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RESEARCH ARTICLE

Local agricultural by-products for rearing superworm larvae (*Zophobas morio*): a Greek example

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Abstract

As the industrial insect production sector is rapidly growing, the high production cost of insects forms a constraint for the further growth of the insect industry. One of the main factors that greatly contributes to the total insect production cost and affects the profitability of insect farms is feed costs. Various agricultural by-products have been successfully evaluated and proposed as low-cost, alternative, insect feedstocks. In this context, this study evaluates a variety of Greek agricultural by-products as feeding substrates for *Zophobas morio* (F.) (Coleoptera: Tenebrionidae) larvae. More specifically, eleven agricultural by-products derived from the seed cleaning process of barley, oats, peas and vetch and the production of cotton, sugar beet and sunflower, were utilized singly and as components of compound isonitrogenous diets at two nitrogen levels (2.7% and 3.2% on dry matter basis). The results showed that larvae reared on sunflower meal, oat and barley (class II) by-products performed well in terms of larval growth and survival. In contrast, for most other diets tested high mortality rates were recorded, indicating the necessity for the formulation of diets that meet the nutritional requirements of the larvae. Our study highlights the potential of locally available by-products for the rearing of *Z. morio* larvae and contributes to determining their nutrient requirements.

Keywords

edible insects – feed conversion efficiency – insects as food and feed – larval development – nutritional value

1 Introduction

The edible insect production sector is rapidly growing and projected to grow from 2022 to 2030 at a compound annual growth rate of 28.3% in terms of value to exceed US $9.6 billion by 2030 (Meticulous Market Research, 2022). One constraint for further adoption of insects as food and feed, and subsequent growth of the insect industry is the high production cost of insect-based products (Arru *et al.*, 2019; Cadinu *et al.*, 2020).

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Apart from labour and energy costs, feed cost represents the largest portion of production cost affecting the profitability of insect farms (Niyonsaba *et al.*, 2021; Van Huis *et al.*, 2021). Therefore, a considerable amount of research is directed at identifying suitable, low-cost by-products and organic wastes suitable as insect feedstock (Fowles and Nansen, 2020; Ojha *et al.*, 2020). Agricultural side-streams and wastes represent a source of largely unexploited resources (Duque-Acevedo *et al.*, 2020) that could be efficiently valorized and upcycled through insect rearing (Van Peer *et al.*, 2021). This would increase the sustainability and circularity of the insect-producing systems (Cadinu *et al.*, 2020) and reduce production costs (Ites *et al.*, 2020). Insect bioconversion of wastes and side-streams has been investigated for several insect species. So far, the scientific and the industrial community have primarily focused on the black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) (Gligorescu *et al.*, 2020; Raksasat *et al.*, 2020; Galassi *et al.*, 2021; Schreven *et al.*, 2021), and the yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) (Oonincx *et al.*, 2015; Van Broekhoven *et al.*, 2015; Silva *et al.*, 2021; Van Peer *et al.*, 2021; Montalbán *et al.*, 2022).

Another species with great potential as a nutrient source is the superworm, *Zophobas morio* (F.) (Coleoptera: Tenebrionidae) (Rumbos and Athanassiou, 2021). Larvae of *Z. morio* are rich in nutrients with high protein and lipid contents, as well as minerals and vitamins (Finke, 2015; Araújo *et al.*, 2019; Dragojlović *et al.*, 2022). *Zophobas morio* has also been widely tested as a feed ingredient due to its high nutritional value and is considered a promising nutrient source for aquafeeds (Fontes *et al.*, 2019; Alves *et al.*, 2020; Mikołajczak *et al.*, 2020; Prachom *et al.*, 2021; Chainark *et al.*, 2022), poultry diets (Kierończyk *et al.*, 2018; Benzertiha *et al.*, 2019; 2020; Józefiak *et al.*, 2020), and ruminants (Toral *et al.*, 2022). Wheat bran and related amylaceous commodities are commonly used for its rearing (Quennedey *et al.*, 1995; Rumbos and Athanassiou, 2021). However, there is potential to rear this species on diets composed of organic wastes and by-products (Van Broekhoven *et al.*, 2015, Harsányi *et al.*, 2020). For instance, Van Broekhoven *et al.* (2015) evaluated the growth of *Z. morio* larvae fed diets based on by-products of the beer brewing, bread/cookie baking, potato processing and bioethanol production and reported variable larval performance and feed conversion efficiency for the various diets tested. High variability in *Z. morio* larval growth has been reported also by Harsányi *et al.* (2020) who evaluated vegetable and garden waste, as well as cattle and horse manure. The nutritional requirements of *Z. morio* larvae are largely unknown. One recent study suggests an optimal protein:carbohydrate ratio between 1.5:1 and 2:1 (Choi and Lee, 2022). However, it is well possible that the carbohydrate source used (sucrose) was not so suitable for *Z. morio* larvae, leading to an overestimate of their protein requirement. This should be verified in further studies to better our understanding of the nutritional needs of *Z. morio* larvae. Besides this fundamental approach, more applied studies evaluating the suitability of certain substrates are needed for sector development. Concerning the origin of insect feeding substrates, local agricultural by-products represent a choice with good perspective. Through the utilization of locally produced feed, the insect production systems may benefit economically, while reducing their environmental impact.

Hence, the present study evaluates a variety of Greek agricultural by-products, singly and as components of compound isonitrogenous diets, as feeding substrates for *Z. morio* larvae by determining growth performance and chemical composition. Compound diets were tested as these often lead to better performance via nutrient compensation.

2 Materials and methods

Insects

Larvae of *Z. morio* were purchased in the beginning of 2018 from a local retailer (FEEDERS, Thessaloniki, Greece). Colonies of *Z. morio* were then setup and maintained at the Laboratory of Entomology and Agricultural Zoology in the University of Thessaly continuously for two years until the beginning of experimentation. Their diet comprised of 90% wheat bran (purchased from a local store) and 10% dry instant yeast (Angel Yeast Co. Ltd., Yichang, China), supplemented with fresh potato slices twice a week, which served as moisture source. As *Z. morio* larvae require solitude to pupate (Tschinkel and Willson, 1971), late-instar larvae were individually placed in cylindrical plastic containers (85 mm × 30 mm) with a 15 mm opening on the lid covered with muslin gauze for air exchange. After eclosion, adults were kept in plastic boxes (48 cm length × 28 cm width × 10 cm height) with a rectangular screened opening (19 cm × 27 cm) on the top cover to allow air circulation. They were provided with the same diet as the larvae, egg cartons for shelter and paper towel cardboard tubes (22 cm length, 5 cm diameter) to oviposit. The tubes with eggs were removed and placed

TABLE 1 Proximate composition (%DM), energy content (Kj/g DM) and cost (€/ton) of a control diet and eleven by-products based on duplicate analysis

By-product	Dry matter (%)	Nitrogen (% DM)	Lipids (% DM)	Ash (% DM)	Energy (Kj/g)	Price (€/ton)	Supplier
Wheat bran (control)	86.7	2.7	2.2	4.6	16.7	170	Mallias N., Bros O.E.
Sugar beet pulp meal [1]	93.7	4.4	1.8	7.5	17.3	210	Mallias N., Bros O.E.
Cotton cake	91.0	4.2	6.8	4.5	18.5	240	Mallias N., Bros O.E.
Cotton seed meal	92.0	4.1	14.0	4.2	20.0	350	Local farmer (Region of Thessaly)
Sunflower meal [1]	91.7	4.4	1.6	7.0	17.1	220	Mallias, N., Bros O.E.
Barley byproduct (class I) [2]	90.3	1.3	0.6	13.5	14.5	100	Fyto-Animal Services
Barley byproduct (class II) [3]	88.6	1.9	0.6	3.3	16.2	140	Fyto-Animal Services
Oat byproduct [4]	91.2	3.1	3.4	9.3	16.9	120	Fyto-Animal Services
Pea byproduct (class I) [5]	91.0	1.7	0.0	23.1	10.9	100	Fyto-Animal Services
Pea byproduct (class II) [6]	91.0	4.1	1.2	4.6	16.3	220	Fyto-Animal Services
Vetch byproduct (class I) [7]	89.5	3.5	3.0	11.8	15.1	100	Fyto-Animal Services
Vetch byproduct (class II) [8]	91.9	4.0	1.0	11.4	14.7	270	Fyto-Animal Services
Yeast (control ingredient)	97.1	8.0	1.7	6.7	19.9	8000	Angel Yeast Co., Ltd

[1]From ground pellets; [2]Remains in the sieves of the barley seed cleaner during the seed cleaning process; [3]Remains of the barley seed cleaning process (beard hairs, husks and other non-seed materials); [4]Remains of the oat seed cleaning process (beard hairs, husks, small seeds, etc.); [5]Remains of the pea seed cleaning process (bean pods and other non-seed materials, few small and broken seeds); [6]Remains of the pea seed cleaning process (small, broken seeds, etc.); [7]Remains of the vetch seed cleaning process (bean pods and other non-seed materials, small and broken seeds); [8]Remains of the vetch seed cleaning process (small and broken seeds).

in separate containers with 50 g of white wheat flour (Loulis Mills, Organic category M soft wheat flour) to acquire newly-hatched larvae. Larvae passing through a 500 µm-openings sieve and remaining within a 250 µm-openings sieve were selected for experimentation. This fraction includes *Z. morio* larvae between the first and third instar based on their body length (Kim *et al.*, 2015). All rearing and experimental boxes were kept at 26 ± 1 °C, 55 ± 5% relative humidity (RH) under continuous darkness.

By-products

Eleven agricultural by-products, i.e, side-streams of the seed cleaning process of barley, oats, peas and vetch and the production of cotton, sugar beet and sunflower were evaluated via two different bioassays. A detailed description including their nutritional composition and price is provided in Table 1. These by-products were selected due to their high local availability, as they stem from major crops largely cultivated in Greece. Indicatively, Greece is the main EU cotton (*Gossypium hirsutum* L.) grower, with 80% of European cotton area, and a major cotton exporter (USDA, 2023; EC, 2024). Prior to experimentation, by-products were ground (Thermomix TM31-1C, Vorwerk Elektrowerke GmbH & Co. K, Wuppertal, Germany), sieved by hand through a 0.5-mm sieve and kept under experimental conditions (26 ± 1 °C, 55 ± 5% RH) for 7 d, to equilibrate with the relative humidity level.

Bioassay I – single by-products

Plastic cylindrical vials (Rotilabo®-sample tins with snap-on lid; 7.5 cm in diameter, 8.8 cm in height; Carl Roth Gmbh & Co. Kg, Karlsruhe, Germany) were filled with 4 g of each by-product and a group of 20 newly-hatched larvae was added. Each group of larvae was

TABLE 2 Inclusion percentages of by-products in two sets of compound isonitrogenous by-product diets (A = 2.7% N; B = 3.2% N)

By-product	Diet									
	A1 (control A)	A2	A3	A4	A5	B1 (control B)	B2	B3	B4	B5
Wheat bran	100					90				
Barley by-product (class I)			41.0	49.0				15.0	30.0	
Barley by-product (class II)		55.0			44.5		31.5			10.0
Oat by-product			23.0		27.5			42.0		28.0
Pea by-product (class I)										
Pea by-product (class II)		9.0		25.5			38.0		39.0	
Vetch by-product (class I)		36.0			28.0		30.5			62.0
Vetch by-product (class II)			36.0	25.5				43.0	31.0	
Yeast						10				

TABLE 3 Proximate composition as a % of dry weight basis and energy content (Kj/g DM) of two sets of compound isonitrogenous by-product diets (A and B)

Diet	Dry matter (%)	Nitrogen (% DM)	Lipids (% DM)	Ash (% DM)	Energy (Kj/g)
A1	86.7	2.7	2.2	4.6	16.7
A2	89.2	2.7	1.5	6.5	15.8
A3	91.1	2.7	1.3	11.8	15.1
A4	90.9	2.7	0.9	10.5	15.0
A5	89.6	2.7	1.9	7.4	16.1
B1	87.7	3.2	2.2	4.8	17.0
B2	89.8	3.2	1.6	6.2	15.9
B3	91.4	3.2	1.8	10.8	15.6
B4	91.1	3.2	1.0	9.2	15.3
B5	89.9	3.2	2.8	10.3	15.7

weighed at the beginning of the bioassay and their weight was recorded. Larvae were provided with fresh carrot slices (0.6 ± 0.1 g) three times per week, and old carrot pieces were removed. A mixture of wheat bran with dry yeast (9:1) served as a control. Each dietary treatment was replicated six times. Larvae were allowed to feed undisturbed *ad libitum* for four weeks. After this interval, all vials were routinely monitored three times per week to prevent larvae from running out of feed. If feed was totally consumed, new feed was weighed, added and recorded. After 4 weeks, larval weight as a group and survival were determined bi-weekly until 50% of the larvae reached or exceeded a body length of at least 5 cm, as previously described (Van Broekhoven *et al.*, 2015). After harvesting, larvae were fasted for 24 h, killed by freezing and kept at −20 °C until further analysis. Development time, feed utilization parameters, growth rate and feed costs per ton of weight gain were assessed (see section "Calculation of indices").

Bioassay II – composed isonitrogenous by-product diets

Two sets of isonitrogenous diets were composed at two N levels (2.7% and 3.2% N) with selected by-products previously evaluated in Bioassay I, as shown in Tables 2 and 3. The N levels correspond to the N level of wheat bran (2.7%), which is a feedstock commonly used for *Z. morio* rearing (Rumbos and Athanassiou, 2021) and the respective level of a 9:1 wheat bran:yeast mixture (3.2%). Wheat bran alone or mixed with dry yeast (9:1) were used as control for set A and B, respectively. The same experimental procedure described for Bioassay I was followed.

Proximate composition

Proximate composition and energy content of *Z. morio* larvae were determined based on methods described in AOAC (1995). Dry matter content (DM) was determined by drying in an oven at 105 °C until constant weight. Nitrogen content was determined by Kjeldahl (Behr Labor-Technik GmbH, Düsseldorf, Germany, K12-block standard digestion system, Programmable infrared digestion device, S4 distillation unit), whereas crude fat was determined by exhaustive Soxhlet extraction using petroleum ether (40-60 °C, BP) using a Soxtherm Multistat/SX PC (Sox-416 Macro, C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Ash content was determined by dry ashing in porcelain crucibles in a muffle furnace (Nabertherm L9/12/ C6, Lilienthal, Germany) at 600 °C for 5 hr, and gross energy content was determined adiabatically using an IKA oxygen bomb calorimeter (C5000; IKA Werke GmbH, Staufen, Germany).

Calculation of indices

The number of live larvae at each evaluation interval was divided by the initial number of larvae to calculate the survival rate. As development time, we calculated the time from the initiation of the bioassays until the termination of the trial for each vial, namely when 50% of the larvae in each vial reached 5 cm body length. For feed utilization parameters, it was assumed that all provided feed was consumed, whereas the weight of provided carrots was excluded from the calculations. The following equations were used to calculate feed conversion and growth parameters (Waldbauer, 1968):

1. Feed conversion ratio (FCR; on fresh weight) = Feed provided/Live weight gained
2. Efficiency of Conversion of Ingested food (ECI; on dry weight) = (Weight gained/Feed ingested) × 100%
3. Specific Growth Rate (SGR; on fresh weight) = 100 × (ln Final Body weight – ln Initial body weight)/days, as previously described by Psofakis *et al.* (2020)
4. Nitrogen efficiency (N-ECI; on dry weight) (Amount of N in the insect at harvest/Dietary N provided during the experiment) × 100%

We refer intentionally to nitrogen rather than crude protein, as no nitrogen-to-protein conversion factor (Kp) has been proposed for *Z. morio* individuals and the Jones' default conversion factor of 6.25 that is routinely used is inaccurate for most feed materials and insects (Mariotti *et al.*, 2008; Janssen *et al.*, 2017; Boulos *et al.*, 2020).

The Economic Conversion Ratio (ECR), namely the feed cost per kilogram of live larvae, was calculated by multiplying the FCR with the feed cost per ton (Psofakis *et al.*, 2020). In Bioassay II, ECR was calculated based on the inclusion level of each by-product in each diet.

Statistical analysis

Data were first checked for normality and homogeneity of variances using Shapiro-Wilk and Levene's tests, respectively. Since bioassay I data violated the assumptions of parametric analysis, a non-parametric test was used to detect whether there were statistically significant differences among treatments. Specifically, significant differences among treatments for chemical analysis of larvae reared on the by-products (i.e. dry matter, nitrogen, lipids, and energy), final larval survival, FCR, ECI, N-ECI, SGR and ECR were detected via the Kruskal-Wallis H test at a significance level of 0.05, followed by Dunn multiple comparisons for post-hoc testing. The Kaplan-Meier method was applied to analyze development time and a Mantel-Cox test was used to determine differences among dietary treatments. The Pearson correlation test was used to determine correlations between development time and feed conversion parameters (FCR, ECI, N-ECI and SGR). Concerning bioassay II, data for chemical analysis for larvae reared on the compound diets (i.e. dry matter and lipids) met the assumptions for parametric analysis and therefore were submitted to ANOVA to determine differences among diets ($P < 0.05$). Additionally, bioassay II data for final larval survival, FCR, ECI, N-ECI, SGR and ECR that did not meet the assumptions for parametric analysis, were analysed as previously reported for bioassay I data. All analyses were performed using SPSS 26.0 (IBM Corporation, Armonk, NY, USA).

3 Results

Bioassay I – single by-products

Single by-products led to a high variation in larval survival and growth among the by-products tested (Figures 1 and 2). Apart from the wheat bran & yeast control, barley (class II), oat by-product, and sunflower meal resulted in high survival rates (>67% at the end of the bioassay; Figure 1). In contrast, for vetch by-product (class I), sugar beet pulp meal and cotton seed meal reduced survival was apparent at the first evaluation interval (4 weeks) and was further reduced at the end of the bioassay (<10%). No larvae survived on pea (class I and II) and vetch (class II) by-product. The final weight of larvae provided with barley by-products (class I and II) and sunflower meal was similar to the control, attaining over 652 mg of live weight per larva (Figure 2). Dietary treatment affected larval development time (Mantel-Cox $\chi^2 = 39.3$, df = 7, $P < 0.001$) and varied between 144 (control) and 199 d (cotton cake) (Table 4). Larvae developed as fast on sunflower meal (152 d) as on the control, and slightly slower on barley by-products (class I, 170 d; class II, 171 d) and oat by-product (174 d).

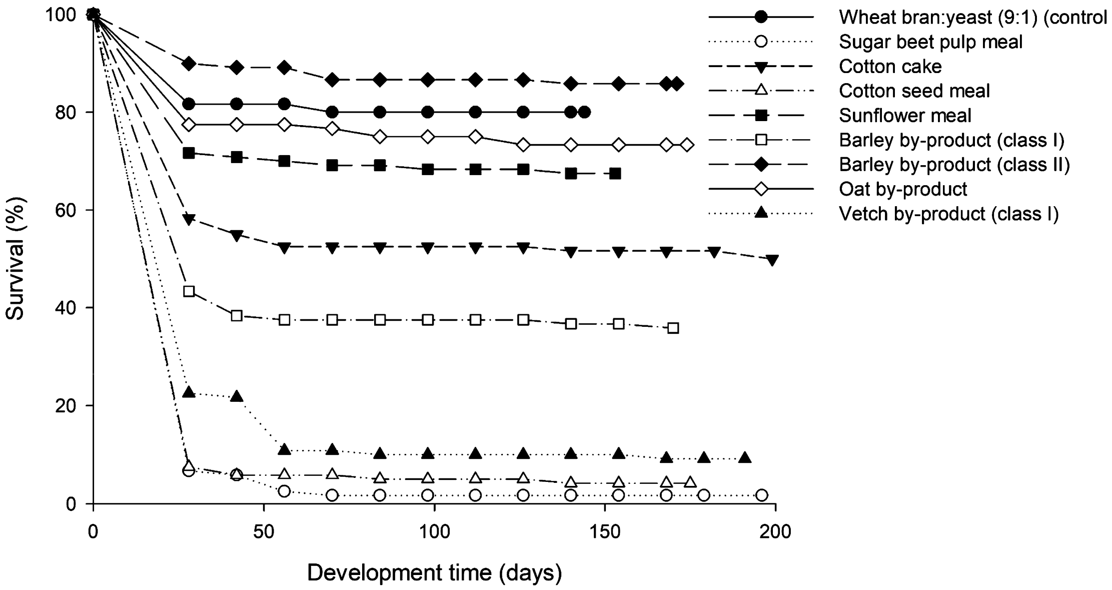

FIGURE 1 Survival rate (%) of *Zophobas morio* larvae reared on an agricultural by-product or a mixture of wheat bran with dry yeast (9:1, control) (Bioassay I). In all cases, values represent means (n = 6).

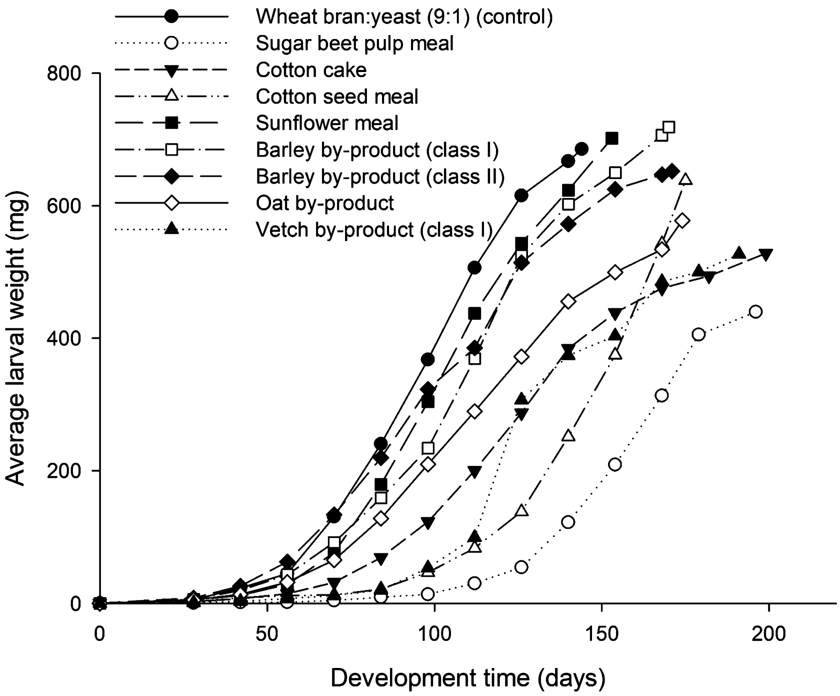

FIGURE 2 Average larval weight (mg) of *Zophobas morio* larvae reared on an agricultural by-product or a mixture of wheat bran with dry yeast (9:1, control) (Bioassay I).

TABLE 4 Development time (days), Feed Conversion Ratio (FCR), Dry matter Efficiency of Ingested Food Conversion (ECI, %), Nitrogen Conversion Efficiency (N-ECI, %), Specific Growth Rate (%) and Economic Conversion Ratio (ECR, (€ ton^{-1} larvae) of *Zophobas morio* larvae reared on an agricultural by-product or a mixture of wheat bran with dry yeast (9:1, control) (Bioassay I)

Substrate	Final survival (%)	Development time (days)	FCR	ECI	N-ECI	SGR (%/day)	ECR (€ ton^{-1} larvae)
Wheat bran:yeast (9:1) (control)	80.0 ± 6.1[ab]	144.3 ± 4.6[c]	2.4 ± 0.1[c]	19.0 ± 0.5[ab]	42.3 ± 1.1[ab]	5.3 ± 0.2[a]	2598 ± 45[c]
Sugar beet pulp meal	1.7 ± 1.7[c]	196.0	18.5	1.8	3.5	3.4	5718
Cotton cake	50.0 ± 9.5[ab]	199.7 ± 13.7[a]	3.9 ± 0.4[ab]	8.9 ± 1.8[c]	18.0 ± 3.6[c]	3.8 ± 0.3[c]	1309 ± 130[bc]
Cotton seed meal	4.2 ± 1.5[c]	175.0 ± 3.0[ab]	10.5 ± 3.4[a]	4.8 ± 1.3[c]	9.1 ± 2.5[c]	4.3 ± 0.1[c]	3374 ± 1073[c]
Sunflower meal	67.5 ± 5.0[ab]	152.7 ± 8.1[bc]	3.1 ± 0.2[bc]	13.5 ± 0.7[bc]	25.3 ± 1.4[bc]	5.0 ± 0.2[ab]	680 ± 50[a]
Barley by-product (class I)	35.8 ± 4.0[bc]	170.0 ± 3.9[b]	7.4 ± 0.6[a]	5.7 ± 0.4[c]	30.2 ± 2.2[bc]	4.5 ± 0.1[abc]	1489 ± 120[b]
Barley by-product (class II)	85.3 ± 4.5[a]	171.0 ± 1.3[b]	2.8 ± 0.1[bc]	17.5 ± 0.6[ab]	49.5 ± 1.8[a]	4.4 ± 0.1[bc]	770 ± 33[ab]
Oat by-product	73.3 ± 5.1[ab]	174.0 ± 7.3[ab]	2.4 ± 0.[c]	19.2 ± 1.1[a]	39.9 ± 2.2[ab]	4.3 ± 0.1[c]	666 ± 37[a]
Pea by-product (class II)	0.0 ± 0.0[d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pea by-product (class II)	0.0 ± 0.0[d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Vetch by-product (class I)	9.2 ± 8.2[cd]	191.5 ± 18.5[ab]	7.8 ± 5.0[ab]	14.8 ± 9.4[abc]	n.d.	3.7 ± 0.5[c]	1018 ± 644[ab]
Vetch by-product (class II)	0.0 ± 0.0[d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Within each column, means followed by the same lowercase letter are not significantly different. In all cases, values represent means ± SEM [for Final larval survival: n = 6; for the rest of the parameters n = 6, with the exception of sugar beet pulp meal (n = 1, excluded from the statistical analysis), cotton seed meal (n = 4) and vetch by-product (class I) (n = 2)]; n.d.: not determined.

Feed utilisation parameters varied considerably among dietary treatments (Table 4). For oat and barley (class II) by-products, as well as for sunflower meal, the recorded FCR was similar to the control, ranging between 2.4 and 3.1, indicating a good feed utilization. If carrot consumption was taken into account, FCR values would have been slightly higher, as previously shown (Van Broekhoven *et al.*, 2015). Faster growth, expressed as a shorter development time, correlated with higher N-ECI values ($r = -0.425$, $P = 0.001$), indicating that higher nitrogen utilization reduces development time. Lower FCR values correlated with lower ECR values ($r = 0.686$, $P < 0.001$), suggesting that higher feed conversion efficiency decreases feed costs. The dry matter content of *Z. morio* larvae reared on the tested by-products varied considerably among treatments and ranged between 31.7 and 42.3%. Similar plasticity was recorded regarding the nitrogen (5.5-8.5% DM), lipid (27.8-46.5% DM) and ash content (2.4-3.9% DM), as well as larval gross energy content (26.0-30.9 Kj/g DM) (Table 5).

Bioassay II – composed isonitrogenous by-product diets
Larval survival and growth varied considerably among the diets tested, the highest survival rates being recorded for the controls (A1 and B1) (Figures 3 and 4). All larvae fed the B4 diet died within 4 weeks. Similarly for the other diets, most of the mortality occurred during the first 4 weeks after which most specimens survived. The growth of larvae reared on most of the Group A diets followed a similar to the control pattern over time, while larvae reared on A4 diet gained less weight. Concerning the larvae reared on diets of Group B, variation in growth was distinct (Figure 4). The larval development time was affected by diet (Mantel-Cox $\chi^2 = 32.8$, df =

TABLE 5 Proximate composition (g/kg diet on dry weight basis) (dry matter, nitrogen, lipid and ash content) and energy content (Kj/g DM) of Zophobas morio larvae reared on an agricultural by-product or a mixture of wheat bran with dry yeast (9:1, control) (Bioassay I)

Substrate	Dry matter (%)	Nitrogen (% DM)	Lipids (% DM)	Ash (% DM)	Energy (Kj/g)
Wheat bran:yeast (9:1) (control)	40.2 ± 0.4^{abc}	7.1 ± 0.1^{ab}	38.2 ± 0.5^{ab}	3.3 ± 0.1	28.6 ± 0.3^{ab}
Sugar beet pulp meal	32.0	8.5*	n.d.	n.d.	n.d.
Cotton cake	28.4 ±^{d}	8.5 ± 0.3^{a}	30.1 ± 3.5^{b}	3.9** ± 0.3	26.0 ± 0.2^{b}
Cotton seed meal	39.1 ± 1.5^{bcd}	7.8*	29.8*	n.d.	n.d.
Sunflower meal	37.4 ± 1.1^{cd}	8.3 ± 0.3^{a}	27.8 ± 4.4^{b}	3.7 ± 0.5	27.1 ± 0.3^{b}
Barley by-product (class I)	37.0 ± 0.4^{d}	6.8 ± 0.2^{ab}	44.5 ± 4.1^{a}	2.5*	28.4 ± 0.4^{ab}
Barley by-product (class II)	42.3 ± 0.4^{a}	5.5** ± 0.0^{b}	46.5 ± 7.8^{a}	2.4 ± 0.3	30.9 ± 0.1^{a}
Oat by-product	41.1 ± 0.5^{ab}	6.4 ± 0.1^{b}	41.7 ± 1.2^{a}	3.4** + 0.1	29.5 ± 0.1^{a}

n.d. = not determined due to insufficient sample. Values are presented as means [for DM n = 6, with the exception of sugar beet pulp meal (n = 1), barley by-product (class I) (n = 5) and cotton seed meal (n = 3); for the rest of the variables n = 3 unless indicated with one (*, n = 1) or two asterisks (**, n = 2)].

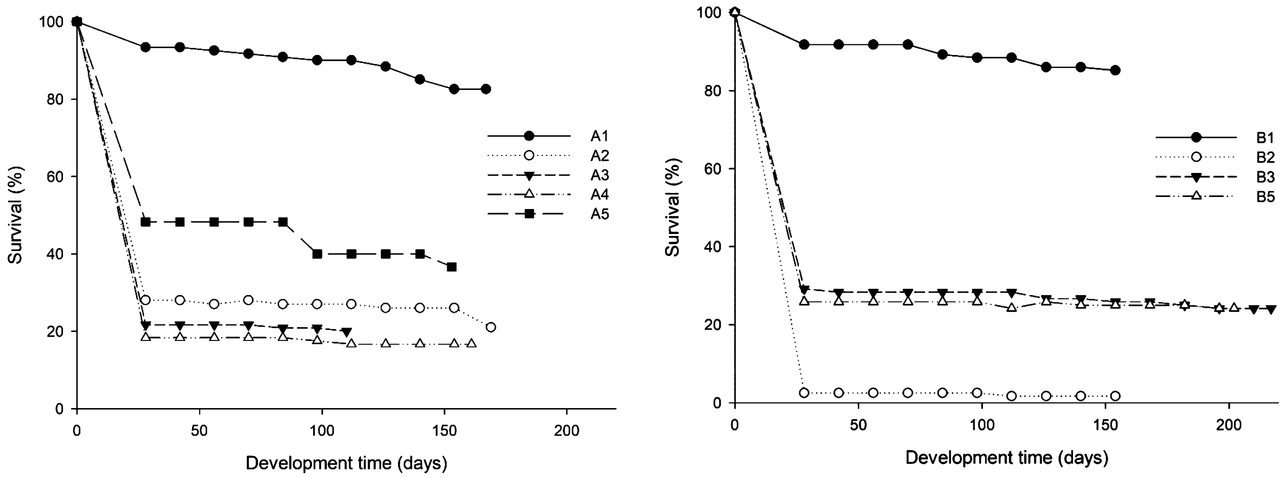

FIGURE 3 Survival rate (%) of *Zophobas morio* larvae reared on two groups of compound isonitrogenous by-product diets (A = 2.7% N, B = 3.2% N). In all cases, values represent means ± SEM (Bioassay II) (n = 6). Refer to Table 2 for the composition of diets A1-A5 and B1-B5.

7, $P < 0.001$) and varied between 153 and 217 d over treatments (Table 6). In general, higher FCR values were recorded for the compound by-product diets compared to controls, showing a lower feed conversion efficiency (Table 6). Lower FCR values correlated with lower ECR values (r = 0.892, $P < 0.001$), indicating that higher feed utilization efficiency decreases feed cost. The proximate composition of the larvae fed the different diets tested is shown in Table 7. The nitrogen content varied between 6.9 and 8.4% DM, whereas lipid content fluctuated between 27.7 and 37.3%. Marginal differences were reported only for dry matter and lipid content.

Discussion

In the pursuit of sustainably producing insects as food and feed, the valorisation of agricultural by-products as cost-effective and environmentally friendly feeding substrates could serve as a pivotal strategy. The advantages of that strategy lie in the affordability of agricultural by-products, and the promotion of sustainable waste management. Thus, in the present study, agricultural by-products were utilized as feeding substrates for *Z. morio*. There was considerable variation among the by-products tested when they were provided singly; the best results in terms of larval survival and growth were obtained for sunflower meal, oat and barley (class II) by-products. Clearly, these have good potential as feed for

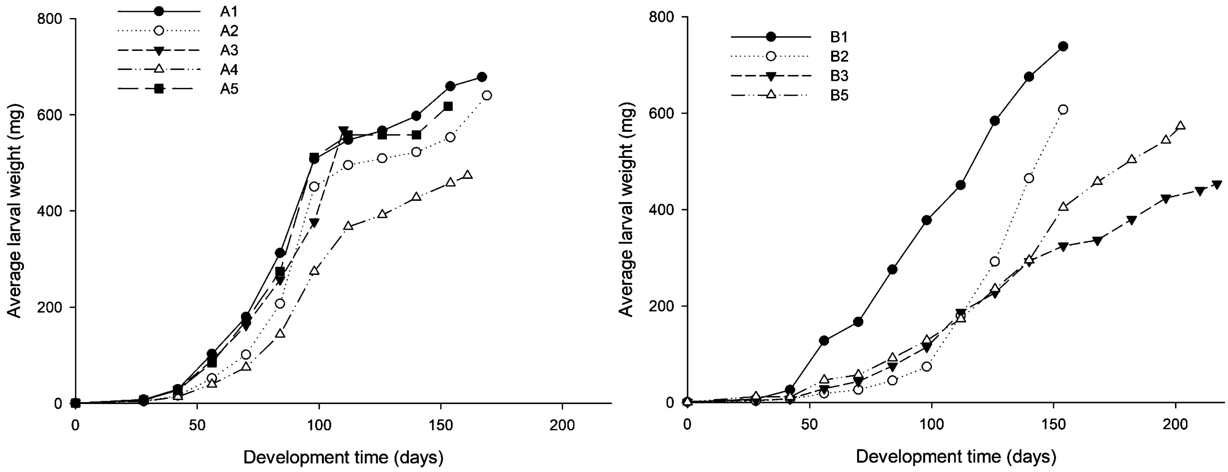

FIGURE 4 Average larval weight (mg) of *Zophobas morio* larvae reared on two groups of compound isonitrogenous by-product diets (A = 2.7% N, B = 3.2% N). Values presented as means ± SEM (n = 6) (Bioassay II). Refer to Table 2 for the composition of diets A1-A5 and B1-B5.

TABLE 6 Development time (days), Feed Conversion Ratio (FCR), Dry matter Efficiency of Ingested Food Conversion (ECI, %), Nitrogen Conversion Efficiency (N-ECI, %), Specific Growth Rate (%) and Economic Conversion Ratio (ECR, (€ ton^{-1} larvae) of *Zophobas morio* larvae reared on two groups of isonitrogenous diets (A = 2.7% N, B = 3.2% N). (Bioassay II)

Diet	Final survival (%)	Development time (days)	FCR	ECI	N-ECI	SGR (%/day)	ECR (€ ton^{-1} larvae)
A1	82.6 ± 4.5^{a}	167.0 ± 1.4^{b}	3.7 ± 0.3^{bc}	12.0 ± 1.5^{ab}	36.0 ± 4.4^{a}	4.3 ± 0.1^{ab}	1078 ± 97^{c}
A2	21.0 ± 8.0^{bc}	169.0 ± 1.0^{b}	12.7 ± 6.9^{a}	5.0 ± 1.3^{cd}	13.6 ± 3.7^{bc}	3.4 ± 0.3^{bc}	2797 ± 1531^{abc}
A3	20.0 ± 2.9^{b}	110.8 ± 14.4^{ab}	6.6 ± 1.0^{ab}	6.9 ± 1.0^{bcd}	18.4 ± 2.7^{bc}	5.5 ± 0.6^{a}	1616 ± 243^{abc}
A4	16.7 ± 4.8^{bcd}	161.6 ± 16.6^{ab}	13.1 ± 3.5^{a}	3.8 ± 1.0^{d}	10.5 ± 2.7^{c}	3.4 ± 0.2^{bc}	2913 ± 772^{ab}
A5	36.7 ± 11.0^{b}	153.6 ± 14.9^{ab}	4.6 ± 0.6^{bc}	10.4 ± 1.3^{abc}	29.2 ± 3.7^{ab}	4.2 ± 0.3^{ab}	1089 ± 150^{c}
B1	85.2 ± 3.4^{a}	154.0 ± 0.0^{a}	2.3 ± 0.1^{c}	19.1 ± 0.8^{a}	42.1 ± 1.7^{a}	4.5 ± 0.1^{ab}	2482 ± 96^{a}
B2	1.7 ± 1.7^{cd}	154	6.7	6.1	13.2	3.0	1407.4
B3	24.2 ± 10.8^{bc}	217 ± 13.9^{c}	11.6 ± 4.1^{a}	5.7 ± 1.9^{cd}	14.9 ± 4.9^{bc}	2.3 ± 0.2^{c}	3057 ± 1075^{abc}
B4	0.0 ± 0.0^{d}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B5	24.2 ± 9.1^{b}	202.5 ± 11.2^{c}	8.6 ± 2.4^{ab}	6.7 ± 1.6^{d}	16.0 ± 3.9^{bc}	2.5 ± 0.1^{c}	1615 ± 443^{bc}

Within each column, means followed by the same lowercase letter are not significantly different. In all cases, values represent means ± SEM means [for Final survival: n = 6, for the rest parameters: n = 6, with the exception of B2 (n = 1, excluded from the statistical analysis), A2 (n = 4), A4 and A5 (n = 5)].

superworm larvae. These by-products are also suitable for *T. molitor* rearing (Rumbos *et al*., 2021a), indicating similar nutritional requirements between these two tenebrionid species. Consequently, data collected for *T. molitor* could prove beneficial for subsequent investigations into *Z. morio*. Although a first screening of single by-products can provide useful information on their suitability as insect feedstocks, single by-products may not always cover the insect nutritional requirements. Therefore, composed diets based on the nutritional requirements of each insect species should be designed and evaluated. In this context, in the present study, we tested two groups of compound isonitrogenous diets at two nitrogen levels (2.7% and 3.2% DM) for *Z. morio* larvae. In most cases these diets led to high mortality rates. The same diets, however, were mostly suitable for *T. molitor* larvae (Rumbos *et al*., 2021a). When *Z. morio* larvae were fed diets of various protein:carbohydrate ratios, the best results regarding larval performance were obtained with diets of P:C ratio of 2:1, whereas larval growth dropped drastically for diets with P:C ratio below 1:1, showing a preference for protein-biased diets (Choi and Lee, 2022). In our study all tested diets were carbohydrate-biased, which could partly explain the reduced performance of larvae fed on these diets. Apart from the dietary nutrient qual-

TABLE 7 Proximate composition (g kg^{-1} diet on dry weight basis) (dry matter, protein and lipid content) of *Zophobas morio* larvae reared on reared on two groups of isonitrogenous diets (A = 2.7% N, B = 3.2% N) (Bioassay II)

Diet	Dry matter (%)	Nitrogen (% DM)	Lipids (% DM)	Ash (% DM)	Energy (Kj/g)
A1	36.7 ± 0.5^{ab}	8.0 ± 0.5	34.6 ± 1.1^{a}	2.9 ± 0.1	28.0 ± 0.0
A2	32.2 ± 6.7^{b}	7.2 ± 0.3	37.3*	n.d.	n.d.
A3	37.4 ± 0.6^{ab}	7.1 ± 0.1	37.7 ± 0.3^{a}	2.5 ± 0.0	28.1 ± 0.1
A4	34.2 ± 1.3^{ab}	7.4 ± 0.1	35.5*	n.d.	n.d.
A5	39.5 ± 0.2^{a}	7.5 ± 0.2	37.0 ± 0.9^{a}	2.4 ± 0.2	28.1 ± 0.1
B1	38.9 ± 0.2^{a}	7.0 ± 0.1	37.0 ± 1.3^{a}	3.0 ± 0.6	28.1 ± 0.5
B2	36.7*	6.9*	n.d.	n.d.	n.d.
B3	34.6 ± 1.2^{ab}	8.4*	27.7 ± 0.6^{b}	n.d.	26.1*
B5	37.1 ± 1.3^{ab}	7.6 ± 0.1	33.8 ± 0.5^{a}	2.3*	27.2 ± 0.2

n.d. = not determined due to insufficient sample. Within each column, means followed by the same lowercase letter are not significantly different. Values are presented as means [for DM n = 6, with the exception of B2 (n = 1), A2 (n = 4), A4 and A5 (n = 5); for the rest of the variables n = 2, unless indicated with an asterisk (*, n = 1)].

ity though, the high mortality rates observed for several of the tested diets may also be partly attributed to the strong cannibalistic behavior of *Z. morio* larvae, especially under unfavorable conditions (Tschinkel, 1981; Ichikawa and Kurauchi, 2009). In general, whereas the nutritional requirements for most livestock animals or farmed fish are well studied and defined, this is not the case for most farmed insect species. Therefore, in our opinion, defining the nutrient requirements of the most common edible insects should be one of the first research priorities of the insect sector.

Our results also showed that the vetch by-product, the cotton seed meal and the sugar beet pulp caused high mortality and can be deemed unsuitable. Although not determined in this study, this might be due to the presence of certain antinutritional factors (ANFs). For instance, common vetch seeds contain c-glutamyl-b-cyano-alanine, b-cyano-L-alanine vicine, phenolics, trypsin inhibitor, tannins and convicine, which are neurotoxic to monogastrics (Huang *et al.*, 2017). The high mortality rates reported for larvae of *T. molitor* and the lesser mealworm, *Alphitobius diaperinus* (Panzer) (Colepotera: Tenebrionidae), fed a similar vetch by-product were attributed to the ANFs of common vetch seeds (Rumbos *et al.*, 2021b). Therefore, ANFs may have to be reduced or inactivated during processing before they can be used as feed (Huang *et al.*, 2017).

Diet can greatly affect insect body composition and subsequently modify the suitability of insect meal for food and feed applications (Oonincx and Finke, 2020). Studies that have comparatively evaluated the diet effect on the body composition of *Z. morio* larvae are scarce. In one of the few studies available, Latney *et al.* (2017) grew *Z. morio* larvae on a high-calcium cricket feed and reported significant differences in the larval body calcium content. In the present study the nitrogen content of *Z. morio* larvae fed the by-products singly or in compound diets fluctuated considerably among the dietary treatments (5.5-8.5%). The lowest nitrogen content was recorded for the barley by-product (class II) (5.5%) and was similar to the nitrogen content previously reported for larvae fed a high protein and high starch diet (5.2%), as well as a high protein and low starch diet (6.2%) (Van Broekhoven *et al.*, 2015). However, in all cases the nitrogen larval content was in the range previously reported for *Z. morio* larvae (6.2-8.6%) (Bosch *et al.*, 2014; Van Broekhoven *et al.*, 2015; Adámková *et al.*, 2017; Rumbos and Athanassiou, 2021). Whereas larval fat content varied over dietary treatments, values concurred with previously published studies (Finke, 2002; Benzertiha *et al.*, 2020; Rumbos and Athanassiou, 2021; da Cruz *et al.*, 2022). The higher lipid content was recorded for the barley by-products (44.5 and 46.5% for class I and II, respectively), two substrates poor in lipids (0.6% for both) but rich in starch (Gous *et al.*, 2015), which is metabolically broken down into glucose and stored as fat (Cohen, 2003). Differences in the fat content, as well as in the lipid profiles (e.g. fatty acid profiles) due to dietary changes have also been previously reported for *Z. morio* larvae fed by-product-based diets (Van Broekhoven *et al.*, 2015; Nascimento *et al.*, 2022), however, the metabolic pathways behind these changes are still vague, indicating that there are still many things to learn about the lipid metabolism and lipogenesis of this species (Toprak *et al.*, 2020). Differences in the body composition of *Z. morio* larvae due to different substrates show the potential for producers to manipulate their composition through the diet (Oonincx and Finke, 2020); but also show the challenges in creating a consistent product when using diets with varying nutrient profiles, as can be the case with by-products and wastes (Van Peer *et al.*, 2021).

Acquiring a better understanding of the nitrogen efficiency of farmed insects is closely related to understanding their nutritional requirements. Optimizing nitrogen efficiency by formulating appropriate diets would improve the productivity of the insect sector. In the present study, results reveal a high variability in the nitrogen efficiency of *Z. morio* larvae for the by-products and evaluated diets, with N-ECI values ranging from 3.5 to 49%. To the authors knowledge, this is the first work to evaluate the nitrogen efficiency for *Z. morio* larvae. High variability in nitrogen efficiency is known for *T. molitor* (22-83%) (Oonincx *et al.*, 2015; Rumbos *et al.*, 2021a). Interestingly, the highest nitrogen efficiency for *Z. morio* larvae was for the oat (40%) and barley (class II) (49%) by-products, which also resulted in a high nitrogen efficiency for *T. molitor* (59 and 83%, respectively) (Rumbos *et al.*, 2021a). This further attest similar nutritional requirements of these two related species and the high digestibility and suitable amino acid profile of these substrates.

To conclude, the present study brings to the fore the potential of several Greek by-products for the rearing of *Z. morio* larvae. Moreover, the reduced performance on compound diets highlights the need for better insight in the nutritional requirements of *Z. morio*. In the context of sustainably producing insects decreasing environmental impact associated with their feed is of utmost importance. A means to tackle this is by exploiting locally available agricultural by-products. This expected decrease in environmental impact should be verified via a life cycle assessment including the utilization of locally produced feed (Oonincx and De Boer, 2012).

Conflict of interest

The authors declare that they have no conflict of interest. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the authors.

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