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Biochemical composition and protein accumulation dynamics in dipteran larvae reared on heterogeneous organic waste substrates

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Abstract

Dipteran larvae of the family Chironomidae accumulate substantial quantities of protein over their short developmental cycle, yet the way substrate composition governs the rate and ceiling of that accumulation has received limited biochemical scrutiny. This research tracked the proximate and amino acid composition of *Chironomus plumosus* larvae reared on four organic waste substrates municipal sewage sludge, abattoir waste, mixed vegetable waste, and paper mill sludge at the Dresden Institute of Applied Biosciences, Germany, from February to July 2024. Larvae were sampled every three days over a 24-day rearing period, freeze-dried, and subjected to Kjeldahl nitrogen analysis, Soxhlet lipid extraction, amino acid hydrolysis, and fatty acid profiling by GC-MS. Crude protein rose from an initial 26.1–30.7% (day 3) to a plateau of 48.9–64.7% of dry mass by day 21, with the mixed vegetable waste substrate producing the highest terminal protein value ($64.7 \pm 1.9\%$). Protein accumulation followed a logistic curve rather than a linear trajectory, reaching half-maximal content between days 10 and 14 depending on substrate. Essential amino acid indices exceeded 0.80 on all substrates except paper mill sludge (0.71). Lipid content ranged from 6.8% to 11.2% of dry mass at harvest, and oleic acid was the dominant fatty acid across all treatments. These results position mixed organic vegetable waste as an especially effective rearing medium for chironomid protein biomass intended for animal feed applications.

Keywords: Biochemical composition, protein accumulation, *chironomus plumosus*, organic waste, proximate analysis, amino acid profile, dipteran larvae, logistic growth, fatty acid composition, animal feed

Introduction

The search for alternative protein sources to replace fishmeal in livestock and aquaculture feeds is no longer a fringe research interest it sits at the centre of global food security planning ^[1]. Insect larvae, particularly those of black soldier fly (*Hermetia illucens*) and mealworm (*Tenebrio molitor*), have received the most commercial attention, but the family Chironomidae offers a parallel track that is ecologically distinct and potentially complementary ^[2]. Chironomid larvae colonise organic waste spontaneously, grow rapidly under warm conditions, and accumulate protein levels competitive with the best-performing insect species, yet their biochemistry during growth has been explored less systematically ^[3]. What makes the protein accumulation pattern of chironomid larvae different from that of other insects? Unlike holometabolous beetles (Coleoptera) that store protein gradually over weeks of larval life, chironomid larvae complete their entire growth cycle in roughly three to four weeks, during which crude protein content can more than double on a dry-weight basis ^[4]. Whether this accumulation follows a linear, exponential, or sigmoidal trajectory and how the inflection point shifts with substrate quality has practical value for timing harvest to maximise protein yield per rearing batch. Organic waste streams vary enormously in their nitrogen, carbon, and micronutrient profiles, and these differences propagate through the microbial biofilms that chironomid larvae actually consume ^[5]. Substrates rich in readily mineralisable nitrogen (such as abattoir waste and sewage sludge) are expected to support faster microbial turnover and, consequently, faster larval protein gain. Carbon-rich, nitrogen-poor substrates (such as paper mill sludge) may constrain microbial protein availability and slow larval development ^[6]. But direct biochemical time-series comparing multiple substrates under identical conditions are rare.

This research therefore had three aim

To chart the temporal trajectory of crude protein accumulation in *C. plumosus* larvae across four chemically distinct organic waste substrates; to characterize the amino acid and fatty acid

Profiles of the resulting larval biomass at harvest maturity; and to identify which substrate yields the biochemically most suitable product for downstream animal feed formulation.

Material and Methods

Reagents and Chemicals

All chemicals used in proximate and amino acid analyses were of analytical reagent grade. Concentrated sulphuric acid (H_2SO_4 , 98 %), sodium hydroxide (NaOH), boric acid (H_3BO_3) petroleum ether (40–60 °C fraction), and hydrochloric acid (HCl, 6 M) were purchased from Merck KGaA (Darmstadt, Germany). The Kjeldahl catalyst tablets (copper sulphate–potassium sulphate mixture) were sourced from Fisher Scientific (Schwerte, Germany). Amino acid standard solution (Sigma A-9906, 17 amino acids) and norleucine internal standard were obtained from Sigma-Aldrich (Taufkirchen, Germany). FAME standard mix (Supelco 37-Component, CRM47885) for fatty acid GC-MS calibration was purchased from Supelco (Bellefonte, USA). Boron trifluoride–methanol (14% w/v) for fatty acid methylation was from Sigma-Aldrich. All solvents (methanol, hexane, diethyl ether) were HPLC grade from VWR International (Darmstadt, Germany). Deionised water (18.2 M Ω ·cm) was produced in-house using a Milli-Q system (Millipore, Germany).

Extraction Protocol and Sample Preparation

Larval samples collected at each three-day interval were immediately flash-frozen in liquid nitrogen and stored at -80 °C until analysis. For each time point and substrate, three pooled replicate samples (each comprising approximately 200 larvae, ~2.5 g wet weight) were prepared. Samples were freeze-dried (Christ Alpha 1-2 LDplus, Martin Christ GmbH, Osterode, Germany) for 48 hours at -55 °C and 0.05 mbar, then ground to a fine powder using a ball mill (Retsch MM 400, Haan, Germany) at 30 Hz for 2 minutes. Crude protein was determined by the Kjeldahl method (AOAC 2001.11) using a Büchi K-446 digestion unit and a B-324 distillation unit (Büchi Labortechnik, Flawil, Switzerland), with nitrogen-to-protein conversion factor 6.25. Crude lipid was measured by Soxhlet extraction with petroleum ether for 8 hours using a Büchi E-816 extraction system. Moisture was determined by drying at 105 °C for 24 hours, and ash by incineration at 550 °C for 6 hours in a muffle furnace (Nabertherm L9/11, Lilienthal, Germany). Amino acid composition was analysed after acid hydrolysis (6 M HCl, 110 °C, 24 hours under nitrogen) using a Biochrom 30+ amino acid analyser (Biochrom Ltd, Cambridge, UK) with postcolumn ninhydrin derivatisation. Tryptophan was determined separately by alkaline hydrolysis with lithium hydroxide [7].

Quality Control and Calibration

Analytical quality was maintained through a series of internal and external checks. Each batch of Kjeldahl analyses included a certified reference material (BCR-274, single-cell protein, Community Bureau of Reference, Brussels) with an accepted nitrogen content of 8.36±0.13 %. Recovery of nitrogen from the CRM averaged 99.1±0.7 % ($n = 12$ batches). Amino acid analyser calibration was verified before each run using a standard amino acid mixture (Sigma A-9906) at five concentration levels; correlation coefficients for all 17 amino acids exceeded $r^2=0.998$. GC-MS fatty acid identification was confirmed by retention time matching against the Supelco 37-Component FAME standard and spectral library matching (NIST 2020). Method detection limits for individual fatty acids

ranged from 0.02 to 0.05 mg ± bg⁻¹ dry mass. All analyses were performed in triplicate on independent sample pools, and results are reported as mean ± standard deviation.

Material

Four organic waste substrates were sourced from facilities within 50 km of the Dresden Institute of Applied Biosciences, Saxony, Germany. Municipal sewage sludge (MSS) was collected from the dewatering stage of a secondary wastewater treatment plant. Abattoir waste (AW) consisted of rumen content and blood meal residue from a cattle processing facility. Mixed vegetable waste (MVW) was obtained from a wholesale produce market and comprised unsold leafy greens, root vegetables, and fruit remnants in approximately equal proportions. Paper mill sludge (PMS) was sourced from the primary clarifier of a recycled-paper manufacturing plant. Each substrate was homogenised, adjusted to 75% moisture content, and distributed into 48 plastic rearing trays (40cm×30cm×10cm) at a loading rate of 1.0 kg wet weight per tray. Trays were placed in a climate-controlled rearing room maintained at 25±1 °C 70±5% relative humidity, and a 16:8 h light:dark photoperiod. *Chironomus plumosus* egg masses were obtained from the institute's established laboratory colony and distributed at a standardised density of approximately 500 eggs per tray. Each substrate treatment had 12 replicate trays, with three trays designated for destructive sampling at each of eight time points (days 3, 6, 9, 12, 15, 18, 21, and 24 post-hatch).

Methods

Larval sampling was carried out every three days beginning on day 3 post-hatch and ending on day 24, by which time the majority of larvae had reached the fourth instar and were approaching pupation. At each sampling point, all larvae from three designated trays per substrate were harvested by sieving the substrate through a 250 µm mesh, rinsing with deionised water, and transferring to labelled sample bags. Larvae were counted, weighed (wet), and immediately frozen as described in the extraction protocol above. Substrate chemical properties total nitrogen (Kjeldahl), total organic carbon (dry combustion, Elementar Vario EL III), and C:N ratio were measured at the start and end of the rearing period. Protein accumulation curves were fitted to a three-parameter logistic model: $P(t) = P_{max} / (1 + \exp(-k(t - t_{50})))$, where $P(t)$ is crude protein at time t , P_{max} is the asymptotic maximum, k is the growth rate constant, and t_{50} is the time at which protein reaches half its maximum. The essential amino acid index (EAAI) was calculated using the method of Oser [8], with whole-egg protein as the reference. Statistical differences among substrates at each time point were assessed by one-way ANOVA with Tukey's HSD post-hoc test ($\alpha = 0.05$) in R v4.3.2 [9].

Results

Table 1: Substrate chemical properties at the start and end of the 24-day larval rearing period

Parameter	MSS	AW	MVW	PMS
TN start (%)	3.67	3.21	3.94	1.08
TN end (%)	2.14	1.89	2.31	0.76
TOC start (%)	42.8	38.6	42.6	36.9
TOC end (%)	31.4	28.3	29.7	31.2
C:N start	11.7	12.0	10.8	34.2
C:N end	14.7	15.0	12.9	41.1

MSS = Municipal sewage sludge; AW = Abattoir waste; MVW = Mixed vegetable waste; PMS = Paper mill sludge. TN = total nitrogen; TOC = total organic carbon.

Table 2: Correlation matrix between substrate properties and larval biochemical parameters at harvest (day 21)

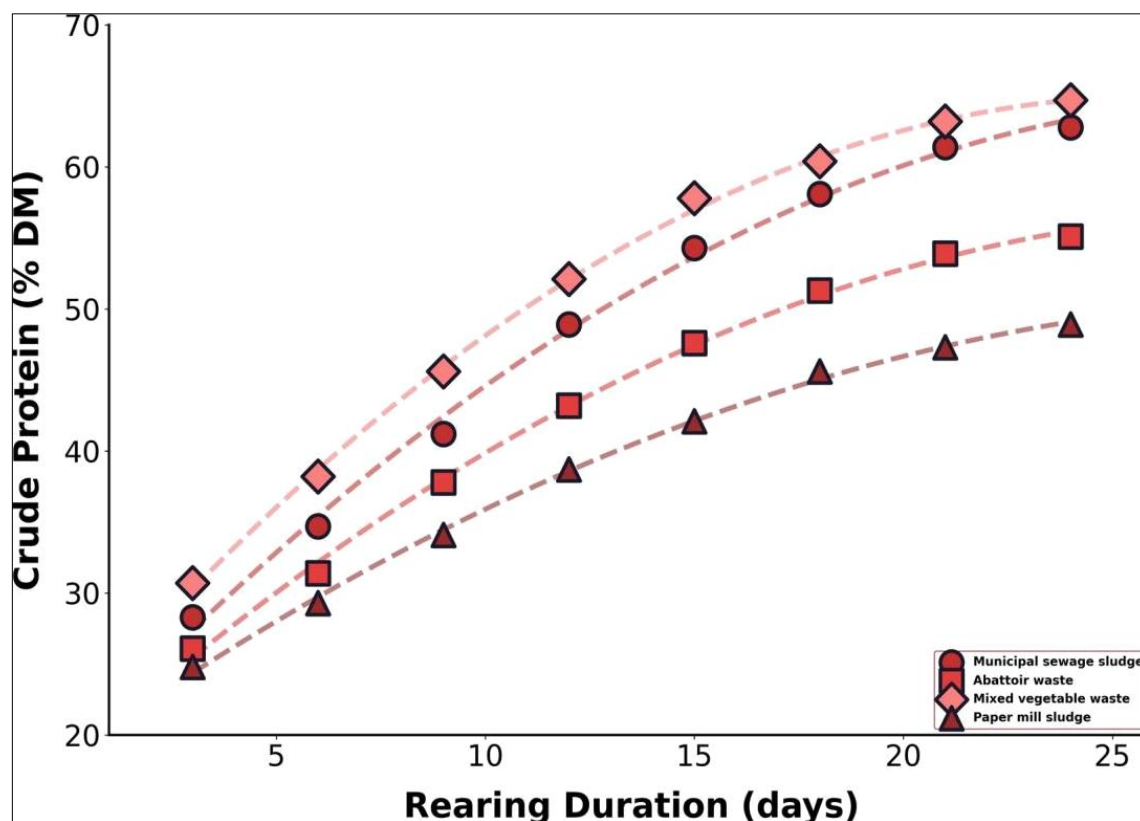
	Crude protein	EAAI	Lipid content
Substrate TN	0.94**	0.87**	0.41 ns
Substrate C:N	-0.91**	-0.83**	-0.29 ns
Substrate TOC	0.38 ns	0.31 ns	0.62*
Crude protein	1.00	0.96**	0.33 ns

$p < 0.01$; * $p < 0.05$; ns = not significant. Pearson correlation coefficients ($n=4$ substrates $\times$ 3 replicates).

Table 3: Amino acid composition (g per 100 g protein) of chironomid larvae at harvest (day 21) by substrate

Amino acid	MSS	AW	MVW	PMS
Leucine	5.6	5.1	6.1	4.8
Lysine	5.3	4.7	5.7	4.2
Valine	4.1	3.8	4.4	3.6
Isoleucine	3.4	3.1	3.7	2.9
Methionine	1.8	1.6	2.1	1.4
Threonine	3.2	2.9	3.5	2.7
EAAI	0.84	0.79	0.87	0.71

Substrate chemical analysis at the start of the rearing period showed marked differences in nutrient content. Mixed vegetable waste had the lowest C:N ratio (10.8:1) and highest total nitrogen (3.94%), while paper mill sludge had the highest C:N (34.2:1) and lowest nitrogen (1.08%). These substrate properties correlated directly with the rate and ceiling of protein accumulation observed in the larvae over the 24-day rearing period. Protein accumulation in larvae reared on all four substrates followed a sigmoidal (logistic) trajectory rather than a linear one. On mixed vegetable waste, crude protein rose steeply between days 6 and 15, reaching its half-maximum (t_{50}) at day 10.3, and plateaued near 64.7% DM by day 21. Municipal sewage sludge produced a similar curve shape but with a lower asymptote (62.8% DM) and a slightly later inflection ($t_{50}=11.7$ days). Abattoir waste larvae peaked at 55.1% DM, while paper mill sludge supported the slowest protein gain, reaching only 48.9% DM by day 24 a value still well above the 40% threshold considered adequate for fish feed formulation [10]. The logistic growth rate constant (k) was highest for mixed vegetable waste (0.31 day^{-1}) and lowest for paper mill sludge (0.19 day^{-1}).

**Fig 1:** Crude protein accumulation dynamics in *Chironomus plumosus* larvae reared on four organic waste substrates over a 24-day period

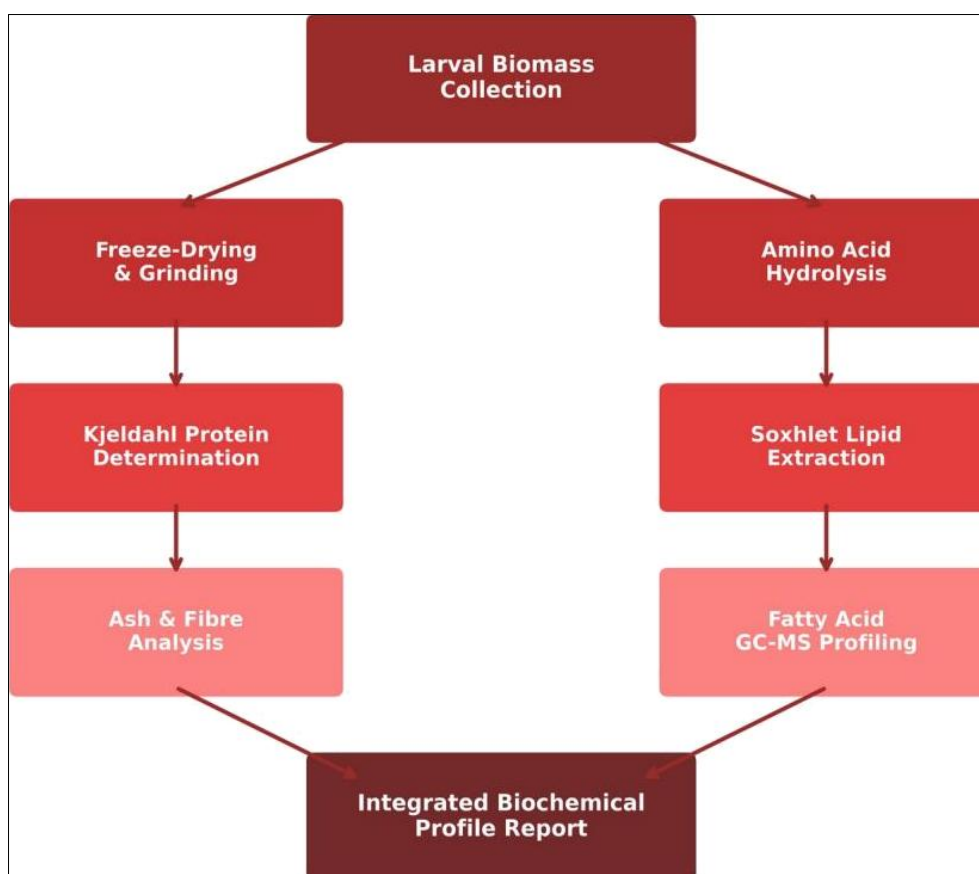


Fig 2: Schematic of the biochemical analysis workflow from larval biomass collection to integrated compositional profiling

Amino acid profiling at harvest (day 21) revealed that larvae from all substrates contained a full complement of essential amino acids. Leucine was the most abundant essential amino acid across all treatments (4.8–6.1 g per 100 g protein), followed by lysine (4.2–5.7 g) and valine (3.6–4.4 g). The essential amino acid index (EAAI) was highest for mixed vegetable waste larvae (0.87) and municipal sewage sludge larvae (0.84), and lowest for paper mill sludge (0.71). This positions MVW-reared chironomid larvae close to the EAAI of fish meal (0.89), making them a competitive protein ingredient [11].

Lipid content at harvest ranged from 6.8% DM (paper mill sludge) to 11.2% DM (abattoir waste). Fatty acid GC-MS profiling identified oleic acid (C18:1n-9) as the dominant fatty acid in all treatments (28.4–35.6% of total fatty acids), followed by palmitic acid (C16:0, 19.3–24.1%) and linoleic acid (C18:2n-6, 11.7–16.8%). Omega-3 fatty acids primarily α -linolenic acid (C18:3n-3) comprised 2.1–4.3% of total fatty acids, with the highest proportion in municipal sewage sludge larvae. The omega-6:omega-3 ratio ranged from 3.9:1 (MSS) to 7.1:1 (PMS), values that fall within acceptable ranges for animal feed but are higher than those typical of marine-origin feed ingredients [12].

Discussion

The sigmoidal protein accumulation pattern observed here with a clear lag phase, exponential gain, and plateau has not been explicitly reported for chironomid larvae before, though it parallels growth models well established for black soldier fly larvae and mealworms [2]. The biological basis is straightforward: young instar I and II larvae invest heavily in structural growth (chitin, cuticle) before redirecting metabolic effort toward protein and lipid storage in the later instars that

precede pupation [3]. The practical takeaway is that harvesting before day 18–21 sacrifices a substantial fraction of the achievable protein yield, while delaying beyond day 24 risks pupation losses and diminishing returns. Substrate C:N ratio proved to be a reliable predictor of both the rate constant (k) and the asymptotic protein ceiling (P_{max}) in the logistic model. This aligns with the findings of Sulistiyarto and Bakrie [4], who documented faster chironomid growth on chicken manure a low-C:N substrate compared with fish waste. The mechanistic link runs through microbial mediation: low-C:N substrates release ammonium nitrogen that fuels bacterial biofilm proliferation, and it is this biofilm, rich in bacterial protein, that chironomid larvae actually ingest [5]. Paper mill sludge, with a C:N of 34.2, promotes cellulose-degrading fungi rather than protein-rich bacteria, and this microbial community shift appears to cap the protein available to the larvae.

The amino acid profiles at harvest confirmed that chironomid larvae are not merely proteindense but protein-complete containing all essential amino acids at levels adequate for monogastric animal nutrition [11]. The EAAI of 0.87 recorded for MVW-reared larvae is nearequivalent to that of commercial fish meal and well above soybean meal (typically 0.72–0.75), reinforcing the potential of chironomid biomass as a fishmeal replacement ingredient [6]. The relatively high leucine and lysine concentrations are especially valuable, as these are the first-limiting amino acids in many cereal-based livestock diets. A limitation of this research is the use of a single species (*C. plumosus*) under controlled laboratory conditions. Field-reared larvae encounter variable temperatures, predation, and mixed-species competition that may alter biochemical outcomes. And the present work did not track tryptophan dynamics across the full time series only at harvest which leaves a gap in understanding how this heat-labile amino acid accumulates

relative to other essentials. Future research should incorporate in-vivo digestibility trials to confirm that the high in-vitro protein values translate to real nutritional benefit in target animal species ^[13].

Conclusion

Organic waste composition directly and measurably governs the rate, timing, and ceiling of protein accumulation in chironomid larvae a relationship that this research quantified through replicated biochemical time-series across four substrates under controlled conditions.

Mixed vegetable waste emerged as the most effective rearing medium, supporting a peak crude protein content of 64.7% DM and an essential amino acid index of 0.87, values that approach those of commercial fish meal. Municipal sewage sludge performed comparably in protein terms (62.8% DM) but raises regulatory considerations for downstream feed use that were beyond the scope of this work. Paper mill sludge, while the least productive substrate (48.9% DM protein), still generated biomass above the 40% protein threshold for basic feed formulations.

For practitioners aiming to produce chironomid protein biomass from organic waste, the key recommendation is to select or blend substrates targeting a C:N ratio below 15:1 and to time harvest between days 18 and 21, when the logistic accumulation curve has plateaued but pupation has not yet begun. Doing so maximises both protein yield and amino acid quality per rearing cycle.

Looking ahead, scaling these findings to pilot-level continuous rearing systems and conducting in-vivo digestibility trials with poultry and fish will be necessary to bridge the gap between laboratory biochemistry and commercial feed ingredient production.

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