Using gas exchange measurements to monitor growth, energy expenditure, and body composition of black soldier fly larvae (*Hermetia illucens*) on diets varying in protein-to-carbohydrate ratio

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Running header: Metabolism and growth of BSFL on diets varying in protein-to-carbohydrate ratio

Abstract

Optimizing black soldier fly larvae (BSFL) production requires a better understanding of how diet composition shapes growth, nutrient allocation, and metabolic efficiency. This study investigated whether respiratory measurements could serve as predictive, non-invasive tools for BSFL performance on diets differing in protein-to-carbohydrate (P:C) ratio. Larvae were reared for seven days on four experimental diets, with growth, survival, gas exchange (O_2 consumption, CO_2 production, respiratory exchange ratio (RER)), and body composition (crude protein and lipid contents) measured across seven replicates per treatment. O_2 consumption correlated strongly with final larval biomass, while RER was closely associated with protein and lipid deposition. Larvae on low-protein, high-carbohydrate diets accumulated proportionally more lipid, whereas protein-rich diets increased crude protein content but required higher energetic investment. This demonstrates that lipid-rich larvae grew more efficient in energetic terms per unit accumulated biomass than protein-rich larvae. Across diets, gas exchange variables reliably reflected growth dynamics, metabolic activity, and nutrient assimilation. Integrating respirometry into rearing systems could enable real-time monitoring of insect biomass yield and nutrient composition.

Keywords: carbon dioxide, dietary macronutrient composition, energetic cost, growth monitoring, metabolism, oxygen, respiratory exchange ratio

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Introduction

The world faces pressing environmental challenges that impact global food production, including climate change, soil degradation, habitat loss, and the depletion of natural resources (Foley *et al*., 2011). More sustainable food systems are essential to meet the increasing food and feed demand driven by population growth while simultaneously mitigating the impacts associated with greenhouse gas emissions, land-use, water scarcity, and food wastage (FAO, 2009; Foley *et al*., 2011). One component of this more sustainable food and feed production is the use of black soldier fly larvae (BSFL; *Hermetia illucens* (L.)) for converting low quality organic material into nutrient-rich biomass (Broeckx *et al*., 2021; Gold *et al*., 2020). This biomass can supplement traditional unsustainable protein sources (e.g. soybean and fishmeal) in livestock feeds (van der Heide *et al*., 2021) and pet foods (Bosch & Swanson, 2021), and as an added benefit, the remaining frass after harvest of larvae represents a high-quality fertilizer (Fuhrmann *et al*., 2022; Lomonaco *et al*., 2024).

The ability of BSFL to feed on a wide range of organic materials is a major reason why this species has become increasingly important commercially (Purkayastha & Sarkar, 2022). Nevertheless, BSFL growth rates, energy conversion efficiencies, and body compositions can vary considerably with diet quality and macronutrient balance (Barragan-Fonseca *et al*., 2021; Eggink *et al*., 2023), and with other conditions such as moisture content (Bekker *et al*., 2021) or temperature (Schøn *et al*., 2024). Diet composition, in particular the protein-to-carbohydrate (P:C) ratio and not just the total diet mass, has been shown to strongly affect larval growth and body composition. Studies have demonstrated that nutrient-deficient diets that deprive larvae of adequate protein or carbohydrate supply, as well as diets with unbalanced P:C ratios, reduce larval growth and alter nutrient deposition patterns (Barragan□Fonseca *et al*., 2019; Berggreen *et al*., 2025; Eggink *et al*., 2023). As a result, differences in diet can lead to substantial variation in BSFL body composition, with larvae ranging from 32–58% crude protein and 15–38% crude lipids on a dry matter (DM) basis (see review by Gold *et al.*, 2018). Because variations in macronutrient composition (encompassing both the levels and specific forms of protein, fat, and carbohydrate) affect larval development, size, and composition, it is important to understand in more detail how these dietary factors influence metabolism and energy conversion during growth. Similarly, there is a growing interest in developing methods to monitor the progression of growth in actively rearing larvae.

Measurements of gas exchange (O□ consumption and CO□ production) as well as the respiratory exchange ratio (RER, VCO□/VO□) are powerful indicators of metabolism, energy expenditure, and nutrient assimilation under different dietary conditions (Parodi et al., 2020;

Schøn *et al*., 2024; Talal *et al*., 2021). BSFL utilizes aerobic energy both for general maintenance of basal physiological processes, but also in association with the direct cost of growth and nutrient assimilation (Ferrannini, 1988; Talal *et al*., 2021) and studies have previously found a close association between O_2 consumption or CO_2 production and total larval mass gain (Eriksen, 2024; Schøn *et al*., 2024). However, this association may be affected by the rearing conditions, including the dietary conditions, as the energetics of growth is expected to differ depending on the ratio of lipid and protein assimilation and because different nutritional quality may affect the relative magnitude of maintenance metabolism versus growth metabolism in ectotherms (Goodrich *et al*., 2024; Laganaro *et al*., 2021). This may be further complicated by the fact that in the BSFL rearing system the diet and digestive tract microbiome also contribute to overall gas exchange rates (Bekker *et al.*, 2021; Parodi *et al.*, 2020). Nevertheless, continuous monitoring of gas exchange rates holds the potential to provide important information on growth and body composition in production systems. In this context, RER may be particularly relevant to examine, as this metric reflects the combined input from a range of metabolic processes related to both anabolism and catabolism. RER reflects the catabolism of macronutrients where oxidation of lipid, protein and carbohydrate results in RER of 0.7, 0.83 or 1.0 (Schmidt-Nielsen, 1997). In addition, RER will increase as a consequence of lipogenesis from carbohydrates or amino acids (Ferrannini, 1988; Talal *et al*., 2021), and RER measurements therefore hold potential to indirectly monitor which macronutrients are metabolized and assimilated.

The main objectives of this study were: (*i*) to assess how altering diet macronutrient composition affects the growth, metabolism, and body composition of BSFL, and (*ii*) to determine whether gas exchange measurements during rearing are predictive of BSFL production output in terms of growth rate, quantity, and quality. To address these objectives, larval growth, gas exchange (O_2 and CO_2) and body composition (crude protein and lipid content) were measured on BSFL reared on diets with varying P:C ratio.

Materials and methods

Black soldier flies

BSFL of 5-6 days upon hatching (4th or 5th instar) (Gligorescu *et al*., 2019) were supplied from a commercial BSFL facility (ENORM Biofactory A/S, Flemming, Denmark). After collection at the factory, the larvae were transported (1 h) to Aarhus University, Denmark, where all experiments were performed. For each experimental replicate, 600 counted larvae with average individual mass of 5.0 ± 0.9 mg were placed in 1 L respirometer chambers (15 cm

diameter, 6 cm height) each containing approximately 340 g of fresh diet. This resulted in a density of ~1.76 larvae per g wet diet or ~0.133 g dry diet per larva.

Experimental protocol

The study investigated how BSFL metabolism is affected by dietary macronutrient composition by measuring gas exchange, growth, and body composition of BSFL during 7 days of rearing on four experimental diets. Experiments were performed over four consecutive weeks using a blocked design where one or two replicates of each dietary treatment was included every week. This resulted in 7 replicates of each dietary treatment. The experimental system allowed for hourly recordings of O_2 consumption rate (V_{O_2}) and CO_2 production rate (V_{CO_2}). Larval growth was estimated daily by sub-sampling and weighing 15 randomly selected larvae from each replicate. At termination of the 7-day experiment, larvae from each replicate were separated from the frass using wet sieving, and larvae were subsequently quickly dried with paper towels. Larvae were then weighed and photographed to count the number of survivors using CountThings (Dynamic Ventures Inc., California, US). All experiments were performed in a climate cabinet at 27 °C (Termaks KB8182, Bergen, Norway) and constant darkness. Larvae sampled at the termination of the experiment were stored at -20 °C before analyses of DM, crude lipid, and crude protein contents. Samples of the initial seed larvae were also collected and stored for analysis.

Experimental diets

The ingredients and nutritional composition of the four experimental diets are shown in Table 1. All four diets contained commercial chicken feed (KylleKræs 1, Danish Agro, Galten, Denmark). The reference (REF) diet consisted of 100% chicken feed and was used as a high-protein diet control to monitor if the system supported growth and metabolism to a similar degree as previous studies (e.g., Berggreen *et al*., 2025; Eggink *et al*., 2023; Nguyen *et al*., 2015). The semi-artificial diets where mixed to represent different ratios of protein to non-fiber carbohydrate (NFC) (P:C). Specifically, P:C 1:3, P:C 1:5, and P:C 1:9 diets contained 35% chicken feed to ensure supplementation of all required micronutrients, and the remaining 65% was included to create as semi-synthetic diets of desired macronutrient contents following Berggreen *et al.* (2025) (Table 1). These treatment diets contained different proportions of a protein mix (casein and peptone, 1:1) and a carbohydrate mix (sucrose and dextrin, 1:1) to achieve desired P:C ratios. A lipid mix (rapeseed oil, sunflower oil, and lard, 1:1:1) and a mix of additives including vitamins (Vanderzant modification vitamin mixture), minerals (Wes-

sons salt mixture), fatty acids (linoleic acid and oleic acid, 4:1), cholesterol, and nipagin (1:6:1:1:1), as well as cellulose and agar were added at similar concentrations in the three mixed diets (Table 1). Cellulose and agar were added as structural components, and nipagin was added as a preservative. All experimental diets were prepared by mixing 320 g of agar solution (agar dissolved in boiling water and then cooled to 40 °C) with 80 g of pre-mixed dry diet ingredients. The diets were then left to solidify during which water evaporated, leaving 341.3 ± 6.3 g wet diet (~77% water) in the chambers at the onset of the experiment.

Table 1

Gas exchange measurements

The open-flow respirometry setup and protocol for gas exchange measurements were similar to that described with greater detail in Schøn *et al.* (2024). Briefly, 8 custom built, airtight respiration chambers (15 cm diameter, 6 cm height, 1 L volume) were placed in the climate cabinet. Each chamber was provided with a controlled and constant flow of room air (~600 mL min^{-1}) that continuously provided oxygen and removed water vapor and CO_2. The air flow for the 8 sequential chambers was regulated and recorded using a Flowbar-8 Mass Flow Meter System (Sable Systems).

To measure V_{O_2} and V_{CO_2}, air was sequentially sub-sampled at a rate of ~200 mL min^{-1} using a SS-4 Sub-Sampler Pump (Sable Systems). Sequential shifting between chambers was performed using an eight-channel RM-8 Respirometry Flow Multiplexer (Sable Systems). The sampled air passed through a calcium chloride column for removal of water vapor, and syringe filters (Acrodisc syringe filter, 25 mm, 0.2 µm Supor membrane, Pall Corporation) filtered the air before O_2 and CO_2 concentrations were analysed using an O_2 analyser (FC-2 Differential Oxygen Analyser (Oxzilla), Sable Systems) and a CO_2 analyser (LI-850 Gas Analyzer, LI-COR Biosciences), respectively. The multiplexer shifted between each of the eight respiration chambers (7 experimental chambers and one empty control chamber) every 7.5 minutes, providing hourly measurements from each chamber over the entire 7-day period. Unstable recordings of O_2 or CO_2 as well as outliers were removed and replaced by interpolation from preceding and subsequent samples of high quality using the data quality assessment protocol described in Schøn *et al.* (2024).

V_{O_2} and V_{CO_2} were calculated hourly from the product of the airflow rate and the difference in gas concentration between the experimental chambers and the empty control chamber. The total volumes of O_2 consumed (VO_2) and CO_2 produced (VCO_2) were cal-

culated by summation of hourly measurements of VO_2 and VCO_2 over the entire 7-day period. The respiratory exchange ratio (RER) was calculated hourly from V_{CO_2}/V_{O_2} or cumulatively from total VCO_2 / total VO_2.

Growth analysis

Respiration chambers with growing larvae were daily removed briefly from the temperature cabinet to track changes in total mass (frass and larvae) between days. On average the replicates lost 27.3 ± 3.4 g day^{-1}, mainly due to removal of water vapor and CO_2. During this procedure, 15 randomly selected larvae were quickly collected and weighed to assess average larval mass within each replicate. Three of the 15 larvae were sampled and frozen, and the other 12 larvae were returned. Thus, during the experiment 18 larvae were removed from each replicate. At the termination of the experiment all remaining larvae were counted and weighed to enable calculation of survival and total mass gain.

The total daily larval mass was estimated by multiplying the average daily larval mass with the estimated number of live larvae on each day. The estimate of live larvae accounted for the daily removal of 3 larvae and assumed that mortality was evenly distributed across the 7-day rearing period. Specific growth rates (SGR) were calculated as the difference in the natural logarithm of average body mass between two time points divided by the elapsed time:

$$SGR = \frac{\ln Final\ wet\ mass - \ln Initial\ wet\ mass}{Time}$$

Water, lipid, and protein content

At the termination of the experiment, all harvested larvae from each replicate were oven-dried at 60 °C for three to four days until they had reached a stable mass. Water content was calculated from the difference between wet mass and DM, and sub-samples of dried larvae were taken to estimate lipid and protein contents. To obtain crude lipid content, samples of 1.76 ± 0.01 g dried larvae were weighed before they were washed 5 times in 25 mL petroleum ether to remove the lipid fraction (Jensen *et al*., 2010). Each round of washing lasted 48 h and after the final round the defatted samples were again oven-dried at 60°C until stable mass to allow for estimation of the crude lipid fraction (calculated as the difference between initial DM and lean DM). Nitrogen analysis of defatted, finely ground sub-samples (4.2–7.2 mg per sample) was performed using a combustion analyser (Na 2000, Italy). The proportional crude protein content was then estimated by multiplying the measured nitrogen content by a factor of 4.76, a standardized conversion factor for the nitrogen-to-protein ratio in BSFL (Janssen *et al*.,

2017). Total protein was estimated from lean DM and expressed as a fraction of the total DM (before lipid removal).

Analysis of gross cost of growth and assimilation

To examine the relationship between energy consumption and energy assimilation, mass gain was converted into energy equivalents based on mass gain of lipid and protein. For these calculations an assumption of 37 kJ per g lipid and 17 kJ per g protein as energy conversion factors was used (FAO, 2002), respectively. Further, to assess the energy used for aerobic metabolism VO_2 was converted to energy expenditure using a standard caloric conversion factor of 20.5 kJ per L O_2 (Schmidt-Nielsen, 1997).

Data analysis

Separate one-way ANOVA were performed to analyse BSFL growth, gas exchange, and body composition between treatment groups. Assumptions of normality and homogeneity of variances were assessed using Shapiro-Wilk tests, visual inspection of Q-Q plots, and Levene's test. Tukey's Honest Significant Difference (HSD) test was used for post hoc comparisons, with statistical significance set at $p < 0.05$.

Linear regressions were used to evaluate the relationships between final larval DM and VO_2, as well as between assimilated energy from lipid and protein and the estimated aerobic energy expenditure. Pearson correlation coefficients were calculated to assess the strength and direction of these relationships. In selected analyses, linear models were constrained to pass through the origin (0,0) to test the assumption of a direct proportional relationship between the dependent and independent variables.

Data processing and visualization were performed in RStudio version 2024.04.2 with R version 4.3.2 (R Core Team, 2023; RStudio Team, 2023).

Results

Effects of diet on growth and body composition of BSFL

Larvae survived well and developed successfully on all diets, with average survival rates ranging from 89–97% and with no significant difference among diets (Table 2). While all diets supported substantial larval growth, the final mass of larvae was significantly different across diets after the 7-day growth period (Figure 1A). Total larval yield was highest with the REF diet, followed by the mixed diets with highest protein fraction, P:C 1:3, P:C 1:5 and P:C 1:9 diets, respectively (Figure 1A, Table 2). The difference in growth between diets was also

evident from our analysis of specific growth rates (SGR) (Figure 1B, Table 2). From the SGR (Figure 1B), it is possible to see the change in estimated total biomass between two consecutive days, where the biggest increase for P:C 1:3, P:C 1:5, and P:C 1:9 was found to be from day 3 to 4, and thereafter a more moderate increase (and even slight decrease for the REF diet) in SGR per day.

The larvae reared on different diets had significantly different body compositions at harvest (Table 2). Larvae reared on the P:C 1:9 diet had significantly lower water content compared to those from the other three diets. While the proportions of crude protein and crude lipid in the DM did not differ significantly between REF and P:C 1:3, they were significantly higher than found in diets with lower protein content (P:C 1:5 and P:C 1:9) (Table 2). As the P:C ratio increased in the experimental diets, larval crude protein levels rose and lipid levels dropped, resulting in larvae from the low protein diets exhibiting significantly elevated proportional lipid fraction and reduced protein fraction (Table 2).

Effects of diet on gas exchange and metabolism

The dynamic changes in V□O□ and V□CO□ over the 7-day growth period followed a broadly similar pattern (see Figure 2A and Figure S1). Here, gas exchange data is presented with a focus on VO□ and RER, showing that gas exchange rates generally increased over the 7-day growth period but with different temporal progressions and distinct patterns depending on dietary treatment (Figure 2). For the commercial high-protein diet, REF, the V□O□ increased slowly during the first two days, after which the rate accelerated. V□O□ spiked at three distinct time points: on day 1 (0.7 mL O□ min^{-1}), day 3.5 (2.2 mL O□ min^{-1}), and day 5.5 (4.6 mL O□ min^{-1}), before V□O□ declined considerably during the last 1.5 days of the experiment. A somewhat similar pattern was found with P:C 1:3 and P:C 1:5, but here the spikes occurred later and were more moderate. P:C 1:9 displayed an even more delayed and reduced response with a small spike around day 1.5-2, another by day 5.5, with indications of a third increase in V□O□ at the end of the experiment. Total VO□ for REF was significantly higher than for all other diets, and the two mixed diets with highest protein content (P:C 1:3 and P:C 1:5) were associated with a significantly higher total VO_2 compared to P:C 1:9 (Figure 2A, Table 2).

RER values were >1 across diets when estimated from the total gas exchange of the chambers over the 7-day growth period (Figure 2B, Table 2). RERs differ significantly among all

four diets with lowest RER for the REF and increasing RER for the P:C 1:3, P:C 1:5, and P:C 1:9 diet, respectively.

Respiratory variables as predictors of growth and body composition

Combining the data of growth and body composition with the data from gas exchange measures revealed several strong statistical correlations. First, there was a strong positive linear correlation between VO and final total larval DM when data was analysed across all diets (R^2 = 0.93, Figure 3A, black solid line). This correlation persists to be strong also when the linear regression model is forced through origo (0,0) (R^2 = 0.99, Figure 3A, red dashed line). As seen in Figure 3B, the correlation of energy consumption per energy assimilation results in a significant, albeit weaker, correlation (R^2 = 0.42, Figure 3B, black solid line) suggesting that across diets 0.91 kJ are expended for each kJ assimilated. Importantly, the gross cost of growth (amount of metabolic energy used in relation to assimilation of 1 unit of energy as biomass) varies significantly and considerably among diets (Figure 3B). The cost of growth was significantly higher for the REF and lowest for the low protein diet (P:C 1:9). To examine this further, the correlation between overall energy consumption and protein energy assimilation was analysed. This analysis also found a strong positive correlation among all treatments rendering an overall energetic cost of 1.88 kJ per kJ of protein assimilated (R^2 = 0.87, Figure 3C, black solid line). Importantly when analysed within each dietary treatment the cost of protein assimilation varied significantly between diets (Figure 3C) such that protein energy assimilation was cheapest on the high protein diets and more costly in the low protein diets.

Because RER is influenced by both catabolic and anabolic processes (i.e. lipogenesis) we hypothesized that this metric could reveal information about the body composition of growing larvae. Thus, lipid rich larvae should be characterized by higher proportional CO_2 contribution from lipogenesis than more lean larvae. As seen in Figure 4, there was a strong correlation between RER and lipid and protein contents when analysed across larvae from all treatments (R^2 = 0.65 and R^2 = 0.64, respectively, Figure 4, black lines). Interestingly, when this correlation was investigated within each diet, the correlations were weaker and, in some cases, opposite to the expected pattern (see Supplementary material for statistical analysis).

Discussion

The purpose of this study was to evaluate BSFL growth performance, metabolic activity, and energy conversion efficiency (energetic cost of growth) using respirometry as a monitoring tool in larvae reared on diets with varying protein-to-carbohydrate (P:C) ratios. Four experi-

mental diets were used; three semi-artificial diets with identical formulations except for varying P and C content to different ratios, and a reference diet of 100% chicken feed. Over a 7-day period, measurements for larval biomass gain, oxygen consumption, carbon dioxide production, and final crude protein and lipid content in the larvae was obtained. As expected, diets with a higher P:C ratio accelerated larval growth and development by increasing protein deposition. We could then link diet composition to metabolic activity and nutrient allocation and evaluate whether gas exchange patterns can provide useful information on larval output in terms of both larval biomass and nutritional body composition. We find that gas exchange measurements are powerful indicators of both biomass yield and body composition in BSFL, capturing not only the intensity but also the pulsatile nature of metabolic activity over time.

Effects of diet on growth and body composition of BSFL

The present study confirms previous findings that higher dietary protein levels support greater larval biomass and protein content, whereas carbohydrate-rich diets increase lipid deposition and produce smaller larvae (Barragan-Fonseca *et al*., 2021; Eggink *et al*., 2023; Gold *et al*., 2020). In our experiments, the protein-rich diet with a P:C ratio of 1:3 (P:C 1:3) resulted in 69.0% higher larval DM and 59.1% higher crude protein content compared to larvae reared on the carbohydrate-rich diet (P:C 1:9) (Table 1). P:C ratios around 1:2–1:3 appear optimal for biomass yield (consistent with Eggink *et al*., 2023), while diets richer in carbohydrate promoted lipid deposition, reaching 52.7% in this study and >44% in Eggink *et al*. (2023). These results reflect how BSFL (and other insects) metabolically can channel surplus carbohydrates in an unbalanced diet into lipid storage by lipogenesis (Schow-Madsen *et al*., 2025; Talal *et al*., 2021) (Further addressed in the last section of the discussion).

The highest larval yield and protein accumulation in the present study was observed for the reference diet (REF). While the REF diet (100% commercial chicken feed) is a valuable reference, it differs from the semi-synthetic diets (35% commercial chicken feed) in nutritional aspects other than the P:C ratio (Table 1). The macronutrient content alone does not capture the full nutritional complexity of a diet, which is also shaped by the types of protein, carbohydrates, and micronutrients it contains (Schow-Madsen *et al*., 2025). Therefore, the three semi-synthetic diets, identical apart from their P:C content, provide the best basis for directly comparing P:C effects.

Oxygen consumption as predictor of growth and development in BSFL

Metabolic activity is known to increase with growth in BSFL, as in other insects (Eriksen, 2022, 2024). In our study, higher oxygen consumption was positively correlated with higher larval end-biomass (Figure 3A), reflecting the energetic expenditure of increased nutrient assimilation, tissue synthesis, and maintenance. In this experiment, total oxygen consumption served as a reliable predictor of final biomass following larval growth in BSFL (Figure 3A). Although some of the measured gas exchange may arise from microbial metabolism (Fuhrmann *et al*., 2025), the data still reveals a direct relationship between respiration and BSFL biomass yield.

Examining cumulative daily oxygen consumption in relation to total daily biomass provides insight into the cost of growing and maintaining biomass. Across all diets, larvae on average used less oxygen per g biomass as they grew, declining from ~0.07–0.08 L O_2 per g biomass early in rearing to ~0.04–0.05 L O_2 per g biomass by day 7 (Figure S2). This cost reduction likely reflects a shift in energy allocation from high-cost rapid structural growth in early development to slower growth in later stages, where more metabolism is directed to maintenance and energy storage, reducing the proportion of metabolic energy from respiration needed per unit biomass as larvae approach peak size.

Monitoring temporal respiratory gas exchange showed that diet composition significantly influenced oxygen consumption, corresponding closely with observed biomass growth curves (Figures 1A and 2A). During the 7-day growth period, larvae grew progressively on all diets (Figure 1A) and the signal of metabolic intensity (V_{O_2} and V_{CO_2}; Figure 2A and S1) showed increases and decreases according to growth. These pulsatile growth patterns likely reflect instar transitions, with molting phases temporarily halting feeding and lowering metabolic rates before the next growth phase (Gligorescu *et al*., 2019). Temporal patterns differed among diets: higher protein content accelerated development and shortened instar duration, whereas carbohydrate-rich diets slowed progression and extended development periods (Figure 2A and S1). Although larval instar was not measured directly, the respiration profiles seem to capture the cohort's ontogeny through larval stages, which demonstrate the value of respirometry as a non-invasive tool for monitoring developmental dynamics in BSFL production systems.

Continuous gas measurements identified the last growth phase, and thereby the maximum biomass, for REF diet larvae by day 6, with respiration on the final day likely dominated by non-feeding 7th instar individuals (Figure 1A and 2A) (also observed in Schow-Madsen *et al*., 2025). For larvae reared on diet P:C 1:3 and P:C 1:5 biomass likely peaked between day 6 and 7, and for those on P:C 1:9 it would have been even later. This aligns with Eggink *et al*.

(2023), where the most carbohydrate-rich diet (P:C 1:9) prolonged development time by two days compared to diets with P:C ratios of 1:3 or less.

Shifts in development time may influence end results, as BSFL body composition (e.g., proportions of water, protein, and lipid) changes throughout development (Eggink & Dalsgaard, 2023). If larvae reared on different diets are at different developmental stages at harvest on day 7, this can directly affect measured body composition as well as observed growth and metabolic profiles. Harvesting larvae at the same developmental stage can help control for this but may also introduce other limitations (see discussion on timing of harvest in Deruytter *et al*. (2025). Notably, Eggink *et al*. (2023) found that even when carbohydrate-rich diets (P:C 1:5 and P:C 1:9) were harvested later to match developmental stage, they still produced larvae with significantly different body compositions compared to protein-rich diets, aligning with results from the present study.

Energy expenditure and energy assimilation in BSFL

To evaluate how efficiently the BSFL converted consumed energy into body mass, the gross cost of growth was analysed; the amount of metabolic energy expended per unit of energy assimilated into biomass. Energy expenditure was calculated from VO_2 measurements using a caloric conversion factor (20.5 kJ L^{-1} O_2) (Schmidt-Nielsen, 1997), while biomass gain was converted into energy equivalents based on lipid and protein content (37 kJ g^{-1} lipid and 17 kJ g^{-1} protein) (FAO, 2002). This approach assumes predominantly aerobic metabolism and integrates both growth and maintenance related costs unlike more advanced studies that can partition these costs in individual larvae (Eriksen, 2022, 2024).

Substantial variation in the gross cost of growth was observed among diets. BSFL reared on the high-protein diet (P:C 1:3) spent 0.6 kJ per kJ lipid and protein assimilated, which is 33% more energy than the high-carbohydrate diet (P:C 1:9) (Figure 3B). When considering protein assimilation alone, larvae on P:C 1:3 required ~47% less energy to store one unit of protein than larvae reared on P:C 1:9, who required 4.5 kJ of energy per kJ of protein stored (Figure 3C). However, these differences should be interpreted with great caution, as it is somewhat naive to isolate assimilation of only one component in the BSFL biomass and then correlate this to the overall energy use of the whole bioconversion system. Furthermore, a substantial part of energetic costs is not directly associated with assimilation, but rather with general maintenance (Eriksen, 2022, 2024) and it is possible that protein-rich larvae have higher maintenance costs per mass unit than do lipid rich larvae. Deviations between the unconstrained (black) and origin-forced (red) linear models in Figure 3C further suggest that factors

beyond a simple proportional relationship between metabolic cost and nutrient gain influence these patterns. While the biological interpretation of these energetic balances should be interpreted with caution it remains interesting for producers to evaluate at what cost either protein or energy is assimilated in different diets. Our data show that the energetic cost per unit of assimilated energy varies with diet composition (Figure 3). While both lipid and protein deposition are closely linked to metabolic activity, protein synthesis is inherently more energetically demanding than lipid accumulation. Storing lipid appears to be metabolically less costly per energy unit gained than synthesizing protein (Goodrich *et al*., 2024; Laganaro *et al*., 2021; Talal *et al*., 2021). As a result, larvae allocating more of their nutrient accumulation to lipid biomass effectively grow "cheaper" in energetic terms per unit accumulated biomass than those accumulating more protein.

Respiratory exchange ratio as predictor of BSFL body composition

Oxygen consumption provides a strong measure of total respiratory energy expenditure, while carbon dioxide production reflects both respiratory metabolism and the biochemical conversion of excess dietary carbohydrates (or amino acids) into lipids via lipogenesis (Laganaro *et al*., 2021; Talal *et al*., 2021). The respiratory exchange ratio (RER = VCO_2/VO_2) therefore reflects the combined inputs of multiple metabolic processes related to aerobic and anaerobic anabolism and catabolism of a system. RER values typically range from ~0.7 for lipid oxidation to ~1.0 for carbohydrate oxidation, reflecting the macronutrient used for energy production (Schmidt-Nielsen, 1997). However, in fast-growing systems, such as BSFL, lipogenesis can drive values above 1.0 by producing CO_2 without consuming O_2, making RER a useful indicator relative to anabolic activity associated with lipid synthesis (Ferrannini, 1988; Talal *et al*., 2021; Parodi *et al*., 2020; Schøn *et al*., 2024). In accordance, higher RER values were observed for the high-carbohydrate diet (P:C 1:9) that produced larvae with higher lipid and lower protein content (Figure 4, Table 2). This pattern is consistent with carbohydrate-driven lipogenesis, where surplus dietary energy from carbohydrates is stored as lipid. The correlation between RER values and the larval protein and lipid content for different treatment diets (Figure 4) highlights the potential of RER to indicate final body composition of BSFL.

Since RER is calculated from two separately measured values (CO_2 and O_2), it is sensitive to measurement variability, so averaging RER over several days or the full 7-day growth period may provide a more robust estimate. However, if the data is sampled with sufficient quality RER holds the potential to indicate to what degree larvae are allocating energy primarily toward lipogenesis or other metabolic processes. It should be noted that VCO_2 and VO_2

measurements, and thereby the estimation of RER, can be confounded by CO_2 production and O_2 consumption from diet-associated microbes and the microbial communities within the larval digestive tract. For instance, other studies, such as Fuhrmann *et al*. (2025) have demonstrated substantial diet microbial CO_2 production, which may complicate or reduce the precision of nutrient allocation using RER. In contrast, the CO_2 production curves observed in this present study are typical for bioconversion systems with low microbial respiration from the diet, suggesting a more accurate reflection of larval metabolism. Under these conditions, RER can serve as a reliable indicator of larval metabolism, allowing both cumulative and real-time tracking of energy use and nutrient allocation, and acting as a non-invasive tool to predict nutrient assimilation in BSFL.

Conclusion and perspectives

This study focused on evaluating BSFL diet effects through the lens of respirometry, demonstrating how respiratory measurements can be applied as predictive, non-invasive tools for growth performance, nutrient allocation, and energetic efficiency. Across diets, the volume of O_2 consumed correlated strongly with final larval biomass, while RER reflected differences in protein and lipid deposition, confirming that dietary P:C ratio shapes both body composition and the energetic cost of growth. Lipid-rich larvae on a high-carbohydrate diet grew more efficient in energetic terms than protein-rich larvae on a high-protein diet, highlighting practical implications for feed formulation and economic optimization.

Three key conclusions emerge: (i) dietary P:C ratio influences both growth and body composition, (ii) nutrient balance shapes the cost of growth and metabolic activity, and (iii) respiratory variables (VO_2, VCO_2, RER) are robust predictors of larval performance, energetics, and nutrient allocation. In a BSFL production facility, integrating O_2 with existing CO_2 monitoring could link gas exchange to biomass yield and nutrient profiles, enabling real-time production monitoring and potentially improving decision-making, resource use, and valorisation of organic side streams. Combined with growth data, respirometry may serve as an indicator of larval energy investment, offering prospects for more precise management and the development of efficient, sustainable insect production systems.

Data availability

Data supporting this study will be made available upon publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper. This includes Moritz Gold who was employed at REPLOID Group AG in parallel to ETH Zurich during the writing phase of the presented research.

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Table 1: Macronutrient contents of the four experimental diets. The reference diet (REF) is an entirely commercial chicken feed used as a reference diet supporting high growth. The three other diets are formulated in house and designed to represent different protein-to-carbohydrate (NFC) ratios.

Diet treatment	**REF**	**P:C 1:3**	**P:C 1:5**	**P:C 1:9**
Protein-to-carbohydrate ratio (P:C)	1 : 3.2	1 : 3.2	1 : 5.0	1 : 9.4
Ingredients (% of dry mass)				
Chicken feed (CF)	97.6	34.1	34.1	34.1
Protein mix	0	10.4	5.2	0
Carbohydrate mix	0	33.3	38.6	43.7
Lipid mix	0	2.7	2.7	2.7
Additive mix	0	3.9	3.9	3.9
Cellulose	0	13.1	13.1	13.1
Agar	2.4	2.4	2.4	2.4
Diet composition (% of dry mass)				
Protein	19.9	17.3	12.1	7.0
Carbohydrate (NFC)	63.3	55.5	60.7	65.8
Carbohydrate (fibre, agar)	5.6	16.7	16.7	16.7
Lipid	5	4.5	4.5	4.5
Ash	6.2	6.1	6.1	6.1

The diet composition is based on the ingredient list and the mass used. The non-fibre carbohydrate (NFC) fraction from chicken feed is estimated by subtracting protein, fat, fibre, and ash from the total dry mass.

Table 2: Survival, growth, body composition and gas exchange of black soldier fly larvae after 7 days of rearing on diets with different protein and carbohydrate contents.

	5–6 day old larvae*	**REF**	**P:C 1:3**	**P:C 1:5**	**P:C 1:9**
Growth performance	At onset	After 7 days	After 7 days	After 7 days	After 7 days
Survival rate (%)		97.0 ± 4.4% [a]	94.9 ± 3.6% [a]	92.9 ± 5.5% [a]	88.8 ± 8.1% [a]
Larvae end biomass (g DM per replicate)	0.78 ± 0.15 g	25.9 ± 0.5 g [a]	22.6 ± 0.7 g [b]	20.1 ± 1.1 g [c]	12.6 ± 3.0 g [d]
Specific growth rate (per day)		0.50 ± 0.03 [a]	0.47 ± 0.03 [ab]	0.45 ± 0.02 [b]	0.36 ± 0.03 [c]
Larvae percent dry matter (% DM)	26.3%	27.7 ± 1.5% [a]	27.7 ± 0.8% [a]	29.0 ± 2.2% [a]	32.7 ± 1.5% [b]
Larvae crude protein (% of DM)	40.9%	31.5 ± 0.8% [a]	32.5 ± 0.6% [a]	25.5 ± 2.3% [b]	19.8 ± 0.8% [c]
Larvae crude lipid (% of DM)	10.0%	24.3 ± 1.9 % [a]	27.5 ± 3.5 % [a]	39.2 ± 6.7% [b]	52.7 ± 2.7% [c]
Gas exchange measurements					
VCO□ (L, total for 7 days)		19.5 ± 1.2 L [a]	16.9 ± 1.1 L [b]	16.2 ± 1.6 L [b]	10.8 ± 2.2 L [c]
VO□ (L, total for 7 days)		17.7 ± 1.3 L [a]	14.2 ± 1.2 L [b]	12.9 ± 1.5 L [b]	8.0 ± 1.7 L [c]
RER		1.10 ± 0.02 [a]	1.20 ± 0.03 [b]	1.26 ± 0.04 [c]	1.35 ± 0.06 [d]

* Samples of 5-6-day old seed larvae were sampled for analysis at the onset of the experiment.
DM = dry matter; V = volume; RER = respiratory exchange ratio, calculated from total VCO□/VO_2 over the 7-day experiment. All values are mean ± standard deviation and dissimilar letters indicate significant differences (one-way ANOVA followed by Tukey HSD test, $p < 0.05$, $n = 7$)

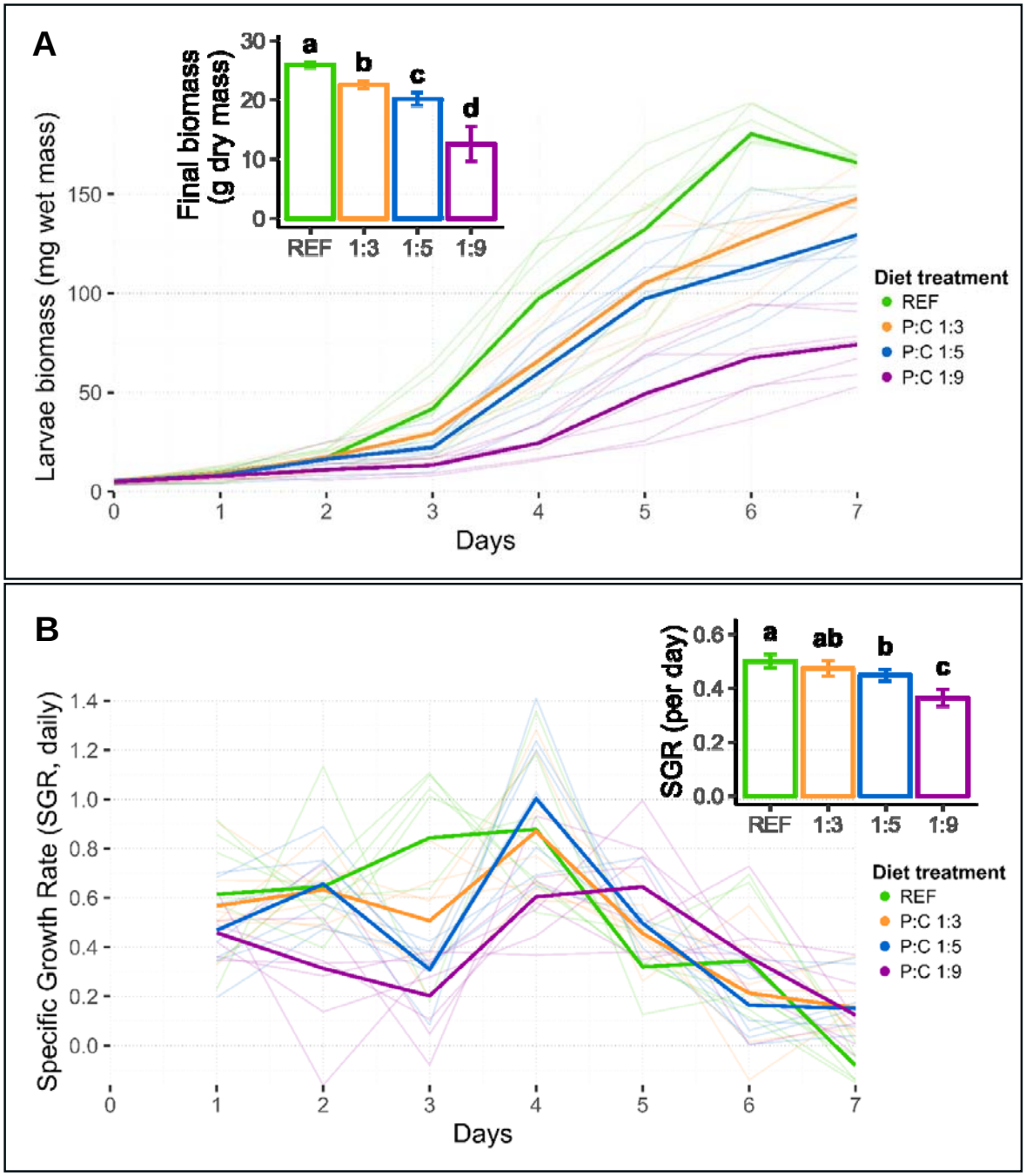

Figure 1: Effect of diets with different protein-to-carbohydrate ratios on total growth and estimated daily specific growth rate (SGR). A) Average individual larvae mass (mg wet mass) over time, where the insert shows the final total dry larvae biomass at the termination of the experiment (approximately 600 larvae). B) Daily estimates of SGR, calculated from the increase in average body mass estimated between two consecutive days, where the insert shows SGR calculated for the entire growth period. Bold lines represent treatment means, while semi-transparent lines show the individual replicates (n=7). Inserted bar plots show treatment means ± standard deviations, and dissimilar letters indicate significant differences (one-way ANOVA followed by Tukey HSD test, $p < 0.05$, $n = 7$).

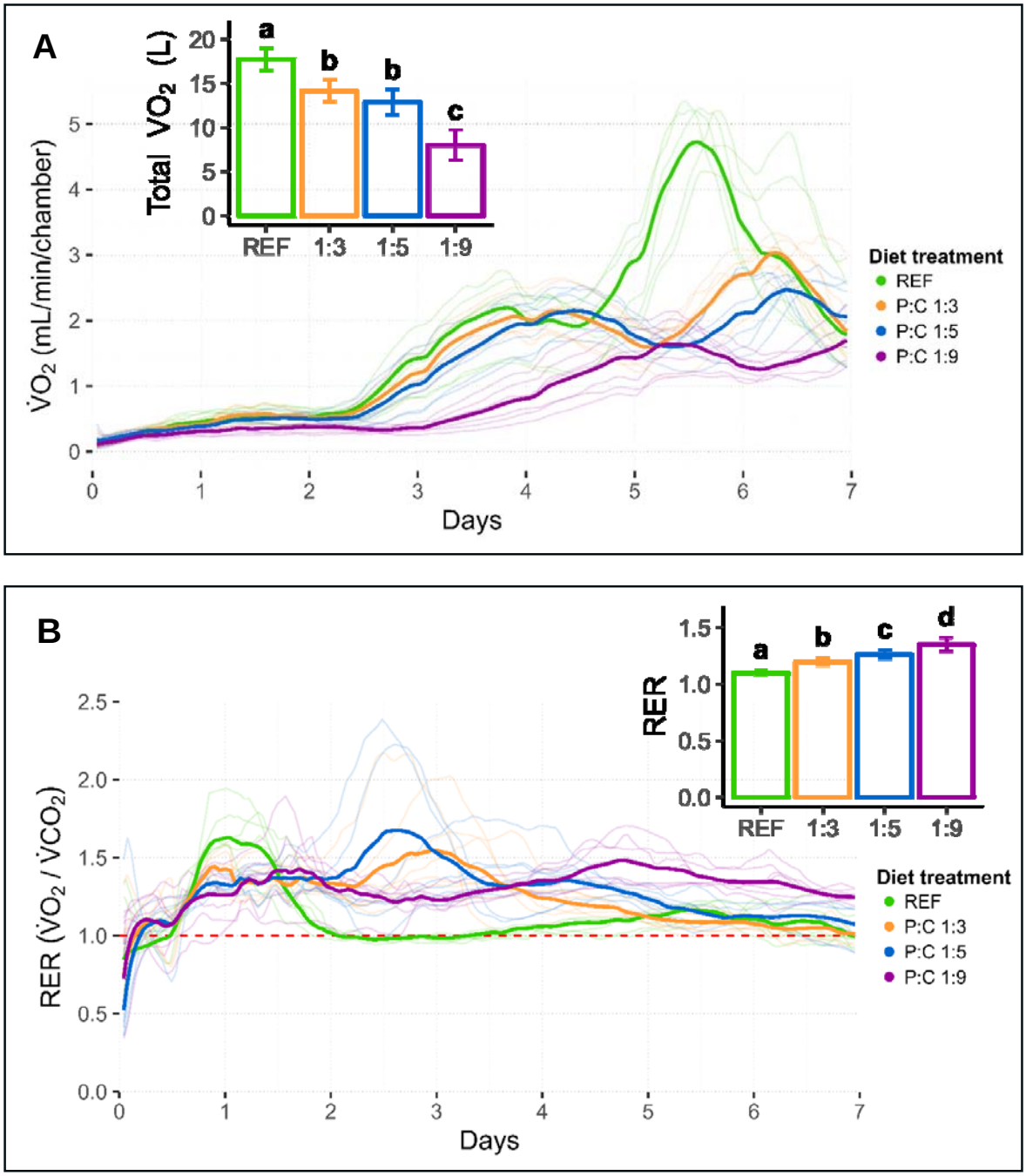

Figure 2: Effect of diet with different protein-to-carbohydrate ratios on gas exchange measurements. A) Change in O□ consumption rate (V̇O□, mL/min/chamber) over time, where the insert shows the total VO□ consumed for the entire period (approximately 600 larvae). B) Change in respiratory exchange ratio (RER) over time V̇CO□/V̇O□ (V̇ is the rate, mL/min), where insert show total RER VO□/VCO□ (V is the volume, L) for each treatment. Bold lines represent treatment means, while semi-transparent lines show the individual replicates (n=7). Inserted bar plots show treatment means ± standard deviations, and dissimilar letters indicate significant differences (one-way ANOVA followed by Tukey HSD test, $p < 0.05$, $n = 7$).

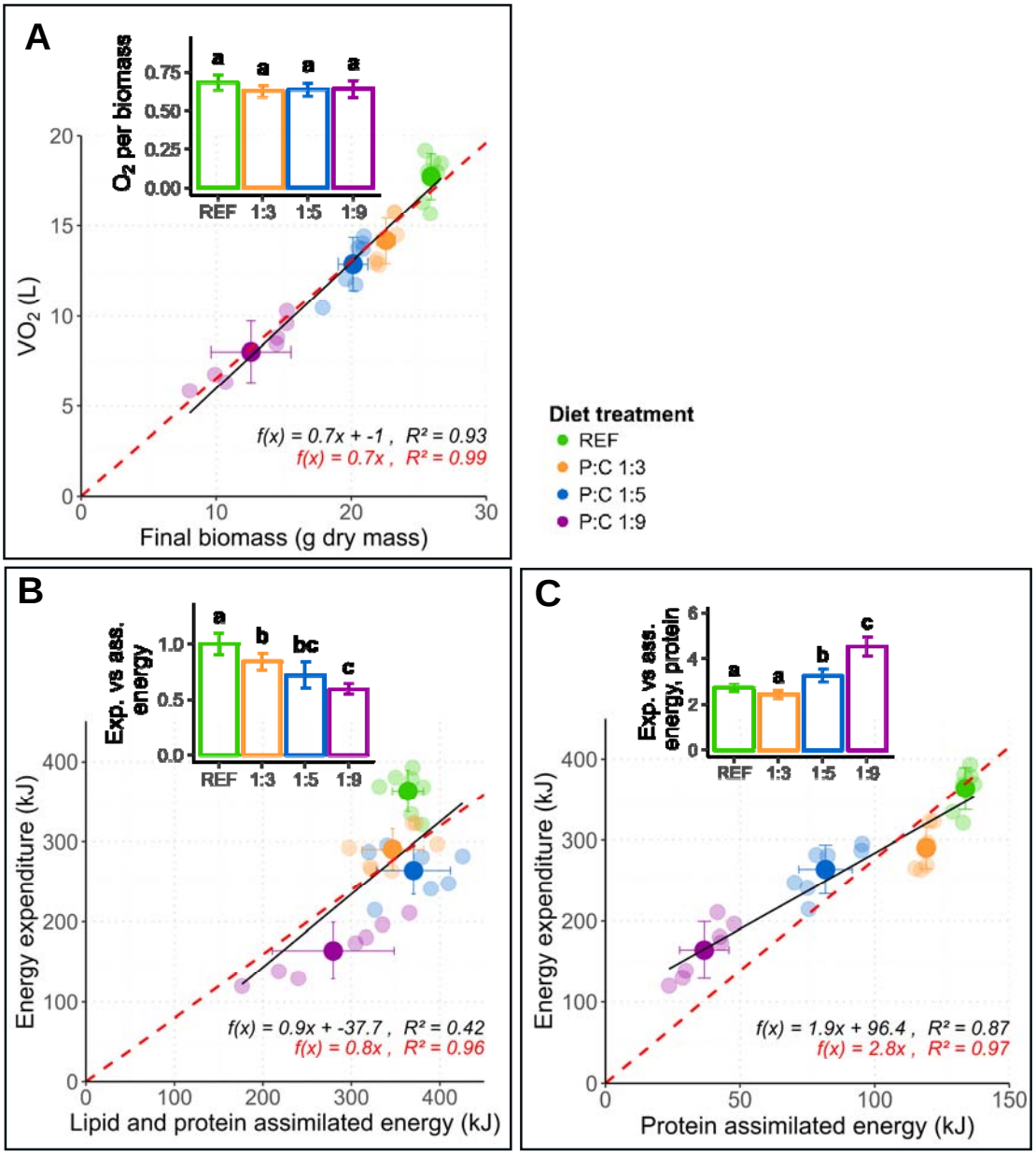

Figure 3: Analysis of the metabolic cost of growth and assimilation in larvae reared on diets with different protein-to-carbohydrate (P:C) ratio. A) Total oxygen consumption over 7 days as a function of final biomass across replicates reared on 4 different diets with varying P:C ratio. Correlation shows a linear relationship between the total volume of O□ consumed (L) and the final dry biomass (g). B) and C) show the energy expenditure of the system (using the energy equivalent of VO□) in relation to the assimilated energy in the biomass (kJ) represented by gain in lipid and protein, or protein only. Bold points represent treatment means with error bars showing standard deviations, and semi-transparent points represent treatment replicates (n=7). Black lines represent a linear model for the replicates (n=28), and red dashed lines represent the linear model when forced through origo (0,0). Linear formulas and R^2 values are displayed for each regression model.

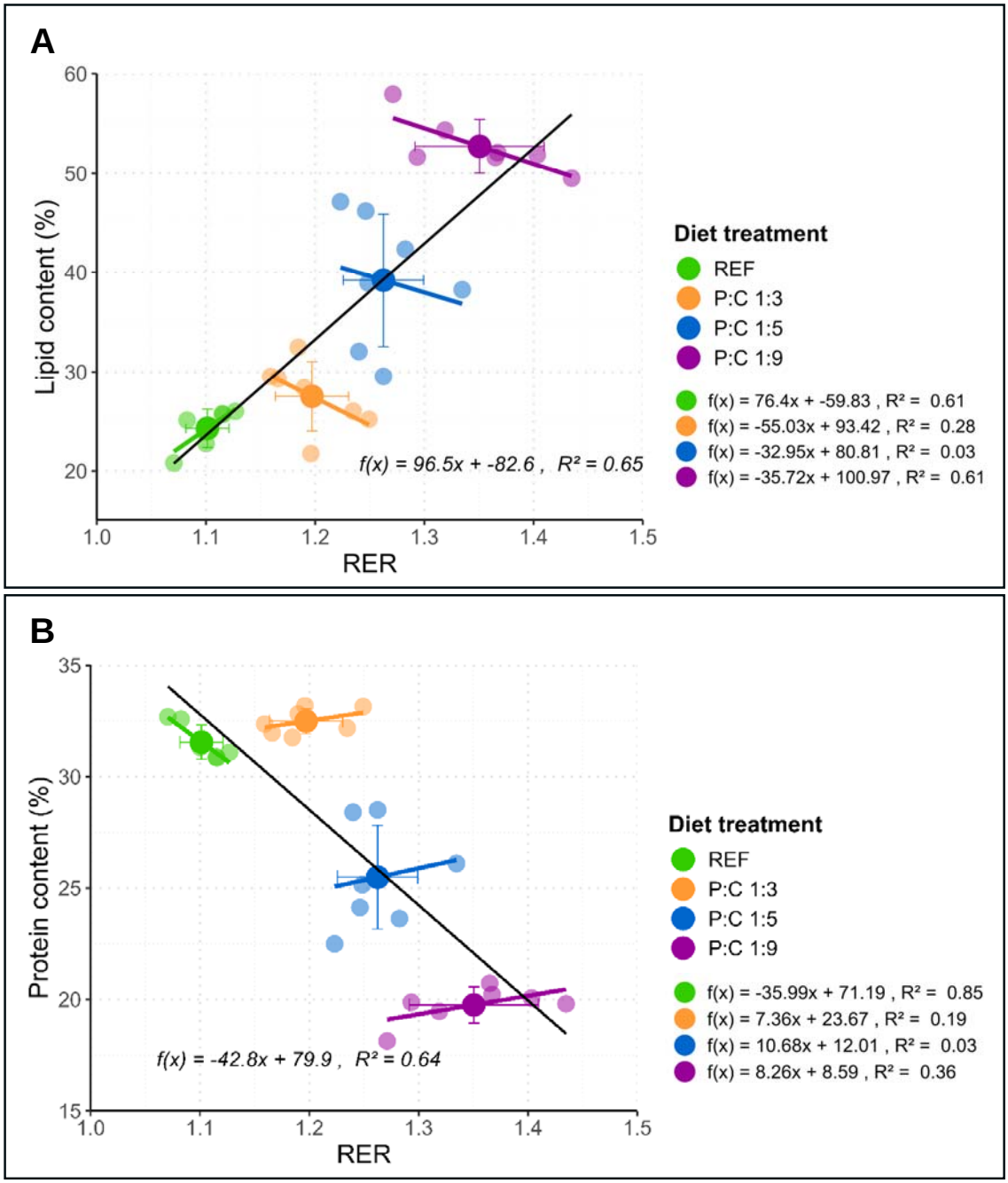

Figure 4: Effect of diets with different protein-to-carbohydrate ratios on larval body composition. Relationship between the measured RER values and A) crude lipid and B) crude protein content (% of dry mass). Bold points represent treatment means with error bars showing standard deviations, and semi-transparent points represent treatment replicates (n=7). The black lines represent a linear model for all the replicate data (n=28). Linear models for each treatment group are shown in their respective colours (n=7). Linear formulas and R^2 values are displayed for each regression model.

Supplementary material

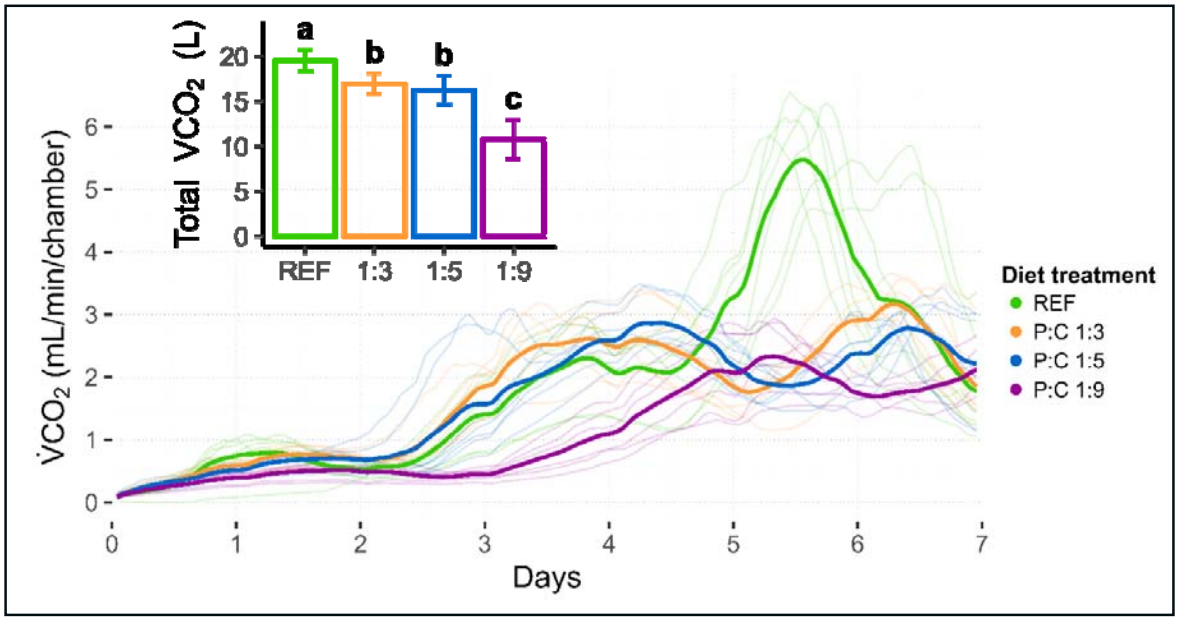

Figure S1: Effect of diet with different protein-to-carbohydrate ratios on CO□ production. Change in CO□ production rate (V̇CO□, mL/min/chamber) over time, where the insert shows the total VCO□ produced for the entire period (approximately 600 larvae). Bold lines represent treatment means, while semi-transparent lines show the individual replicates (n=7). Inserted bar plots show treatment means ± standard deviations, and dissimilar letters indicate significant differences (one-way ANOVA followed by Tukey HSD test, $p < 0.05$, n = 7).

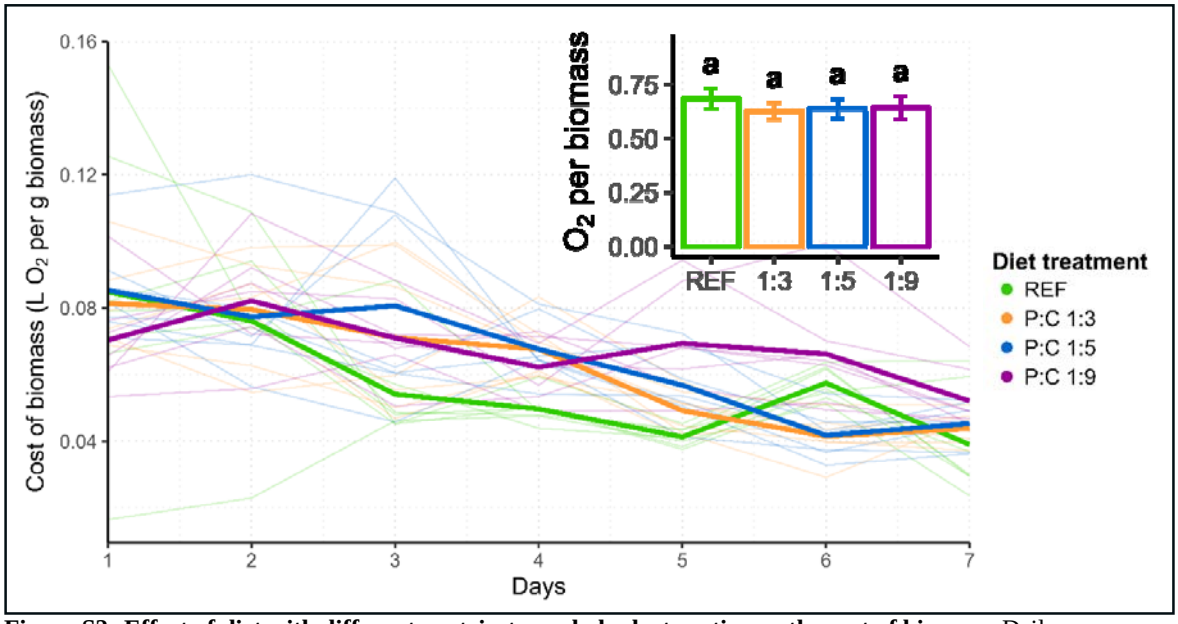

Figure S2: Effect of diet with different protein-to-carbohydrate ratios on the cost of biomass. Daily estimates of the metabolic costs of biomass, when calculated as consumed VO_2 in relation to biomass gain. Bold lines represent treatment means, while semi-transparent lines show the individual replicates (n=7). Inserted bar plot show treatment means ± standard deviations, and dissimilar letters indicate significant differences (one-way ANOVA followed by Tukey HSD test, $p < 0.05$, n = 7).

Tabel S1: Key statistics from the linear regression models shown in Figure 3 and 4. Summarization of function parameters, standard errors, explained variance, and statistical significance. Black regression lines represent a linear model for the replicate values (n=28), and red lines represent the linear model when forced through origo (0,0).

Model illustration	Model formula	Intercept	Slope	Slope SE	R^2	Adj. R^2	Residual SE	DF	F-statistic	p-value	Pearson correlation coefficient
Fig. 3A black	Total VO□ (L) ~ Final larvae biomass (g DM)	-1.04	0.70	0.04	0.933	0.931	1.00	26	363	**$<2.2 \cdot 10^{-16}$**	0.966
Fig. 3A red	Total VO□ (L) ~ Final larvae biomass (g DM) + 0	0.00	0.65	0.01	0.995	0.995	1.02	27	5087	**$<2.2 \cdot 10^{-16}$**	
Fig. 3B black	Energy expenditure (kJ) ~ Protein and lipid energy gain (kJ)	-37.71	0.90	0.21	0.425	0.403	60.18	26	19	**$1.7 \cdot 10^{-4}$**	0.652
Fig. 3B red	Energy expenditure (kJ) ~ Protein and lipid energy gain (kJ) + 0	0.00	0.80	0.03	0.957	0.955	59.37	27	601	**$<2.2 \cdot 10^{-16}$**	
Fig. 3C black	Energy expenditure (kJ) ~ Protein energy gain (kJ)	96.41	1.88	0.14	0.875	0.870	28.07	26	181	**$3.1 \cdot 10^{-13}$**	0.935
Fig. 3C red	Energy expenditure (kJ) ~ Protein energy gain (kJ) + 0	0.00	2.77	0.09	0.974	0.973	46.34	27	1005	**$<2.2 \cdot 10^{-16}$**	
Fig. 4A black	Larvae crude lipid (% DM) ~ RER	-82.59	96.53	14.00	0.647	0.633	7.28	26	48	**$2.5 \cdot 10^{-7}$**	0.804
Fig. 4A treatments	Larvae crude lipid (% DM) ~ RER · Treatment	See Fig. 4A	See Fig. 4A		0.914	0.883	4.10	20	30	**$2.7 \cdot 10^{-9}$**	
Fig. 4B black	Larvae crude protein (% DM) ~ RER	79.86	-42.79	6.34	0.636	0.622	3.30	26	46	**$3.7 \cdot 10^{-7}$**	-0.798
Fig. 4B treatments	Larvae crude protein (% DM) ~ RER · Treatment	See Fig. 4B	See Fig. 4B		0.954	0.938	1.34	20	59	**$5.8 \cdot 10^{-12}$**	

DM = dry matter; V = volume; RER = respiratory exchange ratio, calculated from total VCO□/VO_2 over the 7-day experiment; Treatments = REF, P:C 1:3, P:C 1:5, and P:C 1:9; SE = standard error.