 2023; RAGOZZINO-PAULINO et al., 2025). Surveys of invertebrate prey indicate that calcium is frequently the limiting mineral, whereas phosphorus and several other trace elements tend to be present at adequate or even high concentrations (OONINCX & DIERENFELD, 2012). For many vertebrate species optimal skeletal development and mineral homeostasis depend on an appropriate dietary calcium-to-phosphorus (Ca:P) ratio, typically near 1:1 to 2:1, with approximately 2:1 commonly recommended for growing animals and for prevention of nutritional metabolic bone disease (KLAPHAKE, 2010; HEDLEY, 2012; NIJBOER, 2025). Diets based on non-supplemented *T. molitor* larvae therefore provide an inverted Ca:P ratio and an overall calcium intake that may be insufficient to sustain appropriate bone mineralization. This limitation is widely recognized in

reptile and other exotic-animal husbandry, where exclusive or predominant use of unsupplemented insects has been associated with nutritional metabolic bone disease and related skeletal disorders (MCCLEMENTS, 2003; KLAPHAKE, 2010; HEDLEY, 2012; LATNEY et al., 2017; TREIBER, 2018; AZARGOUN et al., 2025).

To address this constraint, calcium fortification of feeder insects has become routine practice, most commonly through “dusting”, i.e., surface-coating insects with calcium powders immediately before feeding, or through “gut-loading”, in which insects are offered calcium-enriched diets for a defined period before being consumed (KLASING et al., 2000; FINKE, 2003; BOYER & SCOTT, 2019; OONINCX & FINKE, 2021, 2023). Calcium carbonate, calcium citrate, and various calcium phosphates are among the mineral salts most frequently used in these strategies (FINKE, 2003; BOYER & SCOTT, 2019; TREIBER, 2018). However, reported outcomes vary widely across studies and husbandry protocols, partly because mineral measurements can be influenced by diet formulation, feeding duration, and whether insects are analysed immediately after gut-loading or after a post-feeding interval that allows partial gut-content clearance. This distinction is important when interpreting Ca:P benchmarks and comparing fortification approaches under different sampling endpoints.

From a feed-processing and large-scale production standpoint, the way in which inorganic calcium salts are incorporated into insect diets may also affect the practical dispersion of the mineral within the substrate and the efficiency of mineral transfer to the larvae. In practical wheat-bran–based substrates—the standard feed for *T. molitor*—dense calcium carbonate particles differ markedly in density and particle size from the surrounding feed matrix and may segregate during mixing and handling, settling at the bottom of rearing containers and mixing with frass during routine cleaning (FINKE, 2002; LATNEY et al., 2017). This behaviour can lead to losses of supplement and uneven availability of calcium within the diet, so that larvae within the same batch may experience different levels of calcium exposure. Consistent with this, studies assessing the mineral composition of mealworms produced under routine or experimental husbandry have reported within-treatment variability in larval calcium content even when insects were held on fortified diets, suggesting incomplete or uneven access to the supplemented fraction of the feed (LATNEY et al., 2017; NOYENS et al., 2023).

In this context, refining how calcium salts are incorporated into *T. molitor* diets may be as important as the inclusion level itself. Applying $CaCO_3$ as an aqueous dispersion sprayed onto the substrate, followed by controlled drying, could improve adhesion of the mineral to feed particles, reduce segregation risk, and enhance effective exposure of larvae to the fortified fraction. However, empirical data comparing such sprayed diets with conventional dry-powder incorporation in *T. molitor* remain scarce. Therefore, the present study compared two technological strategies for calcium fortification of *T. molitor* larvae—incorporation of $CaCO_3$ as a dry powder versus application as a water-based spray—using wheat-bran diets formulated at defined $CaCO_3$ inclusion levels. The objective was to determine whether spray application improves the analytical calcium content of fortified diets and enhances the magnitude and repeatability of calcium enrichment in *T. molitor* larvae relative to the conventional powdered form. Overall, the results indicate that spray application improves calcium transfer from substrate to larvae and supports more predictable calcium enrichment under the tested preparation protocol.

MATERIALS AND METHODS

Model insect and rearing conditions

T. molitor larvae were sourced from a certified private company (Metamorphosis Biotechnology, São Paulo, Brazil). Prior to the fortification trials, insects were fasted for 48 h with access to demineralized water to allow gut clearance and stabilization. Larvae were maintained on a wheat-bran diet with carrot slices supplied ad libitum as a moisture source. Only healthy, actively feeding larvae of similar size and developmental stage were selected for the experiments.

Experimental design

Experimental design

Wheat bran was used as the basal substrate for all experimental diets (control, CTRL). Calcium fortification was achieved using food-grade calcium carbonate ($CaCO_3$) sourced from a certified private company (Produquímica Indústria e Comércio S/A, São Paulo, Brazil). Two strategies for incorporating $CaCO_3$ into the wheat-bran diets were evaluated.

In the dry powder treatments (POW), $CaCO_3$ was weighed and mixed into pre-weighed wheat bran to obtain diets with nominal inclusion levels of 4, 8 and 12% $CaCO_3$ (w/w; final diet). Diets were prepared in 1.0 kg wheat-bran batches using a 5 L polypropylene container. The required $CaCO_3$ mass was calculated on a final-diet basis as 41.7 g, 87.0 g and 136.4 g $CaCO_3$ per 1.0 kg wheat bran for POW-4, POW-8 and POW-12, respectively (i.e., equation 1). To minimise segregation, $CaCO_3$ was first blended with 10% of the wheat bran (100 g) to form a premix (2 min manual mixing), which was then progressively incorporated into the remaining bran (6 min manual mixing by repeated folding and tumbling), for a total mixing time of 8 min. Diets were subsequently stored in closed containers, handled with minimal vibration, and portioned immediately after mixing to reduce particle-size/density-driven separation.

$$m\ CaCO_3 \quad = \frac{f}{1-f}\ x\ mbran$$

where $m\ CaCO_3$ is the mass of $CaCO_3$ to be added, *mbran* is the mass of wheat bran, and f is the target $CaCO_3$ fraction in the final diet.

In the spray-application treatments (SPR), $CaCO_3$ was first dispersed in deionised water to form a suspension, which was then sprayed onto pre-weighed portions of wheat bran under continuous mixing. The volume of suspension and the amount of $CaCO_3$ were adjusted so that, after drying, the fortified diets matched the same nominal inclusion levels as the corresponding powder diets. For reproducibility, spray diets were prepared in 1.0 kg wheat-bran batches using a 200 g/L $CaCO_3$ suspension maintained under continuous agitation to prevent sedimentation. Based on the same final-diet inclusion targets as POW diets, the required $CaCO_3$ masses were 41.7 g, 87.0 g and 136.4 g per 1.0 kg wheat bran for SPR-4, SPR-8 and SPR-12, respectively, corresponding to spray volumes of 208 mL/kg (SPR-4), 435 mL/kg (SPR-8) and 682 mL/kg (SPR-12). Spraying was performed over 5 min

while mixing, followed by an additional 5 min of mixing to ensure uniform coating of particles prior to drying.

Following spraying, the diets were dried in a PD-15 forced-air oven (Polidryer, Minas Gerais, Brazil) at 50 °C ± 2 °C for 12 h, cooled to room temperature and stored in closed containers until use. Calcium concentration was determined both in the experimental diets and in larvae tissues obtained after exposure to these diets. Treatments were coded as CTRL (control diet, no $CaCO_3$), POW-4, POW-8, POW-12 ($CaCO_3$ supplied as dry powder) and SPR-4, SPR-8, SPR-12 ($CaCO_3$ supplied as sprayed suspension). Calcium and phosphorus contents in both diets and larvae are reported as µg/g dry matter (DM). A schematic overview of the experimental design and the two $CaCO_3$ incorporation strategies (POW vs SPR) is provided in Figure 1.

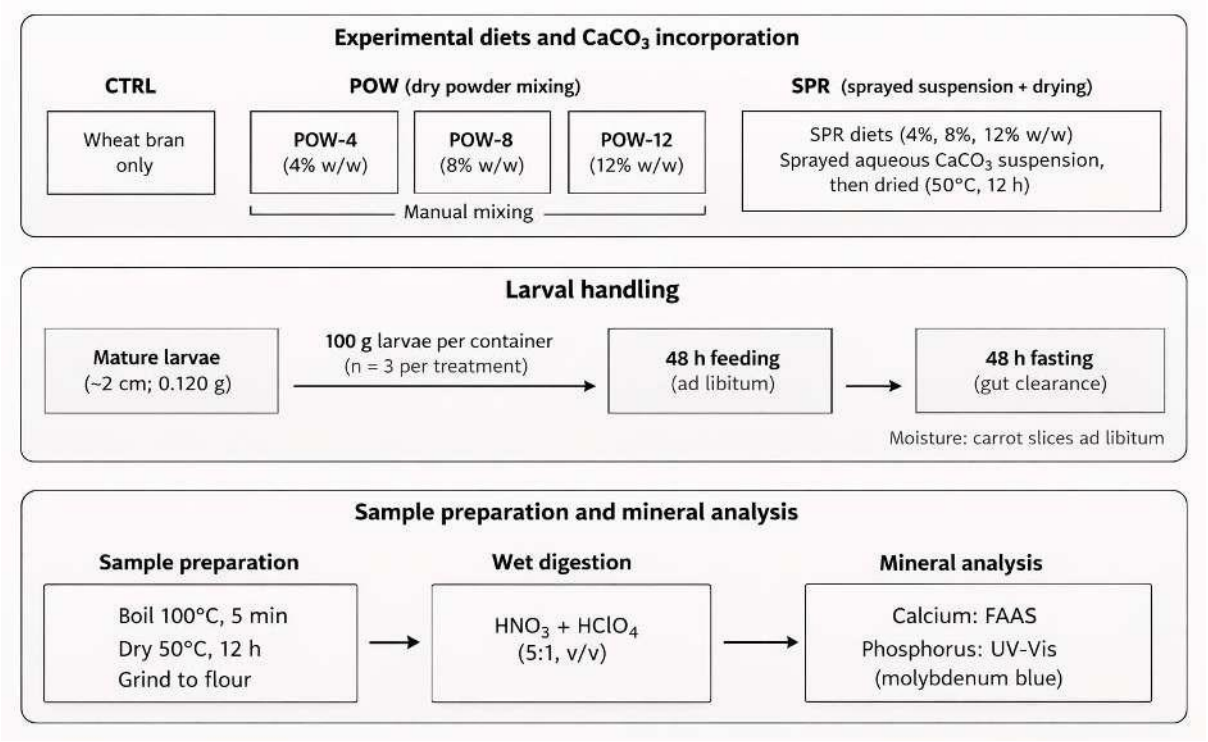


Figure 1. Experimental design and fortification strategies (POW vs SPR).

Larval handling

For the technological comparison, mature larvae (approximately 2 cm in length; mean body mass 0.120 g) were randomly allocated to three independent experimental units per treatment (n = 3), each unit containing 100 g of larvae, and provided with one of the seven diets described above (CTRL, POW-4, POW-8, POW-12, SPR-4, SPR-8, SPR-12) under ad libitum feeding conditions for 48 h. Each experimental unit consisted of a ventilated plastic container (30 × 20 × 12 cm, L × W × H; lid fitted with fine mesh for airflow). Considering the mean larval body mass (0.120 g), each unit contained approximately 833 larvae, corresponding to an areal stocking density of approximately 1.4 larvae/cm², within the range of densities previously tested for *T. molitor* larvae in controlled experiments (DERUYTTER et al., 2022). Environmental conditions were set to standard *T. molitor* rearing conditions, with temperature maintained at 27 ± 1 °C and relative humidity at 60–75%, under darkness

or minimal light exposure, consistent with commonly reported optimal/standard rearing ranges for the species (RIBEIRO et al., 2018).

During the exposure period, moisture was provided ad libitum as equally thin carrot slices across treatments. After the exposure period, larvae were removed from the diets, cleaned of residual substrate, and transferred to clean containers for a 48 h fasting period to allow clearance of gut contents. At the end of the fasting period, insects were frozen at −20 °C and stored until further processing and analysis. For sample preparation, frozen larvae were boiled in water at 100 °C for 5 min, dehydrated in a forced-air oven (PD-15, Polidryer, Minas Gerais, Brazil) at 50 °C ± 2 °C for 12 h, and then ground using a low-speed commercial blender (Skymsen, Santa Catarina, Brazil) to obtain a homogeneous larvae flour for mineral analysis.

Sample preparation and mineral analysis

For mineral analysis, approximately 2 g of sample (diet or larvae flour) were wet digested with HNO_3:$HClO_4$ (5:1, v/v). Calcium (Ca) concentrations were determined by flame atomic absorption spectrophotometry (FAAS) (AOAC, 1985, 2005) and phosphorus (P) concentrations were quantified by UV-Vis spectrophotometry using molybdenum blue method (AOAC, 1995). For calcium, measurements were carried out on a Varian FS2000 AAS equipped with a calcium hollow-cathode lamp, using an air–acetylene oxidising flame with deuterium-lamp background correction for Ca (HORTELLANI, 2013) a lanthanum solution (5% w/v) was used as a releasing agent to minimise chemical interference during Ca determination. The lanthanum solution was prepared by dissolving 11.8 g of La_2O_3 in 50 mL of concentrated HCl and diluting to 200 mL with deionised water; it was added to digested samples at 1 mL per 10 mL of digest (KAUSSHIK, 2023). The limits of detection and quantification (LOD and LOQ) for Ca and P were determined according to the procedure recommended by the National Institute of Metrology (INMETRO, 2019). LOD and LOQ were estimated from seven replicate measurements of a matrix blank processed through the full digestion procedure and spiked with a low-level Ca standard (0.2 μg g^{-1}), using the following equations:

$$LOD = \underline{X} + ts$$

$$LOQ = \underline{X} + 10s$$

where $\underline{X}$ is the mean response of the seven processed blanks, *s* is the standard deviation of the seven processed blanks, and *t* is the Student's t value for six degrees of freedom at $p < 0.05$ ($t = 3.143$). For Ca, occasional negative blank readings were treated as zero when calculating $\underline{X}$. The LOD values were 0.15 μg g^{-1} for Ca and 0.20 μg g^{-1} for P, and the LOQ values were 0.50 μg g^{-1} for Ca and 0.65 μg g^{-1} for P.

Statistical analysis

Calcium (Ca) and phosphorus (P) concentration data in diets and larvae were analysed separately. For each matrix (diets; larvae), a one-way ANOVA with "treatment" as a fixed factor was used to compare all experimental groups (CTRL, POW-4, POW-8, POW-12, SPR-4, SPR-8, SPR-12). When the overall F-test was significant, means were separated using Tukey's honestly significant difference (HSD) test to identify pairwise differences

among treatments. In addition, to explicitly assess the effects of fortification strategy (POW vs SPR) and inclusion level (4, 8, 12% $CaCO_3$), a two-way ANOVA was conducted on the subset of $CaCO_3$-supplemented treatments (POW-4, POW-8, POW-12, SPR-4, SPR-8, SPR-12). In this model, fortification strategy and inclusion level were considered fixed factors, and their main effects and interaction were evaluated. A significance level of $p < 0.05$ was adopted for all tests. The experimental unit was the container, and each treatment consisted of three independent experimental units (n = 3).

RESULTS

Quality control data

The results of the quality control data are present in Table 1.

Table 1. The Ca and P content median ± U (expanded uncertainty with K=2 and 95% confidence interval) (n = 3) and recovery (%) of Ca and P in NIST SRM 1548a (Total Diet).

	Ca (µ g-1)	**P (µ g-1)**
Certified	1740 ± 100	3240 ± 40
Obtained	1739 ± 174	3328 ± 319
Recovery rate (%)	99.9	103

Calcium and phosphorus concentrations in experimental diets

Calcium contents of the experimental diets are summarised in Table 2. One-way ANOVA showed a highly significant effect of treatment on dietary calcium ($F(6,14) = 269.8$, $p < 0.001$). The control diet (CTRL) had the lowest calcium concentration (115 µg/g DM), and all $CaCO_3$-supplemented diets exhibited significantly higher calcium concentrations than CTRL (Tukey's HSD, $p < 0.05$; Table 2). Within powder diets, calcium increased progressively from POW-4 to POW-8 and POW-12, with each treatment differing significantly from the others (Table 2). A similar dose-dependent pattern was observed for sprayed diets, with SPR-4, SPR-8 and SPR-12 all differing significantly pairwise (Table 2). Two-way ANOVA restricted to Ca-supplemented diets (factors: strategy, POW vs SPR; level, 4–8–12% $CaCO_3$) confirmed significant main effects of fortification strategy ($F(1,12) = 81.1$, $p < 0.001$) and inclusion level ($F(2,12) = 327.9$, $p < 0.001$), as well as a significant interaction ($F(2,12) = 25.1$, $p < 0.001$). At 4% and 8% $CaCO_3$, sprayed diets (SPR-4 and SPR-8) had significantly higher calcium concentrations than the corresponding powder diets (POW-4 and POW-8; Table 2). At 12% $CaCO_3$, POW-12 and SPR-12 did not differ significantly, indicating similar dietary calcium concentrations at the highest inclusion level (Table 2).

Phosphorus concentrations in the experimental diets are also reported in Table 2. Dietary P differed among treatments (one-way ANOVA, $F(6,14) = 10.81$, $p = 0.00014$). Overall, most $CaCO_3$-supplemented diets showed higher P values than CTRL (Tukey's HSD, $p < 0.05$; Table 2), whereas POW-12 presented an intermediate value and did not differ significantly from either CTRL or the higher-P fortified diets (Table 2). In the two-way ANOVA restricted to $CaCO_3$-supplemented diets, neither the main effect of inclusion level ($F(2,12) = 2.72$, $p = 0.106$) nor the main effect of fortification strategy ($F(1,12) = 1.93$, $p = 0.190$) reached statistical significance, while the strategy × level interaction showed a

borderline trend ($F(2,12) = 3.60$, $p = 0.060$). Accordingly, dietary phosphorus did not follow a monotonic dose–response pattern across inclusion levels, and differences between POW and SPR were not systematic (Table 2).

Calcium and phosphorus concentrations in *T. molitor* larvae

Calcium concentrations in *T. molitor* larvae differed significantly among treatments (one-way ANOVA, $F(6,14) = 87.2$, $p < 0.001$; Table 2). Control larvae (CTRL) showed the lowest calcium level (428 µg/g DM), and all $CaCO_3$-fortified groups had significantly higher calcium concentrations than CTRL (Tukey's HSD, $p < 0.05$; Table 2). In powder-fed larvae, calcium increased stepwise from POW-4 to POW-8 and POW-12, with each treatment differing significantly from the others (Table 2). In sprayed larvae, calcium was higher in SPR-8 and SPR-12 than in SPR-4, whereas SPR-8 and SPR-12 did not differ from each other (Table 2).

Table 2. Calcium and phosphorus concentrations in experimental diets and in *T. molitor* larvae.

Treatment	Diet Ca (µg g^{-1} DM)	Diet P (µg g^{-1} DM)	Larvae Ca (µg g^{-1}DM)	Larvae P (µg g^{-1} DM)
CTRL	115 ± 1.5^{a}	4919 ± 506^{a}	428 ± 25^{a}	5774 ± 570^{a}
POW-4	21223 ± 515^{b}	8745 ± 952^{b}	715 ± 41^{b}	6782 ± 111^{a}
POW-8	47406 ± 7351^{c}	8690 ± 684^{b}	943 ± 25^{c}	7213 ± 186^{a}
POW-12	97299 ± 1878^{e}	6772 ± 663^{ab}	1173 ± 65^{d}	7017 ± 33^{a}
SPR-4	47652 ± 5130^{c}	8937 ± 618^{b}	1065 ± 64^{d}	7174 ± 554^{a}
SPR-8	76339 ± 4502^{d}	8166 ± 844^{b}	1359 ± 138^{e}	6879 ± 276^{a}
SPR-12	95350 ± 1068^{e}	8720 ± 1067^{b}	1442 ± 34^{e}	7716 ± 1284^{b}

Results are expressed as mean values of three independent experimental units per treatment (n = 3) ± standard deviation (SD).
Means in each column followed by different letters are significantly different (Tukey's HSD test, $p < 0.05$).
CTRL (control diet without added $CaCO_3$), POW-4, POW-8, POW-12 (diets containing 4, 8 and 12% $CaCO_3$ mixed as dry powder), and SPR-4, SPR-8, SPR-12 (diets containing 4, 8 and 12% $CaCO_3$ applied as sprayed suspension) and corresponding larvae. Calcium was determined by flame atomic absorption spectrophotometry (AAS) and Phosphorus was determined by UV–Vis spectrophotometry. Units: µg g^{-1} dry matter (DM).

Two-way ANOVA restricted to $CaCO_3$-supplemented larvae indicated significant main effects of fortification strategy ($F(1,12) = 106.7$, $p < 0.001$) and inclusion level ($F(2,12) = 53.2$, $p < 0.001$), with no significant strategy × level interaction ($F(2,12) = 1.61$, $p = 0.241$). Across all inclusion levels, larvae from sprayed diets showed significantly higher calcium concentrations than those from powder diets at the same $CaCO_3$ level (SPR-4 > POW-4, SPR-8 > POW-8, SPR-12 > POW-12; Table 2). Larvae phosphorus concentrations are reported in Table 2 and showed a modest treatment effect (one-way ANOVA, $p < 0.05$), driven by the higher P value in SPR-12 relative to all other groups; the remaining treatments

did not differ from each other (Tukey's HSD, $p < 0.05$; Table 2). Accordingly, no consistent dose-dependent pattern was evident for phosphorus across inclusion levels.

Because the biological motivation for calcium fortification is often to correct the intrinsically inverted Ca:P of mealworms, it is also relevant to consider phosphorus as a secondary factor shaping mineral balance. In the present study, larvae P showed only a modest treatment effect overall, whereas calcium increased strongly and monotonically with $CaCO_3$ inclusion within both fortification strategies and, across levels, was higher under the sprayed strategy (Table 2). Accordingly, larvae Ca:P (Ca/P) improved markedly relative to the control, rising from around 0.07:1 in CTRL to about 0.11:1 in POW-4 and reaching roughly 0.19:1 in SPR-8 and SPR-12 (Table 3), with sprayed diets yielding consistently more favourable ratios than powder diets at the same nominal inclusion level. Overall, these results show that short-term $CaCO_3$ gut-loading can substantially raise larvae calcium and improve mineral balance, while further optimisation may be required to approach commonly recommended Ca:P targets (e.g., 1:1–2:1) in larvae tissues.

Table 3. Calcium-to-phosphorus ratio (Ca:P) in *T. molitor* larvae after $CaCO_3$ fortification (POW vs SPR).

Treatment	Larvae (Ca:P)
CTRL	0.07:1
POW-4	0.11:1
POW-8	0.13:1
POW-12	0.17:1
SPR-4	0.16:1
SPR-8	0.19:1
SPR-12	0.19:1

Ca:P values were calculated as the ratio of calcium to phosphorus (Ca/P) using paired replicate measurements (n = 3) for each treatment. CTRL (control wheat bran), POW (dry powder mixing) and SPR (sprayed aqueous $CaCO_3$ suspension followed by drying) at nominal $CaCO_3$ inclusion levels of 4, 8 and 12% (w/w).

DISCUSSION

Calcium concentration in experimental diets

In the present study, wheat-bran diets were successfully converted into high-calcium substrates for *T. molitor* through the nominal inclusion of 4–12% $CaCO_3$ (w/w), increasing dietary calcium from 115 µg/g DM in CTRL to 21223 µg/g DM in POW-4 and up to 97299 µg/g DM in POW-12 (Table 2; Figure 2), corresponding to an approximately 183–841-fold increase relative to CTRL. This confirms that cereal-based substrates can serve as practical carriers for calcium gut-loading and aligns with evidence that *T. molitor* exposed to $CaCO_3$-enriched substrates can markedly increase larval calcium and improve mineral supply to consumers (KLASING et al., 2000). More broadly, the clear dose–response patterns within both the POW and SPR series indicate that the preparation protocols generated consistent diet gradients, which is consistent with the responsiveness of mealworm composition to

controlled changes in rearing substrates (MANCINI et al., 2019; NOYENS et al., 2023; NOYENS et al., 2024; OONINCX, 2020).

From a process standpoint, at 4% and 8% $CaCO_3$ the sprayed diets (SPR-4, SPR-8) contained significantly more calcium than the corresponding powder diets (POW-4, POW-8), whereas at 12% no difference was detected between POW-12 and SPR-12 (Table 2). This pattern is consistent with the tendency of particulate systems to segregate when components differ markedly in particle size and/or bulk density, which can impair uniform distribution of dense mineral fractions in cereal matrices during mixing and handling (ZINN, 2004; DESAI et al., 2024; NUNNENKAMP et al., 2022). Applying $CaCO_3$ as an aqueous dispersion followed by controlled drying likely increased adhesion of mineral particles to bran surfaces and reduced segregation at intermediate inclusion levels, thereby increasing the recovered calcium in the analysed substrate. The convergence between POW and SPR at 12% suggests that, above a certain mineral loading, both methods produce similarly high-calcium substrates, and the relative advantage of spraying becomes smaller. As a secondary consideration, dietary phosphorus differed among treatments but showed no systematic pattern across $CaCO_3$ inclusion levels or between fortification strategies (Table 2), with POW-12 presenting an intermediate value relative to CTRL and the higher-P fortified diets.

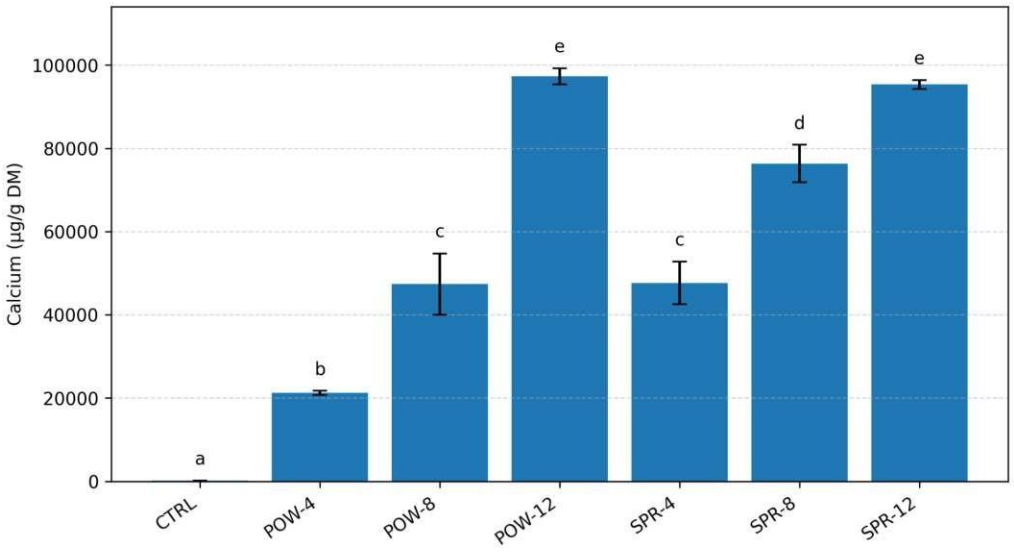


Figure 2. Calcium concentration in experimental diets (DM).
*Calcium concentration in wheat-bran diets: CTRL (control diet without added $CaCO_3$), POW-4, POW-8, POW-12 (diets containing 4, 8 and 12% $CaCO_3$ mixed as dry powder), and SPR-4, SPR-8, SPR-12 (diets containing 4, 8 and 12% $CaCO_3$ applied as sprayed suspension). Values are mean ± SD (n = 3). Different letters indicate significant differences among treatments (Tukey's HSD, $p < 0.05$). Calcium is expressed as µg/g dry matter (DM).

Calcium fortification of *T. molitor* larvae

Beyond their baseline profile, *T. molitor* larvae display pronounced nutritional plasticity, with mineral composition responding measurably to short-term dietary manipulation (OONINCX, 2020; NOYENS et al., 2023; NOYENS et al., 2024). Here, exposure for 48 h followed by 48 h fasting was sufficient to substantially increase larvae

calcium. Relative to control larvae, powder diets increased calcium by approximately 1.7-fold (POW-4), 2.2-fold (POW-8) and 2.7-fold (POW-12), while sprayed diets achieved larger enrichments of approximately 2.5-fold (SPR-4), 3.2-fold (SPR-8) and 3.4-fold (SPR-12) (Table 2; Figure 3). These results reinforce that *T. molitor* can function as an effective vehicle for calcium fortification and that the enrichment outcome depends not only on inclusion level but also on how the mineral is incorporated into the substrate.

The upper range observed in this study is consistent with the feasibility of calcium gut-loading reported for mealworms, although the magnitude of Ca:P improvement can vary markedly with diet formulation, mineral availability, and feeding/handling conditions (KLASING et al., 2000; FINKE, 2003; BOYKIN et al., 2021; LATNEY et al., 2017). Klasing et al. (2000) demonstrated a strong increase in mealworm calcium following exposure to $CaCO_3$-enriched substrates (0–12% Ca from $CaCO_3$), and importantly showed that mealworm calcium increased rapidly during the first 24 h and was influenced by both substrate type (wheat bran vs. chicken starter) and exposure duration. Likewise, the modelling and synthesis presented by Finke (2003) indicated that calcium-enriched gut-loading diets can materially improve insect calcium content and shift Ca:P ratios toward ranges used in husbandry recommendations for many insectivores, with outcomes depending on diet composition and intake. More recently, Boykin et al. (2021) reported that mealworms in a low-calcium state (e.g., fasted or maintained on wheat bran) exhibited very low Ca relative to P, whereas a commercial high-calcium gut-loading diet raised Ca:P ratios to above 3:1 after 24–48 h. Complementing these findings, Latney et al. (2017) experimentally evaluated multiple diets and feeding intervals (2, 7 and 10 days) and found that a high-calcium commercial feed produced the highest calcium content and Ca:P ratios in mealworms among the diets tested, underscoring the importance of formulation and feeding window for mineral outcomes.

Importantly, direct comparison of Ca:P values across studies should account for differences in sampling timing and the extent to which gut contents contribute to “whole-body” mineral measures. In the present work, larvae were intentionally subjected to a 48 h fasting period after the 48 h exposure to promote gut clearance prior to freezing and analysis. This step likely yields a more conservative estimate of calcium retained after clearance, because minerals associated with recently ingested substrate (and their contribution to the overall mineral pool measured in whole larvae) may decline once the gut-load diet is removed and the insects undergo food deprivation. The relevance of “time post gut-loading” has been highlighted in feeder-invertebrate research, where delays after removing gut-load diets are expected to change nutrient composition through excretion of gut contents, nutrient absorption/accumulation dynamics, and metabolic effects of starvation (GORST et al., 2015). Although Gorst et al. (2015) focused primarily on proximate composition and noted that minerals may behave differently and warrant further investigation, the study provides a clear mechanistic framework supporting the likelihood that extended post-feeding intervals can alter the nutritional profile relative to measurements taken immediately after gut-loading. Therefore, the 48 h fasting period used here may partly explain why larvae Ca:P remained below commonly cited “high-calcium” benchmarks measured shortly after gut-loading (BOYKIN et al., 2021), despite clear and substantial increases in larval calcium.

Across all inclusion levels, larvae from sprayed diets showed significantly higher calcium than those from powder diets at the same nominal $CaCO_3$ inclusion, indicating improved transfer of calcium from substrate to larvae when $CaCO_3$ was applied by spraying (Table 2). The higher analysed calcium in SPR substrates at intermediate inclusions supports the interpretation that improved mineral adhesion and effective distribution increased larval exposure during the feeding window, consistent with the broader principle that the physical form of supplements and their practical dispersion in gut-loading matrices can influence nutrient intake by insects (FINKE, 2003; BOYKIN et al., 2021).

Because the biological motivation for calcium fortification is often to correct the intrinsically inverted Ca:P of mealworms, it is also relevant to consider phosphorus as a secondary factor shaping mineral balance. Mealworms are widely recognised as low-calcium feeder insects, with low Ca:P ratios contributing to concerns such as metabolic bone disease risk when fed without correction (YOO et al., 2018). In the present study, larvae P showed only a modest treatment effect overall, whereas calcium increased strongly and monotonically with $CaCO_3$ inclusion within both fortification strategies and, across levels, was higher under the sprayed strategy (Table 2). Accordingly, larvae Ca:P (Ca/P) improved markedly relative to the control, rising from around 0.07:1 in CTRL to about 0.11:1 in POW-4 and reaching roughly 0.19:1 in SPR-8 and SPR-12 (Table 3), with sprayed diets yielding consistently more favourable ratios than powder diets at the same nominal inclusion level. Overall, these results show that short-term $CaCO_3$ gut-loading can substantially raise larvae calcium and improve mineral balance under a post-clearance endpoint, while further optimisation of matrix formulation, exposure/clearance timing, and nutrient delivery may be required to approach commonly recommended Ca:P targets (e.g., 1:1–2:1) in larvae tissues (ANDERSON, 2000; FINKE, 2003; COPELOTTI et al., 2025).

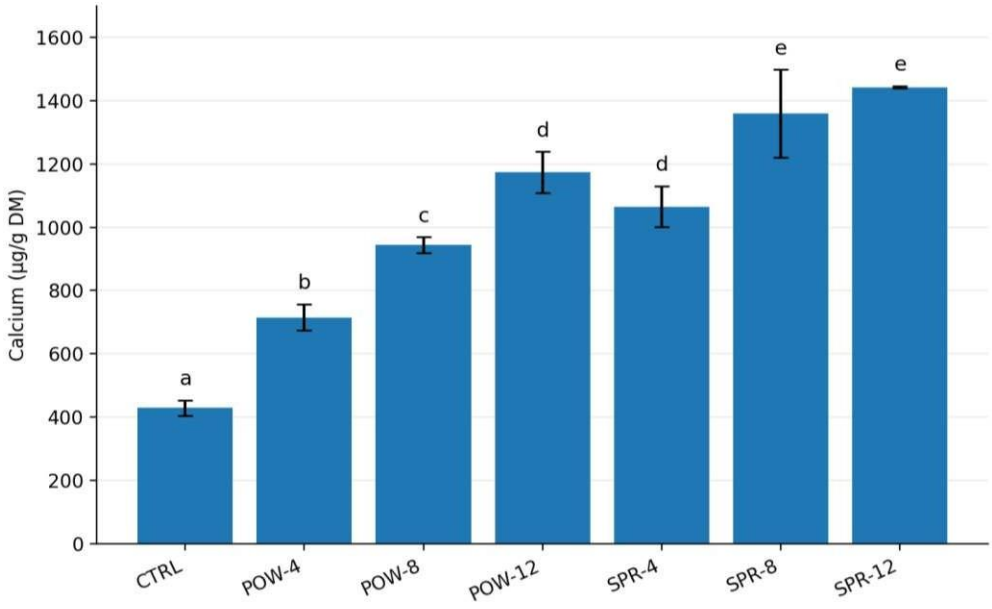


Figure 3. Calcium concentration in *T. molitor* larvae (DM).
*Calcium concentration in *T. molitor* larvae after 48 h exposure to the experimental diets, followed by 48 h fasting (water only). CTRL (larvae fed with control diet), POW-4, POW-8, POW-12 (larvae fed with diets containing 4, 8 and 12% $CaCO_3$ mixed as dry powder), and SPR-4, SPR-8, SPR-12 (larvae fed with diets containing 4, 8 and 12% $CaCO_3$ applied as sprayed suspension). Values are mean ± SD (n = 3). Different letters indicate significant differences among treatments (Tukey's HSD, $p < 0.05$). Calcium is expressed as µg/g dry matter (DM).

Regulatory and safety considerations

Beyond their baseline profile, The use of insect-based foods and feeds is advancing under increasingly structured regulatory frameworks, particularly in the European Union, where whole *Tenebrio molitor* larvae have been authorised as novel foods under the Novel Food framework of Regulation (EU) 2015/2283 (EU, 2015) through specific Commission implementing acts (e.g., Commission Implementing Regulation (EU) 2022/169 (EU, 2022); Commission Implementing Regulation (EU) 2021/882 (EU, 2021), following EFSA-led risk assessment (EFSA NDA PANEL, 2021). Safety reviews underline that substrate choice, process design and hygiene are central determinants of chemical and microbiological hazards in insect production systems, largely because contaminants and hazards may be introduced via the rearing substrate and processing chain (VAN DER FELS-KLERX et al., 2018; MEYER et al., 2021). In addition, EFSA opinions consistently highlight allergenicity as a key consideration for mealworm-derived products, including the potential for sensitisation and cross-reactivity with allergens from crustaceans and house dust mites [46]. Against this background, the fortification strategy evaluated here relies on a simple and widely recognised mineral source ($CaCO_3$) applied to a conventional cereal substrate (wheat bran), both of which are already familiar to food and feed safety authorities.

By demonstrating that a technological adjustment—spray-application versus powder mixing—can enhance calcium incorporation at intermediate inclusion levels without introducing unconventional substrates or additives, this work aligns with the principle of improving insect nutritional quality within established safety paradigms. In practical terms, the ability to produce larvae with more predictable calcium contents may facilitate compliance with compositional specifications and labelling requirements in both feeder-insect markets and formulated feeds. Thus, nutritional plasticity is not only biologically relevant but also technologically and regulatory-friendly: *T. molitor* can be nutritionally "tuned" using ingredients and processes that fit well within current legislative frameworks, supporting broader adoption as a functional feed and food ingredient.

CONCLUSIONS

Wheat-bran substrates fortified with $CaCO_3$ effectively increased dietary calcium and produced rapid calcium enrichment in *Tenebrio molitor* larvae after 48 h of feeding followed by 48 h of fasting. Enrichment was driven by both nominal inclusion level and fortification strategy, with spray application consistently yielding higher larval calcium than powder mixing at the same $CaCO_3$ level, indicating that physical incorporation of the mineral is a key determinant of fortification efficiency. Despite substantial increases in larval calcium, Ca:P (Ca/P) remained below commonly recommended targets (e.g., 1:1–2:1), highlighting the need to optimize exposure time and post-feeding handling when near-unity Ca:P is required. Overall, spray-based $CaCO_3$ application provides a simple, scalable processing adjustment to improve the predictability of calcium-fortified mealworms, supporting their use as feeder insects and as ingredients in precision-formulated feeds. Future studies should evaluate longer or continuous high-calcium feeding regimes, quantify calcium

bioaccessibility, and test the approach across alternative substrates and mineral combinations to maximise enrichment while maintaining production performance.

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