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INSECTS FARMING TECHNIQUES AND VALORISATION OF INSECTS PRODUCTS

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Coordinatore: Ch.ma Prof.ssa Angela Trocino
Supervisore: Ch.ma Prof.ssa Antonella Dalle Zotte
Co-Supervisore: Ch.mo Prof. Marco Cullere

Dottoranda: Bianca Federica Palumbo

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Abbreviations

a*= redness

ADG= average daily gain

b*= yellowness

B. cereus= *Bacillus cereus*

BW= body weight

BWG= body weight gain

CFU= colony forming unit

DHA= docosahexaenoic acid

DM= dry matter

EFSA= European food safety authority

EPA= eicosapentaenoic acid

FA= fatty acid

FAME= fatty acid methyl ester

FCR= feed conversion efficiency

FCR= feed conversion ratio

FI= feed intake

FM= fish meal

FO= fish oil

HUFA= highly unsaturated fatty acid

ICP-OES= inductively coupled plasma-optical emission spectroscopy

L*= lightness

LCA= life cycle assessment

MAPS= animal medicine, production and health

MDA= malondialdehyde

MUFA= monounsaturated fatty acids

PPO= polyphenol oxidase

PUFA= polyunsaturated fatty acid

RH= relative humidity

SFA= saturated fatty acid

SGR= specific growth rate

T0= time 0

T3= time 3

TBARS= thiobarbituric acid reactive substances

TM= *Tenebrio molitor*

TSE= transmissible spongiform encephalopathy

Abstract

The present PhD thesis investigated different aspects of *Tenebrio molitor* (**TM**) farming and valorisation, with the aim of improving rearing efficiency, enhancing nutritional value, optimising processing quality, and assessing the potential of this species as an innovative ingredient for animal feeding. The use of insects, and in particular TM, as a sustainable and valid alternative to conventional protein and fat sources is gaining increasing interest worldwide due to the urgent challenges related to food security, population growth, and environmental impact. However, several challenges still limit the large-scale use of insects, including optimisation of rearing practices, improvement of processing protocols, safety assurance, and consumer acceptance. To address these challenges, the thesis was structured into five experimental contributions.

First contribution. The breeding density of beetles was study to find the optimal value as this parameter represents a key aspect to optimise TM productivity. To this purpose, the study investigated, from an industrial perspective, the impact of 4 breeding densities (D1, D2, D3 and D4, equal to 0.8, 1.1, 1.3 and 1.6 adults/cm^2, respectively) on mortality and reproductive performance of TM adults and larvae. Two weeks after pupae emergence, TM adults were randomly assigned to the four density groups and housed in 48 breeding crates (60 x 40 x 14.5 cm; 12 crates/group). The trial consisted in 4 consecutive weeks of adults breeding (each week represented one oviposition), and 8 weeks of larvae growth. From each week of oviposition, a batch of larvae was obtained for a total of 4 batches of larvae (48 crates/batch). Larvae of each batch were grown until 8 weeks of age, corresponding to the period required to reach the selling size. Larvae were kept in the original crates until the 5th week of age, after which they were divided into additional crates to ensure a density of 4.2 larvae/cm^2 and a final weight of 1500 g of larvae/crate (0.6 g of larvae/cm^2). For each oviposition week, TM adult's mortality, eggs hatchability, body weight (**BW**), feed intake (**FI**), chemical composition and fatty acid (**FA**) profile were evaluated, while ovary weight was measured at week 1 and 4. The number of larvae was monitored at weeks 5 of age, while their chemical composition at week 5 and 8. Larvae FI and BW were monitored over the 8-weeks growth period. Individual FI and BW of adults increased with increasing breeding density ($P<0.0001$ and $P<0.05$, respectively). With increasing adults breeding density, the number of larvae per crate increased ($P<0.001$) while the grams of larvae/gram of adult decreased ($R^2=0.8856$). Larvae from groups D2, D3 and D4 showed higher FI per crate and higher individual and total final BW than D1 ($P<0.001$). However, the feed conversion ratio (**FCR**) worsened with increasing breeding density ($P<0.001$). D4 larvae had higher percentages of protein ($P<0.001$), lipids ($P<0.05$) and cholesterol ($P<0.001$) compared to larvae from other groups. Concluding, a breeding density of 0.8 adults/cm^2 maximised both grams of larvae produced/grams of adults and larvae FCR. However, breeding densities above 0.8 adults/cm^2 resulted in the highest number of produced larvae per crate and with a greater final mass weight, therefore making a density of 1.6 adults/cm^2 the preferable choice from and industrial perspective.

Second contribution. Three experiments were conducted to investigate how beetles size influences reproductive performance of TM. Experiment 1 (1a and 1b) assessed size-based mate preferences among 14-day-old beetles. In Experiment 1a, beetles were divided into four weight categories, each with 18 replicates;

in Experiment 1b, they were assigned to two weight groups with 76 replicates each. Mating behaviour was analysed among mixed-size males and females. Experiment 2 compared reproductive output across three weight classes (80-100 mg, 101-120 mg, 121-140 mg with six replicates per class using groups of 10 males and 10 females.; outcomes included larval number, weight, and daily gain. Experiment 3 investigated the effects of parental BW on offspring development in beetles. In Experiment 3a, 20 pairs were formed with one parent of fixed average weight (male: 116 mg; female: 113 mg) and the other ranging from 90 to 150 mg. In Experiment 3b, the design was expanded to 34 pairs with broader parental weight ranges (80-160 mg, incrementing by 5 mg). The effects of parental weight on larval traits were evaluated, and heritability was estimated. Results indicated that both large and small males preferentially mated with larger females during initial mating events (66.7% and 72.2%, respectively). Male competition to mate first was evident, independent of female size. Beetle size did not consistently affect the number of larvae produced. However, larger beetles generated significantly heavier larvae, with this weight advantage maintained up to 8 weeks (137 mg vs 130 mg and 121 mg), potentially enhancing production efficiency. Paternal weight influenced larval weight more than maternal weight. Heritability of larval weight was estimated at 12.8% (maternal) and 13.2% (paternal), suggesting potential for genetic selection. Findings of the present study suggest that a preference towards large females is present. Furthermore, larger beetles increased larval yield by producing heavier individual larvae, thereby enhancing the efficiency of TM farming.

Third contribution. The effect of supplementing TM diets with camelina and linseed cakes, included at two levels (5% and 10%), was studied on larval performance, chemical composition, FA profile, antioxidant content, oxidative stability, and sensory traits. Six experimental diets were tested: a standard farm diet (STD), a commercial control diet (CON), and the CON diet supplemented with camelina (CAM 5, CAM 10) or linseed (LIN 5, LIN 10) cakes. Each treatment included 12 replicates of five-weeks-old larvae reared under controlled farm conditions. Results indicated that camelina and linseed cakes modulated growth performance and body composition of TM larvae. Larvae from CON and oilseed cakes treated groups showed higher BW throughout all the experimental period compared to the STD group. Treated groups showed also a higher lipid content compared with STD and CON, with CAM 10 achieving the greatest lipid deposition. FA composition was strongly influenced by diet: oilseed cake inclusion enhanced polyunsaturated fatty acids (**PUFAs**) content and markedly reduced the n-6/n-3 ratio, particularly in LIN 10 (4.43 vs. 24.0 in STD). However, high levels of linseed cake (10%) impaired growth and increased susceptibility to lipid oxidation after storage, despite the elevated antioxidant content. Sensory evaluations revealed that LIN 10 larvae received the highest scores for visual, olfactory, and overall acceptance, suggesting that nutritional enrichment may also improve consumer perception. Overall, these findings highlight the dual potential of camelina and linseed cakes as sustainable by-products for valorising agro-industrial streams and as functional dietary ingredients to improve both the nutritional quality and the market appeal of mealworm biomass. Future research should focus on defining optimal inclusion levels to balance growth performance, oxidative stability, and consumer acceptance, thus supporting the sustainable expansion of insect farming.

Fourth contribution. The effect of two killing methods and three drying processes was investigated on physical-chemical, microbiological and sensory characteristics of TM larvae. Nine weeks-old larvae were assigned to four groups: BO, blanching in boiling water at 100 °C for 180 s and drying in oven at 60 °C for 73 h; BM, blanching at 100 °C for 180 s and drying in microwave at 103 °C for 17 min; FM, freezing at -60 °C for 1 h and drying in microwave at 103 °C for 15 min; CA, cryogenic freezing at -90 °C for 12 min and drying in controlled atmosphere at 54 °C for 48 h. Physical characteristics, nutritional composition, and microbial counts of dried larvae were assessed alongside visual, olfactory, and overall acceptance. Groups BO, BM, and FM exhibited lower water activity and higher pH ($P<0.0001$) than CA. On a fresh matter basis, these groups showed higher nutritional value, with increased protein, lipid, energy, mineral, PUFA, and amino acid contents ($P<0.0001$ and $P<0.05$). While group CA preserved fresh larvae's colour, it had elevated Enterobacteriaceae and sulphite-reducing bacteria. Furthermore, larvae from CA and BO received lower sensory scores compared to the other groups. In conclusion, blanching with oven drying, and blanching or freezing with microwave drying effectively preserve nutrition and ensure microbiological safety. However, blanching or freezing with microwave drying emerged as the preferred methods for consumer visual, olfactory, and overall acceptability.

Fifth contribution. The effect of supplementing live TM larvae to laying quails (*Coturnix japonica*) as nutritional enrichment was studied. Live performance, apparent digestibility of nutrients (including that of sole live TM larvae), egg physicochemical quality, sensory traits, and storage stability were considered in this experiment. Sixty laying quails were divided into 2 dietary groups (6 replicated cages/group; 5 quails/cage): a Control group received a basal diet for laying quails and a TM 10 group was fed with the Control diet supplemented with live TM larvae (10% of the expected daily FI). For the digestibility trial, 30 laying quails were divided into 3 dietary groups: the first 2 groups were fed with the Control and TM 10 diets, while the third group received *ad libitum* live TM larvae (TM 100) as a complete replacement for the Control diet. Overall, no mortality was recorded during the trials. Quails fed TM showed a remarkable capability of digesting dietary chitin ($P<0.0001$). TM 100 quails showed the lowest digestibility for dry matter (**DM**), crude protein, and energy, but that of ether extract was the highest ($P<0.001$). The presence of live TM larvae stimulated quails' FI ($P<0.0001$) but did not affect performance traits. Similarly, overall physicochemical quality attributes and storage stability were comparable in Control and TM 10 eggs. The sensory features of quail eggs differed in TM 10 vs. Control groups: TM 10 eggs had the lowest overall flavour ($P<0.01$), sulphur ($P<0.05$) and greasy-oily ($P<0.01$) intensities. Therefore, a 10% TM dietary supplementation is effective in stimulating feeding activity of quails, but it did not provide any productive improvement compared to a standard diet. Further studies should assess the possible beneficial effect of live TM supplementation on quail's gut health. The digestibility trial with the sole live TM larvae allowed to assess the specific nutritional value of this emerging feedstuff which is of utmost importance for future feed formulations.

Overall, the results of this thesis provide a comprehensive overview of TM farming, from rearing optimisation to nutritional and processing strategies, as well as practical applications in animal feeding. The findings contribute to advancing the knowledge required to integrate insects into sustainable food and feed systems, while also identifying directions for future research to overcome the remaining challenges of large-scale implementation.

Riassunto

La presente tesi di dottorato ha indagato diversi aspetti dell'allevamento di *Tenebrio molitor* (TM), con l'obiettivo di migliorare l'efficienza di allevamento, accrescerne il valore nutrizionale, ottimizzarne la qualità tecnologica e valutare il potenziale di questa specie come ingrediente innovativo per l'alimentazione animale. L'impiego degli insetti, e in particolare del TM, come alternativa sostenibile e valida alle fonti proteiche e lipidiche convenzionali sta suscitando un crescente interesse a livello mondiale, in risposta alle urgenti sfide legate alla sicurezza alimentare, alla crescita della popolazione e all'impatto ambientale. Tuttavia, diversi fattori limitano ancora l'impiego su larga scala degli insetti, tra cui l'ottimizzazione delle pratiche di allevamento, il miglioramento dei protocolli di trasformazione, la garanzia di sicurezza e l'accettazione da parte dei consumatori. Per rispondere a tali problematiche, la ricerca è stata sviluppata attraverso cinque studi sperimentali.

Primo contributo. Il presente studio ha avuto come obiettivo l'individuazione della densità di allevamento ottimale degli adulti di TM, un parametro chiave per massimizzare la produttività a livello industriale. A tal fine, sono state confrontate quattro densità di allevamento (D1=0,8; D2=1,1; D3=1,3; D4=1,6 adulti/cm²), valutandone l'impatto sulla mortalità e sulle performance riproduttive degli adulti e produttive delle larve. Due settimane dopo la trasformazione in pupe, gli adulti sono stati assegnati in modo randomizzato ai quattro trattamenti e distribuiti in 48 cassette di allevamento (60 × 40 × 14,5 cm; 12 cassette per gruppo). L'allevamento degli adulti è durato 4 settimane, ciascuna corrispondente a un ciclo di ovideposizione. Da ogni settimana è stato ottenuto un lotto di larve (per un totale di 4 lotti e 48 cassette/lotto). Le larve sono state allevate fino a 8 settimane di età, corrispondente al raggiungimento della taglia commerciale. Fino alla quinta settimana le larve sono rimaste nelle cassette originarie; successivamente sono state redistribuite per raggiungere una densità standardizzata di 4,2 larve/cm² per cassetta, con un peso finale di 1500 g di larve/cassetta (0,6 g/cm²). Per ciascuna settimana di ovideposizione sono stati registrati: mortalità degli adulti, schiudibilità delle uova, peso corporeo, ingestione alimentare, composizione chimica e profilo degli acidi grassi; il peso ovarico è stato registrato alla settimana 1 e 4. Il numero di larve è stato rilevato alla quinta settimana, mentre la composizione chimica è stata analizzata alla quinta e all'ottava. Peso corporeo e consumo alimentare delle larve sono stati monitorati durante l'intero periodo di crescita. I risultati hanno evidenziato che l'aumento della densità ha determinato un incremento sia del consumo alimentare individuale sia del peso corporeo degli adulti ($P<0,0001$ e $P<0,05$). Parallelamente, il numero di larve per cassetta è aumentato ($P<0,001$), mentre la resa larvale (g di larve/g di adulti) è diminuita ($R^2=0,8856$). Le larve dei gruppi D2, D3 e D4 hanno mostrato un maggiore consumo alimentare per cassetta e un peso corporeo finale (individuale e totale) superiore rispetto a D1 ($P<0,001$). Tuttavia, l'indice di conversione alimentare (FCR) è peggiorato con l'aumentare della densità ($P<0,001$). Le larve D4 hanno inoltre presentato percentuali significativamente più elevate di proteine ($P<0,001$), lipidi ($P<0,05$) e colesterolo ($P<0,001$). In conclusione, la densità di 0,8 adulti/cm² ha garantito le migliori performance in termini di resa relativa (g di larve/g di adulti) e indice di conversione alimentare delle larve. Tuttavia, densità superiori hanno consentito

una produzione complessiva più elevata di larve per cassetta, rendendo la densità di 1,6 adulti/cm² la scelta più vantaggiosa in un'ottica di produzione industriale.

Secondo contributo. Tre esperimenti sono stati condotti per indagare in che modo il peso degli adulti influenzi le performance riproduttive del TM. L'Esperimento 1 (suddiviso nelle prove 1a e 1b) aveva l'obiettivo di valutare le preferenze di accoppiamento in base alla taglia di adulti di 14 giorni di età. Nella prova 1a, gli adulti sono stati suddivisi in quattro gruppi, ciascuno con 18 repliche; nella prova 1b, in due gruppi con 76 repliche ciascuno. Nell'Esperimento 2, la resa riproduttiva degli adulti è stata confrontata tra tre classi di peso (80-100 mg, 101-120 mg, 121-140 mg), in gruppi di 10 maschi e 10 femmine, valutando il numero, il peso e la crescita giornaliera delle larve. L'Esperimento 3 mirava invece a verificare l'effetto del peso dei genitori sul numero e sul peso della prole e a stimarne l'ereditabilità. I risultati hanno mostrato che sia i maschi grandi sia quelli piccoli preferivano accoppiarsi con femmine di taglia grande (66,7% e 72,2% nei primi accoppiamenti). È emersa inoltre una forte competizione tra maschi per accoppiarsi per primi, indipendentemente dalla taglia della femmina. Il peso degli adulti non ha influenzato significativamente il numero di larve prodotte; tuttavia, gli individui più grandi hanno generato larve mediamente più pesanti, vantaggio mantenuto fino a 8 settimane (137 mg vs 130 mg e 121 mg), con potenziali benefici sull'efficienza produttiva. Il peso paterno ha inciso in misura maggiore sul peso larvale rispetto a quello materno. L'ereditabilità del peso larvale è stata stimata al 12,8% per la componente materna e al 13,2% per quella paterna, suggerendo la possibilità di selezione genetica. In sintesi, lo studio ha evidenziato una preferenza riproduttiva verso femmine di taglia grande e ha dimostrato che adulti più pesanti incrementano l'efficienza produttiva generando larve più pesanti.

Terzo contributo. Nel presente studio è stato valutato l'effetto della supplementazione della dieta di larve di TM con panelli di camelina e lino, inclusi al 5% e al 10%, sulle performance di crescita, la composizione chimica, il profilo lipidico, il contenuto in antiossidanti, la stabilità ossidativa e le caratteristiche sensoriali delle larve. Sono state testate sei diete: una dieta standard aziendale (STD), una dieta di controllo (CON) e la stessa dieta di controllo arricchita con camelina (CAM 5, CAM 10) o lino (LIN 5, LIN 10). Ogni trattamento prevedeva 12 repliche. Le larve, di 5 settimane di età, sono state allevate in condizioni controllate fino al raggiungimento della taglia commerciale (9 settimane) e alimentate con le rispettive formulazioni. I risultati hanno evidenziato che camelina e lino hanno influenzato significativamente crescita e composizione delle larve. In particolare, larve appartenenti al gruppo CON e quelle alimentate con panello di camelina e lino hanno mostrato un peso corporeo superiore rispetto a STD per tutta la durata della prova. Inoltre, i trattamenti con camelina e lino hanno determinato un maggior contenuto lipidico delle larve rispetto a STD e CON, con CAM 10 che ha registrato il valore più elevato. La dieta ha inciso in maniera marcata sul profilo lipidico delle larve: l'inclusione dei panelli ha aumentato la quota di acidi grassi polinsaturi (PUFA) e ridotto il rapporto n-6/n-3, con una riduzione particolarmente evidente in LIN 10 (4,43 vs 24,0 in STD). Tuttavia, l'inclusione di lino al 10% ha compromesso la crescita larvale e aumentato la suscettibilità all'ossidazione, nonostante l'alto contenuto di antiossidanti.

Le valutazioni sensoriali hanno mostrato che le larve del gruppo LIN 10 hanno ottenuto i punteggi migliori per aspetto, odore e accettabilità complessiva, suggerendo che l'arricchimento nutrizionale possa anche migliorare la percezione dei consumatori. Nel complesso, i dati confermano il duplice potenziale dei panelli di camelina e lino: da un lato valorizzare sottoprodotti agro-industriali, dall'altro migliorare le caratteristiche nutrizionali e commerciali della biomassa larvale. Futuri studi dovranno definire i livelli di inclusione ottimali, in grado di bilanciare performance produttive, stabilità ossidativa e gradimento sensoriale.

Quarto contributo. Nel presente studio è stato valutato l'effetto di due metodi di abbattimento e tre processi di essiccazione sulle caratteristiche fisico-chimiche, microbiologiche e sensoriali delle larve di TM. Larve di 9 settimane di età sono state suddivise in quattro gruppi sperimentali: BO: sbollentamento in acqua a 100 °C per 180 s ed essiccazione in forno a 60 °C per 73 h; BM: sbollentamento in acqua a 100 °C per 180 s ed essiccazione in microonde a 103 °C per 17 min; FM: congelamento a -60 °C per 1 h ed essiccazione in microonde a 103 °C per 15 min; CA: congelamento criogenico a -90 °C per 12 min ed essiccazione in atmosfera controllata a 54 °C per 48 h. Sono stati analizzati: caratteristiche fisiche, composizione nutrizionale, carica microbica e accettabilità sensoriale delle larve. I gruppi BO, BM e FM hanno mostrato un'attività dell'acqua inferiore e un pH più elevato rispetto a CA ($P<0,0001$). Tali gruppi hanno evidenziato un profilo nutrizionale più favorevole, con maggiori contenuti di proteine, lipidi, energia, minerali, PUFA e amminoacidi ($P<0,0001$ e $P<0,05$). Il trattamento CA ha garantito la miglior conservazione del colore delle larve fresche, ma ha presentato livelli più elevati di Enterobacteriaceae e batteri solfito-riduttori. Dal punto di vista sensoriale, le larve dei gruppi CA e BO hanno ricevuto i punteggi più bassi. In conclusione, lo sbollentamento seguito da essiccazione in forno o in microonde, così come il congelamento seguito da essiccazione in microonde, si sono dimostrati metodi efficaci nel preservare i valori nutrizionali e la sicurezza microbiologica delle larve di TM. Tra questi, i trattamenti con microonde hanno ottenuto i migliori risultati in termini di accettabilità visiva, olfattiva e complessiva

Quinto contributo. L'impiego di larve vive di TM come arricchimento nutrizionale nella dieta di quaglie ovaiole (*Coturnix japonica*) è stato valutato considerando le performance produttive delle quaglie, la digeribilità (inclusa quella specifica delle larve vive), la qualità fisico-chimica e sensoriale delle uova, nonché la loro stabilità in conservazione. Sessanta quaglie sono state suddivise in due gruppi sperimentali (6 gabbie replicate per gruppo; 5 quaglie/gabbia): un gruppo Controllo, alimentato con dieta basale, e un gruppo TM 10, alimentato con la stessa dieta supplementata con larve vive pari al 10% dell'ingestione giornaliera prevista. Per la prova di digeribilità, ulteriori 30 quaglie sono state distribuite in tre gruppi: Controllo, TM 10 e TM 100 (alimentato esclusivamente con larve vive). Non è stata registrata mortalità durante la prova. Le quaglie alimentate con larve di TM hanno mostrato un'elevata capacità di digerire la chitina ($P<0,0001$). Nel gruppo TM 100, la digeribilità della sostanza secca, delle proteine e dell'energia è risultata inferiore, mentre quella dell'estratto etereo è stata la più elevata ($P<0,001$). La supplementazione con larve vive ha stimolato l'ingestione ($P<0,0001$), senza tuttavia influenzare le performance produttive. La qualità fisico-chimica e la stabilità in conservazione delle uova non sono state influenzate dalla supplementazione, mentre le analisi sensoriali hanno evidenziato differenze significative: le uova del gruppo TM 10 hanno ricevuto punteggi

inferiori per intensità del flavour complessivo ($P<0,01$), zolfo ($P<0,05$) e grasso-untuoso ($P<0,01$). In sintesi, la supplementazione con larve vive di TM al 10% dell'ingestione giornaliera stimola l'appetito delle quaglie senza apportare benefici produttivi, ma modifica il profilo sensoriale delle uova. La prova di digeribilità con larve vive al 100% (TM 100) ha inoltre permesso di stimare il valore nutrizionale specifico di questa nuova materia prima, dato cruciale per lo sviluppo di future formulazioni alimentari.

Nel complesso, i risultati di questa tesi forniscono una visione integrata dell'allevamento del TM, che spazia dall'ottimizzazione gestionale alle strategie nutrizionali e tecnologiche, fino alle applicazioni pratiche in ambito zootecnico. Le evidenze raccolte contribuiscono ad ampliare le conoscenze necessarie per favorire l'integrazione degli insetti nei sistemi alimentari e mangimistici sostenibili, mettendo in luce al contempo le prospettive di ricerca future utili ad affrontare le sfide connesse alla loro applicazione su larga scala.

Introduction

Why farming insects?

Sustainability and other advantages

Over the last few years, insects farming has become a major topic of discussion regarding food and feed alternatives, as well as economic and environmental sustainability. While the consumption of edible insects is widespread in several countries as part of culinary culture, its application remains limited in Western cultures. The interest to insects can reside to the significant increase in the world population expected by 2050 (United Nations, 2019). Consequently, the demand for animal protein will rise, making traditional sources of food supply unsustainable. The agricultural sector, indeed, is a significant contributor to climate change and the entire food system accounts for 29% of greenhouse gas emissions (Campbell et al., 2017). Giving that, the predicted population growth will exacerbate current environmental challenges. Furthermore, a large part of the world population currently lacks access to affordable and healthy foods, along with a growing deficiency in micronutrients (Von Grebmer et al., 2014). Meet the global demand for food will require a 70% increase in food production, additional strain on already limited raw materials, escalating costs, intensifying competition for land, and exacerbating climate-related risks (Moruzzo et al., 2021).

Given these challenges, a transition toward agricultural solutions and food systems based on the principles of the "Green Deal" is necessary. This approach emphasises ecological resilience, environmental sustainability, and universal access to nutritious foods and nutrients (Patel and Goodman, 2020). Therefore, it is essential to identify new strategies for producing nutritious and sustainable foods that are environmentally, economically, and socially viable. These strategies should focus on shortening supply chains, reducing waste, and minimising environmental impact.

Insects have been proposed as a viable protein alternative in human, livestock and fish (Van Huis et al., 2013). They are typically harvested from nature in tropical countries, but they can also be semi-domesticated to increase production or easily reared in confined industrial facilities ranging from small to industrial scale (Oonincx and Boer, 2012).

From an environmental sustainability perspective, the life cycle assessment (**LCA**) of insects can be compared to conventional protein sources such as meat, milk, fish meal (**FM**), and soy as observed for TM, *Hermetia illucens*, *Acheta domesticus* and *Musca domestica* (Oonincx and de Boer, 2012; Miglietta et al., 2015; Halloran et al., 2017). This comparison is influenced by the high energy consumption required to maintain optimal temperatures for rearing insects (van Huis and Oonincx, 2017). Nevertheless, considering direct emission levels, insects are far more efficient than conventional livestock, producing up to 100 times lower total greenhouse gases emission compared to the livestock sector. Mealworms, for instance, generate significantly lower CO_2 equivalents per unit of edible proteins than both broiler chickens and beef (Oonincx and Boer, 2012). Likewise, on a protein-equivalent basis, crickets emit 89% fewer greenhouse gases than poultry production in Thailand (Halloran et al., 2017). Furthermore, Smetana et al. (2016) found that insect-

based products had among the lowest environmental impacts, comparable to soy meal-based alternatives, when assessed in comparison to a range of meat substitutes.

In terms of resource use, insect production requires substantially less land and water than both traditional feed ingredients (Oonincx et al., 2010) and conventional livestock farming. On average, producing one gram of edible protein from yellow mealworms uses 11 times less land and five times less water than producing the same amount from beef. Similarly, one kilogram of mealworm-derived protein requires only 10% of the land needed for beef production (van Huis et al., 2013). Comparisons with poultry show a similar trend: mealworms demand considerably less land and approximately 50% less water to produce one gram of edible protein (Oonincx and De Boer, 2012; Miglietta et al., 2015). An additional factor to consider when comparing livestock and insects is the proportion of edible mass. For example, adult crickets contain approximately 205 g of protein per kilogram of edible mass, compared to 190 g in beef, 150 g in pork, and 200 g in poultry (van Huis et al., 2013).

The environmental sustainability of insect production is further reinforced by their ability to be reared on waste raw materials, effectively converting these substrates into high nutritional value food through bioconversion, thereby putting into practice the principles of the circular economy (van Huis, 2022). This offers a considerable advantage in terms of resource valorisation, as it enables the transformation of organic waste into valuable products rather than its disposal (Fowley and Nansen, 2020). Depending on the insect's species, they can be fed with different waste materials. For instance, TM showed good growth performance and maintained their nutritional composition when reared on a mix of by-products from the food industry, including residues from potato processing, bread and biscuit production, as well as the brewing and biofuel industries (van Broekhoven et al., 2015; Oonincx et al., 2015b). Additionally, no significant differences in larval composition were observed when yellow mealworms were fed with fruit waste, broiler eggshells, and vegetable waste (Tan et al., 2018; Liu et al., 2020).

Beyond their environmental sustainability and ability to valorise food by-products, insects also offer practical advantages for food and feed production. They can be reared easily and efficiently (Makkar et al., 2014) due to their short reproductive cycles and, consequently, rapid production times (Cadinu et al., 2020).

Considering the edible portion of insects, they show a much higher percentage of edibility (e.g. 80% for crickets) compared to both chicken and pork (55%) and cattle (40%) (Nakagaki et al., 1991). As a consequence, insects show a high feed conversion efficiency (**FCE**), double compared to poultry and around four and twelve times higher than pigs and cattle, respectively (van Huis et al., 2013). In addition, insects exhibit a low feed conversion ratio (**FCR**), which varies depending on their diet and life stage. For instance, the FCR for TM larvae ranges from 2.2 to 5.3, while for *Acheta dom*esticus nymphs, it falls between 1.6 and 4.5 (Broekhoven et al., 2014; Mancini and Antonioli, 2022; Bordiean et al., 2022). These values are either lower or comparable to those of conventional livestock, which average around 10 for beef, 5 for pork, and 2.5 for chickens (van Huis, 2013; Berggren et al., 2018). This high efficiency of insects is attributed to their poikilothermic nature, meaning they do not need to use energy to regulate their body temperature. As a result, they can convert feed and energy into high-protein, energy-dense outputs with greater efficiency

(Guiné et al., 2021). However, this efficiency significantly decreases when insects are fed substrates that are also suitable for human consumption. Using waste materials as substrates for insect farming, on the other hand, could eliminate competition for resources typically used in livestock feed, resources that could otherwise be consumed by humans. This approach would also improve the overall FCE of the system (Miech et al., 2016).

Given these considerations, insect farming offers a promising solution to the growing demand for sustainable and efficient food and feed alternatives in the face of global population growth and environmental challenges. Their ability to thrive on waste materials further enhances their role in a circular economy, reducing competition for human food resources while valorising organic waste. Despite some challenges, such as the energy needed for rearing, the overall benefits of insect farming make it a compelling option for addressing food security, environmental sustainability, and economic viability.

Nutritional characteristics

Insects are considered a promising alternative to conventional food and feed sources primarily due to their high nutritional value (Hawkey et al., 2021). Several studies have demonstrated that they provide proteins, lipids, vitamins, and minerals, delivering energy values comparable to those of traditional foods and feeds (Rumpold and Schlüter, 2013; Ravzanaadii et al., 2012). Nutritional composition of insects significantly varies according to species, development stage, feeding substrate, slaughtering, and processing methods. Protein is the major component, ranging from 13% to 75% of dry matter (**DM**), followed by 20-40% lipids and 5-15% chitin (Oliveira et al., 2024). Insect proteins are of high quality, comparable to soybean meal, FM, and animal-derived proteins such as meat and dairy. They contain all essential amino acids, with particularly high levels of phenylalanine and tyrosine, and meet human nutritional requirements (Rumpold and Schlüter, 2013; Osimani et al., 2016). Digestibility is also high, ranging from 77% to 98%, although lower values have been reported for TM (60%) and *Hermetia illucens* (51%) (Ramos-Elorduy et al., 2002; De Marco et al., 2015).

Regarding lipids, insects provide most essential fatty acids (**FAs**). Among saturated and monounsaturated fatty acids (**SFAs** and **MUFAs**) palmitic and oleic acids are the most abundant, respectively (Hawkey et al., 2021). Insects are also a valuable source of polyunsaturated fatty acids (**PUFAs**), particularly omega-6 and omega-3. For instance, TM contain omega-3 and omega-6 fatty acid levels comparable to those found in fish (van Huis et al., 2013), while *Acheta domesticus* has been reported to contain high levels of linoleic and α-linolenic acids (Paul et al., 2017).

Considering macronutrients, insects show a high content of iron, phosphorous, copper, calcium, magnesium, zinc and selenium. Insects, especially TM larvae, contain also high quantities of vitamins of group B such as riboflavin and pantothenic acid and good amount of vitamin B12, even so levels of other vitamins are very low or absent ((Finke, 2002). Chitin represents the commonly form in which raw fibres are present in insects' body of which represents the 10% of the whole dry weight (van Huis et al., 2013; Belluco et al., 2013). Chitin is a long-chain polymer of N-acetylglucosamine and is the main component of the exoskeleton

of insects (EFSA, 2015). Part of the proteins present in insects' body are linked to the chitin determining a decreasing in their digestibility (Rumpold and Schlüter, 2013). Indeed, only a small portion of chitin can be digest by humans thanks to two catalytically active chitinases, the AMCase and chitotriosidase (van Aalten et al., 2001; Synstad et al., 2004).

The great interest to insects from a nutritional point of view is also linked to their antioxidant activity. In fact, many studies demonstrated that the antioxidant capacity of several insect's species for instance, *Calliptamus italicus*, *Bombix mori*, *Acheta domesticus*, *Oecophylla smaragdina* and *Gryllus assimilis* is higher than the conventional vegetal sources rich in polyphenols (Di Mattia et al., 2019; Alagappan et al., 2021; de Matos et al., 2021).

Furthermore, insects are known for their ability to produce antimicrobial peptides, bioactive compounds with specialised antimicrobial function, active against a wide range of pathogens (Wu et al., 2018). This determines the interest in the application of insects as natural antimicrobial and immune-stimulating compounds in animal feeds (Gasco et al., 2021).

Despite it being considered as a waste product, frass of insects represents a rich source of nitrogen (N), phosphorus (P) and potassium (K). Literature reports a NPK balance of 3.5-1.5-1.5 in TM frass making this compound a promising natural fertiliser reducing the use of agrochemicals (Houben et al., 2021).

Challenges and perspectives

Despite the advantages mentioned above, the insect breeding sector still faces several significant challenges. One of the main obstacles is the acceptance of insects as an alternative protein source. In many parts of the world, particularly in Africa, Asia, and Central and South America, insects are already a traditional component of the human diet. The most widely consumed species include crickets, caterpillars, palm weevils, and termites. In fact, more than 2 000 insect species are eaten by various ethnic groups across over 100 countries (Ramos-Elorduy, 2009; Mitsuhashi, 2017; Gahukar, 2020). However, this is not the case in most Western countries, where only 12.8% of men and 6.3% of women report consuming insects as a substitute for meat (Verbeke, 2015). In Western societies, insects are generally perceived as dirty, disgusting, and dangerous due to psychological and emotional factors (Looy et al., 2014; Fukano and Soga, 2021). Acceptance of insects as food is influenced by sensory experiences, cognitive perceptions, and individual traits. To overcome this resistance, strategies such as public awareness campaigns, educating children about entomophagy, and promoting insects in the market could be effective in reducing the aversion to insect consumption (Motoki et al., 2021). As suggested by van Huis et al. (2013), transform insects in more conventional form or including insects-based products in conventional food, could improve the consumers acceptance as people are more willing to consume processed and less-visible insects (Sogari et al., 2018). In line with this, Meyer-Rochow and Hakko (2018) demonstrated that including insects in the form of flour or paste in food preparation increases acceptance by western consumers compared with products containing whole insects. Consumer attitudes are influenced by multiple factors, including cultural background, gender, familiarity with novel foods, and product presentation. Research in Brazil and other regions has shown that

men are generally more willing to consume insects than women, and that younger or more adventurous consumers are more open to experimenting with insect-based products (Schardong et al., 2019; House, 2016). Similar studies in Europe highlight how knowledge and awareness can play a decisive role: in some northern countries, higher levels of information correlate with greater willingness to consume insect-based foods (Piha et al., 2018). Product development strategies also appear crucial in shaping consumer attitudes. Consumers who were served breads which had insect flour, or who ate burgers and snacks where meat or vegetables were partially replaced by insect proteins, were highly positive and willing to pay for the product (Oliveira et al. 2017; Cicatiello et al., 2020). These findings suggest that reducing the visual salience of insects, while emphasising taste, texture, and nutritional benefits, can significantly increase acceptance. Repeated exposure, positive sensory experiences, and targeted marketing approaches can gradually reduce resistance and promote familiarity to insect consume. In this sense, consumer acceptance emerges as a pivotal challenge for the integration of edible insects into modern diets, where nutritional value and sustainability arguments alone are often insufficient without a corresponding shift in perceptions and experiences (Guiné et al., 2020a).

While the acceptance of insects as food remains challenging, people tend to show a greater acceptance towards the use of insects as animal feed (Mancuso et al., 2016; Kostecka et al., 2017). The use of insect proteins as pet food is the main actual market and is expected to growth by 2030 in a contest where the value of edible insects' market is expected to increase by 26.5% by 2027 (van Huis, 2022). Consumer studies confirm this trend: in Italy, 50% of respondents fully accepted the use of insects in fish diets, while an additional 40% partially accepted it (Mancuso et al., 2016). Similar findings were reported in Scotland, where more than half of consumers expressed willingness to eat salmon reared on insect-based feed and only 10% were opposed (Popoff et al., 2017). Likewise, in Denmark, just 23% of consumers rejected fish fed with insect-containing diets (Ankamah-Yeboah et al., 2018). With regard to other animal species, most Belgian consumers showed acceptance of meat from animals fed with insects, giving higher preference scores to poultry and fish compared to pigs or cattle (Verbeke et al., 2015). Similarly, a Brazilian study reported greater willingness to accept the use of insects in feed for fish, with the lowest acceptance observed for cattle (de Domingues et al., 2020). In Italy, 53% of consumers expressed agreement with the incorporation of insects into animal diets and the consumption of meat from such animals (Laureati et al., 2016). As observed for direct consumption of insects, Laureati et al. (2016) also reported higher acceptance of meat from insect-fed animals among males compared to females, and among students and university staff compared to individuals outside the academic environment. Comparable findings were reported by Ankamah-Yeboah et al. (2018), who observed greater acceptance among younger males, while Verbeke et al. (2015) found no significant influence of age.

These findings highlight that consumer acceptance is conditional upon ideological, cultural, safety and quality standards. Consequently, an important challenge the insect breeding sector must face is ensuring a safe, nutritious and high-quality product (Sogari et al., 2019).

As for other foods, it must be ensured that parameters such as harmful microorganisms, antibiotics, parasites, viruses, toxins, heavy metals, hormones and residues of pesticides are monitored (Belluco et al., 2018). In this regard, insects could represent mechanical or biological vectors of pathogenic microorganisms and parasites. Several bacterial genera have been associated with insects, including *Staphylococcus*, *Streptococcus*, *Bacillus, Proteus*, *Pseudomonas*, *Escherichia*, *Micrococcus*, *Lactobacillus*, and *Acinetobacter* (Braide et al., 2011). In particular, TM and *Acheta domesticus* have been found to harbour Enterobacteriaceae and spore-forming bacteria (Klunder et al., 2012). Potential bacterial hazards for humans and animals related to insect consumption may derive from their resident microbiota, rearing conditions and environment, as well as from handling, processing, and storage practices (ANSES, 2015). Insects also naturally carry a wide variety of viruses, most of which are pathogenic only to insects, often leading to disease, mortality, and colony collapse (Eilenberg et al., 2015). These viruses are generally species- or family-specific and therefore not infectious to humans or other vertebrates. Although they pose a significant challenge for insect producers due to potential farming losses, they are regarded as safe for vertebrates, including humans, and some are even intentionally applied in the biocontrol of food and feed crops (Leuschner et al., 2010; Sundh et al., 2012). The risk of pathogen or contaminant transmission to humans and animals can be effectively reduced through careful substrate selection, strict hygiene protocols, and adequate processing techniques. Substrate choice is particularly critical, as it may determine the accumulation of toxins, pesticides, heavy metals, and other hazardous substances. For instance, mealworms have been shown to accumulate chemical contaminants, which represent a potential risk for both human and animal consumption (Houbraken et al., 2004). These substances are particularly dangerous because they do not appear to compromise insect development or survival, thereby remaining undetected (van der Fels-Klerx et al., 2016).

Insects may also cause allergic reactions through contact, inhalation, or ingestion, affecting not only consumers but also farm workers. These reactions, however, are not life-threatening, and an allergy to one insect species does not necessarily imply cross-reactivity to others (Broekman et al., 2017).

In addition to the challenges related to insect consumption and the potential health risks, the insect sector must also contend with high market prices, which may threaten and slow down its expected growth (van Huis, 2022). Compared with conventional fish, meat, and crop products, insect-based foods generally have a higher market price (Baiano, 2020). This is primarily linked to the elevated production costs of insect farming. In many facilities, indeed, only a small part of the production process is automated, while substantial reliance on manual labour persists, thereby increasing overall expenses (Cadinu et al., 2020). In addition, the need to maintain high temperatures and adequate humidity for insect rearing further contributes to the cost of production. Reducing the market price of insects and insect-derived products, by finding an appropriate balance between manual labour, automation, and mechanisation, would enhance the sector's economic competitiveness. Additionally, the use of organic side streams as feed could represent a promising strategy to lower production costs (van Huis, 2022). For this approach to be effective, however, side streams must be both affordable and available in sufficient quantities. Unfortunately, these conditions often do not

align with the requirements of high-quality substrates for insect farming. Cheaper side streams, for instance, are typically rich in lignin and cellulose but poor in protein, which limits their nutritional value (van Huis, 2022).

Beyond substrate selection, several additional factors influence farm productivity. A deeper understanding of how environmental parameters and diet affect insect health, growth rate, and nutritional composition is essential. Optimising these aspects is key to ensuring both the development and long-term sustainability of insect farming (Cadinu et al., 2020; van Huis, 2022). In this perspective, recently, the connection between genetics and insect rearing has been explored in greater depth. Indeed, research shows significant differences among various strains within the same species in terms of body composition and size, reproductive output, virus resistance, developmental time, and the efficiency of converting organic waste into nutritious outputs in different insects' species (Zhou et al., 2013; Pastor et al., 2014; Chieco et al., 2019; Morales- Ramos et al., 2019; de Miranda et al., 2021). Given these findings, genetic selection targeting desirable traits, such as increased body weight (**BW**) or higher fertility, could be implemented to boost farm productivity and efficiency. The short life cycles of insects make them particularly well-suited for studying and applying these improvements.

Although no regulations currently address insect welfare, a key challenge in the insect breeding industry is the need to legislate for the welfare of invertebrates, similar to the protections in place for other farmed animals. The lack of attention to this issue stems largely from the mistaken belief that insects do not experience pain or stress (Horvath et al., 2013). However, research shows that insects are capable of similar neural processing and conscious experiences as vertebrates, and they also demonstrate social and associative learning (Barron and Klein, 2016; van Huis, 2021). For these reasons, efforts should be made to ensure the well-being of farmed insects and to establish the less painful and most ethical methods for their slaughter.

Legislation

The European Authority of Food Safety (**EFSA**) is the main administrative institution concerning the food safety in Europe. In 2015, EFSA has proposed a list of potential insects' species that could be used as food and feed in Europe including *Musca domestica, Hermetia. illucens,* TM*, Zophobas atratus, Alphitobius diaperinus, Galleria mellonella, Achroia grisella, Bombyx mori, Acheta domesticus, Gryllodes sigillatus, Locusta migratoria* and *Schistocerca americana* (EFSA, 2015).

According to the International Platform of Insects for Food and Feed (IPIFF) the insect's species most used in animal feed are the yellow mealworm, the black soldier fly and the common housefly larvae. Furthermore, four species of insects are authorised for human consumption: TM*, Locusta migratoria, Acheta domesticus and Alphitobius diaperinus.*

Insect producers involved in the rearing, processing, handling, or distribution of insects within the food or feed supply chain are subject to several key regulations. These include Regulation (EC) No 178/2002, known as the "General Food Law", Regulation (EC) No 852/2004, which governs food hygiene, and Regulation (EC) No 183/2005, which addresses feed hygiene. When it comes to feeding insects for various applications

(food, feed, and technical uses), they can only be fed materials of either plant or animal origin. This complies with Regulation (EU) No 2017/1017, which provides a catalogue of permissible feed materials, Regulation (EC) No 999/2001, which outlines measures for the prevention, control, and eradication of Transmissible Spongiform Encephalopathies (**TSEs**) in animals, and Regulation (EC) No 142/2011, which governs animal by-products and derived products not intended for human consumption. According to these regulations, insects can be fed hydrolysed proteins derived from parts and blood products of non-ruminants while hides and skins from ruminants are allowed. The use of fishmeal, as well as compound feed containing fishmeal, is permitted, along with dicalcium phosphate and tricalcium phosphate (Regulation (EC) No 999/2001). Additionally, milk and milk-based products, eggs products, honey, and collagen are accepted as substrates for insect feeding (Regulation (EC) No 142/2011). Regulation (EC) No 767/2009 specifies substances that cannot be used as substrates for insect feed. These prohibited materials include former foodstuffs containing meat and fish, catering waste, slaughterhouse by-products, and animal manure. Insects and their derived products fall under "category 3," meaning they can be used in feed for food-producing animals without restrictions related to TSE regulations. In fact, insect proteins, fats, whole insects (both treated and untreated), and live insects are permitted in the diets of pets, fur-bearing animals, and other non-food-producing animals.

Regarding monogastric livestock, in April 2021, the EU Member States authorised the inclusion of processed animal proteins from insects in poultry and pig feed. Additionally, fish can be fed insect proteins, fats, live insects, and hydrolysed insect proteins. However, due to past concerns with TSE, the use of insects in ruminant diets is restricted to insect fats and hydrolysed proteins only.

Regarding human consumption, insects are classified as "Novel Food" under Regulation (EU) No 2015/2283, as they were not widely consumed in the European Union (EU) before May 19, 1997. Since January 2018, any company wishing to market a novel food must submit an application for authorisation to an EU Member State committee, which is then forwarded to the EFSA. The application must include a scientific dossier demonstrating the product's safety. EFSA evaluates the product from various perspectives and is required to issue an opinion within nine months. Following this, the European Commission has an additional seven months to decide whether to authorise the product for the EU market.

The killing and processing of insects are regulated by the EU Animal By-Products legislation, specifically Regulation (EC) No 1069/2009 and its subsequent amendment, Regulation (EU) No 142/2011. As insects fall under "category 3" materials, they must undergo processing before being used in feed for food-producing animals (Regulation (EC) No 1069/2009). According to Regulation (EU) No 142/2011, insects must be processed using methods 1 to 5 or method 7 for thermal treatment of insect ingredients, and they must comply with the requirements outlined in the General Food Law, as well as food and feed regulations. However, the World Organisation for Animal Health general guidelines on the stunning and slaughter of farmed animals do not yet specifically address insects.

Regarding the welfare, Directive (EU) No 98/58 on welfare of animals kept for farming purposes does not include invertebrates. Thus, there are still no directives regulating the welfare of reared insects. Considering

insects by-products (e.g. frass), they belong to the "category 2" materials, according to Regulation (EC) No 1069/2009 which prohibits their use as food or feed. However, Regulation (EC) No 142/2011 outlines a specific treatment process that allows frass to be marketed as an organic fertiliser and soil improver. Additionally, Regulation (EU) No 2021/1925 governs the production and sale of insect frass, stipulating that the content of dead insects in frass must not exceed 5% by volume and 3% by weight.

Tenebrio molitor

Biology and life cycle

Among insect's species, TM was the first one approved by EFSA for human consumption. TM, commonly known as the yellow mealworm, is one of the most studied and produced insect species in Europe (Krzyzaniak et al., 2022). It belongs to the Tenebrionidae family (Coleoptera) and it is typically considered a pest of farinaceous materials, which constitute its primary food source (Ribeiro et al., 2018). Like other insect of the same family, TM is nocturnal and prefers dark and damp places (Ghaly and Alkoaik, 2009). The life cycle of the yellow mealworm comprises four distinct metamorphic stages: egg, larvae, pupa, and beetle (Figure 1).

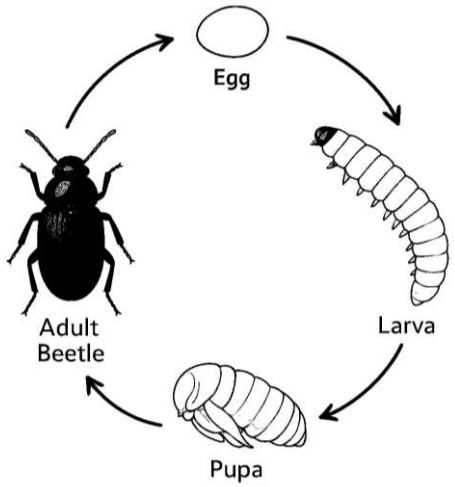

Figure 1. Life cycle of TM.

The entire cycle duration is influenced by environmental parameters such as temperature, humidity and population size. Under optimal conditions, the entire cycle lasts from 60 to 90 days (Spencer and Spencer, 2006) but it could last up to one or two years under uncontrolled environment (Robinson, 2005; Selaledi et al., 2019). Females can produce up to 500 eggs per cycle (Hill, 2002). The eggs (length: 1.7-1.8 mm, width: 0.6-0.7 mm), of white, shiny colour and of beany shape, are laid singly or in small groups, attached to the breeding substrate thanks to a sticky substance covering the eggs. Under an optimal temperature of 26-30 °C, the eggs hatch in about four days, producing small whitish larvae approximately 3 mm long (Cotton, 1927; Ghaly & Alkoaik, 2009; Siemianowska et al., 2013; Selaledi et al., 2019). Larvae are characterised by an elongated cylindrical shape, six legs behind the head and two short appendages at the abdomen's tips (Ghaly & Alkoaik, 2009; Hahn et al., 2018). During the growth phase, which lasts around 60 days under controlled

conditions, the larvae undergo several moults until reaching adult size characterised by an average weight of 0.2 g and a length of 25-35 mm (Aguilar-Miranda et al., 2002). If not utilised for consumption or processed, larvae go against a period of latency and a transformation into pupae, a free-living creature of yellow colour and 1 cm long. The stage of pupae typically lasts about 6 days under farming conditions (Cotton, 1927; Ghaly and Alkoaik, 2009), culminating in the emergence of beetles. First emerged beetles exhibit a whitish colour that gradually transitions to dark brown and black over approximately two weeks, corresponding to the reaching of their sexual maturity (Cotton, 1927; Hill, 2002; Spencer and Spencer, 2006)

Environmental parameters and rearing conditions

Ensuring optimal environmental conditions is crucial in TM rearing to optimise and improve farm productivity. Temperatures commonly applied in yellow mealworm farming range from 25 to 28 °C (Spencer and Spencer, 2006; Koo et al., 2013; Kim et al., 2015). Within this range, the productive performance of both adults and larvae are maximised. Temperatures below 17 °C have been reported to inhibit embryonic development and reduce larval growth rate, with a significant increase in larval development time observed at 20 °C (Koo et al., 2013; Bjørge et al., 2018; Eberle et al., 2022). The highest daily growth rate was reported at 31 °C by Bjørge et al. (2018), whereas Eberle et al. (2022) observed a peak at 30 °C and the lowest rate at 20 °C. However, temperatures above 30 °C led to increased mortality, mainly due to stress conditions in the study of Koo et al. (2013), whereas Eberle et al. (2022) observed the lowest survival rate at 20 °C and the highest at 25 °C and 30 °C. Temperature also affects energy assimilation efficiency, with higher values observed between 23.3 and 31.0 °C. Furthermore, it influences the protein and lipid content of mealworms: Bjørge et al. (2018) reported an increase in lipid content of TM larvae at 31 °C, while the lowest protein level was observed at the same temperature.

Closely related to temperature, relative humidity (**RH**) represents a source of water for insects influencing their adsorption capacity and several factors during the entire life cycle. Optimal RH values of 60% to 75% have been reported in previous research (Manojlovic, 1987; Punzo, 1975; Punzo and Mutchmor, 1980). A combination of optimal temperature and humidity is necessary for oviposition, embryological development, and eggs hatching. Specifically, dry conditions with about 12% RH can cause embryo death due to water loss from the eggs (Punzo, 1975). RH also influences the number and length of instars, adult female activity, and larval growth rates. Larval growth is maximised at RH values of 90-100% and reduced at values around 30% (Punzo, 1975). However, excessively high humidity levels can create an optimal environment for the growth of undesirable pests such as mites.

TM is negatively phototropic, and therefore longer dark exposure in farming appears to be more beneficial for larval growth. In particular, shorter larval development times have been reported under short-day conditions (8 h light:16 h dark) or constant darkness compared to long-day conditions (Eberle et al., 2022). Conversely, Kim et al. (2015) observed reduced development time under long-day conditions (14 h light:10 h dark). Long-day regimes have also been associated with increased eclosion rate and pupation time (Kim et

al., 2015). Nevertheless, Eberle et al. (2022) demonstrated that larvae achieved higher growth rates under constant darkness at 25 and 30 °C compared to long-day conditions.

Another environmental parameter influencing the TM rearing is the availability of oxygen. Specifically, low oxygen concentrations of about 10-15% inhibit growth rates, cause developmental abnormalities in mealworm populations and increase larval mortality (Greenberg and Ar, 1996; Loudon, 1988). Conversely, hyperoxia conditions lead to a lower number of instars and reduced final larval biomass compared to normoxia (Greenberg and Ar, 1996).

Among the factor influencing the productivity and the reproductive success of TM, the breeding density is one of the most significant (Morales-Ramos et al., 2012). High breeding densities of larvae affect the number and the duration of larval moults, resulting in fewer larval instars and smaller larvae (Connat et al., 1991; Morales-Ramos et al., 2012; Morales-Ramos and Rojas, 2015; Tschinkel and Willson, 1971). Weaver and McFarlane (1990) observed a faster larval growth at a density of 0.33 larvae/cm² compared to 0.03 larvae/cm², while larvae reared in isolation produced larger female pupae. Furthermore, in the study of Morales-Ramos and Rojas (2015), increasing the density from 0.44 larvae/cm² to 3.51 larvae/cm² reduced the growth, the feed consumption, and the feed efficiency of TM larvae.

Concerning the breeding density of beetles, high densities are typically linked to increased progeny production overall (Berggren et al., 2018; Deruytter et al., 2019; Zim et al., 2022) (Figure 2). However, when examining progeny production per individual beetles, females raised at lower densities are significantly more prolific than those raised at higher densities (Morales-Ramos et al., 2012; Berggren et al., 2018; Deruytter et al., 2019; Frooninckx et al., 2022) (Figure 3).

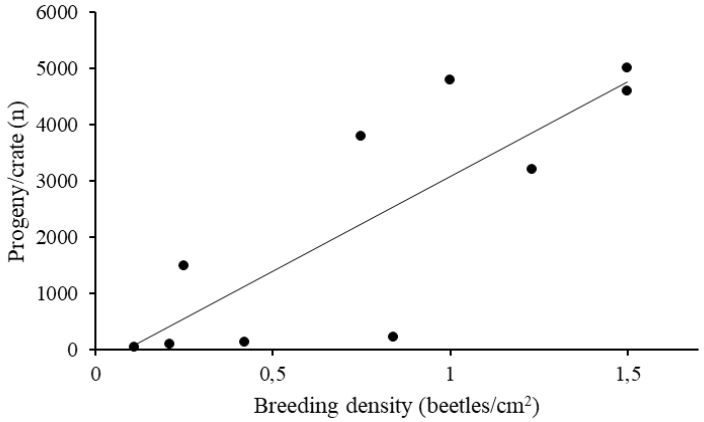

Figure 2. Linear regression of progeny produced per crate according to the beetles breeding density observed by Berggren et al. (2018), Deruytter et al. (2019) and Zim et al. (2022).

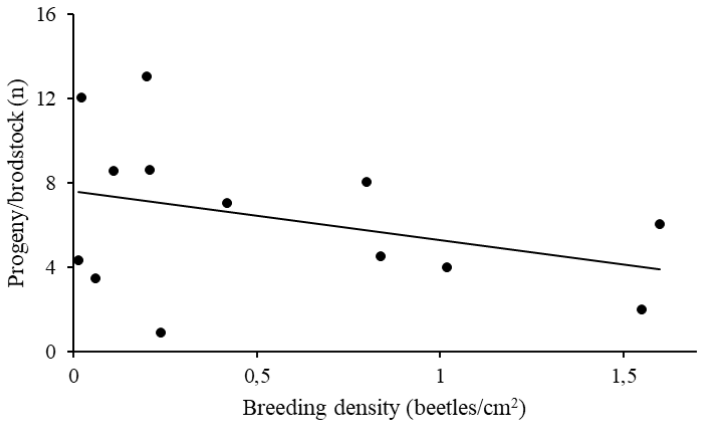

Figure 3. Linear regression of progeny produced daily per beetles according to the beetles breeding density observed by Morales-Ramos et al. (2012); Berggren et al. (2018), Deruytter et al. (2019) and Frooninckx et al. (2022).

Indeed, high breeding density causes crowding stress in insects, which reduces the reproductive capacity of beetles along with an increased mortality due to cannibalism (Berggreen et al., 2018; Gerber, 1984; Morales-Ramos et al., 2012). Previous research has shown that all Tenebrionidae, including TM adults, exhibit cannibalistic behaviour (Kuriwada et al., 2009; Morales-Ramos et al., 2012). Specifically, under mass-rearing conditions, high densities lead to increased cannibalism of eggs, particularly those near the surface of the substrate (Kuriwada et al., 2009; Morales-Ramos et al., 2012). To prevent eggs cannibalism, separating the eggs from the beetles is a viable alternative. This can be achieved by using an oviposition grid, which allows the ovipositor to pass through the holes in the grid and lay eggs deep in the substrate. This way, the adults are unable to reach and eat the eggs. Indeed, as observed by Froonickx et al. (2022), the use of an oviposition grid resulted in a significantly higher yield of eggs.

Feeding of larvae

In addition to environmental conditions, the productivity of TM can be influenced by several factors closely linked to farming techniques. One of the most significant is the choice of feeding substrate. Although TM can be raised exclusively on wheat bran, its diet can be supplemented with additional sources of protein, vitamins, minerals, and organic matter such as carrot, potato, and cabbage to provide essential nutrients. The quality of the feed directly affects oviposition: poor-quality diets can lead to fewer eggs laid and higher female mortality (Gerber and Sabourin, 1984). Van Broekhoven et al. (2015) found that incorporating 40% yeast-derived proteins into the diet of TM larvae shortened larval development time, reduced mortality, and increased weight gain and survival rates. However, this also affected the larvae's FA profile, resulting in a comparable or slightly less favorable n-6/n-3 ratio than a wheat bran-only diet. Feed intake (**FI**) significantly influences various aspects of TM life cycle. For instance, supplementing wheat bran with protein sources at a 1:4 ratio has been shown to shorten development time and improve FCE and fecundity. Similarly, adding carbohydrate sources at the same ratio enhances feed utilisation, growth, development time, survival, and

beetle fecundity (Morales-Ramos et al., 2013). Including protein and carbohydrate sources at a 1:4 ratio with wheat bran also significantly reduces the number of larval instars and the total development time (Morales-Ramos et al., 2010). Furthermore, diets containing 33-39% protein can reduce the time to pupation (Oonincx et al., 2015). Increasing dietary protein by supplementing with pea flour and rice flour has also been shown to raise the protein content of the larvae and to increase their levels of essential amino acids (Kröncke et al., 2023). Additionally, Rumbos et al. (2020) demonstrated that incorporating specific amylaceous commodities with protein contents ranging from 11% to 14%, as well as milk-based feed and layer hens feed with protein contents of 62% on average, significantly increased the larval biomass produced. However, feeding TM larvae legume flour led to a reduction in larval BW, despite the substrate's high protein content. This suggests that while higher dietary protein generally promotes faster insect growth, as Rumbos et al. (2020) noted, the use of legume flours had an inhibitory effect, indicating that other dietary components also play a crucial role in TM larval development.

Including lipids in the diet of TM has also been tested and demonstrated to have beneficial effects at low concentrations, while high concentrations can be detrimental. This is because high lipid levels can cause substrate agglomeration, reduce aeration and subsequently cause respiration problems in mealworms (Alves et al., 2016). The FA content of the diet seems to strongly influence the lipid profile of TM. For instance, Melis et al. (2019) found that rearing mealworms on brewer's spent grain, which is rich in PUFA, increased the levels of omega-3 and omega-6 PUFA in the larvae and improved FCR. Similarly, Lawal et al. (2021) showed that the supplementation of TM diet with 10% chia seeds increased the omega-3 FA favourably reducing the n-6/n-3 ratio. In another study, Park et al. (2023) reported that including sesame cake in the TM diet enhanced the nutritional composition of the larvae. Regarding growth performance, the addition of 7.5% edible oils was found to slow larval growth (Rossi et al., 2022). In contrast, Ruschioni et al. (2020) found that including 25% olive pomace in the diet of TM increased larval and pupal weights, improved survival rates, and extended development time. It also enhanced the protein and amino acid contents of the insects, without affecting their FA profile. Furthermore, the inclusion of 5% and 10% camelina and linseed cake in the TM diet from five to nine weeks of age not only affected the FA profile of the larvae, reducing the n-6/n-3 ratio, but also improved their growth performance by increasing the final body weight and reducing the FCR (personal communication).

Processing techniques

Ensuring the quality and the safety of insects and insect-based products is a significant challenge in industrial TM production. Raw insects typically exhibit a high microbial load, predominantly composed of gram-positive bacteria, including faecal and total coliforms (Belluco et al., 2013). Particularly, previous research showed the possibility to isolate Enterobacteriaceae and spore forming bacteria, not belonging to pathogenic species, from TM fresh larvae (Klunder et al., 2012).

Insects processing includes killing, microbial decontamination by heat, drying and grinding (van Huis and Tomberlin, 2017). Thermal treatments such as boiling, blanching, drying and other cooking methods are

commonly used for microbial inactivation to ensure the safety of insects-based products (Yan et al., 2023). Indeed, boiling TM larvae for 5 minutes was found to be an efficient process to inactivate Enterobacteriaceae while boiling followed by refrigeration from 5 to 7 °C resulted efficient against spore forming bacteria and spoilage (Klunder et al., 2012). Blanching at temperature ranging from 70-150 °C not only affect the microbial load but also ensure the inactivation of enzymes responsible of the browning reaction caused by the oxidation of phenolic compounds (Cardello, 1997; Wang et al., 2017; Cacchiarelli et al., 2022).

In addition to blanching, freezing is a commonly used practice able to stop the microbial growth guaranteeing a gentle and quick death for the insects, thereby reducing their suffering (Lenaerts et al., 2018). Prolonged killing times, in fact, should be avoided to reduce the suffering of the insects and to prevent FA oxidation, which can occur due to the stimulated metabolism of energy reserves (Larouche et al., 2019). The killing method is a crucial stage as it can also influence the proximate composition (Adámková et al., 2017; Leni et al., 2019; Caligiani et al., 2019) and taste of the final product (Farina, 2017). For instance, in *Hermetia illucens*, a reduction in FA content (Caligiani et al., 2019) and certain amino acids (Leni et al., 2019) was observed when freezing was used as the killing method, compared to blanching. Conversely, Adámková et al. (2017) found that freezing best preserved the fat content of TM larvae.

Along with the killing method, the drying process significantly influences the quality of the final product. The application of high temperatures for extended periods are responsible of a visual impairment of the larvae due to pronounced darkening and shrinkage (Purschke et al., 2018; Trukhanova et al., 2022). Furthermore, high temperatures promote the degradation of proteins and vitamins, as well as the oxidation of lipids, which deteriorates the flavour and affects the techno-functional properties of the product (Zamora and Hidalgo, 2005; Kumar et al., 2013; Bußler et al., 2016; Kröncke et al., 2018; Baek et al., 2019). Among the drying processes involving high temperatures, oven drying, and microwave drying are the most commonly applied and tested. These processes are often proposed as alternatives to freeze-drying due to its long operating times and high costs. Freeze-drying is the industrial-scale drying process used for mealworms (EFSA, 2015). This process preserves the texture, the nutritive value, the aroma, and the colour of the product promoting a sublimation of water and avoiding the application of high temperatures (Huang & Zhang, 2012). However, freeze-drying is an expensive technique, highlighting the need for more efficient and cost-effective drying methods.

When compared to freeze drying, microwave drying has been associated with a lower protein solubility (Kröncke et al., 2018). Additionally, microwaved larvae exhibited a reduction in vitamin B_{12} levels, and a darker colour compared to the fresh ones (Lenaerts et al., 2018). A dark brown colour was also observed in larvae dried using oven drying, along with significant shrinkage due to tissue collapse (Purschke et al., 2018). Thus, the optimal processing method is still not defined.

Before their employing, due to the high lipids content of TM larvae, a lipid extraction is necessary before protein extraction. This step is essential not only to isolate the lipids for potential applications but also to enhance protein extraction, resulting in a more concentrated material with improved techno-functional properties and characteristics (Choi et al., 2017). The solvents commonly employed for lipid extraction from

TM larvae include ethanol, hexane-isopropanol mixture or hexane, all of which demonstrated high extraction yields (Bußler et al., 2016; Zhao et al., 2016; Purschke et al., 2017; Kim et al., 2020). However, an alternative and more sustainable method using supercritical CO_2 has shown an impressive extraction yield of 95% (Purschke et al., 2017).

The protein extraction efficiency significantly influences the commercial production of food products rich in TM proteins. The chosen method should ensure a high extraction yield to meet large-scale production demands while preserving the functional properties of the proteins, resulting in a concentrate of protein that is as pure as possible (Zhao et al., 2016). The alkaline extraction followed by isoelectric precipitation is a commonly employed method for various substates. In the case of TM protein extraction, this method resulted in an acceptable yield and a relatively high protein content of the extract (Bußler et al.; 2016; Zhao et al., 2016; Gould and Wolf, 2018; Kumar et al., 2019). Additionally, sonication has been observed as a further method that enhances protein extraction from edible insects (Choi et al., 2017).

Use as food and feed

The nutritional composition of TM larvae closely resembles that of conventional meat products, positioning them as a promising alternative source of nutrients for both human and animal diets. TM larvae are particularly rich in protein, comprising about 50% of their DM, and contain approximately 30% lipids, which are high in beneficial FAs such as oleic, linoleic, and palmitic acids (Kröncke et al., 2019; Trukhanova et al., 2022). TM larvae also supply most of the essential amino acids required daily and serve as an excellent source of minerals, except for calcium, though calcium levels can be enhanced through dietary modifications. Additionally, they are a valuable source of vitamins, including vitamin B_{12} (Nowak et al., 2016). The excellent nutritive composition is emphasised by the easy of breeding and the reduced environmental impact of mealworm production compared to the conventional ones (van Huis et al., 2013). For these reasons, TM larvae are not only a promising alternative to conventional food sources but also a viable substitute for traditional fish and soybean meals used in feed formulations (Makkar et al., 2014).

Poultry

Several authors have investigated the inclusion of TM meal in broiler chickens' diets and found no detrimental effects on growth performance or carcass traits at inclusion levels of up to 10%. Notably, the inclusion of 0.3% full-fat TM meal in broiler diets increased FI and body weight gain (**BWG**), thereby reducing the FCR (Benzertiha et al., 2020). Similarly, Sedgh-Gooya et al. (2020) reported an increase in BWG with a 5% inclusion level of full-fat TM meal, while no significant effects were observed on FI and FCR. Andrade et al. (2023) observed that a 2% inclusion of full-fat TM meal enhanced FI and weight gain in broiler chickens and also modulated the innate immune response. Furthermore, Biasato et al. (2016) and Elahi et al. (2020) found that full-fat TM meal inclusion levels of 7.5% and 8% had no significant impact on growth performance or carcass traits. In contrast, replacing the broiler diet with 5% and 10% of whole dried TM larvae was reported to positively influence BWG, carcass yield, meat composition, and welfare traits (Vasilopoulos et al., 2023). Higher inclusion levels of full-fat TM meal have been associated with negative

effects on the cecal microbiota of broiler chickens, particularly at 10% and 15% inclusion levels (Biasato et al., 2019). Additionally, Bovera et al. (2016) reported that a 29.7% inclusion of full-fat TM meal reduced ileal digestibility of DM, organic matter, and crude protein. Moreover, TM larvae can also be offered live as environmental enrichment for broilers; Bellezza Oddon et al. (2021) found that a 5% inclusion of live larvae had no negative impact on growth performance or health status. In laying hens, the effects of TM meal supplementation have shown some variability. Rahmawati et al. (2022) reported that dietary supplementation with 2% and 5% full-fat TM meal enhanced the omega-3 content of eggs and increase egg weight, without significant effects on physical egg quality. However, Ait-Kaki et al. (2021) observed that a 5% inclusion level reduced both the final body weight of hens and egg weight, while shell breaking strength increased. Ko et al. (2020) found no significant effects on egg production with full-fat TM meal inclusion levels ranging from 1% to 3%; however, 3% inclusion level led to a more intense yolk colour and an increase in histidine content. Expanding beyond chickens, the inclusion of live TM larvae has been tested as a nutritional enrichment in laying quails. Dalle Zotte et al. (2024) reported that an inclusion of 10% did not compromise growth performance, indicating potential applications across various poultry species.

Rabbit

While the majority of research on TM as a feed ingredient has focused on poultry species, its potential applications extend to other livestock, including rabbits. In particular, incorporating 4% full fat TM meal into the diet of New Zealand White rabbits was found to increase final BW and carcass yield (Kowalska et al., 2020). While the FA profile remained unchanged, a difference in amino acid content was observed, with higher levels of isoleucine and methionine in rabbit fed with TM meal. Similarly, in a subsequent study, Kowalska et al. (2021) reported that replacing 4% of soybean meal with full fat TM meal increased the rabbits' BW and muscle fat content, supporting the potential for partial replacement of soybean meal in rabbit diets. Additionally, the inclusion of 4% full fat TM meal was found to increase the apparent total tract digestibility of ether extract, acid detergent fibre, and acid detergent lignin, as well as to enhance the activity of caecal and colon bacterial N-acetyl-β-D-glucosaminidases (Strychalski et al., 2021). However, this inclusion level was associated with a reduction of enzyme activity and lower short-chain FA concentrations in the hindgut. The full replacement of soybean meal with 30 g/kg of TM meal has been shown to maintain rabbit performance without adverse effects (Volek et al., 2021). Furthermore, rabbits fed diets containing TM larvae meal exhibited reduced nitrogen losses in urine and decreased total nitrogen excretion. These findings were supported by a subsequent study, in which no adverse effects on growth performance or nitrogen output were observed (Volek et al., 2023).

Research on TM larvae fat inclusion in rabbit diets, primarily as a substitute for soybean oil, has also yielded promising results. Partial (50%) and total replacement of soybean oil with TM fat have been found to have no detrimental effects on growth performance, apparent digestibility, gut morphometric indices, or organ histopathology in rabbits. Moreover, this substitution did not negatively impact consumer acceptance of rabbit meat and, notably, reduced the meat's susceptibility to oxidation (Gasco et al., 2019).

Aquaculture

The inclusion of TM in aquaculture feed, either as a partial or complete replacement for FM, has been proposed as a promising strategy to enhance the sustainability of the aquafeed industry. Defatting TM meal is generally preferred, as it facilitates the extrusion process, resulting in more stable pellets and reduced degradation (Rema et al., 2019).

The replacement of FM with 15% or 30% of full fat TM meal was evaluated in the diet of rainbow trout (*Oncorhynchus mykiss*), showing no adverse effects on growth performance, protein utilisation, or physiological status. However, at the highest level of FM replacement, a significant reduction in omega-3 FAs in the fillet was observed, along with a decreased viscerosomatic index and increased aerobic catabolism (Melenchón et al., 2020).

Studies on gilthead seabream (*Sparus aurata*) have shown promising results when partially replacing FM with TM larvae meal. Piccolo et al. (2014) reported that substituting 25% of FM with full-fat TM meal resulted in no significant negative effects on BWG or final BW. A subsequent study by the same research group (Piccolo et al., 2017) corroborated and extended these findings. They observed that 25% inclusion level of full-fat TM meal (corresponding to 35% of FM substitution on protein basis) led to statistically significant improvements in BWG, final BW, specific growth rate (**SGR**), and FCR.

Research on higher inclusion levels of TM meal in fish diets has yielded variable results across species. In rainbow trout, inclusion levels exceeding 25% of full-fat TM meal have been associated with adverse effects. FM replacement at 50%, 75%, and 100% with full-fat TM meal has resulted in significant reductions in BWG and final BW, coupled with increased FCR (Valipour et al., 2019). Additionally, a 50% inclusion of full-fat TM meal in replacement of FM has been shown to alter both total and free amino acid profiles in rainbow trout fillets, with notable reductions in free alanine, leucine, isoleucine, and lysine at the highest inclusion level (Iaconisi et al., 2019).

Referring to the European seabass (*Dicentrarchus labrax*), FM replacement of up to 80% with defatted TM meal has not compromised growth performance, nutrient utilisation, flesh quality, or intestinal morphology (Basto et al., 2021). Even at 100% replacement of FM with defatted TM meal, growth performance parameters remained unaffected, although an increase in hepatosomatic index and alterations in FA profile were observed. At 80% replacement, protein and lipid digestibility remained unaltered along with growth performance (Basto et al., 2021).

In rainbow trout, complete replacement of FM with partially defatted TM meal has shown no significant adverse effects on gut and skin microbiota (Terova et al., 2021). High dietary inclusion of defatted TM meal, up to 100% replacement of FM, has been reported to increase lipid and energy content without significantly affecting blood gas pressure, electrolyte balance, or haematocrit (Sakhawat Hossain et al., 2025). These findings collectively suggest that the effects of TM meal inclusion are species-specific and dependent on the processing method of the meal (full-fat vs defatted). While high inclusion levels may be detrimental in some species, others appear to tolerate substantial FM replacement with TM meal without compromising key physiological parameters.

The inclusion of TM meal has also been investigated in Pacific white shrimp (*Litopenaeus vannamei*) diets, yielding promising results, albeit with some variability. Replacing up to 30% of FM with TM meal led to increased BWG, feed efficiency, and hepatopancreas index, whereas higher inclusion levels were associated with reductions in these parameters. Additionally, higher inclusion levels were found to decrease cholesterol, triglycerides, and glucose levels (Sharifinia et al., 2023). Contrasting results were reported by Chung et al. (2015), where FM replacement at 50% and 100% resulted in increased BWG and SGR, along with improved FCR.

The substitution of fish oil (**FO**) with TM oil in the diet of Pacific white shrimp has demonstrated varying effects depending on the level of replacement. While complete substitution of FO with TM oil did not significantly alter shrimp performance parameters (Panini et al., 2017), partial replacement has yielded more favorable outcomes. Eom et al. (2023) reported that replacing 50% to 75% of FO with TM oil resulted in enhanced feed utilisation efficiency. Moreover, their findings suggest that the optimal FO replacement level is dependent on the desired outcome: a 50% substitution was found to be optimal for growth performance, whereas a 25% replacement showed the greatest benefit for disease resistance against *Vibrio parahaemolyticus.*

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Objectives

The yellow mealworm is one of the most studied and reared insect species in Europe. The increasing interest in insects, and in particular in TM, is mainly due to their higher environmental sustainability compared to conventional livestock and their rich nutritional composition. These characteristics make them a promising solution to help mitigate the so-called “protein gap and address future food security challenges associated with the rapidly growing global population and the need to reduce the environmental footprint of food production. Insects, and TM in particular, can contribute to sustainable food systems by reducing greenhouse gas emissions, lowering land and water use, and enabling the circular valorisation of organic side-streams and food industry by-products, which can be efficiently converted into high-value nutritional products.

Despite these advantages, several challenges still need to be overcome before TM can be fully integrated into large-scale food and feed supply chains. Critical aspects include the optimisation of rearing practices to maximise productivity and FCE, the improvement of reproductive performance and growth rates, and the identification of suitable diets that can enhance the nutritional quality of larvae without compromising sustainability. Equally important are the development of processing technologies that ensure product safety and preserve nutritional and sensory quality, as well as strategies to increase consumer acceptance of insect-based products. Furthermore, research is required to assess the potential of TM as a functional ingredient in animal nutrition, which could open new opportunities for diversifying feed resources and reducing dependency on conventional protein sources such as soybean meal and fishmeal.

The main objective of the present PhD thesis was to investigate different aspects of TM production and use, with the aim of improving rearing efficiency, nutritional value, processing quality, and its potential application in animal feeding. By targeting these key stages of the production and utilisation chain, the thesis aims to contribute to the development of TM as a sustainable protein source able to partially replace conventional feed ingredients and thereby address global protein supply constraints. Particular attention was paid to optimise reproductive and growth performance under farming conditions, enhancing larvae nutritional profile through dietary manipulation, identifying effective processing methods to guarantee safety and consumer acceptance, and evaluating the potential role of TM as a functional ingredient in poultry feeding.

This general aim was pursued through five specific contributions, each addressing a different key step of TM production chain and its applications. The overall research framework of the thesis is illustrated in Figure 4, showing how the five contributions address key steps of the TM production chain and relate to global protein supply and sustainability challenges.

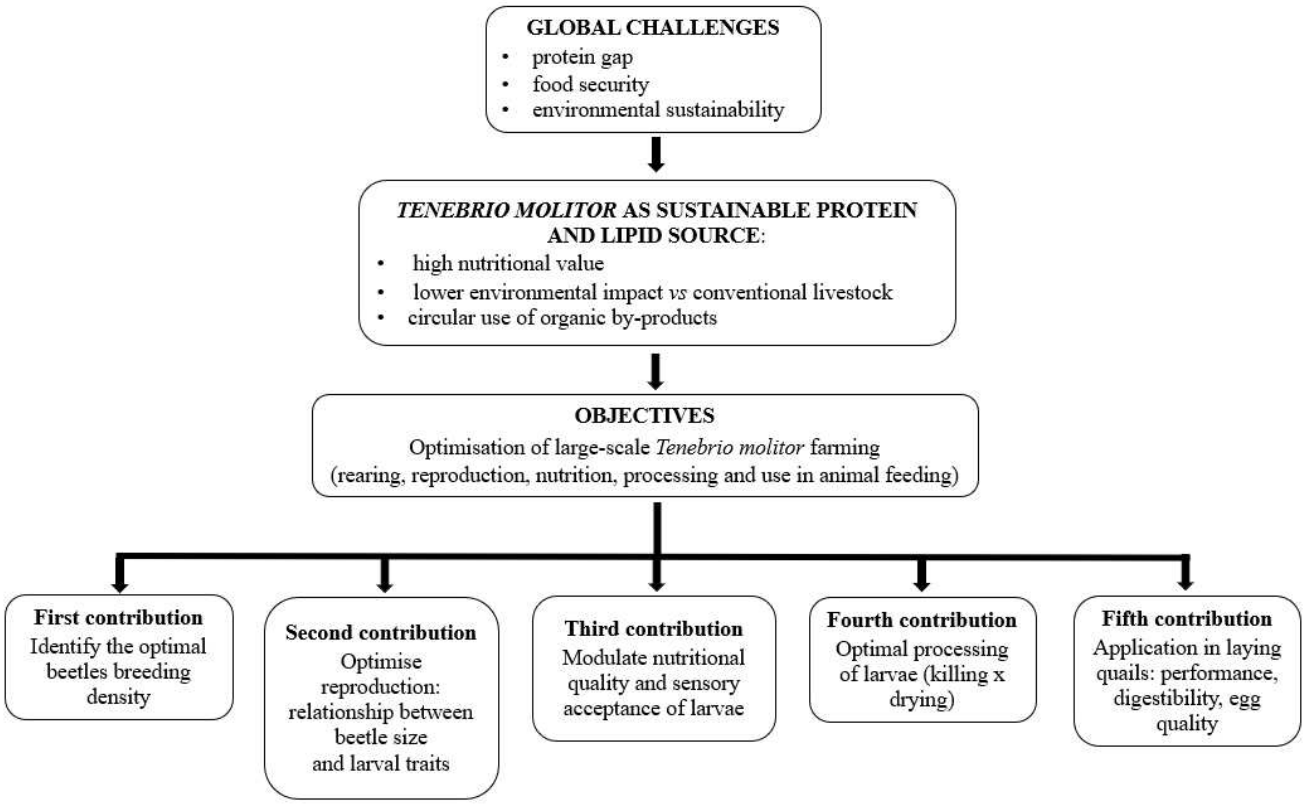

Figure 4. Overall research framework.

First contribution

The first study investigated the effect of different adult breeding densities on the reproductive performance of TM and on subsequent larval growth and development. Four breeding densities were tested under industrial rearing conditions. Adult mortality, ovary development, reproductive output and FI were monitored over four consecutive oviposition weeks. From each oviposition, a batch of larvae was obtained and reared until commercial size, allowing the evaluation of larval growth, feed consumption and chemical composition. This study contributes to the optimisation of TM mass-rearing conditions, which is essential to ensure a reliable and efficient supply of insect protein for food and feed applications.

Second contribution

The second study focused on the influence of beetle size on reproductive efficiency and larval characteristics. Through three experiments, mate preferences and mating behaviour were evaluated across different beetle weight categories, reproductive output was assessed in groups of different sized parents, and the relationship between parental weight and larval traits was analysed. In addition, heritability of larval BW was estimated to provide insights into the potential for genetic improvement in TM. By clarifying how beetle size affects mating behaviour and reproductive output, this contribution provides practical indications to optimise breeder selection and thereby increase the overall productivity of TM farming system

Third contribution

The third study examined the effect of dietary inclusion of camelina and linseed cakes at two levels (5% and 10%) on the nutritional profile and consumer perception of TM larvae. Six diets were tested and larvae were reared from the 5th to the 9th week of age. At the end of the rearing period, larvae were subjected to nutritional and FA composition analyses. In addition, a sensory evaluation was conducted with a panel of consumers to assess visual, olfactory and overall acceptance of dried larvae produced with the different diets. This study provides evidence on how sustainable feed ingredients can be used to modulate the nutritional quality and consumer acceptability of TM, thereby enhancing its value as an alternative protein and lipid source.

Fourth contribution

The fourth study aimed to evaluate the impact of different killing methods (blanching, freezing, cryogenic freezing) in combination with drying processes (oven, microwave, controlled atmosphere) on the physicochemical, microbiological and sensory quality of TM larvae. Nine-weeks-old larvae were processed according to the different combinations, and their nutritional value, microbial loads and sensory attributes were analysed to identify effective processing strategies capable of ensuring product safety and quality. By comparing different killing and drying combinations, this contribution identifies processing strategies that can ensure safe, stable and sensorially acceptable TM products for food and feed use.

Fifth contribution

The fifth study assessed the potential of live TM supplementation in the diet of laying quails (*Coturnix japonica*) as a form of nutritional enrichment. Sixty quails were divided into groups receiving either a standard basal diet or the same diet supplemented with live larvae at 10% of the expected daily FI. Productive performance, nutrient digestibility, egg physicochemical quality, and sensory characteristics were evaluated. In addition, a digestibility trial was conducted using diets with increasing levels of TM larvae, including a complete replacement of the standard diet, to better define the nutritional value of this emerging feed ingredient. By evaluating TM in a practical poultry feeding context, this study contributes to the diversification of feed resources and to the reduction of dependence on conventional protein sources, aligning with global efforts to address the protein gap in animal production.

First contribution:

Yellow mealworm: effects of adults breeding density on adults and larval performance from an industrial perspective

B. Palumbo[a]*, M. Cullere[a], Y. Singh[a], E. Pontalti[a], A. Dalle Zotte[a]

[a]Department of Animal Medicine, Production and Health, University of Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro, Padova, Italy

*Corresponding Author: Bianca Palumbo. E-mail: biancafederica.palumbo@phd.unipd.it

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Introduction

The demand for sustainable, nutritious, and healthy food sources is becoming increasingly urgent. With the well-known global population projected increase by 2050, the demand for animal-derived foods will surge. However, a livestock sector solely relying on conventional feedstuffs like soybean, FM, corn, wheat etc. is becoming less feasible due to the high feed-food competition, rising production costs and the frequent need of long-distance transport (low environmental sustainability) from the few producing countries to fulfil the worldwide request (United Nations, 2019). To address these challenges, incorporating insects into animal feed presents a promising alternative (Henchion et al., 2017), offering the potential to reduce environmental impact and generate nutrients-dense feed for livestock as well as the opportunity to be directly consumed as food.

Mealworms, especially the yellow mealworms (TM, Coleoptera: Tenebrionidae), stand out as an ideal candidate for mass production, suitable for both feed and food applications. Larvae of TM are notably rich in protein (approximately 50% of DM), lipids (about 30% of DM), and essential minerals like potassium and phosphorus (Van Broekhoven et al., 2015; Ghosh et al., 2017; Hong et al., 2020; Wu et al., 2020). Their amino acid profile is comparable to that of commercially available soybean-based protein sources commonly used in livestock feed (Veldkamp and Bosch, 2015; Khanal et al., 2023). Furthermore, TM larvae provide a good source of B vitamins, including vitamin B12 (Dalle Zotte et al., 2021; van der Heide et al., 2021).

Focusing on the environmental sustainability, producing 1 kg of edible protein from TM larvae showed to require only 10% of the land needed for 1 kg of edible protein from beef (van Huis et al., 2013). Mealworms can also produce less CO_2 per unit of edible protein compared to chickens and beef (Oonincx and Boer, 2012). Consequently, TM larvae can partially replace conventional livestock and fish feed ingredients (Henry et al., 2015; Iaconisi et al., 2017).

In TM breeding, various factors can impact productivity: environmental parameters like temperature, RH, and photoperiod, as well as other factors such as feeding, and oviposition time (Bjørge et al., 2017; Eberle et al., 2022). However, one of the pivotal factors affecting the reproductive success is the density at which adults are reared (Morales-Ramos et al., 2012). Stocking density is a crucial determinant that influences several aspects of animal breeding, particularly productivity, growth, and welfare. The impact of adults breeding density has been examined in different insects species. For instance, while higher adults breeding density in black soldier fly (*Hermetia illucens* L.) showed to maximise the reproductive output, in *Dicyphus tamaninii* an increased adults breeding density led to higher mortality and reduced overall production (Agustì and Gabarra, 2009; Liu et al., 2022). Furthermore, in *Chilo partellus* lower pairing densities were associated with increased longevity and fecundity (Hari et al., 2008). In TM breeding, studies conducted on a laboratory scale highlighted that lower breeding densities seem generally more desirable than higher ones (Frooninckx et al., 2022; Zim et al., 2022). Specifically, Zim et al. (2022) found that a density of 0.25 adults/cm² provided the best adult fertility, while Morales-Ramos et al. (2012) reported that densities between 0.084 and 0.14 adults/cm² produced the highest number of larvae per crate. From existing research on TM, it emerges that high densities often correlate with reduced reproductive capacity, likely attributable to stressful conditions

that lower fecundity and increase cannibalism towards both conspecifics and laid eggs (Berggreen et al., 2018; Gerber, 1984; Morales-Ramos et al., 2012). High densities can also lead to an increased mortality rate (Deruytter et al., 2019; Deruytter and Coudron, 2021; Morales-Ramos et al., 2012). On the other hand, low densities can determine a lower global farm efficiency (Zim et al., 2022). A viable strategy to mitigate mortality and welfare concerns arising from high breeding densities involves the use of oviposition grids. These grids physically segregate adults from the eggs laid in the substrate, effectively preventing cannibalism (Froonickx et al., 2022).

Despite the available information in scientific literature, the optimal breeding density for TM adults remains undetermined, especially considering the industrial context. In fact, all existing studies have been conducted in laboratory settings and/or on a small scale, leaving a significant gap in the understanding of the best solution for industrial-scale production. In the insects rearing sector, like for most livestock species, scale is a critical factor. Data from small-scale studies often find little application into commercial contexts, as seen with factors like breeding substrate, temperature inside the crate, and selected life-history traits in the black soldier fly (McGill, 2010; Meneguz et al., 2018 b; Scala et al., 2020; Biasato et al., 2024). This may also apply to the breeding density of TM adults. Therefore, identifying the optimal breeding density at an industrial level is essential for developing cost-effective and sustainable production practises within the TM insects industry. Based on these premises, the aim of this study was to evaluate, on an industrial scale, the effect of four breeding densities of TM adults on mortality and reproductive performance of adults and on larval growth performance and nutritional quality.

Material and methods

Experimental design and conditions

The experiment was conducted at the INEF (Insect Novel Ecologic Food) insects farm (Piombino Dese, Treviso, Italy). The trial lasted three months, from March to May 2023.

Adults were collected 14 days after the transformation from pupae, age at which they are sexually mature, using cardboard egg containers. After collecting the adults (8.6 kg in total), individuals were selected to remove any malformed or immature specimens, specifically the white and light brown ones. The sex ratio of adults was assumed to be 1:1 (Loudon, 1988). The average individual weight was assessed by weighing 100 individuals and attested at 0.122 g. Then, adults were randomly assigned to four different treatment groups to obtain four breeding densities: 120 g (D1), 160 g (D2), 200 g (D3), and 240 g (D4) of adults per crate (corresponding to 0.8, 1.1, 1.3 and 1.6 adults/cm^2 respectively, considering the area of the oviposition grid). This density range was adopted considering the conventional density (1.1 adults/cm^2) used at the commercial farm where the study took place. Adults were housed in 48 breeding crates (12 replicates per treatment group). Crates were made of plastic material with a dimension of 60 x 60 x 14.5 cm and an area of 2400 cm^2. In each crate it was included a metallic oviposition grid, with a dimension of 47 x 27 x 5 cm and an area of 1269 cm^2.

Breeding of adults

At the beginning of the trial, the breeding crates were filled with ground feed to ensure the *ad libitum* intake for the future larvae. An oviposition grid was placed on top of the feed, and adults were placed on the grid. Adults pelleted feed and fresh carrots (water source) were added. The pelleted feed and carrots were weighed and provided in proportion to the different densities (D1: 375 g + 208 g; D2: 500 g + 277 g; D3: 625 g +347 g; D4:750 g + 416 g, respectively). Twice a week, feed and carrots were added both for larvae and adults to ensure *ad libitum* intake. The chemical composition of the feeds used for adults and larvae are reported in the Supplementary Table S1.

Adults were bred for 4 weeks. Every 7 days, the oviposition grid (with adults inside) was positioned in a new crate to start a new oviposition. Each week represented one oviposition (each oviposition lasted 7 days). The temperature and RH in the hatchery were kept at averaged values of 27.8 ± 2.3 °C and 73.8 ± 10%, respectively. The photoperiod was maintained constant at 8 hours of light and 16 h of dark.

Reproductive performance of adults

The hatching rate of eggs laid by adults was assessed by collecting a weekly sample of eggs from each crate. Fifty eggs per crate were placed in a Petri plate and incubated at 28 °C and 70% RH for 7 days. After this period, the number of larvae that hatched was recorded to compute the hatching rate, calculated according to Equation 1.

$$Hatching\ (\%) = \left(\frac{hatched\ eggs\ (n)}{incubated\ eggs\ (n)}\right) \text{x } 100 \qquad (1)$$

Female adults were dissected to obtain the ovary. The ovary weight was assessed at the beginning and at the end of the 4th week of breeding on 5 adults per crate. The ovary proportion was calculated according to Equation 2.

$$Ovary\ proportion\ (\%) = \left(\frac{ovary\ weight\ (g)}{adult\ weight\ (g)}\right) \text{x } 100 \qquad (2)$$

Productive performance of adults

The average weight of adults was assessed at the beginning of the trial, and then once a week until the end of the breeding period: it was obtained by individually weighing 100 individuals per crate. The mortality rate was assessed weekly by counting and weighing the total number of dead adults in each crate. Dead adults were not replaced to preserve the impact of mortality on productivity as a result of the breeding density. The feed and carrots intake by adults during the 4 weeks of breeding were recorded. Data of feed and carrots intake were reported on DM basis.

Breeding of larvae

Larvae were obtained from each of the 4 oviposition weeks, thus obtaining 4 batches of larvae. Each batch consisted of 48 crates (12 replicates/group) for a total of 192 crates of larvae. Larvae were maintained in the original crates until the 5th week of age when they reached a sufficient size to be sieved and separated from

feed and frass. Larvae were split into different crates to obtain the same density (4.2 larvae/cm^2) in each crate. Density equalisation was performed for each batch of larvae aiming to obtain a total amount of 1500 g larvae/crate at 8 weeks of age and considering the individual larvae weight reached at this age at the INEF farm (0.15 g). Thus, it was calculated a number of larvae equal to 10.000 (1500 g/ 0.15 g). This number was multiplied by the average individual weight of larvae at 5 weeks of age to determine the amount (g) of larvae to be included in each crate (D1= 133 g, D2= 140 g, D3= 174 g, D4=176 g, on average).

After density equalisation, only one of the crates obtained from the original one was monitored for the growth trial, assuming that it was representative of the other crates. A scheme to visualise the breeding process for TM adults and larvae is presented in Figure 1. Feed and carrots were administered *ad libitum*. Temperature and RH in the room for larvae breeding averaged 27.8 ± 2.1 °C and 62 ± 10%, respectively. The photoperiod was set at 8 hours of light and 16 h of dark.

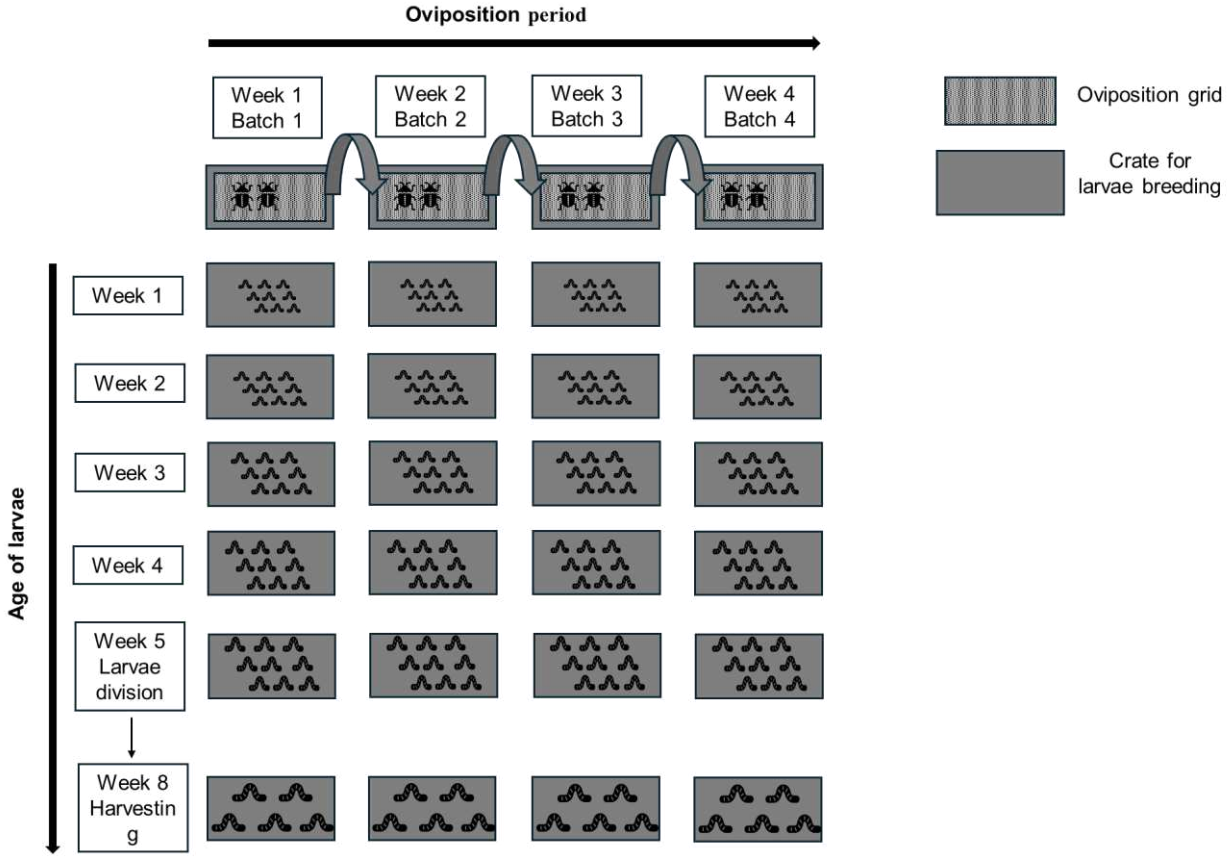

Figure 1. Scheme of the breeding process for *Tenebrio molitor* adults and larvae.

Productive performance of larvae

The total weight of larvae in each crate was registered at the 5th (before and after density equalisation) and at 8th week of age after removing the residual feed and frass. Larvae growth was obtained by subtracting the total weight of larvae at 8 weeks of age and the total weight of larvae at 5 weeks of age, after density equalisation. Moreover, a total of 100 larvae were sampled from each crate and weighted to compute the average individual BW. The number of larvae obtained at 5 weeks of age from each crate was calculated according to Equation 3.

$$Larvae\ (n) = \frac{Total\ weight\ of\ larvae\ 5\ wk\ (g/crate)}{Individual\ weight\ of\ larvae\ 5\ wk\ (g/larvae)} \quad (3)$$

Where the total weight at 5 weeks is that measured before density equalisation. The average number of adults present in each crate at the beginning of the experiment was estimated according to Equation 4.

$$Adults\ (n) = \frac{Total\ weight\ of\ adults\ (g/crate)}{Individual\ weight\ of\ adults\ (g/adult)} \quad (4)$$

The grams of larvae obtained per grams of adults was calculated according to Equation 5.

$$g\ larvae/g\ adult\ = \frac{Total\ weight\ of\ adults\ (g/crate)}{Total\ weight\ of\ larvae\ (g/crate)} \quad (5)$$

The intake of feed and carrots (reported on DM basis) was measured at crate level from week 5 to 8, and the FCR was calculated accordingly.

At 8 weeks of age, the frass was collected from each crate, after separation from the larvae and residual feed.

Chemical analyses

Feed samples of both adults and larvae were collected at each feed batch change. To analyse the chemical composition of adults' bodies, sampling was carried out at the beginning of the trial (100 g) before their assignment to the experimental groups. Other 24 samples (100 g each) of adults were collected from 6 crates per group at the end of the 4th week (end of adults breeding phase). To analyse the chemical composition of larvae, 100 g of larvae were taken from each crate at 5 (at equalisation) and 8 weeks of age and stored at -20 °C. This procedure was repeated for each batch. At the end of the trial, a total of 96 samples (24 samples per group; 48 samples per age) were obtained as pooled larvae from the same crate of different batches. Furthermore, the chemical composition of insect's frass was determined for each batch at 8 weeks of age of larvae. Despite efforts to separate residual feed, finer particles of it persisted in the frass. To determine larvae FI, the starch content of frass and diet was analysed. Considering a starch digestibility of 99% reported for *Hermetia illucens* (Guillaume et al., 2023), the quantity of starch undigested from the diet was used to calculate the grams of residual feed in frass. To analyse the chemical composition of frass, 100 g of frass were collected from each crate at 8 weeks of age of larvae. The procedure was repeated for each batch of larvae. At the end of the trial, a total of 24 samples of frass (6 breeding density) were obtained as pooled frass from the same crate of different batches.

Samples of adults, larvae, frass and diets were transported to the LabCNX laboratory of the Animal Medicine, Production and Health (**MAPS**) Department of (University of Padova) for chemical analyses. Larvae and adults' samples were stored at -20 °C, whereas frass and feed were stored in fridge at +4 °C. Analyses of adults, larvae, frass and diets were performed according to the AOAC (2000) procedures to determine DM (method no. 934.01), crude protein (method no. 2001.11), and ash (method no. 942.05). For adult insects and larvae, the N content was multiplied by the conversion factors 4.76 (Janssen et al., 2017). The ether extract was determined after acid hydrolysis (Commission Directive 98/64/EC, 1998).

Chitin content was determined for adults and larvae according to the method described by Zhang and Zhu (2006), with the modifications provided by Woods et al. (2020). Starch content was analysed in adults and larvae diets, and in frass (amyloglucosidase-α-amylase, method no. 996.11). Lipid extraction for the assessment of the FAs profile in adults and larvae diets (Supplementary Table S2), as well as in adults, was performed by modified accelerated solvent extraction. For adults and larvae diets, the extraction process was carried out using petroleum ether as solvent, whereas for insects the extraction was performed using a binary solvent mixture of chloroform/methanol at a 1:2 ratio, according to the method by Lee et al. (1995). The fat content of samples was determined gravimetrically after vacuum evaporation under nitrogen stream. Then, samples were trans-methylated using a methanolic solution of H_2SO_4 (4%) to determine fatty acids methyl esters (**FAMEs**). An 8 ml of distilled water and 4 ml of *n*-heptane were added to the samples to obtain a biphasic separation. FAMEs were quantified by gas chromatography (Shimadzu GC17A). equipped with an Omegawax (Sigma–Aldrich Co. LLC., Saint Louis, USA) 250 column (30 m × 0.25 μm × 0.25 μm) and flame ionisation detector. Helium was used as the carrier gas at a constant flow of 0.8 ml/min. The injector and detector temperatures were 260 ◦C. Peaks were identified based on commercially available FAME mixtures (37-Component FAME Mix; Supelco Inc., Bellefonte, PA, USA). Results were expressed as % of the total detected FAME. Cholesterol content of adults and larvae was determined according to the method described by Indyk (1990) and Casiraghi et al. (1994).

Statistical analysis

Assumptions of normality were assessed using the Shapiro–Wilk test; variables with a Shapiro–Wilk statistic ≥ 0.90 were considered normally distributed. Data of TM adults (live weight and hatchability rate) were analysed using a mixed model, with breeding density, breeding week and their interaction as fixed effects, and crate (nested within density) as a random repeated effect, using the MIXED procedure of SAS 9.1.3 (SAS Institute, 2008). Mortality of TM adults (non-normally distributed) was analysed using a generalized linear model assuming a Poisson distribution (GENMOD procedure of SAS), with breeding density, breeding week and their interaction as fixed effects, and crate (nested within density) as a random repeated effect. Ovary weight, feed and carrot intake, and the chemical composition of TM adults were analysed by one-way ANOVA, with breeding density as a fixed effect. Data of TM larvae (number, live weight, weight gain, FI and FCR) were analysed by a one-way ANOVA with breeding density as a fixed effect. Chemical composition of larvae was analysed by two-way ANOVA with breeding density and larvae age as fixed effects. Data about the proximate composition of larvae frass was analysed by one-way ANOVA with breeding density as fixed effect. Least square means were obtained, and post-hoc pairwise comparisons were performed using the Bonferroni correction. Statistical significance was declared at $P < 0.05$.

Results

Mortality and reproductive performance of adults

Results about adults' mortality are reported in Table 1. The overall mortality rate showed no significant variation across different breeding densities of TM adults. However, the mortality rate was significantly influenced by the breeding week. The highest mortality rate occurred in week 2 (10.2% on average) across all densities, while lower rates were observed in week 1 (4.31% on average), 3 (3.98% on average) and 4 (2.87% on average) (P<0.001).

Table 1. Effect of the breeding density (D) and breeding week (W) of *Tenebrio molitor* adults on mortality (%).

Breeding density	**Treatments**				**SEM D**	***P-value* D**	**P-value D * W**
	D1	**D2**	**D3**	**D4**			
N. of crates	12	12	12	12			
Mortality:							
Week 1	3.69^{x}	4.02^{x}	4.23^{x}	5.29^{x}	0.61	0.2879	
Week 2	11.4^{w}	9.36^{w}	9.65^{w}	10.5^{w}	0.93	0.4148	0.0537
Week 3	3.76^{x}	3.74^{x}	3.88^{x}	4.55^{x}	0.58	0.7416	
Week 4	2.61^{x}	2.50^{x}	2.86^{x}	3.50^{x}	0.50	0.4976	
SEM W	0.64	0.62	0.64	0.69			
***P-value* W**	<0.0001	<0.0001	<0.0001	<0.0001			
Total Mortality	20.3	18.5	19.6	22.1	1.30	0.2790	

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2.; $^{w-x}$ Values within a column with different superscripts differ significantly at P<0.05.

The results pertaining to eggs hatchability (Table 2) indicate that eggs obtained from density D4 achieved a higher hatching percentage compared to density D3 at week 3 (89.8 *vs* 82.0%; P<0.05), with intermediate values in densities D1 and D2. Conversely, in week 4, D3 had the highest hatching percentage while D4 had the lowest (88.8 *vs* 81.7%; P<0.05), with D1 and D2 being intermediate. However, considering the overall oviposition data, no significant differences were observed among the different breeding densities, and the hatching rate averaged 85%.

The effect of week on the eggs hatchability was significant only for D4, where the highest value was recorded in week 3 and the lowest in week 4 (89.8 vs 81.7%, respectively; P<0.05). The breeding density of adults also influenced the ovary proportion measured at week 4 of breeding (Table 3). Specifically, the ovary proportion was higher in D2 compared to D1 (15.6% *vs* 13.8%; *P<0.05*), whereas intermediate values were outlined for D3 and D4.

Table 2. Effect of the breeding density (D) and breeding week (W) of *Tenebrio molitor* adults on eggs hatchability (%).

Breeding density	**Treatments**				**RSD D**	***P-value* D**	***P-value* D * W**
	D1	**D2**	**D3**	**D4**			
N. of crates[1]	12	12	12	12			
Hatching:							
Week 1	86.7	86.0	86.6	84.7^{wx}	5.47	0.8379	0.0340
Week 2	84.3	83.2	85.2	82.8^{wx}	7.68	0.8818	
Week 3	86.3^{ab}	84.2^{ab}	82.0^{b}	89.8$^{a, w}$	6.67	0.0402	
Week 4	82.2^{ab}	85.0^{ab}	88.8^{a}	81.7$^{b, x}$	6.21	0.0269	
RSD W	6.19	6.48	7.05	6.62			
***P-value* W**	0.2728	0.7539	0.1355	0.0223			
Week 1-4	84.9	84.6	85.6	84.8	6.77	0.8824	

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2;[1]50 eggs/crate; $^{a-b}$ Values within a row with different superscripts differ significantly at $P<0.05$; $^{w-x}$ Values within a column with different superscripts differ significantly at $P<0.05$.

Table 3. Effect of the breeding density of *Tenebrio molitor* adults on ovary weight assessed at the beginning (W0) and at the end of the trial (week 4; W4).

	W0	**W4**				**RSD**	***P-value***
Breeding density		**D1**	**D2**	**D3**	**D4**		
N. of crates[1]		12	12	12	12		
Ovary weight (g)	0.015	0.018	0.020	0.020	0.019	0.005	0.0532
Ovary incidence (% adult weight)	13.5	13.8^{b}	15.6^{a}	15.4^{ab}	14.8^{ab}	3.64	0.0419

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2; [1]1 crate of 50 ovaries; otherwise 25 ovaries/crate; $^{a-b}$ Values within a row with different superscripts differ significantly at $P<0.05$.

Adults BW and FI

The breeding density of adults significantly influenced their BW throughout the oviposition period (Table 4). The highest BW was observed in D4 adults and the lowest in D2 adults (0.1263 *vs* 0.1246 g; $P<0.05$), with intermediate values observed in D1 and D3.

Regarding the effect of the oviposition week, a continuous increase in the average BW of adults was observed. For all densities, the weight peaks were reached in the fourth week (Table 4). As expected, both total and daily individual intake of pelleted feed and carrots increased with breeding density (Table 5).

Table 4. Effect of the breeding density (D) and breeding week (W) of *Tenebrio molitor* adults on their body weight (g/adult).

Breeding density	Treatments				RSD	*P-value*	*P-value*
	D1	D2	D3	D4	D	D	D * W
N. of crates[1]	12	12	12	12			
BW (g/adult):							
Week 1	0.1227^{x}	0.1236^{x}	0.1242^{x}	0.1246^{x}	0.002	0.1731	0.5597
Week 2	0.1236$^{ab, x}$	0.1223$^{b, x}$	0.1247$^{a, x}$	0.1244$^{a, x}$	0.002	0.0075	
Week 3	0.1265^{w}	0.1264^{w}	0.1271^{w}	0.1269^{w}	0.002	0.8754	
Week 4	0.1272^{w}	0.1267^{w}	0.1285^{w}	0.1276^{w}	0.002	0.1552	
RSD W	0.002	0.002	0.002	0.03			
***P-value* W**	<0.0001	<0.0001	<0.0001	0.0008			
Week 1-4	0.1252^{ab}	0.1246^{b}	0.1257^{ab}	0.1263^{a}	0.02	0.0145	

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2; [1]100 adults/crate; $^{a-b}$ Values within a row with different superscripts differ significantly at $P<0.05$, $^{w-x}$ Values within a column with different superscripts differ significantly at $P<0.05$.

Table 5. Effect of the breeding density of *Tenebrio molitor* adults on feed and carrots intake (mg/adult) in 4 weeks of breeding.

Breeding density	Treatments				RSD	*P-value*
	D1	D2	D3	D4		
N. of crates	12	12	12	12		
Total pelleted feed intake	259^{c}	276^{b}	283^{b}	304^{a}	12.2	<0.0001
Total carrots intake[1]	186^{ab}	182^{b}	182^{b}	190^{a}	5.13	0.0015
Daily pelleted feed intake	9.24^{c}	9.87^{b}	10.1^{b}	10.9^{a}	0.44	<0.0001
Daily carrots intake	6.63^{ab}	6.51^{b}	6.52^{b}	6.79^{a}	0.18	0.0015

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2;[1]The consumed carrots were calculated on dry matter basis.

Chemical composition and FAs profile of adults

The breeding density of TM adults did not affect the water, protein, ash and chitin contents of the larvae (Table 6). Conversely, D1 adults displayed a higher lipid content compared to D3 adults (7.89 *vs* 7.38 g/100 g insect; $P<0.01$), with D2 and D4 showing intermediate values. A similar pattern was observed for cholesterol content, with D1 adults having higher amounts than D3 adults (657 vs. 612 mg/kg insect; $P<0.01$). The breeding density of TM adults had a marginal effect on their FAs profile (Table 7). A significant difference resulted in the heptadecenoic acid (C17:0) content, with the highest value observed in group D3 and the lowest in group D2 (0.33% *vs* 0.28%, respectively; $P<0.01$). Among the SFAs, stearic acid (C18:0) was the most abundant, with the highest value showed by adults reared at D1 and the lowest in those reared at D4 (4.56% vs 4.29%, respectively; $P<0.05$) The sole MUFA affected by breeding density was C17:1, which was higher in adults reared at D4 compared to D1 and D2 (0.30% *vs* 0.21% and 0.22%, respectively; $P<0.05$).

Table 6. Effect of the breeding density of *Tenebrio molitor* adults on their proximate composition (g/100 g insect), cholesterol (mg/kg insect) and chitin (g/100 g insect) contents.

Breeding density	Treatments				RSD	*P-value*
	D1	D2	D3	D4		
N. of crates[1]	6	6	6	6		
Water	60.1	59.9	60.2	59.8	0.58	0.7095
Protein[2]	18.0	18.5	17.7	18.4	0.80	0.3128
Lipids	7.89[a]	7.63[ab]	7.38[b]	7.40[ab]	0.22	0.0019
Ash	1.22	1.23	1.23	1.24	0.02	0.3239
Cholesterol	657[a]	626[ab]	612[b]	630[ab]	20.2	0.0079
Chitin	5.65	5.68	5.65	5.54	0.25	0.8262

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2; [1]N=50 g of adults/crate; [2]N x 4.76; [a-b] Values within a row with different superscripts differ significantly at for $P<0.05$.

The total amount of PUFAs was higher in adults reared at the lowest density (D1) than in those reared at densities D2 and D4 (28.5% *vs* 27.7% for both; $P<0.05$). This result was attributable to the proportion of *n*-6 FA, with specific emphasis on two FAs: linoleic acid and arachidonic acid ($P<0.05$), whereas the total *n*-3 FA remained unaffected by adult density. Despite this, the *n*-6/*n*-3 ratio was similar in all the groups, with an average value of 41.9.

Table 7. Effect of the breeding density of *Tenebrio molitor* adults on their fatty acids profile (% total Fatty Acid Methyl Esters).

Breeding density	Treatments				RSD	*P-value*
	D1	D2	D3	D4		
N. of crates	12	12	12	12		
C14:0	2.00	1.95	2.03	1.95	0.08	0.2621
C15:0	0.16	0.16	0.17	0.17	0.03	0.9303
C16:0	19.7	19.5	19.6	19.5	0.27	0.3597
C17:0	0.29[ab]	0.28[b]	0.33[a]	0.32[ab]	0.03	0.0248
C18:0	4.56[a]	4.45[ab]	4.46[ab]	4.29[b]	0.15	0.0396
C20:0	0.15	0.12	0.08	0.07	0.06	0.1501
C22:0	0.16	0.15	0.20	0.19	0.04	0.0550
Total SFAs	27.1	26.7	26.9	26.6	0.39	0.1503
C16:1	1.38	1.47	1.44	1.43	0.06	0.1246
C17:1	0.21[b]	0.22[b]	0.25[ab]	0.30[a]	0.03	0.0004
C18:1 *n*-9	40.2	40.9	40.8	40.3	0.59	0.1195
C20:1 *n*-9	0.39	0.48	0.44	0.47	0.07	0.1809
C22:1 *n*-9	0.05	0.03	0.01	0.05	0.04	0.3148
Total MUFAs	42.2	43.1	43.0	42.6	0.60	0.0656
C18:2 *n*-6	27.6[a]	26.9[b]	27.0[ab]	26.9[b]	0.45	0.0366
C18:3 *n*-6	0.07	0.09	0.09	0.05	0.03	0.0837

Table 7. Continue.

Breeding density	Treatments				RSD	*P-value*
	D1	D2	D3	D4		
C18:3 *n*-3	0.63	0.62	0.61	0.59	0.03	0.0966
C20:2 *n*-6	0.01	0.08	0.06	0.08	0.05	0.0593
C20:4 *n*-6	0.08[a]	0.01[b]	0.00[b]	0.00[b]	0.03	0.0001
Total PUFAs	28.5[a]	27.7[b]	27.8[ab]	27.7[b]	0.47	0.0207
n-6	27.8[a]	27.1[ab]	27.2[ab]	27.1[b]	0.44	0.0237
n-3	0.70	0.64	0.65	0.63	0.06	0.1683
n-6/*n*-3	39.8	42.1	42.3	43.2	3.50	0.3842
Identified	97.8	97.5	97.7	96.9		

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2; SFAs= saturated fatty acids; MUFAs= monounsaturated fatty acids; PUFAs= polyunsaturated fatty acids; [a-b] Values within a row with different superscripts differ significantly at $P<0.05$.

Larval productive performance

As expected, the total weight of larvae at 5 weeks of age (before density equalisation) augmented with increasing density, reaching the highest value in density D4 and the lowest in density D1 (595 g *vs* 336 g, respectively; $P<0.0001$) (Table 8). At 5 weeks old, larvae exhibited higher individual weights in densities D3 and D4 (0.0175 g on average) compared to densities D1 and D2 (0.0135 g on average) ($P<0.05$; Table 8). The total number of larvae per crate increased with the increasing density (26 100 *vs* 31 530 *vs* 35 970, for D1, D3 and D4, respectively; $P<0.0001$). At 8 weeks of age, the total weight of larvae increased from density D1 to density D2 (1 082 g *vs* 1 101 g; $P<0.01$). Differently, the total weight of larvae was similar among groups D2, D3 and D4. Overall, at 8 weeks of age, larvae from density D4 and D3 exhibited higher individual BWs compared to those from density D1 and D2 (0.112 *vs* 0.104 g and 0.106, respectively; $P<0.0001$). Larvae FI from week 5 to week 8 was lower in density D1 (1 681 g) compared to the other three densities (1 763 g, on average; $P<0.001$; Table 9). However, the breeding density of adults did not affect the weight gain of larvae (Table 9). Density D1 demonstrated a lower FCR (1.78) compared to densities D3 and D4 (1.86; $P<0.001$), with an intermediate value was observed for density D2.

Chemical composition of larvae

The breeding density of adults had a notable impact on the proximate composition of larvae. Specifically, at 5 weeks of age, larvae from D1 and D2 exhibited higher water content compared to those from D3 and D4 (69.0 *vs* 67.1 g/100 g, on average, respectively; $P<0.0001$; Table 10). Conversely, both protein and lipid contents increased with rising breeding density ($P<0.0001$). Furthermore, larvae from density D4 displayed the highest cholesterol content ($P<0.05$). By the time they reached 8 weeks of age, breeding density no longer influenced the water, protein, or lipid content of the larvae. However, ash content was the highest in density D1 and the lowest in the other three densities (1.33 *vs* 1.28 g/100 g, on average, respectively; $P<0.0001$). Moreover, cholesterol and chitin contents decreased with increasing breeding density ($P\leq0.01$).

Table 8. Effect of the breeding density of *Tenebrio molitor* adults on larval weight (at 5th and 8th weeks of age, respectively) and number of larvae (at 5th weeks of age) of batch 1-4.

Breeding density	Treatments				RSD	*P-value*
	D1	D2	D3	D4		
N. of crates[1]	12	12	12	12		
Total weight of larvae before density equalisation at 5 weeks (g/crate)	336[d]	444[c]	514[b]	595[a]	58.1	<0.0001
Individual weight of larvae at 5 weeks:(g/larvae)	0.0131[b]	0.0140[b]	0.0174[a]	0.0176[a]	0.03	<0.0001
Total larvae at 5 weeks (n/crate)[2]	26 100[c]	32 440[ab]	31 530[b]	35 970[a]	7 399	<0.0001
Total weight of larvae at 8 weeks (g/crate)	1 082[b]	1 101[a]	1 133[a]	1 126[a]	63.9	0.0003
Individual weight of larvae) at 8 weeks (g/larvae)	0.104[b]	0.106[b]	0.110[a]	0.113[a]	0.06	<0.0001

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2; [1]100 larvae/crate; [2]Calculated considering the total weight of larvae at 5 weeks of age, before density equalisation; [a-b] Values within a row with different superscripts differ significantly at $P<0.05$.

Table 9. Effect of the breeding density (D) of *Tenebrio molitor* adults on feed intake, weight gain and feed conversion ratio of larvae from 5 to 8 weeks of age (after density equalisation).

Breeding density	Treatments				RSD	P-value
	D1	D2	D3	D4		
Feed intake[1] (feed and carrots) (g/crate)	1 681[b]	1 747[a]	1 779[a]	1 764[a]	36.2	<0.0001
Weight gain (g/crate)	949	960	959	950	31.5	0.1776
FCR (per crate)	1.78[b]	1.82[ab]	1.86[a]	1.86[a]	0.05	0.0008

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2; FCR= feed conversion ratio; [1]the intake of feed and carrots was calculated on dry matter basis; [a-b] Values within a row with different superscripts differ significantly at $P<0.05$.

Chemical composition of frass

Results presented in Supplementary Table S3 reveal that the breeding density of TM adults influenced the chemical composition of frass. Specifically, the lipid content was higher in group D2 and D3 compared to group D1, whereas D4 exhibited an intermediate value ($P<0.01$). Frass obtained from density D1 showed lower ash content compared to D3 ($P<0.01$) and higher starch content compared to D3 and D4 ($P<0.01$).

Table 10. Effect of the breeding density (D) of *Tenebrio molitor* adults and larval age (A) on proximate composition (g/100 g), cholesterol (mg/kg) and chitin (g/100 g) contents of larvae.

Breeding density	Treatments				RSD	*P-value*	*P-value*
	D1	D2	D3	D4	D	D	D * A
N. of crates[1]	12	12	12	12			
Water							
Week 5	69.2[a]	68.8[a]	67.3[b]	66.9[b]	0.80	<0.0001	<0.0001
Week 8	65.3	65.5	65.5	65.4	0.42	0.4253	
RSD A	0.65	0.65	0.71	0.52			
P-value A	<0.0001	<0.0001	<0.0001	<0.0001			
Week 5-8	67.2[a]	67.2[a]	66.4[b]	66.2[b]	0.64	<0.0001	

Table 10. Continue.

Breeding density	Treatments				RSD	*P-value*	*P-value*
	D1	D2	D3	D4	D	D	D * A
Protein[2]							
Week 5	15.0[c]	15.1[bc]	15.4[ab]	15.6[a]	0.27	<0.0001	<0.0001
Week 8	12.4	12.5	12.4	12.4	0.05	0.4420	
RSD A	0.17	0.29	0.17	0.21			
***P-value* A**	<0.0001	<0.0001	<0.0001	<0.0001			
Week 5-8	13.7[c]	13.8[bc]	13.9[ab]	14.0[a]	0.21	<0.0001	
Lipids							
Week 5	5.70[b]	5.77[b]	6.55[a]	6.76[a]	0.49	<0.0001	0.1310
Week 8	10.9	10.5	10.5	11.2	0.25	0.3847	
RSD A	0.29	0.66	1.69	0.48			
***P-value* A**	<0.0001	<0.0001	<0.0001	<0.0001			
Week 5-8	8.30[ab]	8.13[b]	8.50[ab]	8.99[a]	0.95	0.0135	
Ash							
Week 5	1.62	1.62	1.60	1.61	0.03	0.0665	0.0279
Week 8	1.33[a]	1.29[b]	1.27[b]	1.27[b]	0.03	<0.0001	
RSD A	0.02	0.03	0.03	0.03			
***P-value* A**	<0.0001	<0.0001	<0.0001	<0.0001			
Week 5-8	1.48[a]	1.45[ab]	1.43[b]	1.44[b]	0.03	<0.0001	
Cholesterol							
Week 5	719[b]	729[b]	752[ab]	795[a]	27.1	0.0004	<0.0001
Week 8	616[a]	598[ab]	600[ab]	592[b]	11.5	0.0140	
RSD A	19.9	19.6	12.7	0.26			
***P-value* A**	<0.0001	<0.0001	<0.0001	<0.0001			
Week 5-8	667[ab]	663[b]	676[ab]	694[a]	20.8	0.0045	
Chitin							
Week 5	3.01	2.91	3.00	3.14	0.53	0.9005	0.5444
Week 8	2.27[a]	2.24[ab]	2.09[ab]	2.05[b]	0.10	0.0020	
RSD A	0.66	0.27	0.12	28.2			
***P-value* A**	0.0813	0.0014	<0.0001	<0.0001			
Week 5-8	2.64	2.57	2.55	2.60	0.38	0.9491	

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2; [1]100 g of larvae/crate; [2]N x 4.76; [a-b] Values within a row with different superscripts differ significantly at $P<0.05$.

Discussion

Effect of adult breeding density on adults' performance and reproduction

Existing scientific literature offers useful information on the optimal breeding density for TM adults. However, most studies were conducted on a small or laboratory scale, thus limiting the applicability of the results, the latter remaining a significant gap yet to be filled in the industrial-scale context. Therefore, identifying the optimal breeding density for TM adults is crucial to ensure a cost-effective and sustainable

insects production. Findings of the present study revealed a significant impact of the TM adults breeding density on their individual BW. The higher individual BW of TM adults in group D4 compared to group D2 could be attributed to an increased FI, as demonstrated by the results. Previous research showed that high breeding densities expose insects to an increased competition for feed, leading to a stressful condition that decreases FI (Morales-Ramos and Rojas, 2015). However, in the present study, both feed and carrots were provided in proportional amounts to each density to ensure the possibility for TM adults to feed *ad libitum*, thus minimising any potential competition.

Independently to the potential competition for feed, overcrowding during breeding can lead to stress, which could explain the lower content of lipids observed in adults reared at high densities compared to low-density ones. This hypothesis could not be verified with other trials on insects, since this aspect has not been investigated yet. However, in several fish species too it was observed that overcrowding conditions are source of stress (Huntingford et al., 2017), leading to decreased body lipid content, mobilisation and utilisation (Basrur et al., 2010; Tolussi et al., 2010; Ren et al., 2017).

Similarly, as observed for lipids, cholesterol content in adults also decreased with increasing stocking density, although the difference was significant only between D1 and D3. In insects, cholesterol serves as the primary sterol for synthesising vitamin D3 and ecdysteroids, a class of steroid hormones. Stressed insects are expected to increase cholesterol utilisation as an energy source to sustain vital functions (Mlček et al., 2019), which could explain the current finding. Another hypothesis to explain this finding could be linked to an increased reproductive activity in TM adults reared at high densities compared to those reared at lower ones. A higher reproductive activity determines a higher depletion of body reserves (Skowronek et al., 2021), including cholesterol to synthesise ecdysteroids. A more intense reproductive activity in TM adults kept at higher densities could also partly be corroborated by the results on ovary incidence calculated at the end of the reproductive period.

According to previous studies, stress derived from overcrowding can lead to cannibalism phenomena (Morales-Ramos et al., 2012; Deruytter et al., 2019; Deruytter and Coudron, 2021). However, in the current study, mortality rates were similar across different breeding densities, thus not confirming previous findings. The peak in mortality observed during the second breeding week remains unexplained. One possibility is that this result could be attributed to the initial handling of young and fragile adults, which was required to start the experiment. However, if this was the case, a high mortality rate should have also been observed during the first breeding week, which was not. Following the second week, TM adults mortality decreased, aligning with previous findings (Morales-Ramos et al., 2012).

When TM adults were reared at high stocking densities, some authors (Berggreen et al., 2008; Morales-Ramos et al., 2012; Zim et al., 2022) reported a depressed reproductive activity, with a reduced number of eggs produced per adult (Froonickx et al., 2022) and decreased fertility (Zim et al., 2022). Accordingly, in this study, the ratio grams of larvae/crate to grams of adults decreased from 2.80 to 2.48 ($R^2 = 0.8856$; Figure 2) as the breeding density increased from group D1 to group D4. This substantiates the negative correlation between eggs production and adult breeding density (Frooninckx et al., 2022).

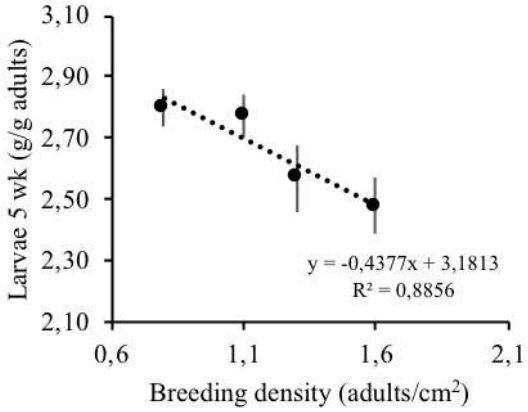

Figure 2. Linear regression of *Tenebrio molitor* larval production (g larvae/g adult) according to adults breeding density.
wk= week.

On the other hand, no differences in hatchability rates were observed across the different breeding densities. The average hatchability rate was 85%, which greatly differed from data obtained by Frooninckx et al. (2022), who reported an average hatchability value of 97%. These discrepancies are not surprising since, as showed by Adamaki-Sotiraki et al. (2022), the reproductive outcomes of different TM strains can greatly differ. This emphasises the need to conduct a proper genetic selection of TM strains to enhance the overall TM production cycle efficiency.

The weight of adults' ovary, along with its impact on adults' weight, could serve as indicator of fertility and reproductive activity. Although the weight of the ovary itself did not vary among the different rearing densities examined, its relative impact on adults' weight was lower at the lowest rearing density and the lowest in absolute terms considering numerical values (13.8 in D1 *vs* 15.3% averagely considering D2, D3 and D4). One possible explanation for this finding is that an increased farming density of TM adults may have stimulated the reproductive activity which could have determined the observed ovary incidence trend. In fact, previous research highlighted that TM adults bred at a density of 1.5 adult/cm^2 (very similar to D4: 1.6 adults/cm^2) displayed the highest fecundity (Zim et al., 2022). Conversely, another possibility is that the increased stress of TM adults kept at densities > 0.8 adults/cm^2, depressed the reproductive activity thus reducing ovary incidence (% adult weight). This would support studies were it was observed that increasing adults breeding densities led to a reduction in the reproductive performance in TM (Morales-Ramos et al., 2012). The latter seems therefore an aspect that merits further study.

Given these considerations, increasing the breeding density of TM adults did not impact overall mortality. Instead, higher densities led to greater total FI. Although the ratio of grams of larvae per crate to grams of adults decreased as breeding density increased, this resulted in a higher total weight of larvae produced, with individual larval weight also increasing. These findings align with existing research on the topic (Berggreen et al., 2018; Deruytter et al., 2019; Liu et al., 2022; Zim et al., 2022). In the present research, the number of larvae produced decreased as adults became older (from breeding cycles 1 to 4), confirming the negative correlation between adult age and eggs laid (Morales-Ramos, 2012; Breggreen et al., 2018).

Effect of adult breeding density on larval performance and chemical composition, and on frass characterisation

As the breeding density of TM adults increased, the quantity of larvae produced/crate, and their final weight, showed a remarkable increment. Different densities affected feed and carrots intake from weeks 5 to 8, with higher densities leading to increased consumption. This finding contrasts with the results of Morales-Ramos and Rojas (2015), who observed a depressed larval FI under high density conditions. In the present study, the amount of feed and carrots provided to the larvae was adjusted according to breeding density, thereby preventing potential feed shortages and ensuring *ad libitum* feeding.

The initial hypothesis was that adults reared at density D4 would have produced approximately twice the number of larvae compared to those reared at density D1 and the quantity of *ad libitum* feeding was calculated in this perspective. However, this was not observed probably due to a combination of density-related factors such as egg cannibalism or reduced adult fertility, as depicted in previous research (Zim et al., 2022).

As a consequence, larvae reared at densities D3 and D4 probably had access to a proportional greater quantity of feed and carrots compared to larvae at densities D1 and D2, which could have contributed to their increased FI and, as a consequence, augmented individual larval weight at week 5 (Figure 3: rise in individual larvae weight with increasing adult breeding density, R^2=0.8404). To further corroborate this speculation, the body of 5-weeks-old larvae showed to be enriched in protein, lipids and cholesterol with increasing TM adults breeding density (Table 10), thus suggesting a higher maturity degree.

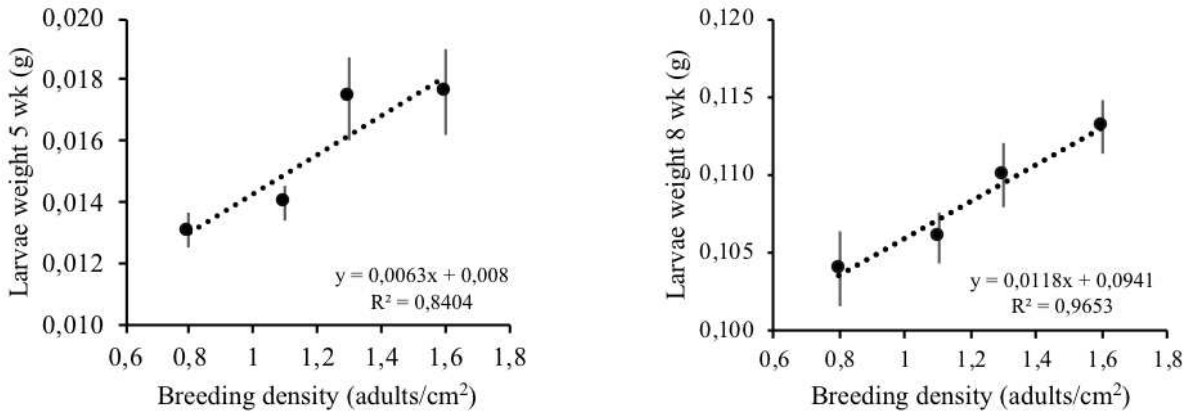

Figure 3. Linear regression of the individual weight of *Tenebrio molitor* larvae at 5 and 8 weeks of age (average of 4 batches) according to adults breeding density.
wk= week.

The difference in larval size recorded at 5 weeks, before equalisation, was maintained throughout the growing process and was detected again at 8 weeks (rise in individual larval weight). Despite larvae had been equalised at 5 weeks of age, thus all groups started the growing part of the cycle at the same conditions, at 8 weeks a strong correlation between individual larval weight with increasing density was again observed (Figure 3: R^2=0.9653). This finding could be explained by the performance results (larvae FI, weight gain and FCR from 5 to 8 weeks). The biggest larvae (D2, D3 and D4) were able to consume more feed than D1 but showed a similar weight gain. Consequently, from 5 to 8 weeks larvae size was maintained throughout

the second part of the cycle. From an efficiency point of view, present findings indicated that increasing adult breeding density worsened production efficiency of larvae in the second part of the cycle too (Figure 4: in the period 5-8 weeks FCR was positively correlated with adult breeding density, R^2=0.8663). Literature provides a heterogeneous scenario in this sense, where some research also showed that larval efficiency decreased with increasing the breeding density of adults (Bordiean et al., 2022), but others found no or minimum variations in FCR as a consequence of breeding density (Deruytter and Coudron, 2022: 0.6 *vs* 2.5 adults/cm^2).

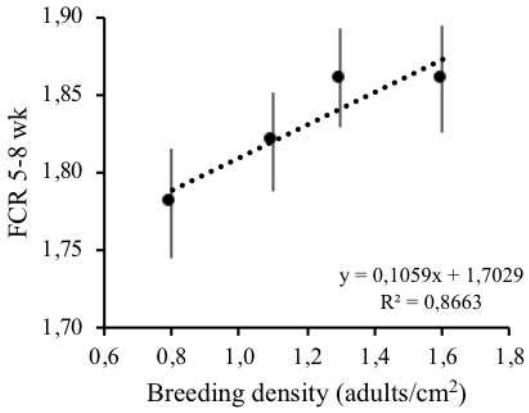

Figure 4. Linear regression of the FCR of *Tenebrio molitor* larvae (average of 4 batches) according to adults breeding density.
FCR= feed conversion ratio.

At 8 weeks of age, larvae showed a similar chemical composition, with the exceptions of cholesterol and chitin contents: in both cases, the amounts decreased from D1 to D4 and from week 5 to 8. The lower cholesterol content of larvae at 8 weeks of age compared to 5 weeks may be linked to their development. For instance, as observed in rabbits, cholesterol content decreases with age due to a reduced proportion of cell membranes, where cholesterol is a key component, relative to the total cell mass (Dalle Zotte, 2002). Differently, the declining chitin content of larvae from D1 to D4 density and from week 5 to 8 remain to be explained. In fact, from existing knowledge on the variations in TM body composition in relation to different developmental stages, chitin should increase along with development (from 7.2 to 11.8% in dry weight; Yu et al., 2021), reaching maximum values at the adult age.

Independently to the considered treatment groups, at 8 weeks of age, all TM larvae had a nutritional composition in line with literature data (Pasini et al., 2022). The breeding density of TM adults influenced also the chemical composition of frass. To this regard, it is worth mentioning the results on the starch content: since insects do not contain starch, its presence is attributable to the presence of feed. A higher amount of starch could be the result of a lower FI which is coherent with present feed Findings, since D1 larvae exhibited the lowest FI thus leaving a higher residual amount of starch in the frass.

Conclusions

Overall, the present research offers valuable insights for the optimisation of TM breeding on an industrial scale. Variations in adults breeding densities (ranging from 0.8 to 1.6 adults/cm²), had no influence on adults mortality. Adults reared at the lowest density (0.8 adults/cm²) exhibited increased production efficiency, namely an improvement in larval production/adult and a reduction in larvae FCR. However, given the limited cost of feed for TM adults and thus the relatively low incidence on cycle profitability, from an industrial perspective the higher yield of larvae/crate and their greater final weight at a density of 1.6 adults/cm² seem to indicate this option the preferred choice to maximise overall farm productivity.

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Supplementary materials

Supplementary Table S1. Chemical composition (g/kg as is) of diets administrated to *Tenebrio molitor* adults and larvae, respectively.

Items	Adults diet	Larvae diet
Chemical composition		
Dry matter	851	880
Crude protein	206	154
Ether extract	51.1	37.5
Ash	41.4	40.9
Starch	213	252

Supplementary Table S2. Fatty acids profile (% total Fatty Acid Methyl Esters) of diets administrated to *Tenebrio molitor* adults and larvae.

Fatty acids	Adults diet	Larvae diet
C4:0	0.01	0.04
C6:0	0.03	0.01
C8:0	0.03	0.00
C13:0	0.03	0.00
C14:0	0.05	0.05
C15:0	0.06	0.07
C16:0	18.8	17.8
C17:0	0.09	0.08
C18.0	2.02	1.20
C20:0	0.23	0.22
C22:0	0.24	0.15
C23:0	0.00	0.04
C24:0	0.60	0.46
Total SFAs	22.2	19.5
C14:1	0.09	0.00
C15:1	0.02	0.00
C16:1	0.58	0.18
C17:1	0.05	0.03
C18:1 *n*-9	20.6	17.4
C18:1 *n*-11	0.13	1.45
C20:1 *n*-9	0.67	0.66
C22:1 *n-9*	0.09	0.14
C24:1 *n-9*	0.12	0.17
Total MUFAs	22.3	20.0
C18:2 *n*-6	47.4	54.6
C18:3 *n*-3	3.28	3.51
C20:2 *n*-6	0.09	0.05
C20:5 *n*-3	0.05	0.06
C22:2 *n*-6	0.04	0.05
C22:6 *n*-3	0.18	0.18
Total PUFAs	51.0	58.4
Total *n*-6	47.5	54.7
Total *n*-3	3.52	3.75
n-6/*n*-3	13.5	14.6

Identified	95.5	98.0

SFAs: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids.

Supplementary Table S3. Effect of the breeding density of *Tenebrio molitor* adults on proximate composition (g/kg frass) of frass.

Breeding density	**Treatments**				**RSD**	***P-value***
	D1	**D2**	**D3**	**D4**		
N. of crates	12	12	12	12		
Moisture	132	136	133	134	8.22	0.8789
N[1]	2.05	2.06	2.07	2.09	0.03	0.2005
Lipids	13.2[b]	15.1[a]	14.9[a]	14.0[ab]	0.94	0.0091
Ash	72.1[b]	73.2[ab]	74.8[a]	74.4[ab]	1.18	0.0035
Starch	108.2[a]	94.9[ab]	88.9[b]	91.5[b]	7.91	0.0020

D1= 0.8 adults/cm^2, D2= 1.1 adults/cm^2, D3= 1.3 adults/cm^2, D4= 1.6 adults/cm^2; [1]Nitrogen percentage; [a-b] Values within a row with different superscripts differ significantly at P<0.05.

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Second contribution:

Optimisation of *Tenebrio molitor* reproduction: assessing the impact of different factors on larval yield, performances and mating preferences of beetles

B. Palumbo[a]*, D. Deruytter[b], B. Contiero[a], A. Dalle Zotte[a]

[a]Department of Animal Medicine, Production and Health, University of Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro, Padova, Italy

[b]Insect Research Centre, Inagro, Ieperseweg 87, 8800 Rumbeke-Beitem, Belgium

*Corresponding author: biancafederica.palumbo@phd.unipd.it

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Introduction

The yellow mealworm is one of the most produced and studied insect species in Europe, emerging as a promising alternative to conventional food and feed sources. Optimising the productivity at industrial level is one of the main challenges for TM farmers, involving several key factors including environmental conditions, oviposition period, stocking density, and the composition of the rearing substrate (Morales-Ramos et al., 2012; Deruytter et al., 2019; Eberle et al., 2022). While many of these factors have been extensively studied, other aspects, such as genetic knowledges, remain underexplored. Recent studies have demonstrated the influence of genetics on performance of TM (Rumbos et al., 2021; Adamaki-Sotiraki et al., 2022b; Adamaki-Sotiraki et al., 2023). Specifically, significant variability in growth performance, development time, larval survival, and FCE has been observed among different strains (Rumbos et al., 2021). Additionally, cross-breeding practices have been proposed as a valuable strategy for developing traits of economic importance such as the number of produced eggs and the final weight of larvae (Adamaki-Sotiraki et al., 2023). High productivity could be automatically associated with quantitative traits such as size, development time and FCE, all of which are significantly influenced by environmental factors (Koo et al., 2013; Morales-Ramos and Rojas 2015; Johnsen et al., 2021). Particularly, insects body size is usually correlated with longevity, fecundity, metabolic rates and abiotic stress tolerance (Atkinson, 1994). The selection of breeding animals based on phenotypic traits represent a practice to enhance desirable characteristics. This process involves choosing animals with superior traits to parent the next generation, thereby gradually improving the genetic quality of the population (Dekkers and Hospital, 2002). A 39% of increase in larval weight was observed in *Hermetia illucens* (Diptera: *Stratiomyidae*) after 16 generation of selection for this trait (Facchini et al., 2022). Accordingly, an increase of about 70 mg of weight was achieved in TM pupae after eight generation of selection (Morales-Ramos et al., 2019). Parental development time and pupal weight were found to strongly influence these same traits in TM progeny, as observed by Morales-Ramos et al. (2022). Specifically, a heritability of 40.7% was observed for pupal weight (Morales-Ramos et al., 2022). However, limited information is available on the influence of beetle size on larval and beetle weight. The maternal effect on development time and beetle size, along with the heritability of these traits, remains poorly understood. Furthermore, it is still unclear if exist a relationship between parent size and the number and size of progeny. Morales-Ramos et al. (2012) found no influence of female weight on progeny production per female, while the age of the females impacted fecundity, with reproductive output peaking in the 2nd and 3rd weeks of age.

In addition to the factors discussed above, reproduction in TM could be affected by the presence of mating preferences. Across various taxonomic groups, males typically select more fecund females to maximise offspring production (Honěk, 1993; Pincheira-Donoso and Hunt, 2017). On the other hand, females tend to choose males capable of offering direct and indirect benefits, leading to more attractive and viable offspring (Kokko et al., 2003; Clutton-Brock, 2017). In this context, female size is positively associated with fecundity (Aquiloni et al., 2008). Some evidence suggests that females are more attracted to healthy males (Worden et al., 2000; Worden and Parker, 2005). Body size has also been shown to influence mating success in various

insect species. For example, in *Hermetia illucens*, larger males have greater mating success than smaller ones, while larger females are more fecund (Jones and Tomberlin, 2021). Similar patterns have been reported in other species: in Ephemeroptera, larger males achieve higher mating success, and in *Cotesia urabae* (Hymenoptera: Braconidae), females prefer males with larger wings (Avila et al., 2017; Flecker et al., 1988. In contrast, Manas et al. (2024) reported that in *Hermetia illucens*, the size of males and females had no effect on mating success, nor was there any correlation between male size and sperm quantity. However, there remains limited information regarding the influence of size on mating in TM beetles.

A deeper investigation into these factors and their potential interactions is crucial for optimising TM reproductive performance, with the possibility to maximise the larvae yield in terms of number and individual weight. Therefore, this study aimed to explore the potential preference in beetle mating based on size, the effect of beetle size on the number and weight of produced larvae, and the heritability of beetle's BW.

Material and methods

Insects and general maintenance

The experiments were conducted at the Inagro insect research centre (Rumbeke-Beitem, Belgium) from March to June 2024. TM pupae used in the present study were obtained from larvae reared at a temperature of 26 ± 1 °C, 60 ± 5% of RH, in dark condition except during feeding and manipulation. Larvae were fed *ad libitum* with INSECTUS Mealworm Grow (Mijten nv, Belgium) and chopped and fermented chicory roots. Pupae of 48-72 h were harvested and sexed using a digital microscope (Dino-Lite digital microscope) with 40x magnification, to separate females and males. Once sexed, pupae were kept in plastic crates of 60 x 40 cm with a layer of wheat bran until beetles emerging and reached sexual maturity, approximately two weeks later. After emerging, the beetles were provided with agar-agar gel as a source of water (25 g/L). Since all the experiments focused on testing the effect of beetle size, the size variability within the population was assessed by individually weighing 100 newly emerged beetles prior to each experiment. This procedure allowed for the determination of the average, minimum, and maximum weights, which were then used to define the weight classes for the experimental groups. The same approach and weight ranges were applied across all experiments, as the weight distribution of the beetles remained consistent. A sample size of 100 individuals was selected, following the standard commonly used in previous studies by these research groups. Sample sizes and replicates were chosen to balance statistical reliability with practical feasibility. All experiments were conducted using 14-day-old beetles, as this age corresponds to sexual maturity and peak progeny production (Morales-Ramos et al., 2012).

Throughout all the experiments, temperature and RH were monitored to maintain stable conditions inside the facility. These parameters were controlled by an automated heating/cooling and humidification system and were checked daily. The temperature was maintained at 26 ± 1 °C and the RH at 60 ± 5%.

Experiment 1a

Beetles of 14 days of age were visually selected, weighed to assess the average weight (128 mg for males and 124 mg for females) and categorised into two weight groups: i) small females and males, weighing between 80 and 100 mg; ii) large females and males, weighing between 120 and 140 mg. To differentiate between sexes and weight categories, beetles were marked with coloured dots, following a previously established method (Carazo et al., 2004): large and small females were marked with red and green dots, respectively, while large and small males were marked with yellow and white dots, respectively. Beetles were divided into four treatment groups, each with 18 replicates. Each treatment consisted of a "primary chooser" (small or large) and two "choices" small and large beetle of the opposite sex, as detailed in Table 1. Beetles were placed in Petri dish lined with paper, which was replaced after each session to eliminate any traces from previous beetles. The "choices" were placed first, followed by the "primary chooser" in the middle of the Petri dish.

Table 1. Treatment groups of experiment 1a.

Experimental groups	Primary chooser	Choice 1	Choice 2
1	Small male	Large female	Small female
2	Large male	Large female	Small female
3	Small female	Large male	Small male
4	Large female	Large male	Small male

During the experiment, in treatments 3 and 4, it was observed that the males acted as the choosers, meaning that the choice was determined by the males rather than the female. Beetles were observed during mating and the following data were recorded: i) the preference shown in the first approach (without mating) by the "primary chooser" toward one of the two options in treatments 1 and 2, as well as which male, small or large, first approached the female (either small or large) in treatments 3 and 4; ii) the choice made during the first mating in treatments 1 and 2, and which male (large or small) first mated with the female (either small or large) in treatments 3 and 4; iii) the choice made during the second mating in treatments 1 and 2, and which male (large or small) mated for the second time with the female (either small or large) in treatments 3 and 4; iv). During observations, operators assigned a value of 0 when a large individual was selected and 1 when a small individual was selected. Due to the lengthy observation period, detailed data, including the time required for the first and second mating events, their durations, and the interval between them, were recorded for only four replicates. To minimise operator bias, the observers underwent two weeks of training in observing the reproductive behaviour of beetles.

Experiment 1b

Beetles aged 14 days were visually selected and weighed; the average weights were 128 mg for males and 124 mg for females. Subsequently, beetles were categorised by size and weighed to distinguish between small and large individuals. They were then divided into two treatment groups, as outlined in Table 2, with each group comprising 76 replicates.

Table 2. Treatment groups of experiment 1b.

Experimental groups	Primary chooser	Choice 1	Choice 2
1	Regular male	Large female	Small female
2	Regular female	Large male	Small male

As in Experiment 1a, beetles were placed in Petri dish. The "choices" beetles were positioned first, followed by the "primary chooser," placed at the centre of the Petri dish. The weight difference between the "choice 1" and "choice 2" was recorded. These beetles were selected such that the weight difference between them progressively decreased from replicate 1 to replicate 76 in both treatment groups 1 and 2. During mating observations, the beetles were monitored to determine whether the primary chooser selected the smaller or larger beetle to mate with. The effect of the weight difference on this final selection was then analysed. During observations, operators assigned a value of 0 when a large individual was selected and 1 when a small individual was selected.

Experiment 2

Beetles of 14 days of age were visually selected, weighed and categorised into three weight groups considering an average weight of 111 mg, determined by weighing 100 individuals. The three weight groups were as follow: i) small females and males, weighing between 80 to100 mg; ii) regular females and males, weighing between 101 to 120 mg; iii) large females and males, weighing between 121 to 140 mg. Beetles were assigned to three treatment groups with six replicates and one reserve each. Each treatment consisted of 10 female beetles and 10 male beetles. The treatments were as follow: i) 1: 10 small females and 10 small males; ii) 2: 10 regular females and 10 regular males; iii) 3: 10 large females and 10 large males. For the experiment, a total of 21 plastic cups with a diameter of 9.3 cm and a height of 8.2 cm, were filled with 30 g of INSECTUS feed, representing the feed for future larvae. An oviposition grid of 7.5 cm of diameter was placed above the feed and an additional 15 g of INSECTUS feed was placed on top of the grid to serve as food for the beetles. Beetles were placed on the grid and bred for four weeks. Every seven days, the oviposition grid, along with the beetles, was moved to a new cup with fresh feed to initiate a new oviposition cycle, resulting in a total of four oviposition periods. Mortality was monitored daily, and any dead beetles were replaced with individuals from the reserve group. Agar-agar gel was provided *ad libitum*. One batch of larvae was obtained from each oviposition week for a total of four batches.

Larvae from each batch were grown until they reached 8 weeks of age. Starting from 2 weeks of age, agar-agar gel was provided *ad libitum*. Larvae were kept in their original cups until they were 4 weeks old, at which point they were redistributed, placing 100 larvae from each cup into a new one filled with 50 g of wheat bran. This step was done to ensure the same larval density in each cup. The number of larvae was assessed at 3 weeks of age, taking two random subsamples from each cup after a gently homogenisation. The average weight of larvae was also registered. At 4 weeks of age, the average individual weight was assessed by weighing 100 larvae while the individual weight of each larva was recorded at 6 and 8 weeks of age. The total weight gain of larvae from week 3 to 8 was calculated along with the average daily gain (**ADG**).

Experiment 3a

Beetles of 14 days of age were visually selected, weighed and categorised according to their weight. A total of 20 pairs of beetles were formed. In 10 pairs (units 1 to 10), the male had an average weight of 116 mg (determined from a sample of 100 individuals), while the female's weight ranged from 90 to 150 mg, increasing incrementally across the units. In the remaining 10 pairs (units 11 to 20), the female had an average weight of 113 mg (also determined from a sample of 100 individuals), and the male's weight ranged from 90 to 150 mg in ascending order. Beetles were placed in plastic cups prepared similarly to those in Experiment 2 but filled with 15 g of INSECTUS feed instead of 30 g. Agar-agar gel was provided *ad libitum*. After one week of breeding, the beetles were removed, and larvae were allowed to grow for 3 weeks. At the end of this period, the number and average weight of the larvae were recorded. Mortality was monitored daily, and any dead beetles were replaced with ones of similar weight.

Experiment 3b

Due to a slight influence of maternal weight on larval weight was observed, Experiment 3 was repeated, changing some conditions, and extended to track larval development up to 8 weeks of age and their subsequent metamorphosis into beetles. Beetles of 14 days of age were visually selected, weighed and categorised according to their weight. A total of 34 beetle pairs was formed. In 17 pairs (units 1 to 17), the male had an average weight of 110 mg (determined from a sample of 100 individuals), while the female's weight ranged from 80 to 160 mg, increasing incrementally by 5 mg for each subsequent unit. In the other 17 pairs (units 18 to 34), the female had an average weight of 100 mg (determined from a sample of 100 individuals), and the male's weight ranged from 80 to 160 mg, also increasing incrementally by 5 mg. Beetles were placed in plastic cups prepared as described in Experiment 3a and were bred for one week. Mortality was monitored daily, with any dead beetles replaced by others of similar weight. After the breeding period, the beetles were removed, and the larvae were grown until 3 weeks of age. At this point, the number of larvae and their average weight were recorded. At 4 weeks of age, larvae were redistributed to equalise density, with 50 larvae placed in each crate. Their average weight was recorded. Individual larval weights were measured at 6 and 8 weeks of age. Additionally, the individual weights of beetles emerging from each crate were recorded to assess the heritability of beetle weight, considering paternal and maternal effects separately. Heritability was calculated using the method described in Experiment 2.

Statistical analysis

Data from Experiment 1a on mating preferences were analysed using a two-proportion z-test to compare the proportion of matings with a large individual between the two independent options (large vs. small mate). This test was used given the binary outcome, the independence of the choices and the sufficiently large sample size. A one-way ANOVA was conducted to analyse the latency and duration of the first and second mating events, as these variables met the assumptions of normality and homogeneity of variances; the experimental group was included as a fixed effect using the GLM procedure of SAS 9.1.3 (SAS Institute,

2008). In Experiment 1b, the differences in weight between the two choices of beetles were categorised into four weight classes (C1: 5-20 mg; C2: 21-30 mg; C3: 31-45 mg; C4: > 45 mg). A χ-square test was performed to assess whether the observed distribution of beetle choices across these weight classes differed significantly from the expected distribution, treating the weight classes as fixed categories. The χ-square test was appropriate because it tests for differences between observed and expected frequencies across categorical classes, without assuming normality. To analyse the probability of mating with a large individual compared to mating with a small one (assumed to be 50%), a PROC FREQ procedure in SAS 9.1.3 was used. This method was chosen because it tests whether the observed frequency of matings with large individuals deviates significantly from the expected frequency under the null hypothesis.

Data from Experiment 2 on the number and average weight of larvae were analysed by one-way ANOVA, with experimental group as a fixed effect; the same approach was used to analyse ADG and total weight gain of larvae For Experiments 3a and 3b, linear regression analyses were conducted, with parental weight (mother and father) as the independent variable (x) and the average weight and number of larvae as dependent variables (y). Parent–offspring regression was applied to estimate the heritability of beetle weight, by fitting linear regression models with parental weight as the independent variable (x) and individual progeny weight as the dependent variable (y). Statistical significance was declared at $P < 0.05$.

Results

Experiment 1

Results of Experiment 1a are reported in Table 3. Regarding the first approach, in group 1, the small male significantly preferred a large female over a small one (72.2%; P<0.001). A similar trend was observed in group 3, where the large male approached the female more frequently than the small male (66.7%; P=0.008). No significant preference was observed in groups 2 and 4.

Table 3. Effect of *Tenebrio molitor* beetles' size on the preference (%) for large versus small beetles during the first approach, first mating and second mating. Data analysed by a two-proportion z-test.

	Experimental groups			
	1	**2**	**3**	**4**
N. of replicates	18	18	18	18
First approach				
Large beetle	$72.2^{X} \pm 0.45$	61.1 ± 0.49	$66.7^{X} \pm 0.47$	61.1 ± 0.49
P-value	<0.001	0.31	0.008	0.31
First mating				
Large beetle	55.6 ± 0.50	$66.7^{X} \pm 0.47$	$66.7^{X} \pm 0.47$	55.6 ± 0.50
P-value	0.74	0.008	0.008	0.74
Second mating				
Large beetle	$72.2^{X} \pm 0.45$	61.1 ± 0.49	55.6 ± 0.50	$27.8^{X} \pm 0.45$
P-value	<0.001	0.31	0.74	<0.001

1= primary chooser: small male, choice 1: large female, choice 2: small female; 2= primary chooser: large male, choice 1: large female, choice 2: small female; 3= primary chooser: small female, choice 1: large male, choice 2: small male; 4= primary chooser: large female, choice 1: large male, choice 2: small male.

During the first mating, in group 2, the large male preferred the large female over the small female (66.7%; P=0.008) and in group 3, the large male mated more with the small female compared to the small male (66.7%; P=0.008). During the second mating, in group 1, the small male mated more with the large female (72.2%; P<0.001), consistent with the preference expressed during the first approach. Additionally, contrary to what observed during the first mating, no differences were found in group 3 during the second mating. In group 4, however, the small male mated more with the large female compared to the large male (72.2%; P<0.001). No significant differences were observed between groups in terms of latency of first mating and the duration of the first mating which were 151 s and 89.0 s on average respectively (Table 4). Considering the second mating, the latency was significantly longer in group 1 and 2 compared to group 3 and 4 (1290 s vs 199 s, on average, respectively; P<0.001). No significant differences emerged in the length of the second mating, which averaged approximately 83.1 s across all groups.

Table 4. Effect of the mating preference of *Tenebrio molitor* beetles on latency of first and second mating (s) and first and second mating duration (s). Data analysed by a one-way ANOVA.

	Experimental groups				**SE**	***P-value***
	1	**2**	**3**	**4**		
N. of replicates	4	4	4	4		
LTF	168	99.0	158	180	43.8	0.59
FMD	71.4	108	126	50.4	40.8	0.59
LSM	1386^{A}	1194^{A}	284^{B}	113^{B}	119	<0.001
SMD	84.6	93.0	97.8	57.0	15.6	0.30

1= primary chooser: small male, choice 1: large female, choice 2: small female; 2= primary chooser: large male, choice 1: large female, choice 2: small female; 3= primary chooser: small female, choice 1: large male, choice 2: small male; 4= primary chooser: large female, choice 1: large male, choice 2: small male. Abbreviations: LFT= latency first mating; FMD= first mating duration; LSM= latency second mating; SMD= second mating duration.

Results of Experiment 1b are presented in Table 5 and 6. No effect of the weight difference between the beetles offered as choices was observed on the decisions of the primary chooser. Notably, even when the weight difference between the options was greater (C1), the decisions of the primary choosers (regular male and regular female) were not significantly influenced in either group 1 or group 2 (P=0.85 and 0.51, respectively) (Table 5). Regardless of the weight difference between the beetles, in both group 1 and group 2, the primary choosers significantly preferred the larger beetle, mating with the larger choice 69.7% and 65.8% of the time, respectively (P=0.001 and P=0.01).

Table 5. Effect of the weight difference between large and small females and large and small males of *Tenebrio molitor* beetles on the preference (%) for large females or males during the first mating. Data analysed by a χ-square test.

	Weight Classes				
	C1	**C2**	**C3**	**C4**	***P-value***
N. of replicates	19	19	19	19	
First mating					
Large female	68.0 ± 11.6	76.2 ± 9.29	70.0 ± 10.3	63.2 ± 11.1	0.85
Large male	64.3 ± 12.2	57.1 ± 10.8	78.9 ± 9.35	63.6 ± 10.3	0.51

C1= 90-50 mg; C2= 45-35 mg; C3= 30-20 mg; C4= 20-5 mg.

Table 6 presents the probability of mating with a large individual relative to the expected 50% baseline for random choice. A significant increase in the likelihood of mating with a large male was observed for regular females only when the weight difference between the options fell within the C2 range (P=0.016). Similarly, when the primary chooser was a regular male, the probability of mating with a large female was significantly higher only when the difference between the choices was within the C3 range (P=0.012).

Table 6. Effect of the weight difference between large and small females and large and small males of *Tenebrio molitor* beetles on the probability of mating with a large or small individual. Data analysed by a χ-square test assuming a random choice probability of 50%.

	Primary chooser			**Primary chooser**		
	Female			**Male**		
	Large	**Small**	***P-value***	**Large**	**Small**	***P-value***
C1	9 ± 0.13	5 ± 0.13	0.134	11 ± 0.12	5 ± 0.12	0.285
C2	12 ± 0.11	9 ± 0.11	0.016	16 ± 0.09	5 ± 0.09	0.513
C3	15 ± 0.09	4 ± 0.09	0.074	14 ± 0.10	6 ± 0.10	0.012
C4	14 ± 0.10	8 ± 0.10	0.251	12 ± 0.11	7 ± 0.11	0.201

C1= 90-50 mg; C2= 45-35 mg; C3= 30-20 mg; C4= 20-5 mg.

Experiment 2

The size of TM beetles influenced the average weight of larvae across all developmental stages, considering all the four breeding cycles. While the number of larvae in each crate was not significantly affected by beetle size, groups 2 and 3 exhibited a significantly higher average larval weight compared to group 1 at 3 weeks of age (2.94 vs 2.76 mg on average; P<0.01) (Table 7). This trend persisted at week 4 and 6, with group 1 consistently showing the lowest larval weights (8.74 vs 9.97 mg on average at week 4, and 54.9 vs 60.4 mg on average at week 6; P<0.01). Furthermore, by 8 weeks of age, larvae of group 3 displayed a significantly higher weight compared to group 2, and both exceeded group 1 (137 vs 130 vs 121 mg, respectively; P<0.001).

Table 7. Effects of *Tenebrio molitor* beetle size (males and females) on the number of produced larvae per crate and the average weight of larvae at 3, 4, 6 and 8 weeks of age of larvae (mg). Data analysed by a one-way ANOVA.

	Experimental groups			**RSD**	**P-value**
	1	**2**	**3**		
N. of replicates	24	24	24		
Number of larvae (Week 1-4)	564	574	566	88.5	0.92
Weight of larvae 3 weeks (Week 1-4)	2.76^{B}	2.92^{A}	2.96^{A}	0.20	<0.01
Weight of larvae 4 weeks (Week 1-4)	8.74^{B}	9.83^{A}	10.1^{A}	1.49	<0.01
Weight of larvae 6 weeks (Week 1-4)	54.9^{B}	59.1^{A}	61.7^{A}	3.85	<0.01
Weight of larvae 8 weeks (Week 1-4)	121^{C}	130^{B}	137^{A}	3.71	<0.01

1= 10 small males and 10 small females; 2= 10 regular males and 10 regular females; 3= 10 large males and 10 regular females.

As the average weight of larvae was influenced by beetle size, the ADG varied accordingly (Table 8). Notably, larvae of group 3 consistently exhibited the highest ADG. Between week 3 and 4, group 1 recorded the lowest ADG (0.92 mg/day; P<0.01), while group 2 showed an intermediate value (0.98 mg/day). From week 4 to 6, no significant differences were observed between the ADG of larvae in groups 3 and 2, though

group 1 continued to show the lowest value (3.61 mg/day vs 3.25 mg/day, respectively; P<0.01). From week 6 to 8, group 1 again demonstrated the lowest ADG, followed by group 2, while group 3 recorded the highest value (5.34 mg/day vs 5.08 mg/ vs 4.70 mg/day, respectively; P<0.01). The same trend was observed when considering the ADG from week 3 to 8, as well as total weight gain, which was significantly higher in group 3 compared to groups 2 and 1 (134 vs 128 vs 118 mg, respectively; P<0.01).

Table 8. Effect of *Tenebrio molitor* beetle size (males and females) on the average daily gain(mg/day) and the total weight gain (mg) of larvae. Data analysed using a one-way ANOVA.

	Experimental groups			RSD	*P-value*
	1	**2**	**3**		
N. of replicates	24	24	24		
ADG 3 - 4 weeks (Week 1-4)	0.92^{B}	0.98^{AB}	1.02^{A}	0.09	<0.01
ADG 4 - 6 weeks (Week 1-4)	3.25^{B}	3.54^{A}	3.68^{A}	0.24	<0.01
ADG 6 - 8 weeks (Week 1-4)	4.70^{C}	5.08^{B}	5.34^{A}	0.29	<0.01
ADG 3 – 8 weeks (Week 1-4)	3.37^{C}	3.64^{B}	3.82^{A}	0.11	<0.01
Total weight gain (Week 1-4)	118^{C}	128^{B}	134^{A}	3.75	<0.01

1= 10 small males and 10 small females; 2= 10 regular males and 10 regular females; 3= 10 large males and 10 regular females. ADG= average daily gain.

Experiment 3

Figure 1 presents the results of Experiments 3a and 3b on the average larval weight at 3 weeks of age. In Experiment 3a, a significant effect of the maternal weight was observed on the average weight of larvae at 3 weeks of age (P<0.001) with a linear increase in the average weight of larvae in relation to maternal weight (R^2 = 0.56). In contrast, paternal weight had no significant influence (P= 0.22; R^2 = 0.08). In Experiment 3b, both maternal and paternal weights showed weak effects on the average weight of larvae (R^2 = 0.09 and 0.11, respectively) with no significant differences (P=0.10 and 0.22, respectively).

Regarding the effect of maternal and paternal weight on larval production per crate, no significant influence was observed for either parent in Experiment 3a (P=0.87 and 0.28, respectively; R^2 = 0.001 and 0.03, respectively) (Figure 2a). In Experiment 3b, an increase in maternal weight was associated with an increase in the number of larvae produced per crate, though this trend was not statistically significant (R^2 = 0.26; P=0.12) (Figure 2b). Paternal weight had no effect on larval production in Experiment 3b (P=0.98) (Figure 2b).

Similar to what observed at 3 weeks of age, maternal weight in Experiment 3b had no significant effect on the average larval weight at 4, 6, or 8 weeks of age (P=0.21, 0.20, and 0.38, respectively; R^2 = 0.11 for both weeks 4 and 6, and 0.06 for week 8). Likewise, paternal weight had no effect on the average larval weight at 4 weeks of age (P=0.22; R^2 = 0.21). In contrast, paternal weight significantly influenced the average larval weight at 6 and 8 weeks of age (P=0.03), with a linear increase as the father's weight increased (R^2 = 0.11 and 0.31, respectively) (Figure 3).

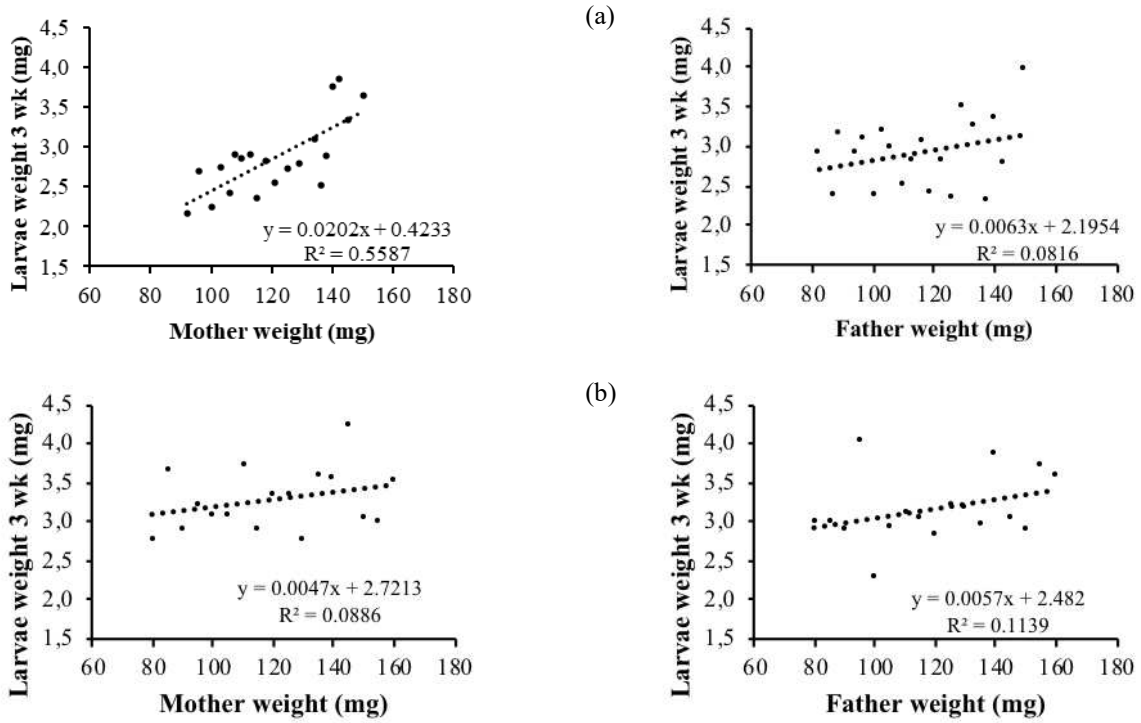

Figure 1. Linear regression of average weight of *Tenebrio molitor* larvae at 3 weeks of age according to weight of parents (mother and father).
(a)= experiment 3 a; (b)= experiment 3 b; wk= weeks

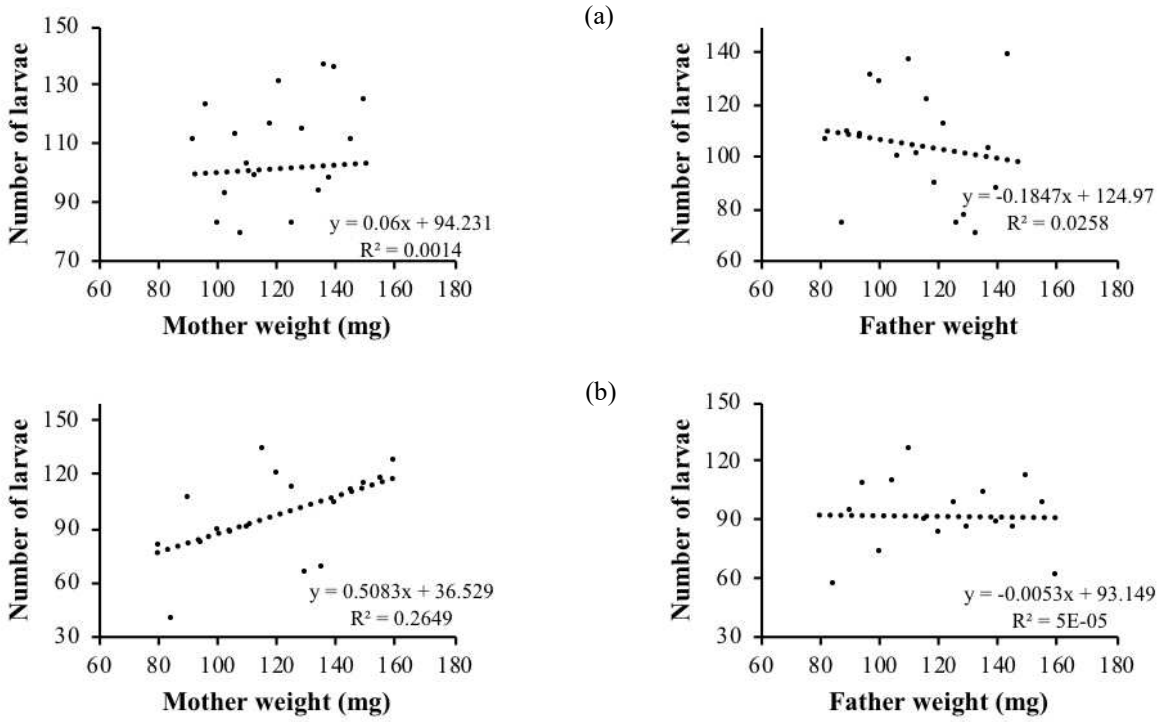

Figure 2. Linear regression of number of *Tenebrio molitor* larvae at 3 weeks of age according to weight of parents (mother and father).
(a)= experiment 3 a; (b)= experiment 3 b; wk= weeks

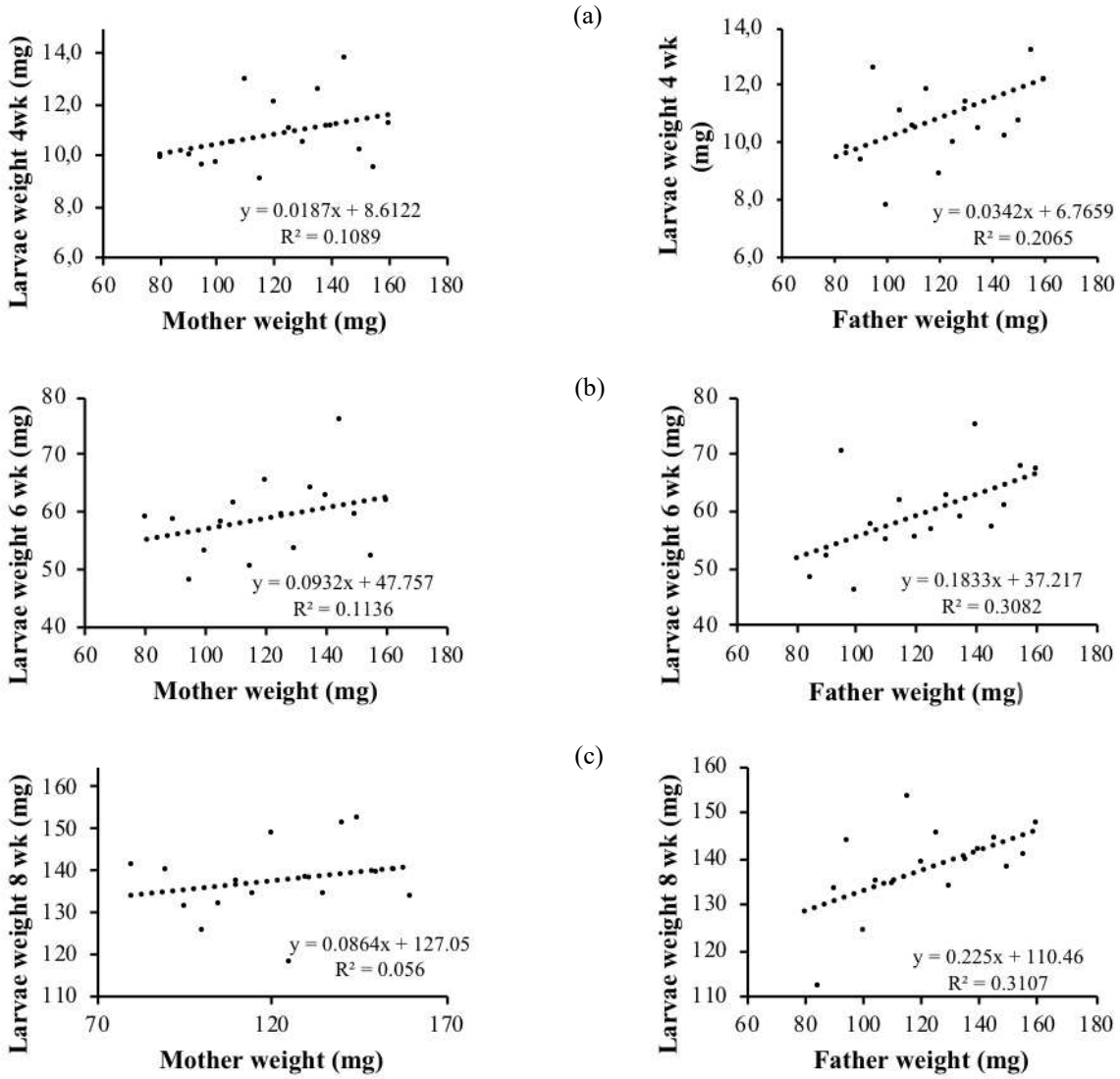

Figure 3. Linear regression of average weight of *Tenebrio molitor* larvae at 4, 6 and 8 weeks of age according to weight of parents (mother and father).
(a)= week 4; (b)= week 6; (c)= week 8; wk = weeks.

Regression analysis of the individual weights of beetles in the progeny, in relation to parental weight, revealed a low correlation between parental weight on progeny weight, both for maternal and paternal contributions (R^2 = 0.03 and 0.05, respectively; $P<0.0001$) (Figure 4). The heritability of beetle weight was estimated at approximately 12.8% from the mother and 13.2% from the father.

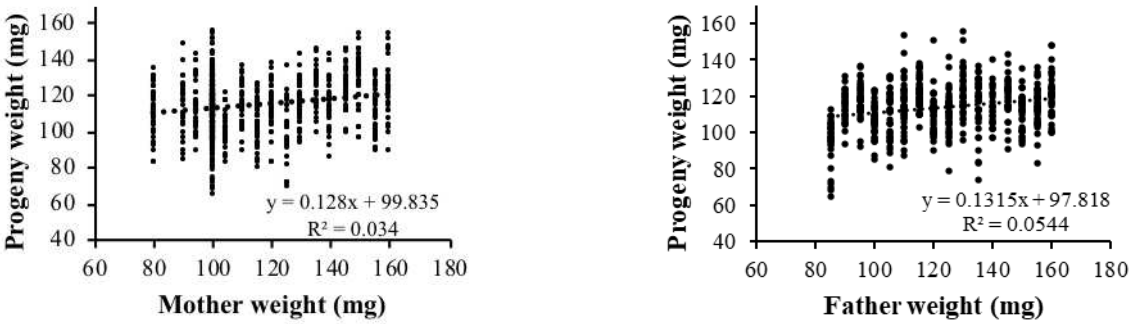

Figure 4. Linear regression of individual weight of *Tenebrio molitor* beetles (progeny) according to weight of parents (mother and father).
wk= weeks

Discussion

Optimising the reproduction of TM is a key strategy for enhancing farm productivity. In particular, genetic selection for traits such as beetle size could serve as an effective tool to optimize and boost reproductive output. In several animal species, indeed, body size significantly influences mating success, affecting both male and female reproductive strategies. For instance, in certain beetle species like *Carabus japonicus* (Coleoptera: *Crabidae*), males of intermediate size may have higher mating success because their genital morphology aligns better with female anatomy, facilitating more effective sperm transfer (Okuzaki, 2021). While larger males often hold an advantage, female body size can also play a pivotal role. In some species, larger females produce more eggs, thereby enhancing their reproductive success. Previous studies have also reported that TM females show a preference for larger males, attributed to their greater attractiveness and their ability to provide genetic or material benefits (Fedorka and Mousseau, 2002). These findings partially align with the results of Experiment 1a, where the 'primary chooser' significantly preferred larger female for the first approach in group 1. A similar pattern was observed also during the first mating for group 2 and during the second mating for group 1, suggesting the potential existence of a preference for mating with larger individuals. Despite these findings, Experiment 1b revealed no significant differences in mating choices, when testing the impact of weight differences between the selected beetles. Even with a more pronounced weight disparity, mating preferences remained unchanged. However, observations revealed that both large and small males generally tended to mate with larger females rather than smaller ones. In some instances, however, small males faced challenges in successfully mating with larger females, particularly when the size difference between the sexes was more pronounced. It is important to emphasize that these findings are observational and not conclusive, as they are based solely on the authors' qualitative assessments.

Regarding the interactions between two males (one large and one small) with a single female, as observed in group 3 and 4, observations revealed the presence of male-to-male competition, including fighting, to gain access to the female for mating, regardless of whether it was large or small. In different insects' species, such as *Cotesia Urabae* ((Hymenoptera: Braconidae), *Bactrocera tryoni* (Diptera: Tephritidae) and *Hermetia illucens*, larger males mate more successfully than smaller ones (Avila et al., 2017; Ekanayake et al., 2017;

Jones and Tomberlin, 2021). Despite the larger size, in the present study, only in group 3 the large male was the predominant during the first approach and the first mating with the female, whereas in group 4, the opposite pattern was observed during the second mating, where small male mated with the female significantly more often. This suggests that a larger male size does not necessarily correlate with greater reproductive success. Fighting between males is also observed in other insect species, such as *Lasiorhynchus barbicornis* (Coleoptera: Brentidae), where larger males typically win such contests. However, smaller males often employ alternative strategies, such as "sneaking" copulations, to achieve mating success. This indicates that while larger size offers advantages in direct competition, diverse mating tactics can also be effective (Tigreros and Lewis, 2011; Painting and Holwell, 2013). Additionally, once a male had mated, it required a recovery period before it could mate again. This explains why smaller males predominantly mated with the female during the second mating in Group 4. Indeed, although this result was not statistically significant, it is likely that during the first mating, the larger male was the first to mate with the female. After mating, the larger male would have required a recovery period, allowing the smaller male to mate next. Furthermore, this recovery time explains the longer latency before second matings observed in Groups 1 and 2 compared to Groups 3 and 4. In the latter groups, the presence of two males allowed them to alternate mating with the female, thereby reducing the time needed to initiate a second mating.

Considering results obtained from Experiment 1, the authors aimed to test whether larger size is also associated with higher prolificacy in TM beetles and to explore the potential correlation between the size of the parents and that of their progeny. In insects, larger individuals tend to have increased energy reserves, enabling them to produce more or larger eggs. Greater energy availability can also support prolonged reproductive periods and more frequent mating events. Additionally, larger insects are often more longer-lived than smaller ones, consequently having more opportunities to reproduce over time (Honěk, 1993; Tammaru and Haukioja, 1996). Despite these considerations, adult size had no effect on the number of larvae produced per crate as emerged from results of Experiment 2 and 3, which aligns with findings of Morales-Ramos et al. (2012). This contrasts with studies in black soldier flies, where a correlation between larger females and a higher number of eggs laid was observed. Specifically, Georgescu et al. (2020) reported that larger females produced significantly more eggs per clutch than smaller females (+ 24.9% of eggs). Similarly, Jones and Tomberlin (2021) observed a 50% increase in egg production by larger females of *Hermetia illucens*, and in the study of Dieng et al. (2016), larger female of *Aedes aegypti* (Diptera: Culicidae) produced 50% more eggs than smaller females. Georgescu et al. (2020) also found that larger *Hermetia illucens* females produced heavier egg clutches compared to smaller ones. Likewise, Morales-Ramos et al. (2012) reported that heavier TM beetles produced larvae with higher growth rates than those from lighter adults. Similarly, larger parents produced heavier offspring in *Spodoptera litura* (Lepidoptera: Noctuidae) (Xu et al., 2018), and the progeny of large parents had the highest adult weights in *Harmonia axyridis* (Coleoptera: Coccinellidae) (Michaud et al., 2020). This is consistent with the results of Experiment 2. Specifically, in the present study, beetles with an average weight of 101 to 120 mg and 121 to 140 mg produced heavier larvae when their average weight was evaluated at 3, 4, and 6 weeks of age. By 8 weeks,

larvae from the heaviest beetles were the largest, showing an average weight difference of 16 mg compared to larvae from smaller beetles. Although the weight of the eggs was not recorded, it is likely that larger beetles produced heavier eggs. Consequently, these heavier eggs likely contained more nutrients for the developing embryos, resulting in larger larvae whose higher weight was sustained until 8 weeks of age. As expected, the ADG of this group of larvae was also the highest, along with the total weight gain.

A possible source of biological variability in this study is the use of beetles from a farm-reared stock population, which is likely to maintain natural genetic diversity rather than being derived from a single inbred line. These factors should be taken into account when considering the reproducibility of the results in other farm contexts.

Previous findings suggest the existence of heritability for the size trait from parents to offspring. Specifically, in the present study, a heritability of 12.8% was observed from the mother and 13.2% from the father, in Experiment 3. Even though these values are modest, they suggest that selecting for this trait could be an effective approach to enhance larval weight and, in turn, boost reproductive performance. For example, sustained selection for greater pupal size in TM has successfully increased the average pupal weight. In a study of Morales-Ramos et al. (2022), the heritability for pupal weight was found to be 40.7%, demonstrating the potential for successfully increasing this trait through selective breeding. Likewise, in *Hermetia illucens*, selecting for larger individuals over 16 generations led to a measurable increase in larval weight (Facchini et al., 2022). Based on the results of Experiment 3, the father weight had a stronger influence on the average weight of larvae compared to the maternal weight, particularly in Experiment 3b both at 6 and 8 weeks of age of larvae. A similar pattern was observed in other species of insects such as *Schistocerca americana* (Orthoptera: Acrididae), where paternal size was positively correlated with the adult body size of the offspring while no effect of the maternal weight was detected (Kosal and Niedzlek-Feaver, 2007). This can be explained by evolutionary pressures favouring paternal contributions to growth-related traits. Additionally, epigenetic marks carried by sperm may influence offspring development, as paternal body size could correlate with epigenetic signals that enhance offspring growth and size (van Otterdijk and Michels, 2016).

Conclusions

In conclusion, the findings of this study represent a further step toward optimising TM reproduction. Results suggest that in TM breeding, selecting larger beetles may be a useful strategy to enhance the reproductive output of the farm. Larger beetles, indeed, appear to be more attractive than smaller ones, however, further research is needed to clearly establish this trend. Although no clear link was observed between beetle size and the number of offspring produced per crate, the results indicate that larger beetles produce larger larvae, with this higher weight being maintained until 8 weeks of age, ultimately increasing yield. Selecting larger beetles for successive generations could enhance larval weight, particularly by selecting larger fathers, as the findings of Experiment 3 suggest that paternal influence was the strongest. To be applied on an industrial scale, some practical operations should be enhanced to reduce the time and costs associated with selecting

beetles by size and sex. Further investigation is needed to evaluate the full potential of implementing these findings to optimise TM farming efficiency.

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Third contribution:

Effect of camelina and linseed cakes supplementation on performance, fatty acid profile, oxidative stability and sensory traits of *Tenebrio molitor* larvae

B. Palumbo[a], M. Cullere[a], E. Pontalti[a], A. Z. Volek[b], A. Dalle Zotte[a]

[a]Department of Animal Medicine, Production and Health, University of Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro, Padova, Italy

[b]*Department of Nutritional Physiology and Animal Product Quality, Institute of Animal Science, Přátelství 815, 104 00, Prague, Czech Republic*

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Introduction

Insects demonstrated to be a promising alternative and sustainable source of food and feed (Makkar et al., 2014; Kröncke et al., 2019; Trukhanova et al., 2022). Among edible insects, TM is one of the most extensively studied and farmed species in Europe. TM larvae are particularly rich in protein, accounting for about 50% of their DM, and provide most of the essential amino acids required in the human diet (Nowak et al., 2016). They also contain approximately 30% lipids, characterised by a relatively low proportion of SFA and a higher content of PUFA, notably oleic, linoleic, and α-linolenic acids (Ghosh et al., 2017; Kröncke et al., 2019; Trukhanova et al., 2022). Although TM can synthesise certain FAs *de novo*, such as linoleic and α-linolenic acids, long-chain PUFA including eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) must be obtained through the diet (Lawal et al., 2021). The feeding substrate influences not only growth performance, fecundity, and survival of TM larvae, but also their chemical composition (Morales-Ramos et al., 2010; Oonincx et al., 2015; Van Broekhoven et al., 2015; Liu et al., 2020). In particular, the FA profile of the diet has been shown to directly affect the FA composition of TM larvae. For example, an increase in n-3 and n-6 FA was reported in larvae fed brewer's spent grain, a PUFA-rich substrate (Melis et al., 2019), while a significant reduction in the n-6/n-3 ratio was observed in larvae reared on diets containing 10% chia seeds, rich in n-3 FA (Park et al., 2023). Furthermore, the inclusion of 25% olive pomace in the diet of TM larvae increased their protein and amino acid contents, while no effect was observed on the FA profile (Ruschioni et al., 2020). Improving the nutritive value of TM larvae represents an important step toward optimising TM production. In particular, enhancing the FA profile of larvae could increase their applicability in both animal and human nutrition. With regard to animal feeding, reducing the n-6/n-3 ratio of the diet to values ≤ 5:1 has been shown to enhance the immune response and performance of broilers, while simultaneously lowering the FCR and enriching meat with long-chain n-3 FA (Qi et al., 2010; Ibrahim et al., 2018). Similarly, in human nutrition, lowering the dietary n-6/n-3 ratio is considered beneficial, as higher ratios have been associated with an increased risk of cardiovascular diseases (Kim et al., 2007).

Within this context, oilseed cakes represent promising dietary supplements for insect rearing. Camelina (*Camelina sativa* L. Crantz) and linseed (*Linum usitatissimum* L.) cakes are by-products of the vegetable oil industry, and their use aligns with the principles of the circular economy by valorising waste streams while offering substrates of high nutritional quality. Camelina cake contains approximately 35% protein, a balanced profile of essential amino acids, and 10-22% residual oil particularly rich in omega-3 FA (Aziza et al., 2014; Singh et al., 2023). Similarly, linseed cake provides around 36% protein, 7-10% residual oil, and is a source of lignans, antioxidants, and minerals (Poreda, 2017).

Therefore, the aim of the present study was to evaluate the effect of including camelina and linseed cakes at two dietary levels (5% and 10%) on the growth performance, chemical composition, FA profile, antioxidant content, oxidative stability and sensory attributes of TM larvae.

Material and methods

Experimental design and conditions

The experiment was conducted at the INEF (Insect Novel Ecologic Food) insects farm (Piombino Dese, Treviso, Italy) from January to March 2025.

Five-weeks-old TM larvae were collected and separated from frass and residual feed by sieving. The average individual larval weight, determined by counting and weighing a subsample of 500 individuals, was 7.68 mg. Larvae were then randomly allocated to six dietary treatments: a diet routinely used at the INEF farm (STD); a control formulation based on wheat bran, corn gluten meal, corn meal, full-fat soybean, soybean meal, and calcium carbonate (CON); the same control diet supplemented with 5% camelina cake (CAM 5); with 10% camelina cake (CAM 10); with 5% linseed cake (LIN 5); and with 10% linseed cake (LIN 10). All diets, except the STD diet, were formulated to be isonitrogenous and isoenergy, and each treatment included 12 replicates. Diets were prepared at the MAPS Department of the University of Padova. The chemical composition of diets, camelina and linseed cakes is reported in Table 1, while the FA profile (% total FAME) of diets and oilseed cakes is presented in Table 2.

Table 1. Chemical composition (g/kg, as is) of camelina cake, linseed cake and diets, and gross energy content (MJ/kg as is) of diets administered to *Tenebrio molitor* larvae.

	Cakes		**Experimental diets**					
	Camelina sativa	***Linum usitatissimum***	**STD**	**CON**	**CAM 5**	**CAM 10**	**LIN 5**	**LIN 10**
Dry matter	892	943	875	885	882	884	883	890
Crude protein	319	208	171	177	176	175	178	181
Ether extract	216	345	27.8	62.0	64.1	74.7	69.3	84.8
Ash	43.5	41.8	62.1	53.0	52.1	48.7	51.1	48.3
Crude fibre	61.5	57.0	53.6	50.9	45.7	41.6	39.4	35.3
Starch	20.1	20.9	209	204	243	254	218	260
Gross energy	-	-	163	174	172	175	174	179

STD: diet commonly used by the farm; CON: standard diet; CAM5: standard diet supplemented with 5% of camelina cake; CAM 10: standard diet supplemented with 10% of camelina cake: LIN5: standard diet supplemented with 5% of linseed cake; LIN 10: standard diet supplemented with 10% of camelina cake.

Table 2. Fatty acids profile (% Total FAME) of experimental diets, camelina cake, and linseed cake administered to *Tenebrio molitor* larvae.

	Cakes		**Experimental diets**					
	Camelina sativa	***Linum usitatissimum***	**STD**	**CON**	**CAM 5**	**CAM 10**	**LIN 5**	**LIN 10**
C16:0	5.91	5.82	16.8	12.9	11.9	10.8	11.4	10.3
C18:0	2.45	3.90	1.39	3.11	2.99	3.07	3.48	3.79
C20:0	13.4	1.67	0.26	0.31	0.51	0.64	0.32	0.41
C22:0	1.99	0.30	0.16	0.30	0.31	0.35	0.32	0.25
C24:0	0.62	0.04	0.23	0.22	-	-	0.28	0.22
Total SFA	24.4	11.7	18.8	16.8	15.7	14.9	15.8	15.0
C16:1 n-9	0.15	0.09	0.16	-	0.22	0.18	0.16	0.18
C18:1 n-7	1.21	0.73	0.85	1.26	1.21	1.20	1.10	1.15
C18:1 n-9	19.3	18.5	18.7	24.2	23.8	24.3	24.2	24.0
C20:1 n-9	0.55	0.07	0.66	0.30	2.69	4.60	0.64	0.85
C22:1 n-9	0.04	-	0.20	0.16	0.34	0.67	-	-
Total MUFA	21.3	19.4	20.6	25.9	28.3	31.0	26.1	26.2
C18:2 n-6	13.6	21.4	55.0	50.9	44.3	38.9	43.5	38.6
C18:3 n-3	36.5	45.6	4.10	5.12	9.97	13.8	13.9	19.3
C20:2 n-6	-	-	-	-	0.23	0.35	-	-
C20:4 n-6	1.06	0.18	-	-	0.17	0.37	-	-
C20:5 n-3	0.23	0.15	-	-	-	-	-	-
C22:6 n-3	0.04	0.02	-	-	0.19	0.25	-	-
Total PUFA	51.4	67.4	59.1	56.0	54.9	53.7	57.4	57.9
Total n-6	14.7	21.6	55.0	50.9	44.7	39.6	43.5	38.6
Total n-3	36.8	45.8	4.10	5.12	10.16	14.1	13.9	19.3
n-6/n-3	0.40	0.47	13.4	9.94	4.40	2.81	3.13	2.00
Identified	97.1	98.5	98.5	98.7	95.9	99.6	99.3	99.1

STD: diet commonly used by the farm; CON: standard diet; CAM5: standard diet supplemented with 5% of camelina cake; CAM 10: standard diet supplemented with 10% of camelina cake: LIN5: standard diet supplemented with 5% of linseed cake; LIN 10: standard diet supplemented with 10% of camelina cake.

Breeding of larvae

Larvae were reared in plastic crates measuring 60 × 40 × 14.5 cm, with a surface area of 2400 cm². Each crate contained 10,000 larvae, corresponding to a density of 4.2 larvae/cm², as commonly applied at the INEF farm. Based on the average weight of five-weeks-old larvae, a total of 76.8 g of larvae was allocated to each crate. Crates were filled with ground feed corresponding to the assigned treatment to ensure *ad libitum* intake throughout the experiment. Larvae were reared for 4 weeks, until 9 weeks of age, when they reached commercial selling size. Fresh carrots, provided as a water source, were added three times per week, and consumption of both feed and carrots was recorded. Environmental conditions in the rearing facility were maintained at an average temperature of 28.0 ± 2.3 °C and a RH of 70.1 ± 10%. The photoperiod was kept

constant at 8 h light and 16 h dark. All the environmental parameters were continuously monitored and maintained automatically using the heating and cooling systems and a humidifier.

Productive performance of larvae

The total larval weight of larvae in each crate was recorded at 9 weeks of age, following the removal of residual feed and frass. Larval growth was calculated as the difference between the total weight at 9 weeks and that at 5 weeks of age. In addition, a subsample of 100 larvae per crate was weighed weekly to determine the average individual BW. Larval FI was estimated by analysing the starch content of both the diet and frass. This approach was used because it is otherwise not possible to separate the residual feed from the frass. Using a starch digestibility of 99% reported for *Hermetia illucens* (Guillaume et al., 2023), the amount of undigested starch in the frass was used to calculate the grams of residual feed. Feed and carrot intake, expressed on a DM basis, was measured at the crate level from week 5 to week 9, and the FCR was calculated accordingly.

Shelf life of larvae

A subsample of larvae from each crate at 9 weeks of age, within each experimental group, was microwave-dried using a Max Industrial Microwave 30B (Max Industrial Microwave, China) at 103 °C (12 kW) for 10 minutes. This drying method, routinely applied at the INEF insect farm, was employed to obtain standardised material for both sensory evaluation and shelf-life assessment. Shelf-life evaluation included the quantification of antioxidant compounds (β-carotene, α-tocopherol, δ-tocopherol, and γ-tocopherol) and the determination of peroxide value. Analyses were performed immediately after processing (T0) and after 3 months of storage at room temperature in plastic closed containers (T3).

Chemical analyses

At the beginning of the experiment, a representative sample of five-weeks-old larvae was collected to analyse proximate composition and FA profile (% of total FAME) (Table 3). At the end of the trial, a sample of larvae (500 g) from each crate was collected for further analyses. Larvae of both five and nine weeks of age collected for chemical analyses were freeze-dried.

Proximate composition of diets, cakes, and larvae was analysed according to AOAC (2000) methods to determine DM (method no. 934.01), crude protein (method no. 2001.11), and ash (method no. 942.05). Ether extract was determined after acid hydrolysis (Commission Directive 98/64/EC, 1998). Gross energy of diets and cakes was measured using an adiabatic bomb calorimeter (ISO 9831:1998). Chitin content of larvae was determined according to the method described by Zhang and Zhu (2006), with the modifications proposed by Woods et al. (2020). Starch content of diets and frass was analysed following the amyloglucosidase–α-amylase method (no. 996.11).

Lipid extraction for the assessment of the FA profile in diets, cakes and larvae diets was performed by modified accelerated solvent extraction. For diets and cakes, the extraction process was carried out using

petroleum ether as solvent, whereas for larvae the extraction was performed using a binary solvent mixture of chloroform/methanol at a 1:2 ratio, according to the method by Lee et al. (1995).

Table 3. Proximate composition (g/100 g DM) and fatty acid profile (% total FAME) of 5 weeks old *Tenebrio molitor* larvae.

Component	**5 weeks old larvae**
Proximate composition	
Water	75.5
Crude protein	17.7
Lipids	1.38
Ash	1.47
Chitin	1.99
Fatty acids	
C14:0	1.02
C16:0	13.7
C17:0	0.67
C18:0	7.66
C20:0	1.44
C23:0	0.37
C24:0	0.39
Total SFA	26.1
C16:1 n-9	0.71
C18:1 n-9	27.0
Total MUFA	27.7
C18:2 n-6	43.6
C18:3 n-6	0.60
C18:3 n-3	0.97
Total PUFA	45.2
Total n-6	44.2
Total n-3	0.97
n-6/n-3	45.6
Identified	99.0

The fat content of samples was determined gravimetrically after vacuum evaporation under nitrogen stream. Then, samples were trans-methylated using a methanolic solution of H_2SO_4 (4%) to determine FAME. An 8 ml of distilled water and 4 ml of *n*-heptane were added to the samples to obtain a biphasic separation. FAMEs were quantified by gas chromatography (Shimadzu GC17A). equipped with an Omegawax (Sigma-Aldrich Co. LLC., Saint Louis, USA) 250 column (30 m × 0.25 µm × 0.25 µm) and flame ionisation detector. Helium was used as the carrier gas at a constant flow of 0.8 ml/min. The injector and detector temperatures were 260 ◦C. Peaks were identified based on commercially available FAME mixtures (37-

Component FAME Mix; Supelco Inc., Bellefonte, PA, USA). Results were expressed as% of the total detected FAME.

The peroxide value of microwave dried larvae was assessed according to AOCS official method Cd 8–53 (AOCS, 2003). This analysis was performed both at the end of the experiment (**T0**) and after 3 months of storage of larvae (**T3**). The contents of ß-carotene, α-tocopherol, δ-tocopherol, and γ-tocopherol in diets, cakes and larvae were determined following the European standards (EN 12822, 2000; EN 12823-2, 2000) for high-performance liquid chromatography, equipped with a diode-array detector (VP series) (Shimadzu, Kyoto, Japan). levels of diets, camelina and linseed cakes are reported in Table 4.

Table 4. Content of α-tocopherol, δ-tocopherol, γ-tocopherol and β-carotene (mg/kg of larvae) in diets, camelina cake and linseed cake administered to *Tenebrio molitor* larvae.

	Cakes		**Experimental diets**					
	Camelina sativa	***Linum usitatissimum***	**STD**	**CON**	**CAM 5**	**CAM 10**	**LIN 5**	**LIN 10**
α-tocopherol	9.00	3.80	10.6	11.7	8.80	6.40	9.80	8.50
δ-tocopherol	13.0	5.44	0.31	9.05	8.26	9.63	10.8	12.3
γ-tocopherol	149	106	8.00	24.1	26.8	29.4	28.2	35.6
β-carotene	0.41	0.31	0.29	0.26	0.18	0.19	0.22	0.26

STD: diet commonly used by the farm; CON: standard diet; CAM5: standard diet supplemented with 5% of camelina cake; CAM 10: standard diet supplemented with 10% of camelina cake: LIN5: standard diet supplemented with 5% of linseed cake; LIN 10: standard diet supplemented with 10% of camelina cake.

Consumers sensory analysis of larvae

For the consumer sensory analysis, a total of 141 employees and students from the University of Padova (Italy) participated on a voluntary basis. Since a preference test was chosen, participants did not require any prior experience with the specific food matrix, or the sensory method used. Instead, each participant received a detailed guide explaining the correct procedure for conducting the evaluation. Each participant then evaluated five TM dried larvae samples, representing the different treatments (labelled A, B, C, D, E, F), for visual, olfactory, and overall acceptance. Each attribute was rated on a scale from 1 (extremely unacceptable) to 7 (extremely acceptable). The sensory evaluation did not include tasting the samples. TM larvae were subjected to microbiological analysis for the most common pathogens before the sensory test to ensure the safe of the product. However, consumers, were not allowed to touch the samples.

Panellists sensory analysis of larvae

For the sensory evaluation, six samples (one per treatment) of dried TM larvae were coded with random three-digit numbers. A total of 22 trained panellists participated in the test, which consisted of a descriptive sensory analysis. They underwent a 2-h pre-test training session to become familiar with the matrices and to select appropriate descriptors. In addition, a list of possible descriptors and off-flavours was prepared. Dried larvae used for the training session were obtained from insects reared on a conventional diet at the INEF farm; they were handled and dried in the same way as the samples used for the subsequent sensory analysis.

For the evaluation, panellists received a list of descriptors to be scored on continuous 15-cm scales, ranging from 1 (lowest intensity) to 10 (highest intensity). The selected descriptors included: colour intensity, size uniformity, general odour intensity, rancid odour intensity, fried odour intensity, peanut odour intensity, unctuosity of whole larvae, unctuosity of minced larvae, and friability. All the evaluations were performed in a room where the temperature was set at 22 °C. Panellists were instructed to sniff, observe, and touch the samples, but not to taste them.

Statistical analysis

Data of performance, chemical composition and FA profile of TM larvae were analysed by a one-way ANOVA using the GLM procedures of SAS 9.1.3 (SAS Institute, 2008), with diets treated as fixed effect. Data of peroxide value and antioxidant of TM dried larvae were analysed by a one-way ANOVA with diet and time as fixed effects.

Sensory analysis data were analysed using a mixed model ANOVA including diets as fixed effect and effect of consumers as repeated random effect. A mixed model (PROC MIXED) was used to detect any dietary influence on sensory analysis scores, therefore considering experimental diet and the panellists as fixed and random effects, respectively. Least squares means were obtained, and post-hoc pairwise comparisons were conducted using Bonferroni correction. Statistical significance was declared at $P < 0.05$.

Results

Productive performance of larvae

Results about larval performance are presented in Table 5. From week 6 to week 9, larvae from the CON, CAM5, CAM 10, LIN5, and LIN 10 groups exhibited higher individual body weights compared with those fed the STD diet (P<0.0001).

Table 5. Effect of camelina and linseed cake dietary inclusion on growth performance, feed consumption and FCR of *Tenebrio molitor* larvae.

	Experimental diets						**RSD**	***P-value***
	STD	**CON**	**CAM 5**	**CAM 10**	**LIN 5**	**LIN 10**		
N. of samples	12	12	12	12	12	12		
Individual weight of larvae (mg)								
6 weeks	11.7^{B}	14.2^{A}	14.6^{A}	14.7^{A}	14.2^{A}	14.6^{A}	1.26	<0.0001
7 weeks	26.7^{B}	32.9^{A}	33.7^{A}	33.7^{A}	32.7^{A}	34.6^{A}	2.54	<0.0001
8 weeks	54.1^{B}	62.4^{A}	63.6^{A}	64.3^{A}	62.4^{A}	62.0^{A}	3.39	0.0001
9 weeks	89.5^{B}	104^{A}	104^{A}	101^{A}	102^{A}	98.6^{A}	6.30	<0.0001
Total weight gain (week 5-9) (g/crate)	854^{B}	965^{A}	989^{A}	946^{A}	953^{A}	871^{B}	48.5	<0.0001
Feed intake (g/crate)	1706^{BC}	1612^{CD}	1811^{A}	1721^{AB}	1650^{BCD}	1592^{D}	80.8	<0.0001
FCR (per crate)	2.00^{A}	1.67^{C}	1.83^{B}	1.82^{B}	1.73^{BC}	1.83^{B}	0.10	<0.0001

STD: diet commonly used by the farm; CON: standard diet; CAM5: standard diet supplemented with 5% of camelina cake; CAM 10: standard diet supplemented with 10% of camelina cake: LIN5: standard diet supplemented with 5% of linseed cake; LIN 10: standard diet supplemented with 10% of camelina cake; $^{A-B}$ values within a row with different superscripts differ significantly for P<0.0001.

Consistently, total weight gain at the crate level was higher in these groups, with the exception of LIN 10, which showed a value similar to STD and lower than the other treatments (863 g vs. 963 g, on average; P<0.0001). FI was the highest in CAM 5 group, followed by CAM 10, STD, and LIN5, while LIN 10 showed the lowest intake (1811 g vs. 1721 g vs. 1706 g vs. 1650 g vs. 1592 g, respectively; P<0.0001). Regarding FCR, the STD group had the highest value, whereas the CON group achieved the lowest (2.00 vs. 1.67, respectively; P<0.0001). The remaining groups showed intermediate values.

Chemical composition and FA profile of larvae

The inclusion of camelina and linseed cakes in the diet of TM larvae significantly influenced their chemical composition (Table 6). An increase in lipid content was observed in all groups supplemented with oilseed cakes, with CAM 10 showing the highest value, followed by CAM5, LIN5, and LIN 10. The STD group exhibited the lowest lipid content (9.70 g/100 g; P<0.0001). As a result, STD larvae had a higher protein content compared with the supplemented groups (17.4 g/100 g vs. 16.9 g/100 g; P<0.0001) as well as a higher water content (66.1 g/100 g vs. 63.9 g/100 g; P<0.0001).

Table 6. Effect of camelina and linseed cake dietary inclusion on the proximate composition (g/100 g) of 9 weeks old *Tenebrio molitor* larvae.

	Experimental diets						**RSD**	***P-value***
	STD	**CON**	**CAM 5**	**CAM 10**	**LIN 5**	**LIN 10**		
N. of samples	12	12	12	12	12	12		
Water	66.1^{A}	64.6^{B}	63.9^{C}	63.0^{D}	64.2^{C}	63.9^{C}	0.29	<0.0001
Protein	17.4^{A}	17.0^{B}	17.0^{B}	16.8^{B}	16.8^{B}	16.9^{B}	0.23	<0.0001
Lipids	9.70^{D}	11.5^{C}	12.3^{B}	13.8^{A}	12.0^{B}	12.4^{B}	0.32	<0.0001
Ash	1.34	1.22	1.30	1.26	1.31	1.23	0.13	0.187
Chitin	1.86^{BC}	1.75^{BC}	2.01^{A}	1.89^{AB}	1.75^{BC}	1.72^{C}	0.12	<0.0001

STD: diet commonly used by the farm; CON: standard diet; CAM5: standard diet supplemented with 5% of camelina cake; CAM 10: standard diet supplemented with 10% of camelina cake: LIN5: standard diet supplemented with 5% of linseed cake; LIN 10: standard diet supplemented with 10% of camelina cake; $^{A-B}$ values within a row with different superscripts differ significantly for P<0.0001.

With respect to chitin, CAM 5 larvae showed the highest content, while the LIN 10 group the lowest (2.01 g/100 g vs. 1.72 g/100 g, respectively; P<0.0001). Diet had no significant effect on the ash content of the larvae.

Considering the FA percentage of total FAME, larvae from the CON group showed the highest proportion of SFA, whereas CAM 5 and CAM 10 exhibited the lowest values (27.5% vs. 23.5% on average; P<0.0001) (Table 7). Among MUFA, the CAM 10 group had the highest content of oleic acid, while LIN 5 showed the lowest (45.3% vs. 43.0%, respectively; P=0.0345), with the other groups displaying intermediate values. A similar trend was observed for total MUFA (47.5% vs. 44.8%, respectively; P=0.0102). Considering total PUFA, larvae fed diets supplemented with camelina or linseed cakes exhibited higher proportions, with CAM 10 and STD showing intermediate values and CON the lowest (29.0% vs. 27.0% vs. 23.6%, on average; P=0.0031). Among individual PUFA, the LIN 10 group showed the highest proportion of α-linolenic acid, followed in decreasing order by LIN 5, CAM 10, CAM 5, CON, and STD (5.28% to 1.10%;

P<0.0001). In contrast, linoleic acid was most abundant in STD larvae and lowest in CON, with the remaining groups showing intermediate values (26.2% vs. 22.4%, respectively; P=0.0117). As a result, the LIN 10 group exhibited a significantly lower n-6/n-3 ratio, whereas the STD group showed the highest (4.43 vs. 24.0, respectively; P<0.0001).

Table 7. Effect of camelina and linseed cake dietary inclusion on fatty acid profile (% Total FAME) of 9 weeks old *Tenebrio molitor* larvae.

	Experimental diets						RSD	*P-value*
	STD	**CON**	**CAM 5**	**CAM 10**	**LIN 5**	**LIN 10**		
N. of samples	12	12	12	12	12	12		
C12:0	0.30^{ab}	0.27^{b}	0.27^{b}	0.32^{a}	0.26^{b}	0.27^{b}	0.04	0.0154
C14:0	2.55^{A}	2.40^{AB}	2.34^{B}	2.47^{AB}	2.27^{B}	2.38^{AB}	0.16	0.0023
C15:0	0.12^{AB}	0.35^{A}	0.07^{B}	0.04^{B}	0.13^{AB}	0.13^{AB}	0.20	0.0049
C16:0	18.2^{A}	19.0^{A}	16.9^{B}	16.6^{B}	16.8^{B}	16.8^{B}	1.07	< 0.0001
C17:0	0.05	0.06	0.04	0.01	0.05	0.03	0.06	0.0017
C18:0	0.17^{A}	0.17^{A}	0.09^{B}	0.09^{B}	0.16^{AB}	0.14^{AB}	0.24	< 0.0001
C20:0	0.31^{B}	0.66^{A}	0.33^{B}	0.32^{B}	0.44^{AB}	0.53^{AB}	0.22	0.0005
Total SFA	26.0^{AB}	27.5^{A}	23.7^{C}	23.3^{C}	23.9^{BC}	24.2^{BC}	1.79	< 0.0001
C16:1 n-9	1.39^{A}	1.24^{C}	1.28^{BC}	1.34^{AB}	1.34^{AB}	1.36^{AB}	0.07	< 0.0001
C17:1 n-9	5.04	6.30	4.49	1.66	6.05	3.35	0.06	0.4253
C18:1 n-7	0.27^{C}	0.52^{A}	0.43^{B}	0.31^{C}	0.31^{C}	0.32^{C}	0.07	<0.0001
C18:1 n-9	44.1^{ab}	44.7^{ab}	44.3^{ab}	45.3^{a}	43.0^{b}	44.3^{ab}	1.48	0.0345
C20:1 n-9	0.19^{C}	0.07^{D}	0.39^{B}	0.57^{A}	0.11^{CD}	0.18^{C}	0.07	< 0.0001
Total MUFA	46.1^{AB}	46.6^{AB}	46.5^{AB}	47.5^{A}	44.8^{B}	46.2^{AB}	1.55	0.0102
C18:2 n-6	26.2^{a}	22.4^{b}	25.8^{ab}	22.9^{ab}	25.7^{ab}	23.1^{ab}	3.27	0.0117
C18:3 n-3	1.10^{D}	1.22^{D}	3.07^{C}	3.85^{B}	4.18^{B}	5.28^{A}	0.50	< 0.0001
Total PUFA	27.3^{AB}	23.6^{B}	28.8^{A}	26.7^{AB}	29.8^{A}	28.4^{A}	3.66	0.0031
n-6/n-3	24.0^{A}	20.7^{B}	8.39^{C}	5.95^{D}	6.16^{CD}	4.43^{D}	2.10	< 0.0001

STD: diet commonly used by the farm; CON: standard diet; CAM5: standard diet supplemented with 5% of camelina cake; CAM 10: standard diet supplemented with 10% of camelina cake: LIN5: standard diet supplemented with 5% of linseed cake; LIN 10: standard diet supplemented with 10% of camelina cake; $^{a-b}$ values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ values within a row with different superscripts differ significantly at P<0.0001.

Peroxide value and antioxidant levels of dried larvae

No significant differences in lipid oxidation were observed among the six experimental groups at T0 (Table 8). However, a significant effect emerged at T3: larvae from the LIN 10 group showed a higher peroxide value compared with the other groups (11.3 vs. 2.93 meq O_2/kg fat, respectively; P<0.0001). Moreover, only in the LIN 10 group, the peroxide value increased significantly over the 3-month storage period, while no changes were detected in the other groups (11.3 vs. 2.83 meq O_2/kg fat; P<0.0001). Regarding antioxidant levels of larvae at T0, the α-tocopherol content was the highest in CAM 10 (7.17 mg/kg) and the lowest in CON (3.79 mg/kg; P<0.0001) (Table 9).

Table 8. Effect of camelina and linseed cake dietary inclusion on peroxide value (meq O_2/kg fat) of dried *Tenebrio molitor* larvae evaluated at time 0 (T0) and after 3 months of storage (T3).

	Experimental diets						RSD	*P-value*
	STD	**CON**	**CAM 5**	**CAM 10**	**LIN 5**	**LIN 10**		
N. of samples	6	6	6	6	6	6		
Peroxide value								
T0	3.52	4.07	4.27	3.61	2.95	2.80	0.96	0.0791
T3	3.63^{B}	2.99^{B}	2.43^{B}	2.81^{B}	2.83^{B}	$11.3^{A,}$	1.53	< 0.0001
RSD	0.55	1.13	1.30	0.77	0.78	2.15		
P-value	0.724	0.1783	0.0596	0.1195	0.8029	< 0.0001		

STD: diet commonly used by the farm; CON: standard diet; CAM5: standard diet supplemented with 5% of camelina cake; CAM 10: standard diet supplemented with 10% of camelina cake: LIN5: standard diet supplemented with 5% of linseed cake; LIN 10: standard diet supplemented with 10% of camelina cake; $^{A-B}$ values within a row with different superscripts differ significantly at P<0.0001.

Table 9. Effect of camelina and linseed cake dietary inclusion on α-tocopherol, δ-tocopherol, γ-tocopherol and β-carotene (mg/kg of larvae) of dried *Tenebrio molitor* larvae evaluated at time 0 (T0) and after 3 months of storage (T3).

	Experimental diets						RSD	*P-value*
	STD	**CON**	**CAM 5**	**CAM 10**	**LIN 5**	**LIN 10**		
N. of samples	6	6	6	6	6	6		
α-tocopherol								
T0	6.32^{AB}	3.79^{D}	5.45^{BC}	7.17^{A}	4.63^{CD}	5.77^{B}	0.61	< 0.0001
T3	5.86^{AB}	4.84^{BC}	6.93^{A}	6.81^{AB}	4.03^{C}	5.11^{ABC}	0.97	0.0001
RSD	0.72	1.06	1.06	0.65	0.73	0.52		
P-value	0.2993	0.1645	0.0465	0.3593	0.1797	0.0541		
δ-tocopherol								
T0	0.23^{C}	4.27^{B}	4.13^{B}	5.27^{A}	4.30^{B}	5.44^{A}	0.47	< 0.0001
T3	0.16^{C}	3.03^{B}	3.70^{AB}	4.77^{A}	3.31^{AB}	4.85^{A}	0.91	< 0.0001
RSD	0.01	1.12	0.63	0.66	0.91	0.44		
P-value	< 0.0001	0.0838	0.265	0.222	0.0912	0.0441		
γ-tocopherol								
T0	2.11^{D}	4.27^{C}	7.21^{B}	11.1^{A}	6.03^{B}	9.82^{A}	0.79	< 0.0001
T3	1.46^{E}	3.54^{DE}	7.56^{BC}	10.6^{A}	5.67^{CD}	7.87^{B}	1.20	< 0.0001
RSD	0.16	0.77	1.34	1.50	0.93	0.73		
P-value	< 0.0001	0.1512	0.6527	0.5652	0.5153	0.0009		
β-carotene								
T0	1.40^{AB}	0.81^{C}	1.20^{B}	1.25^{B}	1.13^{BC}	1.65^{A}	0.20	< 0.0001
T3	1.23^{AB}	0.83^{BC}	1.13^{ABC}	1.24^{AB}	0.79^{C}	1.28^{A}	0.23	0.0011
RSD	0.32	0.14	0.19	0.20	0.19	0.19		
P-value	0.3949	0.8399	0.5376	0.9335	0.0097	0.0067		

STD: diet commonly used by the farm; CON: standard diet; CAM5: standard diet supplemented with 5% of camelina cake; CAM 10: standard diet supplemented with 10% of camelina cake: LIN5: standard diet supplemented with 5% of linseed cake; LIN 10: standard diet supplemented with 10% of camelina cake; $^{A-B}$ values within a row with different superscripts differ significantly at P<0.0001.

At T3, CAM 5 showed the highest value (6.81 mg/kg), whereas LIN 5 recorded the lowest (4.03 mg/kg). Overall, no significant differences were observed between T0 and T3 within each group.

For δ-tocopherol, all groups supplemented with oilseed cakes showed markedly higher levels than STD (0.23 mg/kg) at T0 (P<0.0001). The highest concentrations were observed in LIN 10 and CAM 10 (5.44 mg/kg and 5.27 mg/kg). A similar trend was found at T3, with LIN 10 and CAM 10 maintaining the highest levels and STD diet the lowest (4.81 mg/kg vs 0.16 mg/kg, on average, respectively; P<0.0001).

The amount of γ-tocopherol was strongly influenced by diet. At T0, STD showed the lowest content (2.11 mg/kg), while the highest levels were recorded in LIN 10 and CAM 10 (10.5 mg/kg, on average; P<0.0001). At T3, values decreased in all groups except CAM5, although CAM 10 still showed the highest level and STD the lowest (10.6 vs. 1.46 mg/kg, respectively; P<0.0001).

For β-carotene, STD and LIN 10 larvae displayed the highest initial contents (1.40 and 1.53 mg/kg, respectively), whereas CON showed the lowest (0.81 mg/kg; P<0.0001). At T3, concentrations remained stable, with LIN 10 exhibiting the highest value and LIN 5the lowest (1.28 vs. 0.79 mg/kg; P=0.0011).

Sensory evaluation of dried larvae

Results of the consumer sensory analysis are presented in Table 10. Larvae from the LIN 10 group received the highest score for visual acceptance, followed by CAM 10, whereas CON scored the lowest (5.05 and 4.89 vs. 4.10, respectively; P=0.0032). LIN 10 also achieved the highest score for olfactory acceptance, together with STD, while CON again received the lowest (5.67 vs. 3.76, on average; P<0.0001). The remaining groups showed intermediate values. A similar trend was observed for overall acceptance, with LIN 10 scoring the highest and CON the lowest (5.42 vs. 3.90, respectively; P<0.0001).

Table 10. Effect of camelina and linseed cake dietary inclusion on visual, olfactory and overall acceptance of dried *Tenebrio molitor* larvae.

	Experimental groups						**SE**	***P-value***
	STD	**CON**	**CAM5**	**CAM 10**	**LIN5**	**LIN 10**		
N. of consumers	141	141	141	141	141	141		
Visual acceptance	4.79^{BC}	4.10^{D}	$4.48^{C,}$	4.89^{AB}	4.69^{BC}	5.05^{A}	0.15	<0.0001
Olfactory acceptance	5.68^{A}	3.76^{E}	4.78^{D}	5.29^{BC}	5.10^{C}	5.65^{A}	0.14	<0.0001
Overall acceptance	5.29^{AB}	3.90^{E}	4.72^{D}	5.13^{BC}	4.93^{CD}	5.42^{A}	0.13	<0.0001

STD: diet commonly used by the farm; CON: standard diet; CAM5: standard diet supplemented with 5% of camelina cake; CAM 10: standard diet supplemented with 10% of camelina cake: LIN5: standard diet supplemented with 5% of linseed cake; LIN 10: standard diet supplemented with 10% of camelina cake; value from 1 to 7= 1: extremely unacceptable, 2: very unacceptable, 3: moderately unacceptable, 4: neither unacceptable or acceptable, 5: moderately acceptable, 6: very acceptable, 7: extremely acceptable; $^{A-B}$ values within a row with different superscripts differ significantly at P<0.0001.

Results of the panel sensory analysis are reported in Table 11. Colour intensity was highest in CAM 5(85.9) group, while STD larvae showed the lowest value (61.5; P=0.0032). No significant differences were detected in size uniformity among groups (P=0.8158).

Odour intensity was significantly affected by diet (P=0.0002): LIN 10 larvae received the highest score (97.8), whereas LIN 5 recorded the lowest (67.7). Rancid odour intensity did not differ significantly among

groups (P=0.2536). Conversely, fried odour intensity varied significantly, with CAM 5, CAM 10 and LIN 10 reporting the highest values and LIN 5 the lowest (57.2 vs 39.0, on average respectively; P=0.0028).
Peanut odour intensity was also influenced by diet (P=0.0001): LIN 10 and STD larvae obtained a higher score compared to the other groups (70.5 vs 48.6, on average, respectively; P=0.0001). Concerning unctuousness, whole larvae were judged less unctuous in STD group compared with oilseed cakes supplemented groups, while CON group showed an intermediate value (22.1 vs 38.9 vs 34.4, on average, respectively; P=0.0255). Also fragmented larvae showed significant differences (P=0.0383), with CAM 5 receiving the highest values, and STD the lowest (64.8 vs 44.4, respectively; P=0.038). Finally, friability was strongly affected by diet (P<0.0001). LIN 10 and STD groups obtained the highest scores (109 and 112 respectively), while LIN 5, CON and CAM 5 showed the lowest (55.0).

Table 11. Effect of camelina and linseed cake dietary inclusion on the sensory score of dried *Tenebrio molitor* larvae.

	Experimental diets						RSE	*P-value*
	STD	**CON**	**CAM 5**	**CAM 10**	**LIN 5**	**LIN 10**		
N. of panellists	22	22	22	22	22	22		
Sensory attributes								
Colour intensity	61.5^{C}	81.0^{AB}	85.9^{A}	72.2^{BC}	83.3^{AB}	78.4^{AB}	4.99	0.0032
Size uniformity	62.9	57.1	54.7	57.7	54.0	53.7	6.18	0.8158
Odour intensity	82.1^{B}	77.1^{BC}	88.2^{AB}	79.1^{BC}	67.7^{C}	97.8^{A}	5.94	0.0002
Rancid odour intensity	7.18	13.9	12.6	7.50	14.7	10.2	3.87	0.2536
Fried odour intensity	52.9^{AB}	41.3^{BC}	56.0^{A}	55.5^{A}	39.0^{C}	60.2^{A}	6.51	0.0028
Peanut odour intensity	68.0^{A}	49.0^{B}	49.0^{B}	48.1^{B}	48.4^{B}	72.9^{A}	7.68	0.0001
Unctuosity whole	22.1^{b}	34.4^{ab}	36.0^{a}	35.9^{a}	44.7^{a}	39.0^{a}	5.69	0.0255
Unctuosity fragmented	44.4^{c}	55.1^{abc}	64.8^{a}	51.9^{bc}	59.0^{ab}	56.0^{abc}	5.23	0.038
Friability	112^{A}	59.1^{C}	53.5^{C}	90.8^{B}	52.4^{C}	109^{A}	6.74	< 0.0001

STD: diet commonly used by the farm; CON: commercial diet; CAM5: commercial diet supplemented with 5% of camelina cake; CAM 10: commercial diet supplemented with 10% of camelina cake: LIN5: commercial diet supplemented with 5% of linseed cake; LIN 10: commercial diet supplemented with 10% of camelina cake; value from 1 to 7= 1: extremely unacceptable, 2: very unacceptable, 3: moderately unacceptable, 4: neither unacceptable or acceptable, 5: moderately acceptable, 6: very acceptable, 7: extremely acceptable; $^{a-b}$ Values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ Values within a row with different superscripts differ significantly at P<0.0001.

Discussion

Insect nutrition is increasingly recognised as a key factor influencing not only growth performance but also the nutritional and technological quality of edible species. In this study, the inclusion of camelina and linseed cakes in the diet of TM larvae significantly affected multiple parameters, ranging from productive performance to chemical composition and FA profile along with lipid oxidation and sensory traits of larvae.
Specifically, oilseed cake supplementation increased the individual BW of larvae compared with the STD diet. However, a similar effect was observed in the control group, as no significant differences were found between the control diet and the supplemented diets. Unlike the STD diet, the experimental and the control diets were formulated to be isonitrogenous and isoenergetic. Compared with the conventional diet used at the

INEF farm, they contained higher protein levels and, in particular, a greater fat content (Table 1). This, together with the higher gross energy content of these diets relative to the STD diet, likely contributed to the observed increase in larval BW. Consistent with previous findings, increasing dietary protein in TM larvae has been shown to reduce development time, enhance larval biomass production, and improve FCE (Morales-Ramos et al., 2010; Morales-Ramos et al., 2013; Rumbos et al., 2020; Kröncke et al., 2023). Similarly, an increased dietary lipid content has been linked to higher larval BW, as demonstrated by Copelotti et al. (2025) and Ruschioni et al. (2020). More in detail, supplementation of TM diet with sources of PUFAs has been shown to improve FCR, as reported by Melis et al. (2019), in line with the findings of the present study. Moreover, TM larvae appear to be more efficient when provided with diets containing lower levels of SFAs and a balanced ratio of protein to lipids (Mancini et al., 2019; Montalbán et al., 2022).

The inclusion of 10% linseed cake in the diet of TM larvae had a significant effect on the FI of larvae that resulted the lowest in LIN 10 group compared to the others and a lower weight gain at crate level. Although the diets were formulated to be isoprotein and isoenergy, the LIN 10 diet contained the highest ether extract content, mainly derived from α-linolenic acid. It is likely that larvae reduced FI in response to the higher dietary energy density, reflecting their ability to balance diet intake according to their nutritional needs (Morales-Ramos et al., 2011; Urrejola et al., 2011; Morales-Ramos et al., 2013). In addition, the presence of antinutritional compounds in linseed cake, such as cyanogenic glycosides, phytic acid and tannins (Sun et al., 2021; Talwar et al., 2025), could have further limited nutrient utilisation when included at a 10% level. The fact that LIN 5 performed better than LIN 10 suggests that an inclusion threshold exists, beyond which the negative effects of linseed cake outweigh its nutritional advantages. Nonetheless, also camelina cake is known to contain antinutritional factors such as glucosinolates, phytic acid, erucic acid, sinapine (Tan et al., 2011; Tangendjaja, 2015; Pojić et al. 2015). However, no effects of reduced FI or growth were observed on larvae fed diets including 5% and 10% of camelina cake. The different response observed may be related to the specific nature of their antinutritional compounds. The antinutritional factors present in linseed cake can interfere with nutrient absorption and, in the case of cyanogenic glycosides, even release toxic compounds (Tangendjaja, 2015; Teh and Bekhit, 2015). These factors, combined with the high content of α-linolenic acid, which is prone to peroxidation, may have contributed to the reduced FI and growth performance of LIN 10 larvae. In contrast, the antinutritional factors present in camelina cake may have remained below the threshold at which they could negatively affect FI or growth in TM larvae. Moreover, the lower proportion of HUFA in camelina compared to linseed-containing diets might have further mitigated adverse effects. This finding suggests that the tolerance of TM larvae to different oilseed cakes depends not only on the inclusion level but also on the specific type and relative abundance of antinutritional compounds, as well as the FA profile of the ingredient.

Supplementing the diet of TM with camelina and linseed cakes also promoted higher lipid deposition in larval tissues, at the expense of crude protein content, and improved the PUFA profile. Overall, the FA profile of larvae in the present study resembled the typical FA profile of TM, with palmitic, linoleic, and oleic acids being the most abundant, in line with previous findings (Bordiean et al., 2022; López-Gámez et

al., 2023; Kröncke et al., 2023). Nevertheless, the different diets influenced the relative proportions of specific FA. Indeed, dietary FA profile appears to play a key role in shaping the lipid profile of TM. Notably, TM possesses delta-12 desaturase activity and endogenous FA synthesis capabilities, which may further modulate its FA profile. They can synthesise linoleic and α-linolenic acids *de novo*; however, like other terrestrial insects, they are deficient in producing EPA and DHA. Furthermore, they are able to convert n-9 FA to n-6 FA (Brandstetter and Ruther, 2016). Therefore, the diet remains the main factor influencing the proportion of n-3 FA in this species.

In the present study, a significant reduction in the n-6/n-3 ratio of larvae was observed in the oilseed cake supplemented groups. Similarly, Melis et al. (2019) observed that including brewer spent grain, rich in PUFA, in the larvae diet increased their n-3 and n-6 levels. Consistently, Lawal et al. (2021) demonstrated that supplementing the diet of TM larvae linseed and chia seeds significantly increased the PUFA content and reduced the n-6/n-3 ratio. The incorporation of graded levels of linseed and camelina cakes into TM diets resulted in a proportional increase in the n-3, particularly α-linolenic acid, and a corresponding decrease in linoleic acid, demonstrating an effective strategy to modulate the FA profile and enhance the nutritional quality of insect biomass. Thus, the reduced SFA proportion and the n-6/n-3 ratio, is a favourable outcome for the potential use of mealworms as an alternative feed and food source. High n-6/n-3 ratios are generally associated with negative health effects, as they can promote inflammation, and increase the risk of cardiovascular diseases. For instance, a ratio >10 has been linked to adverse health outcomes (Simopoulos, 2008; Cao et al., 2024), while a favourable n-6/n-3 ratio is recommended to be around 3:1 to 1:1 (Kim et al., 2007). The n-6/n-3 ratio is also a critical factor in animal diets, as it influences growth performance, animal health and quality of the end product. Reducing this ratio, particularly to levels about 4:1 or 2.5:1, through the inclusion of linseed oil or fish oil, has been shown to improve the performance and immune response of broilers, enhancing their overall health and resulting in meat enriched with long-chain n-3 PUFA (Ibrahim et al., 2018). Similarly, decreasing the n-6/n-3 ratio from 30:1 to 5:1 has been shown to reduce the FCR in broilers (Qi et al., 2010). Furthermore, in Heigai pigs, a lower dietary n-6/n-3 ratio reduced triglyceride and cholesterol levels, while increasing the omega-3 FA content in the *longissimus dorsi* muscle (Nong et al., 2020).

Differences in oxidative stability and tocopherol levels further emphasise the role of dietary lipid sources in determining product quality during storage. In the present study, the peroxide value was used to assess the extent of lipid oxidation in dried larvae. The peroxide value is a widely recognised indicator of the degree of lipid oxidation, reflecting the concentration of primary oxidation products in the sample (Ratusz et al., 2018). This parameter was specifically measured to evaluate whether the inclusion of camelina and linseed cakes, both rich in highly unsaturated fatty acids (**HUFA**), could increase the susceptibility of TM larval lipids to oxidative degradation. No specific guidelines are currently available regarding the quality of insect fat; however, EFSA's scientific opinion on blanched and thermally dried TM larvae reports peroxide values in the range of 1.0 to 16.3 mEq O_2/kg. In the present study, the peroxide values of all groups fell within this range at both T0 and T3. Nevertheless, during storage, the peroxide value of the LIN 10 group increased

significantly compared with T0 and was the highest among all experimental groups. This suggests that, although the product remained stable in terms of lipid oxidation, larvae from this group exhibited higher susceptibility after three months of storage. Since the same drying method was applied to all samples, the increased susceptibility of the LIN 10 group can be attributed to the specific composition of the larvae, resulting from the inclusion of 10% linseed cake in the diet. Notably, this group exhibited the highest concentrations of δ-tocopherol (together with CAM 10) at both T0 and T3, the highest γ-tocopherol level (along with CAM 10) at T0, and the highest β-carotene content. This is consistent with the composition of linseed and camelina cakes, which are particularly rich in γ-tocopherol (Table 3). Nevertheless, the higher peroxide value observed in the LIN 10 group at T3, despite its elevated levels of tocopherols and carotenoids, can be explained by the FA profile of the larvae. Linseed cake contains high amounts of α-linolenic acid (45.6%; Table 2), extremely prone to oxidation. Its inclusion in the larval diet markedly increased the α-linolenic acid content of TM larvae (5.28%), a significantly higher value compared to the other groups. Consequently, the high availability of oxidisable substrates in the LIN 10 larvae likely overwhelmed the protective effect of antioxidants, resulting in the observed higher peroxide value. This suggests that the balance between lipid composition and antioxidant content is critical, and that elevated antioxidant levels do not necessarily guarantee oxidative stability when the degree of unsaturation is also high. Furthermore, it cannot be excluded that a portion of the tocopherols might have been modified or oxidised themselves while protecting against feed/food oxidation during storage, thereby reducing their effectiveness in counteracting oxidation.

Despite the limitation observed in the LIN 10 group, the sensory evaluation revealed favourable consumer perceptions. Results of the present study demonstrate that, even though the larvae were not tasted, consumers perceived clear differences in their appearance and olfactory attributes. The inclusion of oilseed cakes enriched the larvae in PUFAs which are likely to influence visual and olfactory traits (Attia et al., 2024; Hui et al., 2025). As a result, LIN 10 larvae obtained the highest overall acceptance scores in consumer tests. More specifically, they achieved the best score for visual acceptability, followed by the CAM 10 group. In terms of olfactory acceptability, however, the LIN 10 larvae shared the top score with the STD group. The sensory panel analysis further confirmed that dietary treatments affected the sensory traits of larvae. However, some findings from both consumers and the panel require further investigation, as certain patterns remain unclear. For example, differences emerged among the groups supplemented with oilseed cakes, despite all TM larvae were dried with the same method. LIN 10 larvae showed lower water activity (a_w) compared to CAM 5 (0.45 vs 0.69; P=0.016), while the other groups displayed intermediate values (average a_w = 0.58). Despite the drying process, residual moisture content in the larvae may have influenced their olfactory traits, potentially leading to the development of less appealing odours. Indeed, some microorganisms can survive even at low a_w values, promoting reactions responsible for odorous compound formation.

Other sensory attributes were also affected by diet. Colour intensity was enhanced in CAM 5 larvae, possibly due to the carotenoid and tocopherol content of camelina cake; however, CAM 10 larvae scored significantly

lower. Odour intensity reached its highest value in LIN 10 larvae, whereas LIN 5 obtained the lowest score. Finally, friability was greatest in LIN 10 and STD groups, suggesting that both high linseed inclusion and the absence of supplementation may affect larval texture. Interestingly, rancid odour did not differ among groups, indicating that despite higher peroxide value in LIN 10, potential off-flavours associated with lipid oxidation were not perceived. Conversely, fried odour intensity was more pronounced in larvae from oilseed cake diets, especially CAM5, CAM 10, and LIN 10, which may reflect differences in lipid composition and their role in Maillard- and lipid-derived volatiles during cooking. Peanut odour was also particularly high in LIN 10 and STD groups, which could be linked to the higher presence of linolenic acid in LIN 10 and specific lipid-derived volatiles naturally occurring in STD. In terms of texture, oilseed cake inclusion increased unctuousness of whole dried larvae, consistent with their higher lipid content. Some inconsistencies in these findings may reflect complex interaction between dietary components and larval metabolism, indicating that the relationship between feed composition and sensory traits is not linear. Additional studies are therefore needed to clarify these effects and to identify the underlying mechanisms.

Conclusions

Insect nutrition is increasingly recognised as a key factor for both growth performance and product quality, making dietary optimisation a strategic step toward the sustainable use of edible insects in food and feed. This study demonstrated that supplementing TM diet with camelina and linseed cakes modulates larval growth, chemical composition, FA profile, oxidative stability, and sensory traits. The incorporation of oilseed cakes in the larvae diet resulted in improved lipid deposition and an enhanced proportion of PUFA, leading to a significant reduction of the n-6/n-3 ratio, especially with a 10% inclusion level. This nutritional improvement, characterised by a more favourably FA profile, has positive implications for both animal and human diets. However, the inclusion level and type of oilseed cake proved critical: while camelina cake did not impair FI or growth, high levels of linseed cake (10%) negatively affected performance. Additionally, the inclusion of 10% linseed cake increased the susceptibility of dried larvae to lipid oxidation, despite their high content of antioxidants. Interestingly, despite the above-mentioned disadvantages, larvae from the LIN 10 group were highly appreciated by consumers for their visual and olfactory attributes, suggesting that nutritional enrichment may also enhance sensory appeal. These findings highlight the dual potential of oilseed cakes: as sustainable by-products that valorise agro-industrial streams and as functional ingredients capable of improving the nutritional and technological quality of insect biomass. Future research should address the balance between inclusion levels, antinutritional factors, and oxidative stability to fully exploit oilseed cakes in insect farming and to advance the use of mealworms as a high-quality and sustainable protein and lipid source for feed and food applications.

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Fourth contribution:

Effect of killing and drying methods on physicochemical traits, microbial count and sensory properties of *Tenebrio molitor* larvae

B. Palumbo[a]*, M. Cullere[a], Y. Singh[a], E. Pontalti[a], A. Bacci[b], B. Contiero[a], A. Dalle Zotte[a]

[a]Department of Animal Medicine, Production and Health, University of Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro, Padova, Italy

[b]FOOBE srl, Via Giuseppe Garibaldi 48/A, 33050 Ronchis, Udine, Italy

*Corresponding Author: Bianca Palumbo. E-mail: biancafederica.palumbo@phd.unipd.it

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Introduction

The use of insects as an alternative source of food and feed is emerging as a promising perspective. With the expected increase in the world population, the sole reliance on conventional raw materials will become unsustainable due to rising production costs and diminished environmental sustainability (United Nations, 2019). Thus, the need to find more sustainable and nutritious source of food and feed is increasingly urgent. The growing interest in insects come from their high nutritional value in terms of proteins, PUFA, vitamins and minerals (Van Huis et al., 2013; Rumpold et al., 2013), along with their higher sustainability compared to livestock in terms of land, water use and produced emissions (van Huis and Oonincx, 2017). In this context, TM is the first insect species approved by EFSA, for human consumption (Turck et al., 2021). Belonging to the Tenebrionidae family (Coleoptera), TM, though recognised as a food pest, is one of the most extensively produced and researched insect species in Europe (Krzyzaniak et al., 2022). TM larvae contain high levels of protein, ranging from 47% to 60% DM, with significant biological value. The essential amino acid profile of TM larvae makes them capable of meeting the human daily intake requirement for nearly all essential amino acids, except for lysine. Notably, TM larvae are rich in leucine, threonine, and valine (Janssen et al., 2017; Costa et al., 2020). The lipid content of TM larvae ranges from approximately 23% to 35% DM, and they contain a substantial amount of beneficial FA, including oleic, linoleic and palmitic acids (35, 22 and 21% of total FAME, respectively) (Paul et al., 2017; Kröncke et al., 2019; Trukhanova et al., 2022). In addition to proteins and lipids, TM larvae are a source of significant amounts of iron (Fe), calcium (Ca), potassium (K), magnesium (Mg), and B-group vitamins (Hong et al., 2020; Lenaerts et al., 2018). For consumption, TM larvae can be used whole, either raw or dried, or processed into flour through a drying and grinding process (Hong et al., 2020; Turck et al., 2021).

A critical aspect of the TM production chain is the possibility to optimise and maintain the quality of the final product. Pretreatments and drying are indispensable techniques in industrial insect production and processing, ensuring the safety and the product shelf-life by inhibiting or slowing down the microbial growth, the enzymatic activity, and browning reactions (Khalloufi et al., 2000; Vandeweyer et al., 2017). These techniques are capable of reducing the high water-activity (a_w) of fresh mealworms (approximately 0.99) below 0.20, blocking any microbial growth and enzymatic activity (Rahman, 2007; Yan et al., 2023). Indeed, edible insects typically harbour a high microbial load, predominantly comprising Gram-positive bacteria, and may also carry pathogenic microorganisms that pose a risk of causing disease in consumers (Belluco et al., 2013). The most diffused bacteria in edible insect are *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., *Proteus* spp., *Pseudomonas* spp., *Escherichia* spp., *Micrococcus* spp., *Lactobacillus* spp. and *Acetobacter* spp. (EFSA, 2015). Thus, maintaining adequate hygiene conditions during insect rearing and applying appropriate processing techniques are essential to ensure the safety of the end product.

Among the pretreatments, the killing method influences chemical and physical properties as well as the microbial load of insects (Larouche et al., 2019). Freezing and blanching are considered the most effective and humane killing methods (van Huis, 2019; El Hajj et al., 2022), since long time of killing could expose insects to stress and a consequent acceleration in oxidative processes (Singh et al., 2020) and cause a

prolonged suffering of the insect. Furthermore, blanching at temperature ranging from 70-150 °C can deactivate enzymes (Cacchiarelli et al., 2022) and affect the microbial loads (Fellows, 2009; Wang et al., 2017), while freezing only halts the microbial growth (Cardello, 1997).

Another aspect that strongly influences the quality of the final product is the drying process. High-temperature techniques can significantly reduce protein solubility due to protein denaturation (Bußler et al., 2016), as well as cause pronounced darkening and shrinkage due to browning reactions and tissue collapse (Purschke et al., 2018). Browning reactions can occur through two different processes: i) enzymatic browning; ii) non-enzymatic browning. The first one is attributed to the activity of polyphenol oxidase (**PPO**), a group of enzymes that catalyse the oxidation of phenolic compounds utilising oxygen and phenols as substrates (Whitaker and Lee, 1995). Non-enzymatic browning occurs due to the breakdown of carbohydrates during the caramelisation process or through a chemical reaction between amino acids and reducing sugars, known as the Maillard reaction. Both processes are promoted by prolonged exposure to high temperatures (Pathare et al., 2013). Browning compromises the visual acceptability for consumers, deteriorates the flavour, reduces protein digestibility and nutritional quality, and affects techno-functional properties (Kumar et al., 2013).

Among the drying techniques, freeze-drying is one of the recommended industrial scale drying processes for mealworms. Indeed, this process, ensures product safety together with high quality by employing low temperatures and removing oxygen, thereby preserving texture, nutritive value, aroma, and colour (EFSA, 2015). However, this process requires high production costs, substantial energy demand and long operation time leading to the necessity to find more sustainable and cheaper drying methods (Huang & Zhang, 2012). Another commonly applied procedure is the conventional oven drying. However, utilising temperatures ranging from 40-80 °C over extended periods is associated with pronounced darkening and shrinkage of larvae (Purschke et al., 2018; Trukhanova et al., 2022). Therefore, several authors have investigated the use of microwave drying for TM larvae (Vandeweyer et al., 2017; Lenaerts et al., 2018; Kröncke et al., 2018; Baek et al., 2019; Trukhanova et al., 2022). Microwaves employ electromagnetic waves to heat the material, expelling moisture from the food in a short operating time, thus ensuring better product quality in terms of colour, aroma, and microbial stability compared to conventional hot air drying (Vadivambal and Jayas, 2007). Nonetheless, the application of high temperatures during microwave drying could lead to the degradation of vitamins (Baek et al., 2019) and proteins (Kröncke et al., 2018).

Several killing and drying techniques with different combination of temperatures and times have been tested by different authors in order to find the best drying method in term of nutritional quality of the end product. Despite that, an optimal process has not been identified yet. The aim of this study was to compare the use of two killing and drying methods, commonly proposed as an alternative to freeze drying, and an innovative method characterised by the application of low temperatures during the killing and the drying phases.

Importantly, this study provides novel insights into sustainable and efficient large-scale processing techniques for TM larvae, therefore contributing to the advancement of insect-based food production.

Specifically, the research evaluates the effects on the physicochemical properties, microbiological safety, and sensory quality of dried TM larvae.

Material and methods

Experimental design and conditions

The experiment was conducted at the INEF (Insect Novel Ecologic Food) insect farm, (Piombino Dese, Treviso, Italy) and at MAPS Department of the University of Padova (Legnaro, Padova, Italy).
TM larvae from the same production batch were selected and fed *ad libitum* with a ground feed made of wheat bran, soybean cake, corn meal, and with carrots as water source. Temperature and RH were maintained at 28 °C and 55-60%, respectively, throughout the growing period. At 9 weeks of age larvae were harvested, separated from the residual feed and frass using a sieve and fasted for 24 hours to ensure the completely intestinal emptying. After this time, larvae were divided in four experimental groups according to a completely randomised design which involved the combination between different killing methods and drying treatments. Larvae were divided in four treatment groups: i) BO: blanching and oven drying; ii) BM: blanching and microwave drying; iii) FM: freezing and microwave drying; iv) CA: cryogenic freezing and drying in controlled atmosphere. For each group, n=8 replicates were performed in independent experiments conducted on different days (for a total of 4 days) resulting in a total of of 32 experimental units.

Blanching and oven drying

A total of 5.6 kg of TM larvae were divided into eight aliquots of 700 grams each, weighed, and blanched by placing them into 5 litres of boiling water (100 °C) for 180 seconds. Blanching parameters were established by modifying those reported in the literature (Lenaerts et al., 2018; Purscke et al., 2018; Baek et al., 2019; Trukhanova et al., 2022). After blanching, the larvae were transferred into water at 20 °C for 120 seconds to decrease the temperature of the samples. They were then drained, weighed, placed on aluminium trays, and inserted into an oven set at 60 °C for 73 hours. Once dried, the larvae were kept at room temperature for 24 hours and weighed again.

Blanching and microwave drying

A total of 5.6 kg of TM larvae were divided into eight aliquots of 700 grams each, weighed, and blanched as described above. Microwave drying was performed in a Max Industrial Microwave 30B with 10 microwave sources of 3kW (Max Industrial Microwave, China) at 103 °C (24 kW) for 17 minutes. Once dried, larvae were maintained at room temperature for 24 hours and weighed.

Freezing and microwave drying

A total of 5.6 kg of TM larvae were divided into eight aliquots of 700 grams each and killed by freezing. Freezing was performed in a shock freezer (Challenge 250 TC, Angelantoni, Perugia, Italy) at -60 °C for 1

hour. After freezing, the larvae were dried in microwave at 103 °C (24 kW) for 15 minutes. Once dried, larvae were maintained at room temperature for 24 hours and weighed again.

Cryogenic freezing and drying in controlled atmosphere

A total of 5.6 kg of TM larvae were divided into eight aliquots of 700 grams, weighed, and killed using cryogenic freezing at -90 °C for 12 minutes. After this, the larvae were dried at 54 °C for 48 hours in a controlled atmosphere of 100% nitrogen, with a complete exchange of atmosphere every 14 minutes. Both cryogenic freezing and drying were performed using an industrial prototype with undisclosed characteristics. Once dried, the larvae were packaged in a controlled atmosphere containing nitrogen and 30% carbon dioxide. They were then maintained at room temperature for 24 hours and weighed again.

Physical analyses: colour evaluation, water activity and pH

Colour measurement was performed on dried and ground larvae obtained from each of the four treatments resulting in a total of 32 samples. The larvae colour was defined by the CIE L*a*b* colour space (International Commission on Illumination, 2004) using a colorimeter (CM 600 d, Konica Minolta, Milano, Italy), with measurements taken in duplicate. Differences in larvae colour among treatments were assessed using four parameters: lightness/darkness (**L***), redness/greenness (**a***), yellowness/blueness (**b***) and Chroma (Equation 1) (Maskan, 2001).

$$Chroma = (a^{*2} + b^{*2})^{\frac{1}{2}} \qquad (1)$$

The a_w of larvae was measured with a AquaLab cx-2 instrument (Meter group, München, Germany). The pH was assessed using a digital pH-meter (Orion Star A214, Thermo Fisher Scientific, Milan, Italy).

Chemical analyses: proximate composition gross energy content, glucose and fructose contents, FA, amino acid and mineral profiles, and lipid oxidative status

Proximate composition of dried larvae was performed according to the AOAC (2000) methods to determine DM (method no. 934.01), crude protein (method no. 2001.11; protein content was calculated as N x 4,76, as proposed by Janssen et al. (2017)), and ash (method no. 942.05). The ether extract was determined after acid hydrolysis (Commission Directive 98/64/EC, 1998). Gross energy was measured with an adiabatic bomb calorimeter (ISO 9831:1998). Glucose and fructose contents were determined after extraction with aqueous solution and determination in HPLC (Shimadzu SCL-10A VP) – RID (Shimadzu RID-10A) – Column Aminex 300 mm*7,8 mm HPX-87C Ion Exclusion Column – Water Flow 0,6 ml/min – Oven 75 °C. Chitin content was determined for adults and larvae according to the method described by Zhang and Zhu (2006), with the modifications provided by Woods et al. (2020). Lipid extraction to determine the FA profile was performed by modified accelerated solvent extraction (M-ASE). Extraction was carried out using chloroform/methanol at a 1:2 ratio as solvent, according to the method of Lee et al. (1995). The fat content of samples was determined gravimetrically after vacuum evaporation under nitrogen stream. Then samples

were trans-methylated using a methanolic solution of H_2SO_4 (4%) to determine FAMEs. 8 mL of distilled water and 4 mL of *n*-heptane were added to the samples to obtain a biphasic separation. These quantities were obtained by making a proportion as the content of extracted lipid was high. FAMEs were quantified via gas chromatography (Shimadzu GC17A). Results were expressed as% of the total detected FAMEs as described by Cullere et al. (2018), and the total lipid content of samples were used for quantitative determination (mg/100g larvae) of FA.

An aliquot of 2.0 g of TM larvae meal was used to analysed lipid oxidation through the evaluation of the thiobarbituric acid-reactive substances (**TBARs**). The absorbance of samples was read at 532 nm using a spectrophotometer (Hitachi U-200, Hitachi, Mannheim, Germany). The TBARs values were calculated from a standard calibration curve of 1,1,3,3-tetraethoxypropane, and the values were expressed as mg of malondialdehyde (**MDA**)/kg dried insect (Botsoglou et al., 1994). TBARs analysis was performed after drying processes.

The mineral profile (macro and trace elements) and heavy metals content of TM larvae was evaluated through an inductively coupled plasma optical emission spectrometry (**ICP-OES**) performed with Spectro Arcos (SPECTRO Analytical Instruments, GmbH, Kleve, Germany) after microwave digestion with Milestone rotor at 64-bar pressure, according to AOAC (2019). Data were expressed as mg/kg as is.

The amino acid composition of TM larvae was determined after acid hydrolysis and pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), separated by RP-HPLC and analysed by UV detection (Agilent 1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) following an adapted method from European Pharmacopoeia (Council of Europe, 2005). The protein content of the samples was hydrolysed with 6M HCl at 105 °C for 24h. Cysteine was determined as the sum of cysteine and cysteine, after reaction with 3,3'-dithiodipropionic acid, producing a mixed disulfide, which then underwent acid hydrolysis accordingly. After hydrolysis, the samples were neutralised with (8M) NaOH, adjusted to volume, and filtered at 0.45 μm. Then, the derivatisation step was conducted according to the manufacturer's instructions (AccQTag Ultra Derivatisation Kit; Waters, Milford, MA).

Microbiological analysis

Microbiological safety of dried larvae was evaluated according to ISO methods to determine the total number of mesophilic bacteria at 30 °C (ISO 4833-1:2013 Amd 1:2022), sulphite reducers bacteria (ISO 15213-1:2003), Enterobacteriaceae (ISO 21528-2:2017), positive coagulated staphylococcal bacteria (ISO 6888-2:2021), *Clostridium perfringens* (ISO 15213-1:2003), *Escherichia coli* (ISO 16649-2:2001), and the presence of *Bacillus cereus* (***B. cereus***) (presumptive) at 30 °C (ISO 7932:2004). The total number of yeasts and moulds was determined according to the method PDP BAT 016 2022 REV. 13. The presence of *Salmonella* spp. and *Listeria monocytogenes* was determined using a qualitative real time PCR (AFNOR BRD 07/06-07/04) and a qualitative real time PCR cultivation method (AFNOR BRD 07/10 - 04/05), respectively.

Consumer sensory analysis

For the consumer sensory analysis, a total of 201 students and staff members from the University of Padova were involved on a voluntary basis. Since the chosen sensory analysis was a preference test, consumers did not require any prior experience or training on the specific food matrix as well as the sensory technique adopted. Instead, every consumer received an individual guide where the correct procedure to conduct the sensory evaluation was explained. Before conducting the sensory evaluation, participants also had to complete a preliminary questionnaire, providing information about their gender, age, familiarity with consuming insects, and the acceptability of insects as feed or food. Subsequently, each consumer had to evaluate a total of n=4 TM larvae samples, representing the different treatments (labelled with letters A, B, C, D), in randomised order for visual, olfactory, and overall acceptance. Each attribute was rated by selecting a number ranging from 1 (extremely unacceptable) to 7 (extremely acceptable). The sensory evaluation did not involve tasting the product.

Statistical analysis

All experimental data of the present study, except those obtained from the sensory analysis, were subjected to a one-way ANOVA using the GLM procedures of SAS 9.1.3 (SAS Institute, 2008), with the combination of killing and drying method as a fixed effect and insect batch as a random effect. Microbiological data were transformed to $\log_{10}$ unit to ensure a normal distribution. For sensory analysis data, a mixed model (PROC MIXED) was performed to detect any influence of personal traits of the consumers (gender, age, confidence in consuming insects and admissibility in human and animal diet) on sensory analysis scores. Larval killing and drying method, and personal traits were used as fixed effects and the consumers as random effects. Least squares means were obtained, and post-hoc pairwise comparisons were conducted using Bonferroni correction. Statistical significance was declared at $P < 0.05$, with highly significant effects reported at $P < 0.0001$ where relevant.

Results

Physical characteristics

The killing and drying methods had a strong influence on the visual aspect (Figure 1) and other physical traits of dried larvae (Table 1). The group CA had the highest a_w due to an unsuccessful drying process, while group BO had the lowest (0.56 *vs* 0.30; P<0.0001). The pH of larvae belonging to group BO, BM and FM ranged between neutral values differently from group CA, characterised by a lower pH (7.14 *vs* 6.25, on average; P<0.0001). Regarding colorimetric characteristics, a significant difference in colour between larvae in group CA and the other groups was observed, with group CA showing higher L* (45.0 *vs* 32.5, on average; P<0.0001). Index of b* varied among treatments, decreasing from group CA to group BO (10.4 *vs* 4.56; P<0.0001). The same trend was also observed for Chroma index (11.8 *vs* 5.44; P<0.0001). No differences were observed in terms of a* between group CA and FM while this value was significantly lower

in both group BM and BO, with the latter showing the lowest value (5.20 *vs* 2.96; P<0.0001, on average, respectively).

Figure 1. TM larvae slaughtered and dried according to different methods: (a) BO: blanching and oven drying; (b) BM: blanching and microwave drying; (c) FM: freezing and microwave drying; (d) CA: cryogenic freezing and drying in controlled atmosphere.

The oxidative status of dried larvae varied between the four groups (Table 1). The TBARs value was the highest in group FM and the lowest in group BO (0.82 *vs* 0.32 MDA/kg; P<0.05), while group BM and CA exhibited intermediate values.

Table 1. Effect of killing and drying methods on water activity (a_w), pH, colour (L*, a*, b*), Chroma and oxidative status (TBARs: mg MDA/kg dried larvae) of TM larvae.

	Experimental groups				RSD	*P-value*
	BO	**BM**	**FM**	**CA**		
N. of crates	8	8	8	8		
a_w	0.30^{C}	0.35^{BC}	0.41^{B}	0.56^{A}	0.05	<0.0001
pH	7.18^{A}	7.14^{A}	7.09^{A}	6.25^{B}	0.07	<0.0001
Lightness, L*	30.9^{Bb}	32.6^{Bab}	34.0^{Ba}	45.0^{A}	1.84	<0.0001
Redness, a*	2.96^{C}	4.30^{B}	4.95^{Ab}	5.45^{Aa}	0.31	<0.0001
Yellowness, b*	4.56^{D}	6.21^{C}	7.54^{B}	10.4^{A}	0.75	<0.0001
Chroma	5.44^{D}	7.55^{C}	9.02^{B}	11.8^{A}	0.77	<0.0001
TBARs[1]	0.32^{b}	0.55^{ab}	0.82^{a}	0.64^{ab}	0.22	0.0188

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; [1]TBARs: thiobarbituric acid-reactive substances, MDA: malondialdehyde.; $^{a-b}$ Values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ Values within a row with different superscripts differ significantly at for P<0.0001.

Chemical composition

Chemical composition of TM larvae was significantly influenced by the different killing and drying methods (Table 2). Group CA exhibited higher water content compared to the other groups (8.20 *vs* 4.03 g/100 g, on average; P<0.0001). Conversely, group CA had a lower protein content compared to the others (34.6 *vs* 36.5 g/100 g, on average; P<0.0001). This trend was also observed for lipids (31.7 *vs* 34.03 g/100 g, on average;

P<0.0001) and gross energy (25.6 *vs* 27.23 MJ/Kg, on average; P<0.0001). The ash content was higher in group BO and FM compared to group CA (3.36 on average *vs* 3.28 g/100 g; P<0.05). Chitin content reached the highest value in group BM and the lowest in group BO (5.65 *vs* 4.85; P<0.0001). Among sugars, group CA exhibited the highest content compared to the other groups, both for glucose (2.82 *vs* 0.58 g/100g, on average; P<0.0001) and fructose (0.23 *vs* 0.03 g/100 g, on average; P<0.0001).

Table 2. Effect of slaughtering and drying methods on proximate composition, chitin, glucose, fructose content (g/100 g as is) and gross energy (MJ/kg) of TM larvae.

	Experimental groups				**RSD**	***P-value***
	BO	**BM**	**FM**	**CA**		
N. of crates	8	8	8	8		
Water	3.93^{B}	3.66^{B}	4.51^{B}	8.20^{A}	1.13	<0.0001
Protein[2]	36.2^{A}	36.6^{A}	36.7^{A}	34.6^{B}	0.46	<0.0001
Lipids	34.4^{A}	34.2^{A}	33.5^{A}	31.7^{B}	0.91	<0.0001
Ash	3.36^{a}	3.33^{ab}	3.36^{a}	3.28^{b}	0.06	0.0266
Chitin	4.85^{B}	5.65^{Aa}	5.19^{Aab}	5.04^{Ab}	0.36	0.0010
Glucose	0.77^{Ba}	0.49^{Bb}	0.49^{Bb}	2.82^{A}	0.17	<0.0001
Fructose	0.01^{B}	0.03^{B}	0.02^{B}	0.23^{A}	0.03	<0.0001
Gross energy	27.4^{A}	27.3^{A}	27.0^{A}	25.6^{B}	0.46	<0.0001

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; [2]N x 4.76; $^{a-b}$ Values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ Values within a row with different superscripts differ significantly at P<0.01.

Mineral profile

Results regarding the mineral profile of dried larvae are presented in Table 3.

Table 3. Effect of killing and drying methods on minerals compositions (mg/kg as is) of TM larvae.

	Experimental groups				**RSD**	***P-value***
	BO	**BM**	**FM**	**CA**		
N. of crates	8	8	8	8		
<u>Macroelements</u>						
K	8605^{A}	8544^{A}	8637^{A}	8156^{B}	134	<0.0001
P	7743^{Aa}	7728^{Aa}	7521^{Ab}	7196^{B}	135	<0.0001
S	3670^{A}	3659^{A}	3622^{A}	3338^{B}	77.2	<0.0001
Mg	1877^{Aa}	1883^{A}	1822^{ABb}	1764^{Bc}	37.0	<0.0001
Na	1429^{A}	1415^{A}	1347^{B}	1269^{C}	29.2	<0.0001
Ca	405^{A}	405^{A}	395^{A}	368^{B}	11.3	<0.0001
<u>Trace elements</u>						
Zn	133^{A}	133^{A}	131^{A}	121^{B}	3.21	<0.0001
Fe	50.8	52.4	46.5	44.5	6.52	0.0796
Cu	22.4^{A}	22.4^{A}	22.1^{ABa}	20.8^{Bb}	0.87	0.0024
Mn	12.6^{A}	12.4^{A}	12.2^{AB}	11.5^{B}	0.52	0.0019

Table 3. Continue.

	Experimental groups				RSD	*P-value*
	BO	**BM**	**FM**	**CA**		
Al	9.24	3.07	5.30	3.90	6.68	0.2803
Sr	3.02^{A}	3.00^{A}	2.99^{A}	2.75^{B}	0.16	0.0071
Ba	1.66	1.64	1.79	1.42	0.36	0.2441
Mo	1.45	1.40	1.37	1.36	0.16	0.6533
Ni	0.93	0.93	0.87	0.90	0.10	0.5751
Cr	0.46	0.35	0.27	0.24	0.23	0.2459
Ti	0.20	0.32	0.68	0.09	0.55	0.1721
Heavy metals						
As	<0.70	<0.70	<0.70	<0.70		
Pb	<0.70	<0.70	<0.70	<0.70		
Se	<0.70	<0.70	<0.70	<0.70		
Hg	<0.05	<0.05	<0.05	<0.05		
Cd	<0.03	<0.03	<0.03	<0.03		

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; $^{a-b}$ Values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ Values within a row with different superscripts differ significantly at P<0.01.

The most abundant among the macroelements was the K, particularly in groups BO, BM, and FM, which exhibited a higher content compared to group CA (8595 *vs* 8156 mg/kg, on average; P<0.0001). This trend was similarly observed for phosphorus (P) (7664 *vs* 7196 mg/kg, on average; P<0.0001), sulphur (S) (8536 *vs* 3338 mg/kg, on average; P<0.0001), and Ca (402 *vs* 368 mg/kg, on average; P<0.0001). The sodium (Na) content varied among treatments, with the highest content observed in groups BO and BM and the lowest in group CA (1422 *vs* 1269 mg/kg, on average; P<0.0001), as well as for Mg (1180 *vs* 1764 mg/kg, on average; P<0.0001), while group FM exhibited intermediate values. Among trace elements, zinc (Zn) and strontium (Sr) content were lower in group CA compared to the others (132 *vs* 121 mg/kg and 3.00 *vs* 2.75 mg/kg, on average, respectively; P<0.0001). Additionally, groups BO and BM displayed the highest values of copper (Cu) and manganese (Mn) compared to group CA (22.3 *vs* 20.8 mg/kg and 12.4 *vs* 11.5 mg/kg, on average, respectively; P<0.0001), whereas intermediate values were observed for group FM. Results shows that no heavy metals were detected in the dried larvae and no differences between the treatments were observed.

FA profile

The different killing and drying methods partially affected the FA profile (% total FAME, Table 4) of TM larvae. Among the SFAs, significant differences were observed in the content of stearic acid (C18:0) and arachidonic acid (C20:0), with the highest values associated to the FM group and the lowest to the CA group (3.66% *vs* 3.22%; P<0.0001; 0.31% *vs* 0.26%; P<0.05, respectively). Conversely, the content of oleic acid (C18:1 n-9) was higher in group CA compared to the others (41.9% *vs* 39.7%, on average; P<0.0001), leading to a similar trend in the total amount of MUFAs (43.7% *vs* 41.4%, on average; P<0.0001). The total

amount of PUFAs was higher in group BM and FM, while the lowest content was observed in group CA (31.8% *vs* 30.7%, on average; P<0.0001). Accordingly, the content of linoleic acid (C18:2 n-6) followed the same trend (30.5% *vs* 29.5%; P<0.0001).

Table 4. Effect of killing and drying methods on fatty acid profile (% total Fatty Acid Methyl Esters – FAME) of TM larvae.

	Experimental groups				**RSD**	***P-value***
	BO	**BM**	**FM**	**CA**		
N. of crates	8	8	8	8		
C12:0	0.30	0.30	0.27	0.29	0.06	0.7707
C14:0	2.89	2.89	2.81	2.82	0.10	0.4109
C16:0	17.2^{b}	17.4^{ab}	17.6^{a}	17.7^{a}	0.36	0.0390
C18:0	3.40^{AB}	3.36^{AB}	3.66^{A}	3.22^{B}	0.22	0.0046
C20:0	0.30^{ab}	0.30^{ab}	0.31^{a}	0.26^{b}	0.03	0.0155
Total SFA	24.1	24.2	24.7	24.3	0.50	0.0941
C16:1	1.53	1.54	1.53	1.54	0.05	0.9786
C18:1 *n*-9	39.5^{B}	39.9^{B}	39.6^{B}	41.9^{A}	0.62	<0.0001
C18:1 *n*-11	0.27	0.16	0.26	0.29	0.10	0.0669
Total MUFA	41.3^{B}	41.6^{B}	41.4^{B}	43.7^{A}	0.63	<0.0001
C18:2 *n*-6	30.3^{AB}	30.5^{A}	30.5^{A}	29.5^{B}	0.57	0.0046
C18:3 *n*-3	1.20	1.22	1.22	1.20	0.05	0.6089
Total PUFA	31.5^{AB}	31.8^{A}	31.7^{A}	30.7^{B}	0.59	0.0053
n-6	30.3^{AB}	30.5^{A}	30.5^{A}	29.5^{B}	0.57	0.0046
n-3	1.20	1.22	1.22	1.20	0.05	0.6089
n-6/*n*-3	25.4	25.0	25.1	24.7	0.88	0.4807
Identified	96.9^{B}	97.6^{AB}	97.8^{AB}	98.8^{A}	0.89	0.0026

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; SFA: Saturated FA; MUFA: Monounsaturated FA; PUFA: Polyunsaturated FA; $^{a-b}$ Values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ Values within a row with different superscripts differ significantly at P<0.01.

The percentage of identified FAs was the highest in group CA and the lowest in group BO (98.8% *vs* 96.9%; P<0.0001). When expressed in mg/100 g of dried larvae the total amount of SFAs was the highest in the BM and FM groups and the lowest in the CA group (22942 *vs* 22302 mg/100 g, on average; P<0.0001), with stearic acid (C18:0) and arachidonic acid (C20:0) levels at 2955 and 235 mg/100 g, respectively (P<0.0001) (Table S1). Among the MUFAs, palmitoleic acid (C16:1) content was the highest in the BM group and the

lowest in the CA group (1483 *vs* 1410 mg/100 g; P<0.05). The CA group also exhibited lower levels of PUFAs, with less linoleic acid compared to the other groups (29233 *vs* 27123 mg/100 g, on average; P<0.0001), and less linolenic acid (C18:3 n-3) than the BM group (1178 *vs* 1100 mg/100 g; P<0.0001).

Amino acids profile

The killing and drying methods had a significant impact on the content of essential amino acids of dried larvae (Table 5).

Table 5. Effect of killing and drying methods on amino acids profile (mg/100 g of larvae as is) of TM larvae.

	Experimental groups				**RSD**	***P-value***
	BO	**BM**	**FM**	**CA**		
N. of crates	8	8	8	8		
Essential amino acids						
Arginine	2367^{b}	2591^{ab}	2607^{a}	2571^{ab}	175	0.0354
Histidine	1915	1956	1900	1910	149	0.8844
Isoleucine	1783^{a}	1753^{a}	1679^{ab}	1618^{b}	102	0.0130
Leucine	3375^{A}	3341^{A}	3246^{A}	2903^{B}	162	<0.0001
Lysine	3547^{A}	3437^{ABa}	3255^{ABab}	3030^{Bb}	278	0.0051
Methionine	713^{A}	708^{A}	683^{A}	626^{B}	27.2	<0.0001
Phenylalanine	1602^{A}	1608^{A}	1600^{A}	1436^{B}	84.7	0.0006
Threonine	1895^{A}	1901^{A}	1811^{AB}	1686^{B}	98,0	0.0004
Tryptophan	268^{Bb}	292^{A}	287^{Aa}	270^{Bb}	11.6	0.0004
Valine	2508^{a}	2510^{a}	2388^{ab}	2292^{b}	142	0.0118
Non-essential amino acids						
Alanine	3313^{a}	3309^{a}	3150^{ab}	2887^{b}	223	0.0019
Aspartic acid	3654^{A}	3659^{A}	3466^{AB}	3183^{B}	28.0	0.0060
Cysteine	373^{A}	372^{A}	346^{AB}	324^{B}	28.6	0.0050
Glutamic acid	6000^{a}	6055^{a}	5866^{ab}	5330^{b}	472	0.0182
Glycine	2404^{A}	2390^{A}	2282^{A}	2105^{B}	122	<0.0001
Proline	2747^{A}	2832^{A}	2685^{AB}	2491^{B}	156	0.0012
Serine	2254^{A}	2245^{A}	2235^{A}	1967^{B}	127	0.0002
Tyrosine	2316^{A}	2279^{A}	2290^{A}	1916^{B}	124	<0.0001
Total	43037^{A}	43239^{A}	41777^{A}	38547^{B}	2261	0.0009

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; $^{a-b}$ Values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ Values within a row with different superscripts differ significantly at P<0.01.

Group FM showed the highest amount of arginine compared to group BO, which had the lowest (2367 *vs* 2607 mg/100 g; P=0.04). Group BO, BM and FM showed a higher amount of isoleucine, leucine, methionine and phenylalanine compared to group CA, that showed the lowest value for all of these amino acids. Also the content of threonine, tryptophan and valine was the lowest in CA group compared to the others (1686, 270 and 2292, respectively; P<0.001 and P<0.05). Additionally, the FM and BM groups had a higher tryptophan

content than the BO group. No significant differences were observed among the groups in histidine content (P=0.88). Among the non-essential amino acids, the BO and BM groups had the highest levels of most amino acids, while the FM group showed intermediate values, and the CA group had the lowest. No significant differences were observed among the BO, BM, and FM groups in glycine, serine, and tyrosine content. As expected, the total amino acid content was lowest in the CA group compared to all other groups (38,547 vs. 42,684 on average; P<0.0001).

Microbiological safety

Table 6 shows results about microbiological safety of TM dried larvae. No trace of *Salmonella* spp., *Listeria monocytogenes* and *Cronobacter* spp. were detected in 25 g of the samples. The presence of *Clostridium perfringens* was detected below 10 colony forming unit (**CFU**)/g as well as coagulase positive staphylococci and *Escherichia coli*, with no difference observed among the experimental groups. Group BO exhibited a higher microbial count of mesophilic bacteria compared to groups BM and FM, which displayed the lowest count (8.08 *vs* 2.59 log CFU/g, on average; P<0.0001). No differences were observed in the count of moulds and yeasts among the treatments. Group CA showed the highest microbial count of sulphite-reducing bacteria and Enterobacteriaceae (4.09 *vs* 1.00 log CFU/g, and 5.91 *vs* 1.22 log CFU/g, on average, respectively; P<0.0001). Conversely, group BO was associated with the highest number of colonies of *B. cereus* compared to the other groups (2.93 *vs* 2.06 log CFU/g, on average; P<0.0001).

Table 6. Effect of killing and drying methods on microbiological safety (log CFU/g) and the presence of *Salmonella* spp. and *Listeria monocytogenes* and *Enterobacter sakazakii* (in 25 g) of TM larvae.

	Experimental groups				**RSD**	***P-value***
	BO	**BM**	**FM**	**CA**		
N. of crates	6	6	6	6		
Mesophilic bacteria	8.08^{A}	2.70^{C}	2.48^{C}	6.42^{B}	0.78	<0.0001
Moulds	2.20	2.10	2.12	2.40	0.29	0.2809
Yeasts[2]	<100	<100	<100	<100		
Sulphite-reducing bacteria	1.00^{B}	1.00^{B}	1.00^{B}	4.09^{A}	0.40	<0.0001
Bacillus cereus (presumptive)	2.93^{A}	2.10^{B}	2.10^{B}	2.00^{B}	0.19	<0.0001
Enterobacteriaceae	1.00^{B}	1.55^{B}	1.10^{B}	5.91^{A}	0.69	<0.0001
Salmonella spp.	n.d.	n.d.	n.d.	n.d.		
Listeria monocytogenes	n.d.	n.d.	n.d.	n.d.		
Clostridium perfringens[1]	<10	<10	<10	<10		
Coagulase positive[1] staphylococci	<10	<10	<10	<10		
Escherichia coli[1]	<10	<10	<10	<10		
Cronobacter spp. (*Enterobacter sakazakii*)	n.d.	n.d.	n.d.	n.d.		

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; n.d.: not detected; [1]CFU/g; $^{a-b}$ Values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ Values within a row with different superscripts differ significantly at P<0.01.

Visual acceptance

The results of the visual acceptance of the dried larvae are summarised in Table S2. When considering the age of the consumers, no significant effect of the killing and drying methods on the visual acceptance of the larvae was observed, except among consumers aged 21 to 30 years. In this age group, the highest score was assigned to group FM, while the lowest score was given to group BO (4.48 *vs* 3.39; P<0.0001). Among the gender, male consumers assigned the worst score to group BO (4.43 *vs* 3.47, on average; P<0.0001) as well as females which preferred the larvae of group FM (4.24 *vs* 3.29; P<0.0001). Consumers who had previously consumed insects assigned similar scores to BM, FM and CA groups and the lowest to group BO (4.69 *vs* 3.30, on average; P<0.0001). Accordingly, consumers who were new to insects' consumption assigned the highest score to group FM and the lowest to group BO (4.32 *vs* 3.40; P<0.0001). Considering the consumer's predisposition, or lack thereof, to accept insects and products containing insects in their own and animal's diet, the trend was the same, with group BO scoring the lowest value in all three cases (3.28, 3.13 and 2.91, on average; P<0.0001). Furthermore, consumers favourable to the consumption of insects, assigned the highest value to group FM, while those not favourable made no distinction between group FM, BM and CA. Whitin each experimental group, no differences in scoring were observed among consumers of different age groups, while male consumers consistently assigned higher scores for all the groups, compared to females. There were no differences between consumers who had previously consumed insects and those who had not, except for treatment FM, where the latter group assigned a higher score (5.06 *vs* 4.32; P<0.0001). Consumers favourable to consuming insects and insects-based products rated all the experimental groups higher, while, except for group BO, no differences were found between consumers favourable or not to the inclusion of insects in animal diet.

Olfactory acceptance

The different killing and drying methods influenced the olfactory acceptance of dried larvae (Table S3). In all the age groups, except among consumers aged 41-50 years, the highest score was assigned to group BM and FM (5.37 and 5.48, on average, respectively; P<0.0001) and only to group FM by consumers of age lower than 20 years old (4.86; P<0.0001). Conversely, the lowest score was assigned to group CA (2.76 on average; P<0.0001). Male consumers assigned higher scores to group BM and FM, while no differences were found between group BO and CA (5.06 *vs* 3.39, on average; P<0.0001). Similarly, female consumers preferred group BM and FM and the lowest score was given to group CA (5.21 *vs* 2.58, on average; P<0.0001). Consumers who had or had not previously consumed insects exhibited the same trend, scoring group CA with the lowest value and group BM and FM with the highest (5.43 *vs* 2.52 and 5.09 *vs* 2.93, on average, respectively; P<0.0001). No differences between group BO and CA were found by consumers not favourable to introduce insects or insects-based products in their own diet, that scored the lowest value contrarily to group BM and FM (4.72 *vs* 2.63 and 4.57 *vs* 2.62, on average, respectively; P<0.0001). Consumers favourable to consuming insects and insects - based products assigned the lowest value to group CA and the highest to group BM and FM (5.39 *vs* 3.17 and 5.26 *vs* 2.92, on average, respectively;

P<0.0001), whereas group BO exhibited intermediate values. The same trend was observed by consumers favourable or not to the use of insects in animal diet (4.15 *vs* 2.69 and 5.2 *vs* 2.88, on average, respectively; P<0.0001). Within each experimental group, no significant differences were observed between consumers of different age classes or genders, except in group BO, where increasing age was associated with higher scores, and in group CA, where females assigned lower scores than males. For groups BM and FM, consumers favourable to the consumption of insects and insects-based products assigned a higher score to all the groups, as well as consumers favourable to include insects in animal diet.

Overall acceptance

Overall acceptance of dried larvae was significantly influenced by the killing and drying methods (Table S4). Indeed, among consumers aged <20 years old, group FM received the highest score while group BO received the lowest (4.29 *vs* 2.57; P<0.0001). Consumers aged 21-30 assigned the lowest scores to groups BO and CA, with no differences between them, contrasting with the higher scores given to groups BM and FM (4.82 *vs* 3.41, on average; P<0.0001). This trend was consistent across both male and female consumers. Whitin each personal traits of consumers, the evaluation results remained consistent: group BM and FM were preferred obtaining the highest score while group BO and CA the lowest. Age did not influence the results within each experimental group, except for group BM, where scores increased with age (P=0.0301). Regarding gender, males assigned higher scores than females in groups BO and CA (3.63 vs. 3.23, $P < 0.05$; and 3.64 vs. 3.07, $P < 0.0001$, respectively).In group FM, consumers who had previously consumed insects assigned higher scores (5.48 *vs* 4.78; P<0.0001), unlike other groups where no differences were found. Consumers who were favourable towards consuming insects and insect-based products assigned higher scores across all groups, while only in groups BM and FM favourability towards introducing insects into animal diets led to better scores by the consumers.

Visual, olfactory and overall acceptance

Significant differences were observed between all the groups concerning visual, olfactory and overall acceptance of dried larvae (Table 7).

Table 7. Effect of killing and drying methods on visual, olfactory and overall acceptance[1] of TM larvae.

	Experimental groups				**RSD**	***P-value***
	BO	**BM**	**FM**	**CA**		
N. of consumers	201	201	201	201		
Visual acceptance	3.38^{C}	$4.25^{AB,}$	$4.44^{A,}$	4.00^{B}	1.21	<0.0001
Olfactory acceptance	3.42^{B}	$5.01^{A,}$	5.25^{A}	2.86^{C}	1.42	<0.0001
Overall acceptance	3.44^{B}	4.71^{A}	4.90^{A}	3.36^{B}	1.07	<0.0001

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; [1] value from 1 to 7= 1: extremely unacceptable, 2: very unacceptable, 3: moderately unacceptable, 4: neither unacceptable or acceptable, 5: moderately acceptable, 6: very acceptable, 7: extremely acceptable; [a-b] Values within a row with different superscripts differ significantly at P<0.05; [A-B] Values within a row with different superscripts differ significantly at for P<0.0001.

Group FM exhibited the highest score for visual acceptance of the product while group BO had the lowest one (4.44 *vs* 3.38; P<0.0001). Intermediate values were observed for group BM and CA. Regarding olfactory acceptance, group BM and FM scored higher compared to the other groups, particularly in comparison to group CA, which showed the lowest value (5.13 *vs* 2.86, on average; P<0.0001). Furthermore, groups BM and FM showed higher overall acceptance compared to group BO and CA (4.81 *vs* 3.4, on average; P<0.0001).

Discussion and conclusions

A primary consideration that emerges from this study is the determination of the protein content in TM larvae. According to the literature, a conversion factor of 4.76 is commonly applied for TM (Janssen et al., 2017). Consequently, proximate composition analyses typically follow the official AOAC (2000) method, calculating protein content as N × 4.76. However, an analysis of the amino acid profile revealed a higher protein content compared with the value obtained using this method. This discrepancy may be related to several factors, including differences in experimental design, the developmental stage of the larvae at harvesting, feeding substrate, all of which could influence nitrogen content and protein estimates.

The killing and drying processes performed in the present study had an impact on physical characteristics of dried TM larvae. Although all four drying techniques reduced the a_w of the samples, none achieved an a_w level below or equal to 0.20. Indeed, the lowest recorded value was 0.30, observed in group BO. Most bacteria require an aw higher than 0.91 for survival, and when a_w falls below 0.60, the product is considered shelf-stable as it inhibits the growth of a wide range of microorganisms (Tapia et al., 2008). It is also known that an a_w between 0.20 and 0.40 slows down both enzymatic activity and non-enzymatic browning (Rahman, 2007). The a_w value seen in group BO differs from previous studies on oven drying, which reported a_w values as low as 0.13 (Kröncke et al., 2018; Krzyzaniak et al., 2022). A possible explanation for this difference is that, in the present study, the dried larvae were left at room temperature for 24 hours to equilibrate with environmental humidity, a step omitted in the studies by Kröncke et al. (2018) and Krzyzaniak et al. (2022). Conversely, the a_w results of group BM and FM were in line with those obtained by Lenaerts et al. (2018) and Vlahova-Vangelova et al. (2023), equal to 0.36 and 0.41, respectively.

Consistent with prior findings (Purschke et al., 2018; Kröncke et al., 2019; Selaledi et al., 2021; Trukhanova et al., 2022), the oven-dried larvae (BO group) exhibited a dark brown colour. Considering the applied processing conditions (boiling at 100 °C for 180 s followed by drying at 60 °C for 73 h), this appearance can be attributed to a combination of residual PPO activity, potentially insufficiently inactivated during boiling, and non-enzymatic browning processes that are promoted during drying (Whitaker and Lee, 1995 This reaction also explains the lowest Chroma value observed in this group. Chroma, in fact, is an index used to determine the degree of difference between a hue and grey colour with the same L* (Phatare et al., 2013) and a low value of Chroma indicate a low colour intensity perceived by the human eye. A low value of chroma was also observed in groups FM and BM. Nonetheless, a lighter brown colour was observed in these two groups compared to group BO. The high temperature applied during the microwave drying process,

inactivated enzymatic reactions, and the short duration of the process minimised the occurrence of non-enzymatic reactions (Trukhanova et al., 2022). However, a significant difference in a*and b* was observed between groups BM and FM. The latter exhibited a more yellow and red colour, probably linked to differences in drying duration. Specifically, due to the higher moisture content of the samples after blanching, microwave drying for group BM lasted approximately two minutes longer, thereby extending the Maillard reaction and resulting in a darker colouration of the larvae (Phatare et al., 2013). Furthermore, as expected, drying treatments involving high temperature determined the highest lipid oxidation, a condition responsible for the loss of nutritional value and the development of a rancid odour and taste (Zamora and Hildago, 2005). Despite group FM showed the highest value of MDA/kg, this value falls into the category of "not rancid" (<1.5 mg/MDA/kg), thereby not significantly affecting the sensory quality of the product (Larouche et al., 2019). Along with colour changes, the proximate composition of dried larvae was also influenced by heat treatments. The proximate composition of oven-dried larvae (BO group) was consistent with those reported in previous studies, as were the values for groups BM and FM (Baek et al., 2019; Selaledi et al., 2021). Exposure to high temperatures can lead to protein denaturation and browning reactions, potentially decreasing protein content (Akonor et al., 2016). Heat treatments, in facts, damage amino acid structures, reducing their bioavailability. In comparison to fresh larvae's amino acid composition reported in an earlier research (Costa et al., 2020), larvae in the present study showed a decrease in most amino acids after drying. Specifically, cysteine, methionine, and tryptophan, which are highly heat-sensitive, were notably affected (Chaudhary et al., 2021). Arginine, commonly involved in the Maillard reaction, binds to sugars, reducing its availability, which aligns with the lower arginine content found in group BO. The high temperatures applied during the drying processing were also responsible of the lower sugar content, particularly glucose and fructose, in the larvae from groups BO, BM, and FM. A reduction in sugar content is typically associated with non-enzymatic browning reactions. Given the relatively low sugar levels naturally present in TM larvae, caramelisation is unlikely to occur; instead, the Maillard reaction in heat-treated groups provides a more plausible explanation for the reduced concentrations of reducing sugars. By contrast, no evidence of such reactions was observed in the CA group, where larvae were subjected to lower temperatures and shorter drying times, which preserved a higher sugar content.

In addition to the above-mentioned effects of thermal treatments on proximate composition of larvae, high temperatures are also known to alter the FA profile of the product in particular leading to the breakage of long chains of PUFAs, promoting the formation of SFAs and MUFAs (Singh et al., 2020). Despite that, larvae treated with thermal treatments in the present study, in particular larvae of group BM and FM resulted in a higher content of PUFAs, consistent with the findings of Lenaerts et al. (2018). The reduced exposure time to high temperatures during microwave drying, in fact, minimised the negative effects of the heating process and preserved the nutritional value of the product. Nevertheless, according to the results and in line with the existing literature, TM larvae were found to be rich in unsaturated fatty acids (**UFAs**) and SFAs (Ravzanaadii et al., 2012; Paul et al., 2017). According to Trukhanova et al. (2022) and Kröncke et al. (2019), larvae were notably rich in oleic, linoleic, and palmitic FAs, with average values of 40.2%, 30.2%,

and 17.5% respectively. Focusing on the mineral composition of dried larvae, the Fe content of groups BO, BM, and FM was consistent with observations from previous studies (Baek et al., 2019; Selaledi et al., 2021). However, the contents of P, Ca, K, and Na were higher in the current study while the Mg and Cu content were lower compared to Baek et al. (2019) and Selaledi et al. (2021), respectively. Differences in the mineral profile of larvae could be attributed to variations in substrate, farming conditions, the analysis methods as well as microbial degradation (Rumpold and Schlüter, 2013) As demonstrated by the present results and according to other authors (Jajić et al., 2019), TM larvae lack in Ca. In fact, the quantity of Ca present in 1 kg of mealworms is insufficient to meet the human recommended daily intake of 1000 mg (World Health Organisation, 2004). However, TM larvae showed to be a good source of K, P, S and trace elements, making them a suitable food choice to combat the growing deficiency in micronutrients (Von Grebmer et al., 2014). According to the results of the present study, among the trace elements, zinc is the most abundant, in accordance with the quantity of zinc observed by Kröncke et al. (2019).

Thermal treatments applied in the present study and in general treatments such as boiling, blanching, roasting, and other cooking methods are necessary to ensure the safety of insects-based products, and they are commonly employed for microbial inactivation. The effectiveness of these treatments depends on factors such as temperature and duration of the process and the microorganisms' characteristics. Nevertheless, in Europe, specific microbiological criteria for insect-based products intended for human consumption have not been established yet (Yan et al., 2023). The count of mesophilic bacteria serves as a crucial parameter for evaluating hygiene practices and identifying potential contamination during food processing. These bacteria grow in a temperature range of 20 to 45 °C, with an optimal growth temperature of 37 °C. Given that most human pathogens fall within this group, monitoring mesophilic bacteria is essential to ensure food safety (Jay et al., 2005). In the present study, microbiological analyses revealed no traces of *Listeria monocytogenes*, *Salmonella* spp, *Cronobacter* spp, *Clostridium perfringens* and *Staphylococcus aureus* in dried larvae. However, colonies of *B. cereus* were observed in dried larvae, especially in group BO with a value of 2.93 log CFU/g. This bacterium has been frequently detected in various insect-based products, with counts ranging from 4 to 6.6 log CFU/g (Fasolato et al., 2018) and exceeding 5 log CFU/g in cricket powder (Osimani et al., 2017). The presence of *B. cereus* in heat-treated products is due to its ability to produce spores that are resistant to heat and drying. Indeed, because of its resistance to industrial treatment and other stresses, *B. cereus* is a major concern in the consumption of edible insects (Fasolato et al., 2018). The presence of Enterobacteriaceae in groups BO, BM, and FM ranged from 1.00 to 1.55 log CFU/g, falling within the range reported by Yan et al. (2023). However, a significantly higher number of these bacteria, along with sulphite-reducing bacteria, was detected in group CA, indicating that the drying process was insufficient. Indeed, while blanching of larvae at 100 °C was able to inactivate microbial load (Wang et al., 2017; Yan et al., 2023), the use of low temperature during the killing, as applied in groups FM and CA, was only able to stop microbial growth without inactivating the microorganisms. The absence of high temperature step during killing and drying of CA larvae explains the elevated counts of Enterobacteriaceae and sulphite-reducing bacteria observed in this group. Additionally, contamination during the handling or

processing of the larvae could be another contributing factor. As demonstration of the ineffectiveness of the drying process applied for group CA, a higher value of a_w was detected for this group of larvae compared to the other groups, along with a lower pH. Some microorganisms are able to reduce the pH of a product due to the production of some organic acids as a result of fermentation processes such as lactic acid, acetic acid and respiratory byproducts (Tsapekos et al., 2017), explaining the slight acidic pH of larvae belonging to group CA. Larvae of this group contained more water and, as a result, less protein, ash, and lipids than the other groups, which contributed to their reduced energy value compared to the average of 27.2 MJ/kg. Despite the low temperature applied during the drying process, the lower content of lipids of group CA could be attributed to the activation of insects' lipases which was previously observed in black soldier fly larvae (Caligiani et al., 2019). Microbial contamination of larvae can explain the lowest content of amino acids of group CA compared to the other groups. Certain microorganisms can break down proteins into peptides and amino acids through the action of proteolytic enzymes. For instance, Enterobacteriaceae fall into this category (Todar, 2020). These intestinal bacteria utilise proteins for growth and energy production, playing a crucial metabolic role in the gastrointestinal tract of animals and humans (Garrity, 2005). Sulphite-reducing bacteria, such as *Desulfovibrio*, are also involved in protein degradation. These bacteria use sulphite reduction as part of their anaerobic metabolism, breaking down proteins to obtain the amino acids necessary for their growth and metabolism (Muyzer and Stams, 2008). Amino acids obtained by protein degradation, indeed, can then be used as sources of carbon and nitrogen, leading to further breakdown into simpler compounds (Feng and Cerniglia, 2017). This explains the reduced levels of certain amino acids, such as isoleucine, leucine, lysine, and threonine, in group CA. Despite the previous considerations, group CA exhibited the best preservation of fresh larval colour. Indeed, this group displayed the highest L*, a* and b* and Chroma values. The application of low temperatures during drying limited the occurrence of non-enzymatic browning reactions, while enzymatic browning by PPO was not expected under the applied nitrogen atmosphere due to the lack of oxygen. These combined factors explain the observed colour retention.

A significant difference in visual, olfactory, and overall acceptance was observed among the four groups of dried larvae, as evaluated through consumers sensory analysis. The highest scores obtained by group FM in all three considered parameters, along with group BM in olfactory and overall acceptance is attributed to the Maillard reaction occurred during the drying process. Indeed, the Maillard reaction leads to the formation of several aromatic compounds such as furans, pyrazines and thiols which confer complex and pleasant aromas to the food, typically associated with roasted and caramelised foods. The presence of these compounds likely explains the high olfactory acceptance scores for groups BM and FM. The Maillard reaction also enhances the visual appeal of the food by giving a slight brown coloration, which human often associate with cooked and tasty food (Martins et al., 2000). The slightly brown colouration of larvae in groups BM and FM was visually appreciated by consumers, in contrast to the dark brown colouration of group BO. The group CA was also not appreciated by consumers, despite it was the best method in maintaining the typical yellowish colour of fresh TM larvae. The reason could be their excessive similarity to live larvae. Indeed, in Western

cultures, most people are disgusted by insects and are afraid to introduce them into their diet (Looy et al., 2014). Transforming insects into more conventional food forms could improve their acceptance (van Huis, 2013). As observed in the present study, the visual acceptance of the product could also be improved by a proper drying method, as seen with groups BM and FM. Furthermore, in addition to a more pleasant colour, larvae in groups BM and FM may have been more appreciated due to their slightly swollen appearance, compared to larvae in group BO which appeared shrunken due to a loss of volume and tissue collapse during the drying process. This shrunken appearance is typically not appreciated by consumers (Mayor and Sereno, 2004).

These findings provide valuable insights into optimising the processing techniques of TM larvae to enhance both their physical and nutritional quality, as well as consumer acceptance. Results highlight the profound impact of different killing and drying methods on physical, chemical, microbiological and sensory characteristics of TM dried larvae. Among the proximate composition, groups BO, BM and FM resulted in higher content of proteins, lipids, ash and energy compared to group CA, when expressed on a fresh weight basis. Furthermore, group BO, BM and FM exhibited a higher content of minerals, PUFAs, essential and non-essential amino acids. Despite group CA preserved the fresh colour of larvae, this drying process was not adequate. The higher water activity (aw) of this group, together with the presence of a high load of Enterobacteriaceae and sulphite-reducing bacteria, negatively affected nutritional composition and consumer acceptance, demonstrating the necessity to refine the method. Concluding, blanching followed by oven drying or microwave drying and freezing followed by microwave drying emerged as valuable processing methods in terms of nutritional properties of the final product. However, blanching or freezing followed by microwave drying emerged as the most advantageous approaches when considering microbiological safety and consumer acceptance, including visual, olfactory, and overall attributes. Future research efforts should focus on refining processing protocols to meet the diverse preferences of consumers and to maximise the nutritional properties of dried TM larvae.

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Supplementary materials

Table S1. Effect of killing and drying methods on fatty acid content (mg/100 g as is) of TM larvae.

	Experimental groups				RSD	*P-value*
	BO	**BM**	**FM**	**CA**		
N. of crates	8	8	8	8		
C12:0	286	285	256	263	58.2	0.6564
C14:0	2722	2783	2686	2589	97.1	0.0039
C16:0	16557	16731	16848	16260	430	0.0568
C18:0	3263^{AB}	3240^{AB}	3498^{A}	2955^{B}	228	0.0007
C20:0	286^{ABa}	288^{ABa}	294^{A}	235^{Bb}	31.1	0.0024
Total SFA	23113^{ABab}	23326^{Aa}	23582^{A}	22302^{Bb}	605	0.0015
C16:1	1471^{ab}	1483^{a}	1463^{ab}	1410^{b}	48.4	0.0275
C18:1 *n*-9	37928	38458	37780	38454	732	0.1589
C18:1 *n*-11	258	157	246	264	92.3	0.0948
Total MUFA	39657	40099	39488	40128	763	0.2618
C18:2 *n*-6	29152^{A}	29411^{A}	29135^{A}	27123^{B}	621	<0.0001
C18:3 *n*-3	1150^{ab}	1178^{a}	1160^{ab}	1100^{b}	47.3	0.0162
Total PUFA	30303^{A}	30589^{A}	30295^{A}	28223^{B}	647	<0.0001
n-6	29152^{A}	29411^{A}	29135^{A}	27123^{B}	621	<0.0001
n-3	1150^{ab}	1178^{a}	1160^{ab}	1100^{b}	47.3	0.0162

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; SFA: Saturated FA; MUFA: Monounsaturated FA; PUFA: Polyunsaturated FA; $^{a-b}$ Values within a row with different superscripts differ significantly at $P<0.05$; $^{A-B}$ Values within a row with different superscripts differ significantly at $P<0.01$.

Table S2. Effect of killing, drying methods, and personal traits of consumers, on visual acceptance[1] of TM larvae.

	Experimental groups				RSD	*P-value*
	BO	**BM**	**FM**	**CA**		
N. of consumers	201	201	201	201		
Age						
<20	3.00	4.00	3.86	4.29	0.98	0.1239
21-30	3.39^{C}	4.27^{AB}	4.48^{A}	3.99^{B}	1.18	<0.0001
31-40	3.57	3.71	4.29	4.14	1.37	0.6276
41-50	3.60	4.75	4.25	4.00	3.12	0.8060
RSD	1.42	1.42	1.45	1.60		
P-value	0.8617	0.6327	0.7107	0.9600		
Gender						
M	3.47^{B}	4.39^{A}	4.64^{A}	$4.27^{A, x}$	1.11	<0.0001
F	3.29^{C}	4.10^{AB}	4.24^{A}	$3.72^{BC, y}$	1.30	<0.0001
RSD	1.41	1.41	1.44	1.56		
P-value	0.3743	0.1341	0.0531	0.0121		
Have you ever consumed insects?						
No	3.40^{C}	4.17^{AB}	$4.32^{A, Y}$	3.93^{B}	1.13	<0.0001
Yes	3.30^{B}	4.64^{A}	$5.06^{A, X}$	4.36^{A}	1.54	<0.0001
RSD	1.42	1.41	1.43	1.58		
P-value	0.7229	0.0823	0.0070	0.1497		
Would you accept insects in your diet?						
No	$2.81^{B, Y}$	$3.68^{A, Y}$	$3.73^{A, Y}$	$3.45^{A, Y}$	1.21	<0.0001
Yes	$3.74^{C, X}$	$4.60^{AB, X}$	$4.89^{A, X}$	$4.34^{B, X}$	1.21	<0.0001
RSD	1.34	1.35	1.34	1.53		
P-value	<0.0001	<0.0001	<0.0001	<0.0001		
Would you accept products containing insects in your diet?						
No	$2.71^{B, Y}$	$3.63^{A, Y}$	$3.61^{A, Y}$	$3.53^{A, y}$	1.02	0.0002
Yes	$3.54^{C, X}$	$4.39^{AB, X}$	$4.64^{A, X}$	$4.11^{B, x}$	1.25	<0.0001
RSD	1.38	1.39	1.39	1.57		
P-value	0.001	0.0028	<0.0001	0.0404		
Would you accept insect-based products for animal diet?						
No	$2.38^{B, Y}$	3.85^{A}	3.85^{A}	4.00^{A}	1.03	0.0006
Yes	$3.44^{C, X}$	4.27^{AB}	4.48^{A}	4.00^{B}	1.21	<0.0001
RSD	1.37	1.41	1.44	1.59		
P-value	0.0084	0.3048	0.1249	0.9906		

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; [1]value from 1 to 7= 1: extremely unacceptable, 2: very unacceptable, 3: moderately unacceptable, 4: neither unacceptable or acceptable, 5: moderately acceptable, 6: very acceptable, 7: extremely acceptable; $^{a-b}$ Values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ Values within a row with different superscripts differ significantly at for P<0.01. $^{x-y}$ Values within a column with different superscripts differ significantly at P<0.05; $^{X-Y}$ Values within a column with different superscripts differ significantly at P<0.01.

Table S3. Effect of killing, drying method, and personal traits, of consumers on olfactory acceptance[1] of TM larvae.

	Experimental groups				RSD	*P-value*
	BO	**BM**	**FM**	**CA**		
N. of consumers	201	201	201	201		
Age						
<20	3.29^{AB}	$3.57^{AB, z}$	4.86^{A}	2.00^{B}	1.09	0.0008
21-30	3.39^{B}	$5.02^{A, y}$	5.25^{A}	2.86^{C}	1.43	<0.0001
31-40	4.00^{ABb}	$5.71^{Aa, x}$	5.71^{Aa}	3.43^{B}	1.02	0.0005
41-50	3.80	5.75^{x}	5.00	3.33	2.93	0.2870
RSD	1.59	1.42	1.29	1.47		
P-value	0.7241	0.0206	0.6449	0.2919		
Gender						
M	3.63^{Ba}	4.84^{A}	5.27^{A}	$3.14^{Bb, X}$	1.45	<0.0001
F	3.21^{B}	5.18^{A}	5.23^{A}	$2.58^{C, Y}$	1.32	<0.0001
RSD	1.57	1.44	1.29	1.45		
P-value	0.0658	0.0969	0.8906	0.0073		
Have you ever consumed insects?						
No	3.34^{B}	4.99^{A}	5.18^{A}	2.93^{C}	1.36	<0.0001
Yes	3.85^{B}	5.12^{A}	5.58^{A}	2.52^{C}	1.60	<0.0001
RSD	1.58	1.45	1.28	1.47		
P-value	0.0889	0.6299	0.1278	0.1375		
Would you accept insects in your diet?						
No	$2.90^{Ba, Y}$	$4.56^{A, Y}$	$4.87^{A, Y}$	$2.35^{Bb, Y}$	1.53	<0.0001
Yes	$3.75^{B, X}$	$5.29^{A, X}$	$5.48^{A, X}$	$3.17^{C, X}$	1.36	<0.0001
RSD	1.53	1.40	1.25	1.42		
P-value	0.0002	0.0004	0.0007	<0.0001		
Would you accept products containing insects in your diet?						
No	$2.63^{B, Y}$	$4.55^{A, y}$	$4.58^{A, Y}$	2.61^{B}	1.44	<0.0001
Yes	$3.61^{B, X}$	$5.12^{A, x}$	$5.40^{A, X}$	2.92^{C}	1.41	<0.0001
RSD	1.54	1.43	1.24	1.47		
P-value	0.0006	0.0300	0.0002	0.1897		
Would you accept insect-based products for animal diet?						
No	2.92^{B}	$4.15^{A, y}$	$4.15^{A, Y}$	2.69^{B}	1.58	0.0051
Yes	3.46^{B}	$5.07^{A, x}$	$5.33^{A, X}$	2.88^{C}	1.40	<0.0001
RSD	1.59	1.43	1.26	1.47		
P-value	0.2418	0.0280	0.0014	0.6647		

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; [1]value from 1 to 7= 1: extremely unacceptable, 2: very unacceptable, 3: moderately unacceptable, 4: neither unacceptable or acceptable, 5: moderately acceptable, 6: very acceptable, 7: extremely acceptable; $^{a-b}$ Values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ Values within a row with different superscripts differ significantly at for P<0.01. $^{x-y}$ Values within a column with different superscripts differ significantly at P<0.05; $^{X-Y}$ Values within a column with different superscripts differ significantly at P<0.01.

Table S4. Effect of killing and drying methods and personal traits of consumers on overall acceptance[1] of TM larvae.

	Experimental groups				RSD	*P-value*
	BO	**BM**	**FM**	**CA**		
N. of consumers	201	201	201	201		
Age						
<20	2.57^{B}	$3.43^{ABab, y}$	4.29^{Aa}	3.00^{ABb}	0.47	0.0014
21-30	3.44^{B}	$4.74^{A, xy}$	4.90^{A}	3.37^{B}	1.06	<0.0001
31-40	4.00	5.14^{x}	5.43	3.57	1.81	0.0541
41-50	3.80	5.00^{xy}	4.99	2.39	2.38	0.1859
RSD	1.38	1.20	1.20	1.40		
P-value	0.2375	0.0301	0.3591	0.5257		
Gender						
M	$3.63^{B, x}$	4.72^{A}	4.97^{A}	$3.64^{B, X}$	1.02	<0.0001
F	$3.23^{B, y}$	4.71^{A}	4.83^{A}	$3.07^{B, Y}$	1.10	<0.0001
RSD	1.37	1.22	1.20	1.37		
P-value	0.0400	1.000	0.4053	0.0045		
Have you ever consumed insects?						
No	3.38^{B}	4.64^{A}	$4.78^{A, Y}$	3.38^{B}	1.05	<0.0001
Yes	3.70^{B}	5.09^{A}	$5.48^{A, X}$	3.25^{B}	1.10	<0.0001
RSD	1.38	1.21	1.17	1.40		
P-value	0.2350	0.0717	0.0019	0.5622		
Would you accept insects in your diet?						
No	$2.89^{B, Y}$	$4.26^{A, Y}$	$4.38^{A, Y}$	$2.77^{B, Y}$	0.98	<0.0001
Yes	$3.77^{B, X}$	$4.99^{A, X}$	$5.22^{A, X}$	$3.72^{B, X}$	1.14	<0.0001
RSD	1.32	1.17	1.13	1.32		
P-value	<0.0001	<0.0001	<0.0001	<0.0001		
Would you accept products containing insects in your diet?						
No	$2.76^{B, Y}$	$4.12^{A, Y}$	$4.00^{A, Y}$	3.06^{B}	0.80	<0.0001
Yes	$3.59^{B, X}$	$4.85^{A, X}$	$5.11^{A, X}$	3.43^{B}	1.12	<0.0001
RSD	1.35	1.18	1.12	1.39		
P-value	0.0008	0.0003	<0.0001	0.0963		
Would you accept insect-based products for animal diet?						
No	2.83^{B}	$4.00^{A, Y}$	$4.00^{A, Y}$	3.33^{AB}	0.74	0.0046
Yes	3.47^{B}	$4.76^{A, X}$	$4.96^{A, X}$	3.37^{B}	1.09	<0.0001
RSD	1.38	1.21	1.18	1.40		
P-value	0.1231	0.0397	0.0070	0.9516		

BO: blanching and oven drying; BM: blanching and microwave drying; FM: freezing and microwave drying; CA: cryogenic freezing and drying in controlled atmosphere; [1]value from 1 to 7= 1: extremely unacceptable, 2: very unacceptable, 3: moderately unacceptable, 4: neither unacceptable or acceptable, 5: moderately acceptable, 6: very acceptable, 7: extremely acceptable; $^{a-b}$ Values within a row with different superscripts differ significantly at P<0.05; $^{A-B}$ Values within a row with different superscripts differ significantly at P<0.01. $^{x-y}$ Values within a column with different superscripts differ significantly at P<0.05; $^{X-Y}$ Values within a column with different superscripts differ significantly at P<0.01.

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Fifth contribution:

Live yellow mealworm (*Tenebrio molitor*) larvae: a promising nutritional enrichment for laying quails

A. Dalle Zotte[a], Y. Singh[a]*, B. Palumbo[a], B. Contiero[a], M. Cullere[a]

[a]Department of Animal Medicine, Production and Health, University of Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro, Padova, Italy

*Corresponding author: yazavinder.singh@unipd.it

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Introduction

Insects and derived products are considered one of the possible alternative feedstuffs to improve the sustainability of the livestock sector thanks to several promising attributes: limited space requirements for farming and the possibility to exploit the vertical space, short productive cycles, limited water needs (i.e. water is often obtained directly from the feeding substrate), and the suitability of some species to mass rearing (Smetana et al., 2021). Last but not least, some insect species are perfect candidates in the perspective to set-up circular economy models (Ojha et al., 2020): insects feed on organic side-streams, including agro industrial by-products, and other feedstuffs with no commercial value and that are considered waste, they up-cycle them into high-value nutrients to be successfully included in the diets of different food-producing animals, including fish (Henry et al., 2015), poultry (Shaviklo, 2023), rabbit (Cullere et al., 2022), and pig (DiGiacomo et al., 2019). Furthermore, the residue of insect farming, that is the frass, can be used as organic fertiliser to replace the use of agrochemicals thus promoting sustainable agriculture (Poveda, 2021). From the nutritional point of view, insects are characterised by high amounts of protein with excellent biological value, lipids, minerals, water-soluble vitamins, and compounds with functional properties such as chitin and antimicrobial peptides (Koutsos et al., 2023). In the European Union, the current legislative framework allows 8 insect species (*Hermetia illucens*, *Musca domestica*, TM, *Alphitobius diaperinus*, *Acheta domesticus*, *Gryllodes sigillatus*, *Gryllus assimilis*, and *Bombyx mori*) which can be farmed to produce feed for food producing animals (Commission Regulation (EU), 2017; 2021) and they can also be fed alive. The possible use of live insects offers the perspective of using them also as environmental enrichment for food-producing animals, aiming at increasing the complexity of the captive environment. One form of environmental enrichment is foraging enrichment, which can increase bird activity, improve leg health and decrease stereotypic behaviours (Laurence et al., 2015; Ipema et al., 2020 a, b). Among food-producing animals, poultry surely represents one of the most promising sectors where live insects can play a relevant role in designing novel feeding strategies, also considering that insects represent a natural food source for them (Koutsos et al., 2023). Previous research indicated that the incorporation of low amounts (5% of the expected daily FI) of live TM and *Hermetia illucens* larvae in the diet of broiler chickens (Colombino et al., 2021) can provide a slight improvement in the cecal microbiota. This was attributed to the bioactivity of chitin, an indigestible polysaccharide constituting insects' exoskeleton, which possesses antimicrobial and immunostimulant properties (Islam et al., 2017). Furthermore, when turkey poults were supplemented with live *Hermetia illucens* larvae (10% of the expected daily FI), FI and BW gain improved, with a reduced incidence of feather pecking (Veldkamp and van Niekerk (2019). Despite these encouraging results, further investigations into the impact of this novel feeding approach on both animal productivity and product quality (live insects are a nutrients-dense feedstuff) have been carried out to a limited extent and exclusively considering *Hermetia illucens* (Tahamtani et al., 2021). On the contrary, no studies considering the impact of live TM larvae on both performance and product quality have been conducted up to now. Furthermore, it is not clear which is the apparent digestibility of live TM larvae and thus their real nutritional value, as well as if a daily provision higher than 5% of the expected daily FI could be feasible. Based on

these premises, the present research tested the effect of a live TM larvae supplementation (10% inclusion) as nutritional enrichment for laying quails (*Coturnix japonica*) on performance, egg physicochemical traits, and shelf-life. Furthermore, an in vivo digestibility trial was conducted to assess, for the first time, the nutritional value of the TM larvae as well as that of the experimental diets. The quail was chosen because it is a popular bird for egg production and is economically interesting for many productive contexts thanks to a series of positive attributes such as early sexual maturity, rapid growth, short generation interval, limited space requirements, and high laying percentage (Dalle Zotte et al., 2019).

Material and methods

Animals and experiment design

The research study received ethical approval from the Ethical Committee of the University of Padova under protocol number 56/2021. The trial was conducted at a farm with which the MAPS Department of the University of Padova has a scientific agreement. A diet, referred to as the "Control" diet (Table 1), was formulated based on the minimum requirements for laying Japanese quails (*Coturnix japonica*) as recommended by the National Research Council, Subcommittee on Poultry Nutrition (NRC, 1994). The laying quails were divided into 2 experimental groups: the first group received the Control diet in mash form, whereas the second group received the Control diet, daily supplemented with live TM larvae (TM 10: 10% of the expected daily FI, which was calculated based on data from previous trials (Dalle Zotte et al., 2019; Singh et al., 2023) on laying quails of the same age). The 8-weeks-old live TM larvae were provided by INEF - Insect Novel Ecologic Food (Via Fossetta, 23, 35017 Piombino Dese, Padova, Italy).

A total of n = 60, 119-day-old laying Japanese quails (30 quails/treatment) were individually weighed and assigned to the 2 dietary treatments ensuring the same average live weight in each group. For each dietary treatment, a total of n = 6 replicated cages (n = 5 quails/each, with a space allowance of 0.13 m^2 per quail) were designed. Quails were housed in battery cages, fed with experimental diets for 5 weeks, and with *ad libitum* access to feed and water throughout the trial. The environmental conditions of the room were monitored: the average RH and temperature were 76.1% and 21.2 °C respectively, and the set photoperiod was 16 h light:8 h dark.

Productive performance

Quails were individually weighed on the first and last day of the experimental trial, to assess the live weight change along the experiment. FI was recorded weekly on a cage basis. Also, morbidity and mortality were monitored along the trial. During the 5 weeks of the experiment, the laid eggs per cage were counted daily and individually weighed; average egg weight and egg production were then calculated. Additionally, defected eggs (i.e., broken, without solid shell, or with unusual shape meaning extremely elongated or rounded) were also daily counted and used to calculate defected egg percentage. The FCR was calculated as kg of feed consumed/kg of egg produced. At the 5^{th} week, the eggs were collected for 7 consecutive days, identified and analysed for physicochemical quality, sensory profile, and storage stability. A total of n = 68

and 85 eggs/treatment (Control and TM 10, respectively) were used for physicochemical analyses, n = 27 eggs/treatment for the sensory analysis, and n = 56 eggs/treatment for the storage stability trial.

Table 1. Ingredients of the experimental diet (g/kg as fed).

Ingredients	**Control**
Corn	495
Soybean meal	335
Wheat flour	30.1
Wheat bran	30.0
Soybean oil	35.0
Calcium carbonate	64.4
Dicalcium phosphate	0.70
NaCl	3.50
Methionine DL	1.30
Vitamin-Mineral premix[1]	5.00

[1]Vitamin and mineral premix provided the following per kg of diet: Vitamin A, 11,500 IU; cholecalciferol, 2100 IU; vitamin E (from dl-tocopherylacetate), 22 IU; vitamin B12, 0.60 mg; riboflavin, 4.4 mg; nicotinamide, 40 mg; calcium pantothenate, 35 mg; menadione (from menadione dimethyl-pyrimidinol), 1.50 mg; folic acid, 0.80 mg; thiamine, 3 mg; pyridoxine, 10 mg; biotin, 1 mg; choline chloride, 560 mg; ethoxyquin, 125 mg; Mn (from $MnSO_4 \cdot H_2O$), 65 mg; Zn (from ZnO), 55 mg; Fe (from $FeSO_4 \cdot 7H_2O$), 50 mg; Cu (from $CuSO_4 \cdot 5H_2O$), 8 mg; I (from Ca $(IO_3)2 \cdot H_2O$), 1.8 mg; Se, 0.30 mg; Co (from Co_2O_3), 0.20 mg; Mo, 0.16 mg.

Digestibility

A separate in vivo digestibility trial was conducted following the procedure described by Dalle Zotte et al. (2021). A total of n = 30, 85-day-old laying quails were individually housed in n = 30 digestibility cages and assigned to one of the following 3 dietary groups: the 2 diets that is, 1) Control and 2) TM 10, and 3) a diet consisting of 100% live TM larvae (aiming at establishing the nutritional value of this insect species, TM 100). With this purpose, quails were weighed and allocated into 3 experimental groups (10 replicates/treatment) ensuring homogenous live weight. During the trial, the environmental parameters were the same of the performance trial. The experimental diets and water were provided *ad libitum* to the quails. The digestibility trial allowed to determine the apparent nutrient digestibility of DM, organic matter, crude protein, ether extract, ash, starch, chitin, and energy. Subsequently, the nutritive value of the experimental diets was calculated based on the obtained digestibility data.

Physical analyses of the eggs

Eggs of the 5-weeks performance trial were collected on a daily basis and transported to the meat and egg quality laboratory of the MAPS Department of the University of Padova (Italy) and were individually weighed to calculate the surface area. Further measurements were recorded: egg equatorial diameter (mm), and egg height (mm), using a digital caliper (Juwel Schraubtechnik, EB, Werkstraße 14, 57537 Wissen, Altenkirchen, Rheinland Palatinate, Germany) (0−150 mm—Juwel). Such measurements were used to calculate the egg shape index (%). After physical measurements, eggs were broken for shell and interior egg quality measurements, and within 30 s albumen height was computed as the arithmetic mean of 2

measurements performed with a Haugh digital micrometer (Baxlo, Barcelona, Spain) to calculate the egg Haugh unit. The pH of the albumen (± 0.1) was determined in duplicate (FG2-Five GoTM; Mettler Toledo, Greifensee, Switzerland - 3 points calibration: pH 4, 7, and 10), and yolk color was evaluated by comparison with the 15-points dsm-firmenich YolkFanTM (DSM, Wurmisweg 576, CH-4303 Kaiseraugst, Switzerland). The albumen and yolk weights were determined to compute the albumen percentage and yolk percentage, as well as the yolk to albumen ratio. Furthermore, the eggshell was dried with a paper towel and weighed (± 0.1 g), then eggshell thickness (mm) was measured at the equatorial level with the digital caliper. The shell percentage was calculated. The weight of the egg and of the edible portion (calculated as egg weight minus shell weight) were used to obtain the edible portion percentage.

Chemical analyses of TM larvae and the experimental Diet

The chemical composition and energy content of TM larvae and experimental diet are shown in Table 2.

Table 2. Chemical composition (g/kg as is), mineral (mg/kg as is) and gross energy (MJ/kg) contents of Control diet and *Tenebrio molitor* larvae.

	Control diet	**TM larvae**
Dry matter	909	307
Crude protein	262	128
Ether extract	48.3	79.3
Ash	104	12.6
Starch	336	-
Chitin	-	31.0
Ca	25.9	0.13
P	3.62	2.11
Ca/P	7.15	0.06
Gross energy[1]	16.4	8.44

[1]Analysed.

Analyses of TM larvae and the experimental diet were carried out in duplicate following the Association of Official Analytical Chemists (AOAC, 2019) methods to determine DM (method no. 934.01), crude protein (method no. 2001.11) and ash (method no. 967.05). For the total nitrogen content of TM larvae, the Kjeldahl method was used; nitrogen content was then multiplied by 4.76 N-conversion factor to obtain the corrected crude protein content (Janssen et al., 2017). Ether extract was determined after acid hydrolysis. Gross energy was measured with an adiabatic bomb calorimeter. Starch (amyloglucosidasea-amylase, method no. 996.11) content was analysed in the experimental diet. The chitin content of TM larvae and freeze-dried excreta was analysed according to the method described by Woods et al. (2019). The chemical analyses of freeze-dried excreta were carried out in accordance with the same AOAC (2019) methods previously described for the experimental diet. The crude protein content of excreta was corrected for uric acid content, which was analysed according to the procedure described by Cullere et al. (2016). The FA profile of TM larvae and experimental diet are presented in Table 3. The lipids extraction of the experimental diet and TM larvae was

performed by M-ASE using petroleum ether and chloroform/methanol 1:2 as solvent binary mixtures, respectively. The fat content of the sample was determined gravimetrically after vacuum-evaporation under nitrogen stream. Samples were trans-methylated using a 4% methanolic solution of H2SO4 in order to FAME. A biphasic separation was obtained by adding 0.5 mL of distilled water and 1.5 mL of n-heptane to each sample. FAME were quantified by gas chromatography (Shimadzu GC17A), equipped with an Omegawax (Sigma-Aldrich Co. LLC., Saint Louis, MO) 250 column (30 m £ 0.25 mm £ 0.25 mm) and flame ionisation detector. Helium was used as the carrier gas at a constant flow of 0.8 mL/min. The injector and detector temperatures were 260°C. Peaks were identified based on commercially available FAME mixtures (37-Component FAME Mix; Supelco Inc., Bellefonte, PA). Results were expressed as% of total detected FAME. The mineral profile (macro and micro elements) and heavy metals content of TM larvae are presented in Table 4. ICP-OES was performed with Spectro Arcos (SPECTRO Analytical Instruments, GmbH, Kleve, Germany) after microwave digestion with Milestone rotor at 64-bar pressure, according to AOAC (2019). Data were expressed as mg/kg as is.

The amino acid profile of the TM larvae and experimental diet are provided in Table 5. The amino acid composition of the TM larvae and the experimental diets were determined after acid hydrolysis and pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, separated by RP-HPLC and analysed by UV detection (Agilent 1260 Infinity, Agilent Technologies, Santa Clara, CA) following a method adapted from European Pharmacopoeia (Council of Europe, 2005).

Table 3. Fatty acid profile (% of total Fatty Acid Methyl Esters, FAME) of Control diet and *Tenebrio molitor* larvae.

	Control diet	**TM larvae**
C6:0	0.24	0.01
C8:0	0.03	0.01
C10:0	0.00	0.01
C12:0	0.00	0.22
C14:0	0.09	3.53
C15:0	0.32	0.14
C16:0	14.0	13.9
C17:0	0.11	0.19
C18.0	3.76	2.73
C20:0	0.33	0.18
C22:0	0.26	0.07
C24:0	0.17	0.00
Total SFA	19.2	21.0
C14:1	0.02	0.12
C15:1	0.13	0.05
C16:1	0.10	1.86
C17:1	0.05	0.18

Table 3. Continue.

	Control diet	TM larvae
C18:1 n-9	23.5	43.0
C18:1 n-11	1.21	0.00
C20:1 n-9	0.09	0.09
Total MUFA	25.1	45.3
C18:2 n-6	49.5	30.8
C18:3 n-6	0.00	0.05
C18:3 n-3	5.38	0.90
C20:2 n-6	0.05	0.09
C20:3 n-6	0.00	0.03
C20:4 n-6	0.03	0.00
Total PUFA	54.9	31.9
n-6	49.6	31.0
n-3	5.38	0.90
n-6/n-3	9.22	34.4
Identified,%	99.3	98.1

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

The protein content of the samples was hydrolysed with (6M) HCl at 105°C for 24 h. Cysteine was determined as the sum of cysteine and cystine, after reaction with 3,30 -dithiodipropionic acid, producing a mixed disulfide, which then underwent acid hydrolysis accordingly. After hydrolysis, the samples were neutralised with (8M) NaOH, adjusted to volume, and filtered at 0.45 mm. Then, the derivatisation step was conducted according to the manufacturer's instructions (AccQ-Tag Ultra Derivatisation Kit; Waters, Milford, MA).

Table 4. Mineral profile (mg/kg, as is basis) of *Tenebrio molitor* larvae.

	TM larvae	EFSA reports[1]	EC No 1881/2006[2]
Macroelements:			
K	2618	-	-
P	2107	-	-
S	819	-	-
Mg	708	-	-
Na	464	-	-
Ca	128	-	-
Zn	28.4	-	-
Fe	11.6	-	-
Mn	2.61	-	-
Microelements:			
Cu	4.25	-	-

Table 4. Continue.

	TM larvae	EFSA reports1	EC No 1881/20062
Sr	1.02	-	-
Al	1.01	-	-
Se	<1.0	-	-
Sn	<1.0	-	(inorganic)
Tl	<1.0	-	-
B	0.66	-	-
Ni	<0.5	-	-
Mo	0.28	-	-
Ag	<0.2	-	-
Ba	0.11	-	-
Cr	0.11	-	-
Be	<0.1	-	-
Co	<0.1	-	-
Li	<0.1	-	-
Sb	<0.1	-	-
V	<0.1	-	-
Ti	0.05	-	-
Contaminants:			
As	<1.0	0.00-0.29	-
Pb	<1.0	0.00-<0.08	0.50 (crustacean)
Cd	0.03	0.00-0.08	0.50 (crustacean)
Hg	0.00	0.00-0.04	0.50 (crustacean)

[1]Safety of dried yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal, 24/11/2020; Safety of frozen and dried yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal, 07/07/2021; [2]Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union L 364/5.

Table 5. Amino acid profile (g/100 g, as is basis) of Control diet and *Tenebrio molitor* larvae.

	Control diet	TM larvae
Essential amino acids:		
Arginine	1.34	0.64
Histidine	0.66	0.53
Isoleucine	0.60	0.41
Leucine	1.61	0.92
Lysine	1.42	0.83
Methionine	0.08	0.02
Phenylalanine	0.98	0.45
Threonine	0.78	0.50
Valine	0.66	0.61
Non-essential amino acids:		

Table 5. Continue.

	Control diet	TM larvae
Alanine	1.06	1.06
Aspartic acid	2.59	1.22
Cysteine	0.17	0.06
Glutamic acid	4.81	1.80
Glycine	0.95	0.80
Proline	1.25	1.05
Serine	1.20	0.69
Tryptophan	0.24	0.17
Tyrosine	0.46	0.78
Total AA	20.9	12.5

Chemical analyses of the eggs

Eggs were collected for 2 consecutive days: 7 eggs from each cage (8 replicates/treatment) were homogenised to 1 sample (pool) and freeze-dried to guarantee enough matrix to perform all the scheduled analyses. The proximate composition of egg samples was analysed in accordance with the same AOAC (2019) method previously described for the experimental diet. The FA profile of the eggs was analysed following the same method previously described for the experimental diet, with the only exception that, for the fat extraction, the binary mixture of solvents hexane/Isopropanol 3:2 was used. Then, the results were expressed as% of total detected FAME. Also, the lipid peroxidability index, atherogenic index, thrombogenic index, and hypocholesterolemic/Hypercholesterolemic index of the eggs were calculated as reported by Dalle Zotte et al. (2019). Additionally, the quantitative determination (mg/100 g egg) of FA was also obtained by using the chromatographic peak area according to the internal standard, that is nonadecylic acid (C19:0), and the total lipid content of the sample. Similarly, the amino acids profile of the eggs was analysed following the same method previously described for the TM larvae and the experimental diet. The obtained results were expressed as mg/100 g egg.

Shelf-life trial

The storage stability trial was carried out on 56 eggs/treatment stored for d 0 (28 eggs/treatment) and d 28 (28 eggs/treatment) with average humidity and temperature of 65 ± 5% and 20 ± 1 °C respectively. Out of 28 eggs, 21 eggs/treatment were used to evaluate the albumen pH (FG2-Five GoTM; Mettler Toledo, Greifensee, Switzerland - 3 points calibration: pH 4, 7, and 10), Haugh unit, and yolk color (dsm-firmenich YolkFanTM), whilst the other 7 eggs/treatment were assigned for TBARs analysis for d 0 vs. d 28. The extent of egg yolk lipid oxidation (TBAR) was evaluated with a spectrophotometer (Hitachi U-2000; Hitachi, Mannheim, Germany) set at 532 nm, that measured the absorbance of TBARs and a 1,1,3,3-tetraethoxypropane calibration curve (Botsoglou et al., 1994). Oxidation products were quantified as MDA equivalents (mg MDA/kg egg yolk).

Sensory analysis

Eggs were assigned to a descriptive sensory analysis, to detect possible difference among the experiment treatments (Control vs. TM 10). A total of 27 eggs/treatment were used and a day analysis was scheduled. Panellists participated in 2 pre-test training sessions of 1 h each to familiarise themselves with the matrix and select appropriate descriptors, which were also drawn from the literature. Additionally, panellists were provided with freeze-dried and ground TM larvae powder to evaluate and select possible descriptors. The quail eggs used for the training sessions were obtained from quails fed with a conventional diet and eggs were processed, stored, handled and cooked in the same manner as the samples which were used for the subsequent sensory analysis. The selected descriptors were: odour intensity, sulphur, and TM, and flavour intensity, sapidity, sulphur, greasy-oily, persistency, TM. For TM odour and flavour, panellists were trained on a TM larvae powder. At the farm, freshly collected eggs were identified with a 3-digit random code and then transported to the MAPS Department. Once there, eggs were boiled for 5 mins, cooled under running tap water, and served to panellists in a random sequence. Each panellist evaluated a total of 8 eggs (4 eggs/treatment), except one panellist received a total of 6 eggs (3 eggs/treatment); the panel received the aforementioned list of descriptors to score on numerical and continuous 15 cm long scales from 1 (the lowest score for each attribute) to 10 (the highest score for each attribute). All the evaluations were performed in a room where the temperature was set at 22 °C. In each sensory session, unsalted crackers and still water at room temperature were available to panellists.

Statistical analysis

Performance data and egg physicochemical traits were analysed by one-way ANOVA using the GLM procedure of SAS 3 (SAS Institute, 2008), with the experimental diet (Control vs. TM 10) as fixed effect. Digestibility data were analysed by one-way ANOVA testing the effect of dietary treatment (Control vs. TM10 vs. TM100) on the relevant traits. For the shelf-life trial, a two-way ANOVA was used to test the effects of dietary treatment, day of storage and their interaction. For sensory traits, data were analysed by one-way ANOVA with experimental diet (Control vs. TM10) as a fixed effect, except for the traits TM odour and flavour. As these variables were not normally distributed, percentages were compared using a z-test for two proportions and a χ-square test. The experimental unit varied: cage for performance data, individual quail for digestibility, and single egg and pooled sample for physical and chemical characteristics of the eggs, respectively. Least squares means were obtained, and post-hoc pairwise comparisons were performed using Bonferroni correction. Statistical significance was declared at $P < 0.05$.

Results

TM *larvae*

Data concerning the chemical composition of fresh TM larvae confirmed once more the nutritional value of this feedstuff. Table 2 shows that TM larvae were rich in protein (12.8 g/100 g), and ether extract (7.93 g/100 g), thus resulting in a notable gross energy content (8.44 MJ/ kg). The FA profile of the TM (Table 3) was

characterised by a prevalence of MUFAs (45.3% of total FAME), followed by PUFAs (31.9% of total FAME), and SFA (21% of total FAME). Oleic acid (C18:1 n-9) was the most abundant MUFA (43.0% of total FAME) while, among PUFA, the n-6 fraction accounted for the almost totality (31% of total FAME) and with linoleic acid accounting alone for the 30.8% of total FAME. From the analysis of the mineral profile (Table 4), it emerged that live TM larvae contain a substantial quantity of macroelements, especially potassium (2618 mg/kg), phosphorus (2107 mg/kg), and sulfur (819 mg/kg). Among microelements, copper was the most represented (4.25 mg/kg). Concerning contaminants, no particular concerns were noted, as values were within legislative limits and values published by the EFSA reports. The protein quality of TM larvae was evidenced by looking at their amino acid content (Table 5): among essential amino acids, leucine (0.92 g/100 g), lysine (0.83 g/100 g), and arginine (0.64 g/100 g) were the most abundant, while glutamic acid (1.80 g/100 g), aspartic acid (1.22 g/100 g), and alanine (1.06 g/100 g) were the most represented non-essential amino acids.

Productive performance

The effect of the dietary inclusion of live TM larvae on the productive parameters of quails and egg measurements are displayed in Table 6. Over the course of the 5- weeks feeding trial the final live weight, FCR, egg weight, egg production, and defected eggs did not differ between the Control and TM 10 groups ($P>0.05$), and no mortality was observed. However, the TM 10 group exhibited a significantly higher ($P< 0.001$) FI compared to the Control group. Additionally, the TM 10 group showed a lower egg shape index compared to the Control group ($P<0.01$).

Table 6. Effect of the dietary inclusion of live *Tenebrio molitor* (TM – 10% of the daily feed intake) larvae on the productive performance of laying quails and egg measurements during a 5-weeks trial.

	Control	**TM 10**	**RSD**	***P-value***
N. of laying quails	30	30		
Initial live weight, g	342	336	26.7	0.3726
Final live weight, g	354	347	31.1	0.4312
N. of replicated cages	6	6		
N. of total egg laid	923	950		
Feed intake (FI), g/day/quail	43.3	51.5[2]	1.55	<0.0001
Egg weight, g[3]	14.3	14.2	1.30	0.1021
Egg production,%	90.0	90.5	5.37	0.8870
Egg mass, g egg/hen/day	13.0	12.9	0.75	0.6964
FCR	3.29	3.50	0.19	0.0943
Egg shape index,%[3]	76.0	75.6	2.81	0.0100
Defected eggs,%	1.26	0.20	1.62	0.2850

[2]Mash feed + live TM larvae. [3]Measurements performed on total eggs laid.

Digestibility

Results presented in Table 7 show the effect of the dietary supplementation of live TM larvae (10% of the expected daily FI - TM 10 or 100% - TM 100) to laying quails on the total tract apparent digestibility of nutrients and nutritive value of diets.

Table 7. Effect of the dietary inclusion of live *Tenebrio molitor* (TM – 10% of the daily feed intake or 100%) larvae on the apparent nutrient digestibility in laying quails and nutritive value of the diets.

	Control	**TM 10**	**TM 100**	**RSD**	***P-value***
N.	10	10	10		
Live weight, g	337	337	339	26.2	0.9817
Feed intake, g	68.6^{B}	82.3^{B}	157^{A}	20.8	<0.0001
Dry matter (DM) intake, g	62.3^{B}	73.4^{A}	48.9^{C}	7.73	<0.0001
DM excreted, g	26.8	29.2	29.5	5.08	0.4412
Apparent digestibility,%:					
DM	57.0^{A}	60.1^{A}	44.0^{B}	5.35	<0.0001
Organic matter	59.7^{A}	62.6^{A}	47.1^{B}	4.96	<0.0001
Protein	50.8^{A}	50.8^{A}	24.0^{B}	10.7	<0.0001
Ether extract	83.9^{B}	85.9^{B}	94.0^{A}	3.60	<0.0001
Starch	96.5	97.0	-	0.83	0.1625
Chitin	-	100^{A}	88.7^{B}	2.37	<0.0001
Energy	65.3	67.9	65.4	3.67	0.2460
Nutritive value of diets:					
Metabolizable protein (MP: g/kg diet)	133^{A}	132^{A}	31.2^{B}	16.3	<0.0001
Metabolizable energy (ME: MJ/kg diet)	10.7^{A}	11.0^{A}	5.38^{B}	0.47	<0.0001
MP/ME	12.4^{A}	12.0^{A}	5.75^{B}	3.35	0.0016

$^{A-B}$ Values within a row with different superscripts differ significantly at P<0.01.

The TM 100 group exhibited the highest FI, followed by the TM 10 group and then by the Control group (TM 100 > TM 10 > Control; P<0.0001). Considering the DM intake, instead, the 3 treatments displayed the following rank: TM 10 > Control > TM 100 (P<0.0001). The TM 10 group showed an apparent digestibility of nutrients comparable to that of the Control diet. Conversely, TM 100 group displayed the lowest digestibility for DM (P<0.001), organic matter (P<0.001), and protein (P<0.001), while ether extract was better digested in TM 100 than in groups Control and TM 10 (P<0.0001). As a result, energy digestibility was similar in the 3 treatment groups. Starch digestibility was not affected by the presence of TM 10. For what concerns chitin, TM 10 showed a 100% digestibility which was higher than the 88.7% of the TM 100 group (P<0.0001). The supplementation of 10% live TM larvae to the diet of laying quail did not alter the nutritive value of diets, whereas the nutritive value of 100% live TM larvae was lower than that of Control and TM 10 diets (P<0.0001).

Egg physical traits and proximate composition

The inclusion of live TM larvae into laying quails' diet (TM 10) did not affect the physical traits of the whole egg and of its parts, including albumen, yolk and shell weight, as well as their proportion to the total egg weight, albumen pH and Haugh index, yolk colour, and shell thickness (Table 8). As it was observed for the physical traits of eggs, also proximate composition was not influenced when live TM larvae (TM 10) were supplemented to the laying quails' diet (Table 9).

Table 8. Effect of the dietary inclusion of live *Tenebrio molitor* (TM – 10% of the daily feed intake) larvae on eggs physical traits.

	Control	**TM 10**	**RSD**	***P-value***
N.	68	85		
Egg weight, g	14.6	14.6	1.13	0.8150
Edible portion,%	88.8	88.5	1.12	0.1611
Surface area, cm2	26.3	26.4	1.45	0.8220
Yolk weight, g	4.37	4.36	0.50	0.9223
Yolk,%	30.0	29.7	2.34	0.4308
Yolk color Fan2	5.41	5.53	0.80	0.3650
Albumen weight, g	8.57	8.60	0.77	0.7928
Albumen,%	58.8	58.9	2.90	0.9182
Yolk to Albumen ratio	0.51	0.51	0.06	0.5724
Albumen pH	8.62	8.68	0.19	0.0891
Haugh unit	95.8	96.3	4.46	0.5180
Shell weight, g	1.63	1.67	0.17	0.1488
Shell thickness, mm	0.25	0.25	0.03	0.5401
Shell,%	11.2	11.5	1.12	0.1611

Table 9. Effect of the dietary inclusion of live *Tenebrio molitor* (TM – 10% of the daily feed intake) larvae on the proximate composition of quail eggs (g/100 g egg).

	Control	**TM 10**	**RSD**	***P-value***
N.	8	8		
Water	72.7	71.3	3.27	0.4231
Protein	13.2	13.6	1.42	0.5424
Lipids	10.6	11.4	1.36	0.2597
Ash	1.01	1.07	0.13	0.3321

Egg FA and amino acid profiles

The effect of the dietary inclusion of live TM larvae into laying quails' diet on the FA profile (% of total FAME) of eggs collected at week 5 is depicted in Table 10. The main FA classes that is, SFA, MUFA, and PUFA remained unaffected by the dietary inclusion of live TM. However, some individual SFAs, such as

myristic acid (C14:0), pentadecylic acid (C15:0) and margaric acid (C17:0) were higher (P<0.001) in TM 10 group than in Control group. In the case of individual MUFAs, the proportion of myristoleic acid (C14:1) and heptadecenoic acid (C17:1) was significantly higher in TM 10 than the Control group (P<0.05).

Table 10. Effect of the dietary inclusion of live *Tenebrio molitor* (TM –10% of the daily feed intake) larvae on the fatty acid profile of quail eggs (% of total FAME).

	Control	**TM 10**	**RSD**	**P-value**
N.	8	8		
C14:0	0.35	0.41	0.02	0.0002
C15:0	0.04	0.05	0.03	0.0013
C16:0	24.8	25.2	0.38	0.1035
C17:0	0.15	0.17	0.01	<0.0001
C18:0	10.7	10.8	0.40	0.8045
C20:0	0.10	0.09	0.01	0.3587
C24:0	1.41	1.33	0.11	0.1433
Total SFA	37.6	38.0	0.53	0.1682
C14:1	0.06	0.07	0.01	0.0483
C16:1	3.21	3.18	0.29	0.8310
C17:1	0.07	0.08	0.03	0.0004
C18:1 n-9	33.5	32.7	1.08	0.1811
C18:1 n-11	1.60	1.41	0.13	0.0148
Total MUFA	38.4	37.5	1.25	0.1485
C18:2 n-6	18.4	18.9	1.08	0.3684
C18:3 n-6	0.28	0.26	0.03	0.3208
C18:3 n-3	0.90	0.83	0.09	0.1513
C20:2 n-6	0.09	0.09	0.03	0.6008
C20:3 n-6	0.12	0.16	0.04	0.0285
C20:4 n-6	2.81	2.73	0.12	0.2589
Total PUFA	22.6	23.0	1.23	0.5332
n-6	21.7	22.1	1.14	0.4379
n-3	0.91	0.85	0.11	0.2464
n-6/n-3	23.9	26.2	2.10	0.0483
AI	0.43	0.44	0.01	0.0089
TI	0.35	0.34	0.02	0.1119
PI	32.1	32.4	1.43	0.7495
hH	2.21	2.16	0.05	0.0686
Identified	98.6	98.5		

PUFA: Polyunsaturated fatty acids; AI: Atherogenicity index = (C12:0 + 4 x C14:0 + C16:0)/[Total MUFA + Total (*n*-6) + total (*n*-3)].TI: Thrombogenicity index = (C14:0 + C16:0 + C18:0)/[(0.5 x total MUFA) + 0.5 x (*n*-6) + 3 x (*n*-3/*n*-6)]; PI: Peroxidability index = (% monoenoic x 0.025) + (% dienoic x 1) + (% trienoic x 2) + (% tetraenoic x 4) + (% pentaenoic x 6) + (% hexaenoic x 8); hH: Hypocholesterolemic/Hypercholesterolemic index = (C18:1 *n*-9 + C18:2 *n*-6 + C20:4 *n*-6 + C18:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3)/(C14:0 + C16:0).

For the vaccenic acid (C18:1 n-11), Control eggs showed a higher percentage than the TM 10 ones (P<0.05). As for individual PUFAs, the TM 10 diet increased the proportion of dihomo-g-linolenic acid (C20:3 n-6) in quail eggs compared to the Control (P<0.05). As a result, the n-6/n-3 ratio increased in eggs belonging to the TM 10 treatment compared to the Control ones (P<0.05).

Table 11. Effect of the dietary inclusion of live *Tenebrio molitor* (TM – 10% of the daily feed intake) larvae on the fatty acid content of quail eggs (mg/100 g egg).

	Control	**TM 10**	**RSD**	***P-value***
N.	8	8		
C6:0	2.26	2.94	0.68	0.0855
C14:0	31.1	38.4	3.99	0.0044
C15:0	4.26	5.10	0.50	0.0081
C16:0	2171	2353	289	0.2674
C17:0	12.7	16.2	1.64	0.0016
C18:0	938	1012	141	0.3541
C20:0	8.60	8.59	1.54	0.9756
C24:0	124	124	19.2	0.9792
Total SFA	3292	3560	451	0.2934
C14:1	5.12	6.33	0.77	0.0115
C16:1	281	296	36.4	0.4452
C17:1	6.45	7.72	0.73	0.0060
C18:1 n-9	2925	3060	372	0.5198
C18:1 n-11	139	132	18.0	0.4506
Total MUFA	3356	3502	418	0.5364
C18:2 n-6	1608	1759	206	0.2001
C18:3 n-6	24.2	24.5	3.58	0.9029
C18:3 n-3	79.3	77.3	11.4	0.7364
C20:2 n-6	8.23	8.06	2.00	0.8675
C20:3 n-6	10.3	15.3	3.51	0.0188
C20:4 n-6	245	256	33.3	0.5653
Total PUFA	1976	2142	252	0.2475
n-6	1896	2063	242	0.2254
n-3	80.2	78.7	11.6	0.8029

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

As a consequence of the limited effect of the dietary supplementation of live TM larvae on quails' egg FA profile, also the health indexes were comparable in Control and TM 10 eggs. The sole exception was the AI, which exhibited the highest value in TM 10 eggs (P<0.01). Looking at the amounts of FAs (Table 11), a similar outcome than that reported for the FA proportions (Table 10) was observed: main FA classes were comparable in eggs of the 2 dietary treatments, while the same individual SFA, MUFA, and PUFA for which a treatment effect was observed with percentage data, were influenced also in the case of quantitative data.

The only exception was the C18:1 n-11 whose amount was similar in Control and TM 10 eggs. Also, the amino acids contents of eggs did not change as a result of the live TM larvae supplementation to the laying quails' diet (Table 12). Control and TM 10 eggs displayed similar amounts for each of the essential and non-essential amino acids, as well as the same sum of amino acids

Table 12. Effect of the dietary inclusion of live *Tenebrio molitor* (TM – 10% of the daily feed intake) larvae on the amino acid profile of quail eggs (g/100 g egg).

	Control	**TM 10**	**RSD**	***P-value***
N.	8	8		
Essential amino acids				
Arginine	0.54	0.50	0.06	0.1981
Histidine	0.34	0.32	0.04	0.2494
Isoleucine	0.35	0.33	0.04	0.4571
Leucine	0.87	0.79	0.10	0.1749
Lysine	0.91	0.81	0.04	0.1189
Methionine	0.14	0.13	0.02	0.1486
Phenylalanine	0.53	0.49	0.06	0.2503
Threonine	0.55	0.50	0.07	0.1514
Valine	0.45	0.41	0.05	0.1097
Non-essential amino acids				
Alanine	0.60	0.55	0.07	0.2075
Aspartic acid	1.19	1.08	0.15	0.1967
Cysteine	0.13	0.12	0.02	0.2230
Glutamic acid	1.58	1.44	0.19	0.1612
Glycine	0.43	0.40	0.05	0.2715
Proline	0.39	0.82	0.05	0.2119
Serine	0.89	0.36	0.10	0.2222
Tryptophan	0.13	0.14	0.02	0.4660
Tyrosine	0.23	0.22	0.03	0.3131
Sum	10.3	9.42	1.22	0.1949

Egg shelf-life

Table 13 shows the effect of the dietary supplementation of live TM larvae into the diet of laying quails on the storage stability of eggs over a 28-d retail display, as well as the effect of storage time on the considered traits within the treatment group, including their interaction. The qualitative parameters monitored along the trial were albumen pH, yolk colour, Haugh unit and oxidative status of lipids (TBARs). As expected, results highlighted that albumen pH increased in eggs of both Control and TM 10 groups at d 28 of storage compared to those at d 0 ($P<0.0001$), and that, conversely, Haugh unit (freshness) and yolk colour decreased ($P<0.001$). However, albumen pH, Haugh unit and yolk colour showed similar values for both Control and TM 10 eggs. In contrast to other traits, the initial oxidative rate of TM 10 egg lipids on d 0 of storage was

lower compared to Control egg lipids (P<0.001). However, after 28 d of retail display storage, eggs from both Control and TM 10 groups showed comparable oxidative status. Notably, within the Control group a significant day effect was evident, with TBARs values on d 28 being lower than those on d 0 (P<0.0001), whereas TM 10 eggs had similar oxidative status at both d 0 and 28 (P > 0.05).

Table 13. Effect of the dietary inclusion of live *Tenebrio molitor* (TM – 10% of the daily feed intake) larvae on the physical traits (albumen pH, yolk colour, Haugh unit) and oxidative status (TBARs[1], mg MDA[2]/kg egg yolk) of quail eggs, assessed over a 28-day display trial.

	Control	**TM 10**	**RSD**	***P*-diet**	***P*-diet*day**
N.	21	21			
Albumen pH					
Day 0	8.64	8.67	0.19	0.5513	0.5002
Day 28	9.59	9.58	0.05	0.6366	
RSD	0.17	0.12			
P-day	<0.0001	<0.0001			
Yolk Colour FAN					
Day 0	5.67	6.00	0.60	0.0750	0.1838
Day 28	4.84	4.81	0.64	0.8739	
RSD	0.71	0.52			
P-day	0.0008	<0.0001			
Haugh Unit					
Day 0	95.9	96.3	4.85	0.7848	0.5510
Day 28	81.5	83.0	2.74	0.1022	
RSD	4.35	3.60			
P-day	<0.0001	<0.0001			
N.	7	7			
TBARs					
Day 0	1.56^{A}	1.21^{B}	0.12	0.0004	0.0001
Day 28	1.14^{B}	1.26^{B}	0.13	0.1210	
RSD	0.11	0.14			
P-day	<0.0001	0.5338			

[1]MDA = malondialdehyde; [2]TBARs = thiobarbituric acid-reactive substances; $^{A-B}$ Values within a row with different superscripts differ significantly at P<0.01.

Egg sensory traits

The dietary supplementation of live TM larvae to laying quails' diet affected the sensory traits of eggs (Table 14). While odour attributes were similar in eggs of the 2 treatment groups, eggs of the TM 10 group had an overall milder flavour than Control eggs. In fact, overall flavour intensity (P<0.01), sulphur (P<0.05), and greasy-oily (P<0.01) flavours scored lower values in TM 10 than in Control eggs.

Table 14. Effect of the dietary inclusion of live *Tenebrio molitor* (TM – 10% of the daily feed intake) larvae on the sensory traits of quail eggs.

	Control	**TM 10**	**RSD**	***P*-diet**
N.	27	27		
Odour:				
Intensity	59.0	54.0	9.50	0.0634
Sulphur	44.0	36.9	13.0	0.0512
Tenebrio molitor[2]	0.22	0.30	-0.31	0.7562
Flavour:				
Intensity	44.8	35.4	10.9	0.0030
Sapidity	30.2	28.5	6.58	0.3435
Sulphur	28.0	22.7	9.12	0.0405
Greasy-oily	59.0	50.9	10.2	0.0061
Persistency	34.9	32.6	9.76	0.4095
Tenebrio molitor[2]	0.22	0.15	0.35	0.7261

[2]For *Tenebrio molitor* a Z-test for 2 proportions was conducted, as the trait was not normally distributed.

Discussion

TM is surely one of the most interesting insect species from the perspective of providing alternative and sustainable feedstuffs for food-producing animals, including poultry. This is testified by the number of research articles that have been published in the last decade, assessing the possible inclusion of its protein (i. e., meal) or fat fractions into the diet of different animal species, including poultry and swine (Hong et al., 2020), fish (Henry et al., 2015), and rabbit (Volek et al., 2021). Despite this, the use of live larvae occupies a marginal part of this literature. Bellezza Oddon et al. (2021) observed that the provision of live TM larvae to broiler chickens (5% of the expected FI) had no effect on growth and carcass traits, hematological and serum parameters, as well as gut morphometric indexes and histopathological alterations. The same research group observed that this feeding treatment did not alter the mucin composition or local immune response of chickens too, while slightly improved cecal microbiota by enhancing a minor fraction of short chain FA-producing taxa (Colombino et al., 2021). Considering other insect species, live *Hermetia illucens* larvae were tested as a possible tool to limit feather pecking in laying hens (Star et al., 2020): a larvae dispenser provided a daily amount of 12 g live larvae/hen (10% of daily FI), and this resulted in hens (65 weeks of age) with better feather condition than control ones, as a result of less pecking. Then, the same insect species was studied by Veldkamp and van Niekerk (2019) on turkey poults and by Ipema et al. (2020a,b) on broiler chickens. In all trials, however, the possible effect of live *Hermetia illucens* on product quality was not assessed. This research represents the first study exploring the impact of live TM larvae as nutritional enrichment for laying quails. In a research conducted on laying hens, feeding live black soldier fly larvae at 0, 10, and 20% of their daily expected DM intake or *ad libitum* access to live black soldier fly larvae for 12 weeks, stimulated hen's interest and FI (Tahamtani et al., 2021), with the whole daily portion of live larvae

being consumed in approximately 5 min. This was confirmed also in the present research; in fact, a first subjective aspect that was observed during both the performance and the digestibility trials on laying quails, but which is of importance in explaining the experimental results, was the remarkable interest and extremely excited response of laying quails when seeing the live larvae in their feeder. Feeding stimulus, constant during the 5-weeks performance trial, was so strong that they consumed the whole portion of larvae within 2 to 5 min from the administration. Scientifically, this was confirmed and highlighted by the results on the FI, both during the performance and during the digestibility trials. Despite the Control diet was nutritionally complete, and this was testified by the satisfactory laying performance of Control quails (Table 6), TM 10 quails exhibited a higher feed consumption that was almost exclusively attributable to the presence of larvae. The average feed consumption of live TM larvae was 4.84 g/quail/d (data not shown). Also, during the digestibility trial, no statistical difference was observed for FI, but Control and TM 10 quails consumed 68.6 vs. 82.3 g feed/d, respectively. Quails of the treatment TM 100 displayed a particular outcome in terms of FI, since it was more than double that of the other 2 groups (157 g/d; $P<0.0001$): on the one hand, this extreme result could be attributable to the inadequate nutritional profile of the TM larvae compared to the Control diet (Table 2), thus triggering quails to maximise FI, as well as to the lower DM content of TM 100 vs. the Control feed. In fact, the DM intake of TM 100 quails was only 48.9 g/d, vs. 62.3 and 73.4 of Control and TM 10 quails, respectively. The digestibility trial allowed to observe that TM larvae were overall less digestible than the Control diet which was expected, since they contain chitin which can act as an antinutritional factor mainly reducing the digestibility of the protein faction (Kroeckel et al., 2012): in the present digestibility trial, TM 100 quails had had an apparent crude protein digestibility of 24.0%. The antinutritional activity of chitin, however, is not always observed in poultry trials, since the effect is dose-dependent and poultry species possess enzymes that can hydrolyse the b-1, 4 glycoside bonds of chitin thus allowing its digestion (Tabata et al., 2018). This was practically demonstrated in the present digestibility trial, where TM 10 quails displayed a complete digestion (100%) of chitin, while it decreased in *ad libitum* TM larvae feeding (TM 100). Despite this, a remarkable 88.7% chitin digestibility was observed. Low chitin amounts can have a probiotic effect on poultry gut health (Bovera et al., 2015) and, in general, no particular drawbacks on productive outcomes have been observed in previous researches on laying hens (Star et al., 2020; Tahamtani et al., 2021), quails (Dalle Zotte et al., 2019), as well as considering meat-producing poultry species (Loponte et al., 2017; Biasato et al., 2018; Zsedely et al., 2022) fed with different insect meals or live larvae. Interestingly, the sole provision of live TM larvae (TM 100) provided the best ether extract digestibility, thus emphasising that the lipid fraction should not negatively be affected by the presence of chitin in the diet. Results dealing with the physical egg traits provided evidence that the live TM larvae can be fed to laying quails, together with a standard diet, without impairing egg quality. Despite no other research studied the impact of the dietary inclusion of live TM larvae into laying quails or hens' diet, feeding hens with 10, 20% or *ad libitum* live *Hermetia illucens* larvae (Star et al., 2020; Tahamtani et al., 2021) did not affect the egg physical quality parameters, except the yolk colour, which was paler in the *ad libitum* group. The latter result was attributed to the reduced intake of the control diet, thus determining a

reduced uptake of carotenoids. In studies where TM meal was included in the diets for laying hens (Ko et al., 2020) or quails (Secci et al., 2021) at different inclusion levels (5-20% dietary inclusion), no drawbacks on egg physical traits were detected. Previous research on the use of insect meals into laying quails' diet highlighted that the dietary treatments had a marginal effect on the FA profile and content of egg lipids (Secci et al., 2018; Dalle Zotte et al., 2019). This was attributable to a noticeable activity of desaturase and elongase enzymatic patterns on SFA, as well as on the synthesis of long chain FA (Güclü et al., 2008; Secci et al., 2021). Furthermore, birds can exploit the acetate/malonate way to synthesise ex novo palmitic acid (C16:0), partly convert it into stearic acid (C18:0) and desaturate both into palmitoleic acid (C16:1 n-7) and C18:1 n-9 (Klasing, 1998). This is coherent with the results observed in the present research, as the lipid fraction of TM larvae was richer in some individual FAs compared to the Control diet, particularly C14:0, myristoleic acid (C14:1), palmitoleic acid (C16:1), C17:1, C18:1 n-9. Conversely, TM larvae displayed a remarkably lower amount of linoleic acid (C18:2 n-6) compared to the Control diet. Despite this, the FA profile and content of egg lipids was only marginally affected by the inclusion of live TM larvae into the quails' diet. Above all, C18:1 n-9, total MUFA and overall n-6 were not different in the eggs of the 2 dietary treatments, despite a notable difference for this FA was noticed in the TM larvae vs. the Control diet. In addition to the metabolic pathways previously discussed, it must be highlighted that quails of the present experiment ingested 4.84 g/larvae/d, roughly corresponding to 0.38 g lipids/d. Conversely, the Control diet contributed to about 2.1 g lipids/d. Therefore, the relative contribution of TM lipids to the overall dietary lipids was relatively low. Eggs contain high quality proteins and are known to be good source of essential amino acids. Literature data (Genchev, 2012) indicate about 49.5% essential amino acids, with lysine and leucine being the main amino acids. This is in line with the results of the present study as quail eggs provided about 45% essential amino acids, and lysine and leucine were the most abundant ones. The comparable results of Control and TM 10 quail eggs in terms of amino acids contents was expected, since TM is known to be an excellent amino acids source (Makkar et al., 2014). Coherently to previous research on eggs obtained from quails fed with different insect sources (Dalle Zotte et al., 2019; Singh et al., 2023), also in the present trial no particular effects linked to the dietary treatment on eggs' shelf-life were observed. The pH values of quail eggs (average: 8.66) at d 0 of shelf-life are typical for this species (Dalle Zotte et al., 2019) and are indicative of freshness. Similarly, also the high initial Haugh unit of both groups (average: 96.1) is a further indication of egg freshness. In fact, at d 28 of shelf-life, pH increased and the Haugh unit decreased along with storage duration. This trend is naturally attributable to the progressive loss of carbon dioxide, and the disruption of the ovomucin-lysozyme complex, which is known to negatively affect albumen consistency (Dogan et al., 2018), ultimately leading to a reduction in egg quality. Despite this, eggs at 28 d of storage quail eggs could be categorised within the best grade, amino acids, in accordance with the classification proposed for hen eggs (Caner and Yüceer, 2015), where Haugh unit >72 is categorised as amino acids grade, 72−60 as A grade, 59−31 as B grade, and <30 as C grade. The progressive attenuation of yolk colour traits during storage is also a natural result of chemical, enzymatic and physical degradation processes for which

also xanthophylls and carotenoids, the main yolk pigments naturally contained into some feed ingredients (i.e. corn and TM), are progressively degraded (Omri et al., 2019).

The supplementation of antioxidant-rich feeding substrates during the laying period reduces egg susceptibility to the oxidative phenomenon, thus offering the potential to increase the oxidative stability of poultry products. In the present study, at d 0 storage, eggs of the TM group displayed lower TBARs values than Control ones, which was unexpected. These differences, however, disappeared at d 28 of storage as the oxidative degree of the Control eggs decreased with storage time, which was also unexpected. As highlighted in previous studies (Finke, 2002; Secci et al., 2018), insect larvae contain tocopherols and b-carotene, which are known for their antioxidant activity and could have potentially improved to oxidative status of TM eggs compared to Control ones along the storage period. This would have also been coherent with the result of Dalle Zotte et al. (2019), where eggs of quails fed with black soldier fly larvae meal exhibited a lower oxidative rate compared to Control ones at the end of a 28-d storage trial. A hypothesis to explain why eggs of the Control group at d 0 of storage displayed the highest TBARs value could be attributed to a limitation of the TBARs test. Specifically, pigments (insect-derived, but more in general derived from feed ingredients) could have interacted with the chromatic reaction resulting from the TBA and the oxidation products, therefore altering the reading at the spectrophotometer. This interference had already been observed when a rooibos (Aspalathus linearis) extract was added in the manufacturing of rabbit meat patties (Cullere et al., 2019), but in that case, a post-mortem additive was considered, and an alteration of the colour of the product was visually evident, which was not the case of the present study. For these reasons, further investigations in to explain present findings about the TBARs values are necessary and caution is suggested to infer on present results. The sensory profile of a food product is a key aspect to ensure consumer liking and thus a successful market placement. In the present research it was observed that TM 10 eggs had an overall milder sensory profile than Control ones, mainly due to lower sulphur and greasy-oily flavours perceptions. This being the first study considering the possible effects of live TM larvae as feed for laying quails on the sensory traits of their eggs, no direct literature comparisons can be performed. A recent research analysing the sensory characteristics of raw and cooked TM larvae (Seo et al., 2020), depicted that the aroma of raw mealworms is primarily composed of hydrocarbons (50.11%) and aldehydes (37.14%), with 4- methyl benzaldehyde being the prevalent individual component. These corresponded to a strong wet-soil-like notes but mild oily, shrimp-like, and sweet-corn-like notes, thus possibly explaining why the overall sensory traits of TM 10 eggs of the present trial did not display peculiar sensory attributes compared to the Control ones. In another study considering the effect of increasing dietary inclusion levels of *Hermetia illucens* meal (10 and 15%) on the sensory characteristics of quail eggs (Dalle Zotte et al., 2019), no relevant changes in the overall sensory profile were observed as well as on specific off-odours and off-flavours perception, with the sole exception of the “feed” off-flavour, which linearly increased with the *Hermetia illucens* meal inclusion level.

Conclusions

Results of the present experiment indicated that supplementing live TM larvae to laying quails at 10% of their daily FI stimulated feeding activity and did not pose any drawback on productive performance and mortality rate, as a result of a satisfactory nutritive value of the TM 10 diet. Also, egg physicochemical quality, sensory characteristics and shelf-life were comparable with conventional eggs, which is a key factor to ensure both health and consumer expectations. The sole administration of live TM larvae allowed the quantification of the specific nutritive value of this emerging feedstuff, which is of pivotal importance to optimise feed formulations for poultry. To complete the scientific evaluation of this emerging nutritional enrichment for quails, future research could be directed in assessing the potential beneficial effect of TM 10 supplementation on quail's gut health, similarly to what it was conducted on broiler chickens, to understand if a 10% supplementation level is adequate or if lower levels provide similar outcomes. In addition, a deeper evaluation of the value of TM as an enrichment tool should be considered.

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General conclusions

The present PhD thesis investigated different aspects of TM farming with the aim of optimising large-scale rearing conditions, increasing production efficiency, improving processing quality, and exploring its potential applications in animal feeding. This objective was addressed through five main research objectives: (*i*) to determine the optimal breeding density of TM adults from an industrial perspective; (*ii*) to examine the role of beetle size in reproductive success, fecundity, larval traits, and the heritability of these characteristics; (*iii*) to enhance the nutritional value of TM larvae through dietary supplementation with camelina and linseed cakes; (*iv*) to identify the most effective combination of killing and drying methods for obtaining larvae with high microbiological safety, nutritional value, and consumer acceptance; and (*v*) to evaluate the effects of supplementing live TM larvae on productive performance, nutrient digestibility, egg quality, and sensory traits in laying quails.

Results obtained in the fifth contributions highlight that:

I. adult breeding density, tested between 0.8 and 1.6 adults/cm², did not affect beetles mortality, but markedly influenced production efficiency. At 0.8 adults/cm², higher larval production per adult and improved FCR were observed, while 1.6 adults/cm² resulted in greater larval yield per crate and higher final larval weight. From an industrial perspective, the latter density appears to maximise overall farm productivity;

II. beetle size proved to be an important factor in reproductive success and progeny traits. Larger beetles were more attractive mates and produced larvae that maintained a higher BW up to 8 weeks of age. Heritability estimates indicated a moderate genetic basis for this trait, with paternal size exerting the strongest effect. Although selection for large beetles would require refinement of sorting practices to reduce costs, these findings suggest that size-based selection could be a useful tool for long-term optimisation;

III. dietary supplementation with oilseed cakes demonstrated clear potential to improve larval nutritional value. Inclusion of 10% camelina cake enhanced n-3 PUFA deposition and improved the n-6/n-3 ratio without affecting performance, whereas 10% linseed cake (LIN 10) improved the FA profile but reduced growth and increased susceptibility to lipid oxidation. Despite these drawbacks, larvae from the LIN 10 group were well appreciated by consumers, confirming that oilseed cakes can both valorise agro-industrial by-products and enrich the nutritional and sensory profile of mealworms;

IV. processing methods had a profound impact on product quality. The combination of cryogenic freezing and drying in controlled atmosphere preserved larval colour but resulted in higher water activity and microbial loads, making it unsuitable. In contrast, blanching followed by oven or microwave drying and freezing followed by microwave drying improved nutritional composition, reduced microbial risks, and were better accepted by consumers. Among these, blanching or freezing combined with microwave drying offered the best balance between safety, nutritional value, and sensory quality;

V. supplementation of live larvae at 10% of daily FI in laying quails stimulated feeding activity and had no adverse effects on mortality, productivity, or egg quality. Eggs from supplemented birds were

comparable to controls in terms of physicochemical, sensory, and shelf-life characteristics, supporting the feasibility of TM as a safe alternative feed ingredient. Future investigations should explore potential benefits for gut health and assess whether lower supplementation levels may yield similar positive outcomes.

The present thesis evidenced the possibility to optimise TM farming through targeted strategies acting at different levels of the production chain, from rearing density and parental selection to dietary enrichment, processing techniques and application in animal feeding. The outcomes confirmed the potential of TM as a sustainable protein and lipid source for both food and feed, while also highlighting some critical aspects that still limit its large-scale integration. In particular, the economic trade-off between density and efficiency, the operational challenges of size-based selection, and the balance between nutritional enhancement and oxidative stability of enriched larvae represent areas that require further refinement. Future investigations should focus on combining genetic selection with nutritional strategies, as well as on developing innovative processing technologies capable of maximising product safety, stability and consumer acceptance. Moreover, the evaluation of TM in animal nutrition should be extended to different livestock species and production systems, in order to fully exploit its role in sustainable farming and in circular economy models.

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