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RESEARCH PAPER

Analysis of the ^{13}C isotope ratios of amino acids in the larvae, pupae and adult stages of *Calliphora vicina* blow flies and their carrion food sources

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Abstract

Adult blow flies are one of the first necrophagous insects to colonize fresh carcasses. The eggs they lay hatch into larvae, which then feed on the decomposing body. Like all organisms, blow flies "are what they eat," meaning that the isotopic composition of their body tissues reflects their diet. This manuscript combines ecology with a forensic application by using isotope ratio mass spectrometry (IRMS) to understand the relationship between the $\delta^{13}C$ of amino acids in different carrion sources and the blow fly that feed on them. We also measure the amino acid-level fractionation that occurs at each major life stage of the blow flies. Adult blow flies from a commercial strain of *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae) oviposited on raw pork muscle, beef muscle, or chicken liver. Larvae, pupae, and adult blow flies from each carrion were selected for amino acid compound-specific isotope analysis. Canonical discriminant analysis showed that flies were correctly classified to specific carrion types in 100% (original rules) and 96.8% (leave-one-out cross-validation [LOOCV]) of cases. Regarding life stages, we obtained 100% and 71% of correct classification in original rules and LOOCV, respectively. The isotope ratios of most of the essential amino acids did not significantly change between life stages (at 95% CI). However, some non-essential amino acids (Ala, Ser, and Glu) and some conditionally essential amino acids (Gly and Pro) were isotopically depleted in the adult stage. Except for the essential amino acids, the amino acids in larvae and pupae were enriched in ^{13}C, and adult blow flies were depleted in ^{13}C relative to the carrion on which they fed. These results make it possible to exclude potential sources of carrion as larval food. Amino acid-specific IRMS could help inform entomologists whether a fly has just arrived from another location to feed on a corpse or has emerged from a pupa whose feedstock was the corpse. Such insight could enhance the significance of blow flies for post-mortem interval determinations. The analytical ability to link organisms from one trophic level to another through the use of compound-specific isotope analysis of amino acids could have wide-reaching consequences in a variety of disciplines.

Keywords Stable isotopes · Isotope ratio mass spectrometry · Amino acids · Carrion · Blow flies · Ecology

Introduction

The use of naturally occurring stable isotopes for the study of arthropods is a small but well-explored field. For ecology applications, most of the studies are based on bulk isotope analysis for diet reconstruction and trophic position estimates [1–6]. Only a few studies have explored compound-specific isotope analysis (CSIA) to link amino acids from insects to their food sources [7–9]. Nonliving organisms (detritus) [10] have been often placed and studied as the base of food chains [11], but limited attention has been given to the category of detritus composed by decaying flesh of animal tissues, also known as carrion [12]. Animal decomposition plays an important role in the ecosystem when it influences the vegetation by

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releasing nitrogen-rich compounds into the soil [13] and increases insect activity in the area surrounding the carcass [12].

Among the necrophilous arthropods, the most commonly encountered and important colonizing insects are blow flies (Diptera: Calliphoridae). In the USA, adult blow flies are typically the first insects to arrive at a carcass, upon which they oviposit a clutch of eggs. The eggs hatch into larvae, commonly known as maggots, which actively feed on the corpse tissues. After feeding, the larvae depart the corpse to burrow into soil or other protective detritus. There, the outermost layer of the larval epithelial chitin hardens into a puparium, inside which the pupa transforms from the larval to the winged adult stage. Once metamorphosis is complete, the adult emerges from the puparium and leaves it behind [14]. Although this process happens in a predictable fashion, the length of the egg, larval, and pupal stages is highly dependent on environmental variables, particularly temperature. As a result, post-colonization interval estimation from immature insects can be highly contentious and is an area of active research in the field of forensic entomology [15–17].

The period of insect activity (PIA) is a proxy for the shortest possible time a corpse has been dead, so the standard practice in forensic entomology is to collect the most mature specimens on or around a corpse, thus avoiding an underestimation of the post-mortem interval (PMI) [18]. However, decaying carrion and the necrophilous insects that feed on it are part of the natural ecosystem and could potentially serve as sources of contamination for a corpse of forensic interest. For example, the presence of larvae, pupae, or empty puparium from a preexisting carrion source could easily cause an overestimate of the PIA [19]. Similarly, adult flies collected from a corpse could be either newly arrived, indicating a short PMI, or might have hatched from larvae that originally fed on the preexisting corpse, indicating a much longer PMI. In cases with insect contamination, it is therefore critically important for a forensic entomologist to be able to positively associate the insects being used for estimating PMI and PIA with the decedent in question.

Forensic DNA analysis of tissues extracted from insect guts is one reliable method used to demonstrate that an insect larva has fed upon a corpse [20, 21]. However, maggot saliva contains enzymes that also reach the gut region (specifically the crop) when combined with food [22], which can degrade DNA in less than 48 hours. Njau and coworkers [23] reported that human DNA could be recovered from larva crops only up to 48 h, or 96 h if maggots were starved. DNA recovery from the pupal or adult insect life stages would be impossible, although one case study did recover DNA from the exterior of a puparium [24]. An alternative method is therefore needed that works with a broader range of insect samples, in different life stages, and with longer PMIs. In this context, stable isotope analysis serves as a prospective methodology to link insects to their originating corpse.

In this study, we demonstrate the potential of stable isotope ratio analysis to link different blow fly life stages to carrion from different species, which could help resolve any uncertainty about the relationship between insects and corpses. Also, not many studies depict how the complex metabolic process that occurs during metamorphosis influence the isotopic signatures of insect life stages [4, 9, 25, 26]. Herein, we also depict how three life stages of a holometabolous insect can be distinguished from each other based on isotopic signatures of their amino acids.

Isotope ratio analysis is a powerful tool of forensic chemistry that has been used, for example, to predict the geographic origin of individuals based on the isotopic ratios of light elements in their hair [27–29]. Because stable isotopes originate from food, "you are what you eat." If, like people, blow flies *are* what they eat, the isotope ratios found in the fly specimens should allow identification of their developmental resource. Isotope ratio mass spectrometry (IRMS) should be particularly effective with blow flies developing on corpses because all of the biomolecules needed to complete blow fly growth and development are typically acquired from the tissue of a single individual. Proof of this hypothesis comes from a recent study using direct analysis in real time coupled to high-resolution mass spectrometry (DART-HRMS) used amino acid profiling of oviposited eggs to identify necrophagous fly species [30].

Several groups have shown that biological chemicals originating from different organs or different metabolic pools can have significantly different degrees of isotopic fractionation within the same organism [2, 6, 31]. However, isolation of these different chemical components of a specimen typically requires pooling specimens and conducting a significant number of wet chemical processes. The resulting number of isotope measurement variables derived from such extensive work is typically limited to 3–4 variables per element, such as $\delta^{13}C$ values of lipids, protein (muscle) and chitin.

To maximize the number of isotopic variables and metabolic pools for chemical comparison, and to minimize the extent of wet chemical methods, we use amino acid-specific isotope ratio analysis as a basis for comparing blow flies with the carrion on which they have fed. We examined the $\delta^{13}C$ values of amino acids derived from the different blow fly life stages, and we show that whereas the extent of $\delta^{13}C$ fractionation varies between amino acids and life stages, there is greater variance in $\delta^{13}C$ values of amino acids in the carrion. The results indicate that, among other capabilities, one may differentiate insects that grew and developed from on carrion from two different species.

Material and methods

Chemicals

Orthophosphoric acid (> 85% purity), sodium persulfate (99% purity), sulfuric acid (> 95% purity), and amino acids

(98–99% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isotope standards NBS-19, LSVEC, USGS-40, USGS-41, and IAEA-600 were purchased from NIST (Gaithersburg, MD, USA), USGS (Reston, VA, USA), and the IAEA (Vienna, Austria), respectively.

Blow fly treatment

Adult blow flies from a commercial strain of *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae) oviposited under controlled conditions on three media. All of these were food-grade animal tissues obtained from a local grocery store: raw beef muscle, raw chicken liver, and raw pork muscle. Larvae were allowed to feed ad libitum and develop normally into adult flies. Arbitrary individual insects were chosen from each treatment in three life stages. These stages were the post-feeding third instar (larvae) stage, the pupal stage consisting of both developing pupa and associated chitonous exterior puparium, and adults. Adult flies were not permitted to feed before analysis.

Sample preparation

We analyzed a total of 12 replicates for each set of carrion type: carrion tissue ($n = 3$), associated life stages larvae ($n = 3$), pupae ($n = 3$), and adult flies ($n = 3$). Samples were individually pulverized in 2-mL polypropylene tubes containing four 3.2-mm chrome steel beads using a minibead beater (Biospec Products Inc., Bartlesville, OK, USA) for 5 min at 3450 rpm [32].

EA-IRMS

For bulk analysis, the different pulverized samples were weighed to approximately 470 μg into tin capsules for posterior bulk $\delta^{13}C$ and $\delta^{15}N$ measurements. Samples were analyzed using a Flash HT Plus Elemental Analyzer (Thermo Fisher Scientific, Bremen, Germany) coupled to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) via a Conflo IV interface (Thermo Fisher Scientific). Gases were ultra-high purity from Airgas (Morgantown, WV): > 99.9999% He, 99.999% N_2, 99.997% CO_2, and 99.999% O_2.

Samples were measured relative to compressed CO_2 working gas and calibrated to the international scale, relative to VPDB, using a two-point normalization based on NBS-19 ($\delta^{13}C = +1.950‰$) and LSVEC ($\delta^{13}C = -46.60‰$). For nitrogen isotope ratio measurements, samples were measured relative to atmospheric air and converted to the "atmospheric nitrogen (air-N_2)" scale using USGS-40 ($\delta^{15}N = -4.52‰$) and USGS-41 ($\delta^{15}N = +47.57‰$) as two-point calibration standards [33]. IAEA-600 (caffeine; $\delta^{13}C = -27.77‰$, $\delta^{15}N = +1.0‰$) was run after every three or four samples as a quality control standard. The measurement uncertainty (95% confidence interval) from pooled replicates is estimated to be ± 0.2‰ for $\delta^{13}C$ measurements and ± 0.3‰ for $\delta^{15}N$ measurements. The following equation was used to express the relative difference of isotope ratios into the delta notation (per mil ‰) [34, 35]:

$$\delta\text{sample}(‰) = \left(\frac{R\ \text{sample}}{R\ \text{standard}} - 1\right)$$

where R_{sample} is the specific isotope abundance ratio of the of the heavier isotope over the lighter for sample and $R_{standard}$ is the abundance ratio of the corresponding international standard.

LC-IRMS

For carbon compound-specific isotope analysis of insect amino acids (AA), we used a Dionex ICS5000 ion chromatography system (ICS) (Dionex, Sunnyvale, CA, USA) coupled through an LC-Isolink interface (Thermo Fisher Scientific, Bremen, Germany) to the same Delta V Advantage isotope ratio mass spectrometer described above. The LC-IRMS procedure used in this work has been used on numerous occasions for compound-specific isotope analysis of amino acids in proteinaceous material [32, 36–41], and more information about the method validation can be found in these previous articles [32, 36–41]. Aliquots of approximately 10 mg of each pulverized sample were hydrolyzed in 6 M hydrochloric acid for 24 h at 110 °C in a vacuum oven. This hydrolysis protocol does not cause significant $\delta^{13}C$ fractionation on the recovered amino acids [42]. The AA mixture was posteriorly filtered with a 0.45-μm PTFE filter and evaporated to dryness at 40 °C in a Mivac evaporator (Genevac, Ipswich, UK). The dry residues were then reconstituted in 1 mL of deionized water and filtered with a 0.45-μm PVDF filter to remove any non-hydrolysable body tissues. Like others, we observed that acid hydrolysis causes conversion of glutamine (Gln) and asparagine (Asn) to their respective dicarboxylic acids: glutamic acid (Glu) and aspartic acid (Asp). For this reason, the delta values reported for Glu and Asp include a small contribution from Gln and Asn, respectively [32, 43]. Methionine and cysteine were not observed after acid hydrolysis.

A mixed mode column Primesep A, 2.1 × 250 mm, 5 μm, 100 Å (SIELC Technologies, Prospect Heights, IL, USA) provided adequate separation of the recovered underivatized amino acids. The mobile phase was composed of 100% solvent A (100% deionized water [17.6 MΩ]) for the first 18.7 min. The mobile phase then underwent a linear gradient to 14% solvent B (0.03 M sulfuric acid in deionized water) at 40 min. The mobile phase then underwent another linear gradient elution to 100% solvent B at 95 min until the end of the separation at 120 min. The flow rate of the mobile phase was 260 μL/min.

The HPLC eluent passed into the LC-Isolink interface, where wet chemical oxidation quantitatively converted all carbon containing compounds to CO_2. Oxidation took place in the aqueous phase at 99.9 °C using 10% sodium peroxodisulphate and 1.5 M phosphoric acid. These reagents were individually pumped into the Isolink two-head pump interface. The resulting gas mixture of CO_2 and water was removed from the cooled solution by a membrane exchanger and passed through two water traps before the effluent CO_2 stream was transferred to the attached IRMS. An Aura solvent degassing system with a 1.5-μm Teflon filter membrane (Sigma-Aldrich, St. Louis, MO, USA) filtered and degassed all mobile phase solvents and oxidation reagents for 60 min prior to use. To remove any external CO_2, all reagent solutions were continuously sparged with UHP helium before and during use.

Similar to the bulk IRMS measurements, samples were measured relative to CO_2 working gas and calibrated to the international scale relative to VPDB by using a two-point normalization based on USGS-40 ($\delta^{13}C = -26.39‰$) and USGA-41 ($\delta^{13}C = 37.63‰$). Caffeine IAEA-600 ($\delta^{13}C = -27.77‰$) was likewise used as quality control standard. Drift corrections were not necessary. Following the principle of identical treatment, a mixture of 17 pure standard amino acids of known isotopic composition was analyzed under the same conditions (except for the acid hydrolysis) to verify that chromatographic separation and instrument performance were being properly achieved without any significant fractionation. The measurement uncertainty (95% confidence interval) of pooled replicates was different for every amino acid (Table 1). More measurements should be performed to better estimate the individual precisions.

Data analysis

For EA and LC-IRMS analyses, Isodat 3.0 software was used for data acquisition (Thermo Fisher Scientific) and SPSS 24 (IBM, Armonk, NY, USA) was used for statistical analysis. Statistical tests such as one-way ANOVA, principal component analysis (PCA), and canonical discriminant analysis (CDA) were performed following the definitions published in reference [32].

Results and discussion

EA-IRMS

Nitrogen

In general, nitrogen fractionation occurred at each life stage of the blow flies. From carrion to larvae, the average fractionation across all carrion sources was + 3.7‰ (Fig. 1), which is slightly higher than the general ^{15}N enrichment of + 3.4‰ observed by others [44–46]. From the larvae to pupae life stages, the blow flies display an average fractionation of − 1.0‰, but the uncertainty in the average fractionation is too large to be significantly different from the larvae. However, paired sample *T* tests revealed that within each carrion source, there was significant depletion in ^{15}N between the larvae and the pupae ($p < 0.05$) (Fig. 2). The metamorphic change from larvae to adult involves complex metabolic processes wherein the body tissues become more enriched in ^{15}N in each life stage due to the release of depleted nitrogenous excreta [4, 47]. In this way, this apparent depletion pattern from larvae to pupae between the carrion sources was a surprising result. It is worth mentioning that we did not separately analyze the puparium or meconium (post-emergence nitrogenous excreta) of the insects, which would provide a better understanding of the nitrogen fractionation during these stages.

Bulk nitrogen isotope ratios increased by an average of + 2.5‰ from pupae to adult flies (Fig. 1). The average enrichment of the adult flies relative to the average pupae or average larvae isotope ratios was significantly different, which was confirmed by paired sample *T* tests ($p < 0.01$) within each carrion source (Fig. 2). The total average fractionation from carrion to adult flies was + 5.2‰, which was very significant (paired sample *T* test, $p < 0.0001$). This overall $\delta^{15}N$ enrichment is usually as a consequence of fractionation during transamination and deamination of nitrogen-containing compounds during the insect development.

McCutchan et al. [48] observed that insects raised in high-protein diets depict a trophic shift (diet to consumer) that is higher than insects raised on invertebrate or plant-based diets. In our experiments, *C. vicina* was raised on a nearly pure protein diet of vertebrate tissues. We speculate that the combination of diet and metamorphic effect might explain the average fractionation higher than the usual + 3.4‰ [48]. Supporting this hypothesis is

Table 1 Measurement uncertainty (95% CI) of $\delta^{13}C$ amino acid measurements when three separate samples of the same type are carried through the entire method (digestion, filtering, evaporation, reconstitution, separation, and analysis). The uncertainties below are pooled uncertainties of triplicate measurements for each of three carrion sources and three life stages. Therefore, for each pooled value below, $N = 36$, and DOF = 21

Amino acid	Asp	Glu	Ser	Thr	Gly	Ala	Pro	Val	Ile/Leu	Tyr	Lys	His	Phe	Arg
± 95% CI (‰)	1.2	0.5	0.5	0.4	0.8	0.6	0.8	1.1	1.3	1.6	1.3	1.5	1.5	1.9

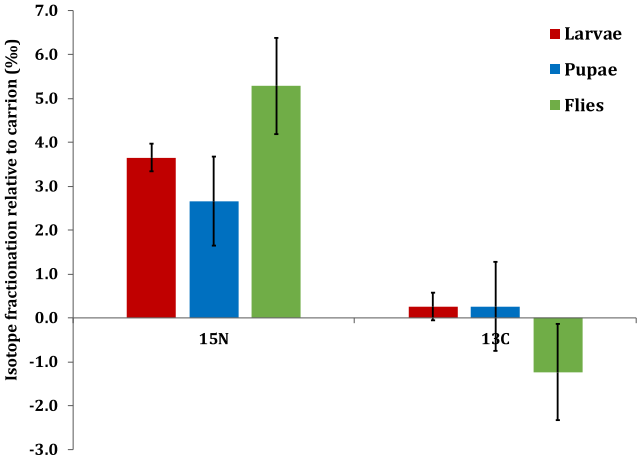


Fig. 1 Fractionation of bulk isotope ratio values for different life stages of blow flies, averaged across carrion sources ($n = 12$). Error bars show the pooled 95% confidence intervals

that each life stage had an isotopic signature that was slightly different for each carrion source. In general, blow flies raised on beef depicted the most enriched nitrogen signatures, followed by blow flies raised on chicken and pork, respectively (Fig. 2).

Carbon

Average bulk carbon isotope values showed an enrichment of + 0.6‰ to + 0.4‰ for larvae and pupae relative to carrion and a depletion of − 1.2‰ for adult flies relative to carrion (Figs. 1 and 2), but only the adults were significantly different at the 95% confidence interval. Figure 1 shows that the average change in bulk carbon isotope ratio values is not significant between carrion and larvae or pupae. Trophic-level carbon fractionation results have been reported previously [48], and the $\Delta^{13}C$ shifts for most insect consumers were reported to be

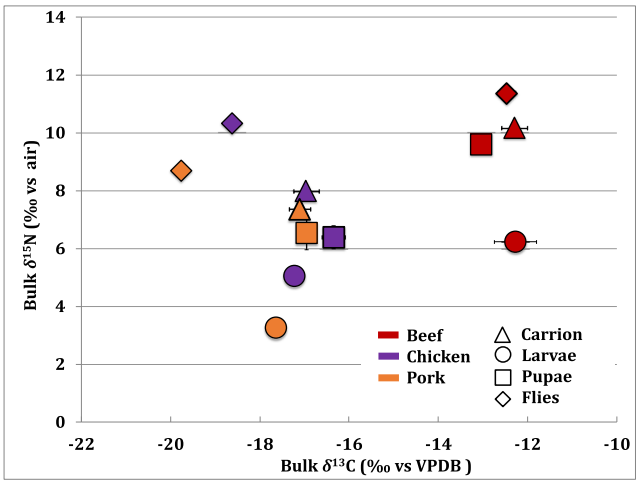


Fig. 2 Bivariate plot of bulk isotope ratio values for different life stages of blow flies that were raised on different sources of carrion ($n = 4$ for each data point). Error bars show the measured 95% confidence intervals

negligible or small, i.e., ranging from − 2.7‰ to + 3.4‰, relative to the food source [6, 49].

In this study, the magnitude of carbon fractionation generally is less than 1‰. On the contrary, the isotope ratio differences between different carrion are as large as 8‰. The small degree of fractionation relative to the large difference between carrion sources suggests that ^{13}C values may be used to exclude certain sources of carrion as potential sources of food for blow flies measured at any life stage. For example, Fig. 2 indicates that any life stage of a blow fly with a bulk ^{13}C value between −12‰ and −14‰ cannot have originated from either of the sources of chicken or pork used in this study. However, given the uncertainty in fractionation, a blow fly with a bulk ^{13}C value between −18 and −20‰ could have originated from either the pork sample or the chicken sample, but not from the beef sample.

These interpretations specifically relate to the individual samples of each food source used in this study, and not necessarily to every specimen of each food source. The average bulk ^{13}C value of the diets of different individuals within a species—and therefore the bulk ^{13}C value of individuals within a species—may vary by more than 10‰ [50], which means that more samples of each species should be analyzed before species-level determinations can be made. Given that dietary variations within species is likely to yield individuals that have similar bulk ^{13}C isotope ratios as individuals of different species, bulk ^{13}C isotope ratio measurements are unlikely to be able to selectively identify the species-level food source of a particular blow fly. However, to provide more variables for statistical comparisons, and to thereby obtain better discriminating power, we explored the use of compound-specific ^{13}C measurements of different amino acids among life stages and tissue types.

Compound-specific isotope analysis

To assess the isotopic fractionation between life stages, we plotted the average change in carbon isotope ratio values against each amino acid and life stage (Fig. 3; also, see Electronic Supplementary Material (ESM) Fig. S1 and Table S1). The results show that the $\delta^{13}C$ values of the essential amino acids (His, Leu/Ile, Lys, Phe, and Thr) at any life stage were not significantly different (at 95% CI) relative to the carrion on which the blow flies fed (Fig. 3). It is widely established that there is a carbon isotope shift of approximately + 1‰ between trophic levels [6, 51, 52]. However, since essential amino acids cannot be synthesized by blow flies, it is reasonable to expect that no carbon fractionation from diet to consumer should exist for the essential amino acids [51], but that a greater extent of fractionation should exist for the non-essential amino acids that can be chemically altered via metabolism in the blow flies. An exception to this trend was the essential amino acid Val, which was significantly different (at 95% CI) relative to the carrion (Fig. 3). Based

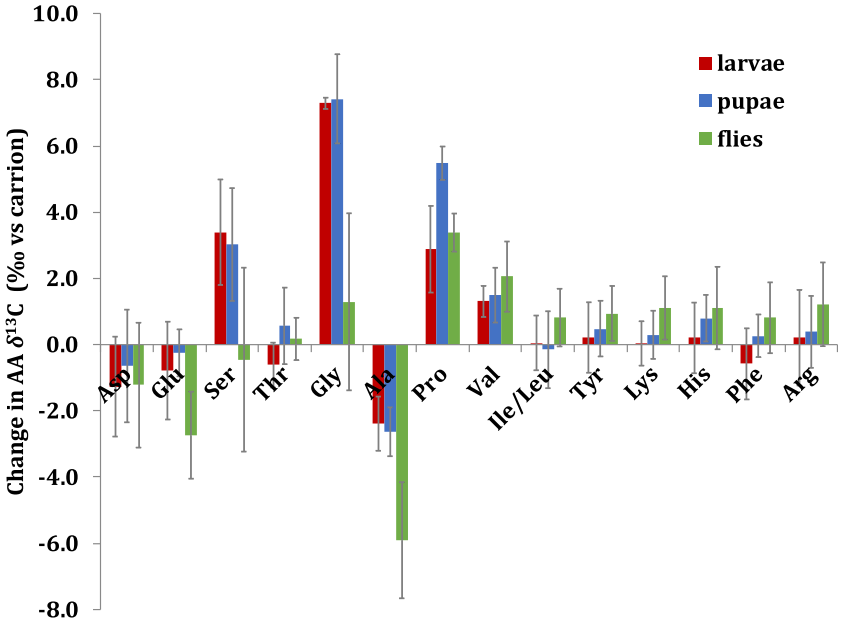


Fig. 3 Carbon fractionation between *C. vicina* life stages. When comparing the carbon isotopic ratio of larvae ($n = 9$), pupae ($n = 9$), and adult blow flies ($n = 9$) relative to carrion, there was no significant difference for essential amino acids in between the different stages (95% CI). Non-essential and conditionally essential amino acids of larvae and pupae were more enriched in ^{13}C while adult flies were more depleted in ^{13}C, both relative to carrion. Bars represent mean ± 95% CI

solely on our results, we could not find an explanation for valine being more enriched than the other essential amino acids. The assumption of an extra valine source coming from the insect's gut is a possible option because many fly species use metabolic products from symbiotic bacteria to enhance growth and development at early metamorphic stages [53]. If gut bacteria provide valine fractionation, one might expect the same valine enrichment in all three types of carrion, but enrichment seems to be less dramatic when insects were feed on chicken (see ESM Fig. S1). To our knowledge, there are no references investigating the role of valine in a fly-bacteria symbiotic relationship. Because the number of replicate measurements is quite small ($N = 3$ within carrion), more experiments must be performed before any drawing and additional conclusions regarding valine metabolism. Nonetheless, it is still generally true that the essential amino acids can act as tracers that link the different fly life stages back to the specific consumed carrion, without concern for major fractionation.

In contrast to the essential amino acids, some non-essential amino acids in the blow flies showed measurably different isotope ratios relative to the food sources. This fractionation is an indication of de novo synthesis rather than direct routing from the diet [54]. For larvae and pupae, the isotopic ratios of serine, proline, and glycine were enriched in ^{13}C relative to carrion by approximately + 3.8‰, + 4.5‰, and + 7.2‰, respectively (Fig. 3). However, alanine showed $\delta^{13}C$ depletion of -2 to -3‰ for larvae and pupae relative to carrion. Although the magnitude of isotope enrichment of specific amino acids exceeds the typical trophic-level ^{13}C enrichment in whole animals [5, 6, 55], our observations are consistent with bulk tissue or trophic-level fractionation, such as those reported by Webb et al. [31] for lipids and proteins (muscle).

We assume that some metabolic shifts during the growth and development of the insects influence the amino acid fractionation at different life stages of this species. De novo synthesis of non-essential amino acids is more energetically consuming than the direct routing of non-essential amino acids from the diet, the latter being favored when the diet contains high-protein content, such as carrion [54, 56, 57]. Our results showed the opposite trend, with a prevalence for the more-energetically consuming de novo synthesis pathway. Similar results were reported with birds [54], where the authors assumed that the non-direct routing from diet could be related to fasting preceding feather synthesis. The metabolic shifts depicted in our results were most dramatic when the blow flies metamorphose from pupae to adult flies. For example, the non-essential amino acids (e.g., Ser, Pro, Gly, Ala, and Glu) all showed ^{13}C depletion between the pupae and adult fly stages; the same pattern can be visualized in the plots for individual amino acids in the various diets (see ESM Fig. S1). The greatest ^{13}C depletions from pupae to adult flies occurred for Ala (~ 3‰), Glu (~ 3‰) and Gly (~ 4‰) (Fig. 3).

The isotope depletions observed between pupae and adult flies do not seem to be correlated with the fractionation occurring between earlier life stages. For example, Glu did not show fractionation at all from carrion to larvae or carrion to pupae, Ala showed depletion in the first two life stages, and other non-essential amino acids (Ser,

Pro, and Gly) all showed enrichment in the first two life stages relative to carrion.

Non-essential amino acids, except tyrosine, are synthesized from one of the intermediates of carbohydrate metabolism, such as pyruvate, oxaloacetate, α-ketoglutarate and 3-phosphoglycerate. Necrophagous insects, including *C. vicina*, rely on diets containing virtually no carbohydrates (animal tissues). Therefore, these insects probably use glucogenic amino acids and lipid catabolism to produce glucose via gluconeogenesis, a common metabolic pathway performed by all insects [58]. In insects, gluconeogenesis primarily occurs in the "fat body," an organ unique to arthropods, which is responsible for most of the synthesis, storage, and utilization of biomolecules during insects' development [59, 60]. Usually, more than 50% of the fat body is composed of lipids [61], which are stored during the larval feeding stages of holometabolous insects such as flies for use during metamorphosis and as initial reserves for the young adults [59]. Thus, the dominance of gluconeogenesis in the larvae is probably responsible for the fractionations observed between carrion and larvae.

Another nutrient present in the fat body is glycogen, the main source of glucose during the post-feeding larval and pupal stages [59]. Within an organism, lipids generally present lower $\delta^{13}C$ values than carbohydrates and proteins because of a fractionation step associated with pyruvate dehydrogenase, the enzyme responsible for the oxidation of pyruvate to acetyl-CoA [62, 63]. The reason the adult flies are more depleted in ^{13}C than their previous stages is therefore likely to be because they mobilize their lipid reserves to synthesize their exoskeleton, organs, wings, and reproductive organs, among other tissues. The known mechanisms do not seem to adequately explain why the earlier stages are so enriched in ^{13}C compared to the adults. One additional explanation is that the fractionation step required for the glycogen mobilization during the earlier stages makes larvae and pupae enriched in ^{13}C due to the constant release of ^{12}C-enriched CO_2. Although the details remain to be resolved, the present results show that the $\delta^{13}C$ values of amino acids in blow flies correlate with that of their diets, but that each amino acid provides a unique pattern of fractionation during insect development.

Pro and Glu showed quite unexpected signatures in that they differ from one another. Pro and Glu were different by 6‰ in larvae and pupae, but approximately 8‰ in adult flies. Proline is biosynthesized via a series of reactions that involve the cyclization of glutamate (deprotonated form of glutamic acid) as the precursor [64]. Because there are no carbon atoms involved in this process, we assumed there should be no carbon fractionation, so we expected Pro and Glu to have similar $\delta^{13}C$ values when Pro is not routed directly from the diet. However, Hare et al. [65] observed similar disparity between Pro and Glu when investigating modern and fossil collagen; they suggest that proline might come directly from the diet rather than de novo synthesis.

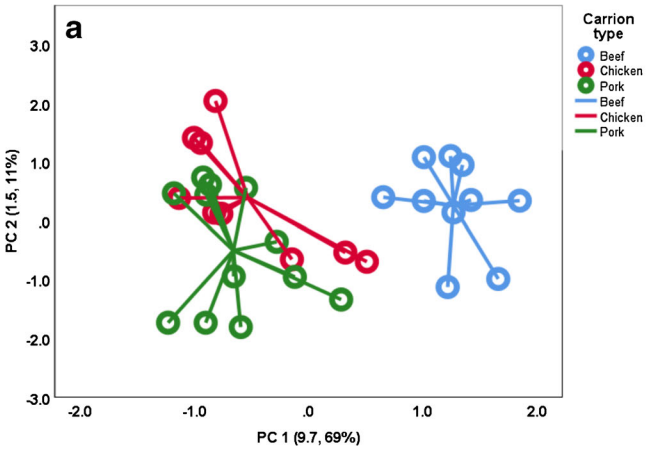
Regardless of the underlying metabolic factors, we were interested to see how effective the amino-acid-specific isotope ratio values were in classifying blow flies with their carrion diets. We therefore performed some relatively simple multivariate approaches to understand the variance in isotope ratios within and between different grouping factors, like life stage.

Classification based on carrion

Individuals were first classified into groups based on the type of consumed carrion. Initially, principal component analysis (PCA) was conducted using the $\delta^{13}C$ values for 15 amino acids extracted from each carrion and blow fly life stages as variables (Fig. 4). The data points include $n = 3$ separate measurements for each sample. A separate measurement implies a unique sample, digestion, and analysis step to provide independent input variables for each sample type.

One-way analysis of variance (one-way ANOVA) followed by Tukey's HSD post hoc test using carrion as the fixed factor (response variable) showed very significant differences between the within-group variance and between-group variance for every amino acid isotopic ratio (explanatory variable) (see ESM Table S2). Ala was the only not-statistically-significant amino acid, with α value of 0.109. All the other amino showed α values less than 0.05. These significant alpha values demonstrate that the variance in isotope ratio values of amino acids between carrion sources is vastly larger than the variance captured within all the samples derived from a carrion source. In other words, fractionation of amino acids by the blow flies is negligible relative to the large difference that exists between the amino acids in the food source.

Principal component analysis (PCA) also showed a high degree of correlation between the variables. This fact was corroborated by the Kaiser-Meyer-Olkin measure of sampling adequacy (0.800) and Bartlett's test of sphericity reaching significance (< 0.05). The variance explained by the first three principle components was 69.3% (PC1), 10.9% (PC 2), and 9.1% (PC3), which together explained ~ 89.3% of the total variance. Some natural clustering showed that individuals fed beef were separated into a distinct group, whereas insects fed pork and chicken were clustered together. Figure 4b is a bivariate plot of $\delta^{13}C$ values of Lys and Phe to show the contribution of just two amino acids to the variance in the isotope ratios between samples (blow flies or carrion source)



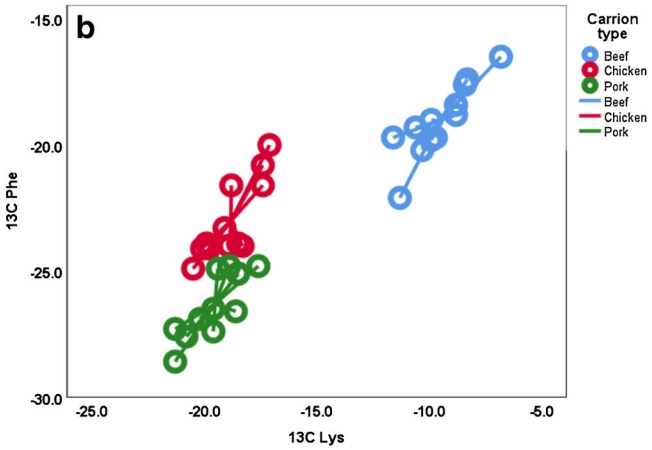


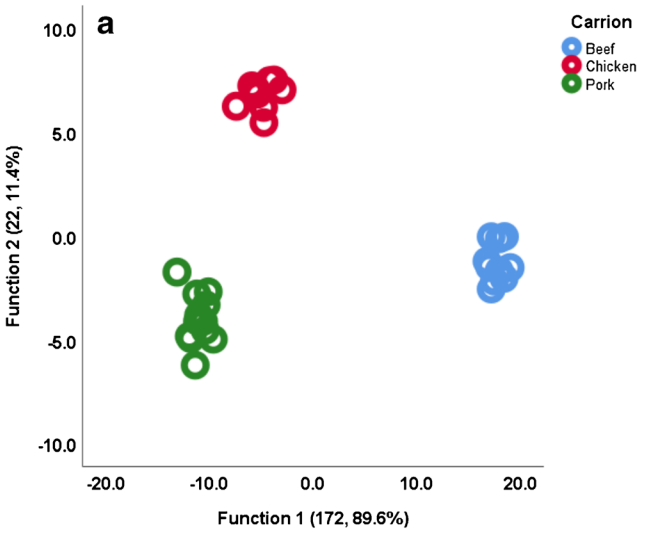
Fig. 4 **a** Principal component analysis (PCA) plot to show some natural clustering when grouping blow fly stages to different carrion types. PCA used the non-averaged carbon isotope ratios of 15 amino acids as the input variables. The absolute and percent variance explained by the first two PCA components is shown in the axis titles. **b** Bivariate plot of $\delta^{13}C$ values of Lys and Phe to show the contribution of just two amino acids to the variance between samples (blow flies or original carrion source) derived from different carrion sources

derived from different carrion sources. One can clearly visualize the relative difference between within-group variance and between-group variance of $\delta^{13}C$ values of these two amino acids.

When using a supervised dimension reduction method —e.g., canonical discriminant analysis (CDA)— to examine the variance of blow flies and carrion, the results provided more useful clustering. Using CDA, all samples derived from chicken and pork carrion could be separated using the first two canonical functions (Fig. 5a). When predicting group memberships—e.g., to the original carrion source—100% of samples were correctly classified based on the CDA original rules, and 96.8% were accurately classified after leave-one-out cross-validation (LOOCV) (Fig. 5b). The CDA original rules include every data point from the sample groups when performing the classification analysis. This approach is considered biased because a particular data point is used to build the classification rules which then attempt to predict its group membership. LOOCV is considered unbiased because the classification rules are based on all-but-one of the data points, and the rules are then used to predict the group membership of the held-out point. The process is repeated for every data point to assess the overall classification accuracy.

When Ala was omitted as a variable, the original-rules accuracy and leave-one-out cross-validation accuracy were both 100% accurate, which indicates that Ala does not provide any additional benefit for linking the blow flies to the carrion. As previously mentioned, one-way ANOVA showed Ala to be the only not significant variable, with an α value of 0.109.

To test the classification scheme and to ensure we are not overfitting the data, samples were assigned to random groups



b **Classification Results**[a,c]

		Carrion	Predicted Group Membership 1.0	2.0	3.0	Total
Original	Count	1.0	10	0	0	10
		2.0	0	9	0	9
		3.0	0	0	12	12
	%	1.0	100.0	.0	.0	100.0
		2.0	.0	100.0	.0	100.0
		3.0	.0	.0	100.0	100.0
Cross-validated[b]	Count	1.0	10	0	0	10
		2.0	0	8	1	9
		3.0	0	0	12	12
	%	1.0	100.0	.0	.0	100.0
		2.0	.0	88.9	11.1	100.0
		3.0	.0	.0	100.0	100.0

a. 100.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 96.8% of cross-validated grouped cases correctly classified.

Fig. 5 **a** Canonical discriminant analysis (CDA) plot of not-averaged carbon isotope ratios of 15 amino acids (14 variables) using carrion type as the classification factor. The absolute and percent variance explained by the first two functions is shown in the axis titles. **b** Assignment to carrion groups based on CDA original rules and leave-one-out cross-validation

instead of their carrion or sample type (i.e., life stage). The leave-one-out cross-validation accuracy for predicting group membership of randomly grouped samples was 25%, which is no better than guessing. As indicated by the PCA results, the successful clustering of all samples to their original carrion source is made possible by the small extent of amino acid fractionation relative to the large differences in amino acid isotope values between carrion. The amino acids that are most useful for carrion classification are those with the largest standardized canonical discriminant function coefficients, which—as one might expect—tend to be the essential amino acids that do not undergo fractionation (Table 2).

The importance of each amino acid variable in the classification of blow flies to carrion source can also be understood through examination of the pooled within-group correlations between discriminating variables—i.e., the amino acids—and the standardized canonical discriminant functions (see ESM Table S3). For example, the essential amino acids Thr, Lys, and Val have the largest correlations with discriminant function 1, with correlations of 0.384, 0.304, and 0.229, respectively. These amino acids are most useful for distinguishing samples originating from beef from the other two carrion sources.

As a general rule, multivariate techniques should employ many more (e.g., > 5× more) data points than variables. The use of fewer variables with successful classification gives strength to the statistical significance of the multivariate approach. Therefore, given the lack of fractionation for the essential amino acids, we repeated the CDA classification using only the eight non-essential amino acids as seven input variables for the 36 data points. Using His, Ile/Leu, Lys, Phe, Thr, Val, and Arg, the first two discriminant functions explained 100% of the variance between samples from different carrion sources, and the leave-one-out cross-validation accuracy was also 100%. Classification accuracy remained at 100% (for LOOCV and original rules) when using the four essential amino acids Val, Arg, His, and Phe. Classification works with fewer variables because there is a strong correlation between all the amino acids. For example, the isotope ratios of essential amino acids correlate with coefficients (R) that range between 0.785 for His and Phe to 0.976 for Arg and Lys. In contrast, the use of only the non-essential amino acids (Ala, Asp, Glu, Ser, Gly, and Pro) resulted in a LOOCV classification accuracy of 71%, probably because the metabolic fractionation of the non-essential amino acids adds extra variance to the isotope ratios at each life stage.

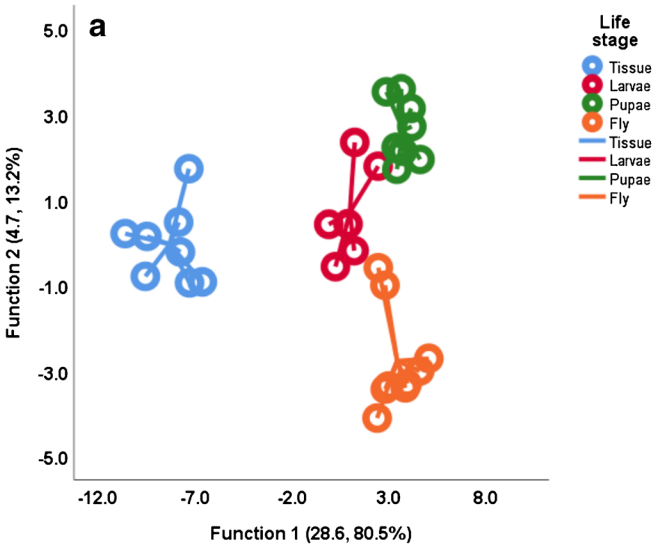
Cluster techniques were also investigated. Hierarchical cluster analysis showed that all the samples derived from beef readily clustered after the first branch points (see ESM Fig. S2). The samples derived from pork and chicken did not naturally cluster at the next few branch points, presumably because of the similarity between the sample means for several of the amino acids.

Table 2 Standardized canonical discriminant function coefficients for carrion as a discrimination factor. Variables with large absolute coefficient values have a bigger impact on the separation of carrion groups and successful classification. Highlighted variables are essential amino acids

Standardized canonical discriminant function coefficients		
	Function	
	1	2
His	− 1.689	− .426
Ile/Leu	758	2.268
Lys	2.146	− 1.243
Phe	058	2.829
Thr	1.825	660
Val	198	− .103
Asp	− .140	− .537
Glu	− .180	.280
Ser	2.167	− .348
Ala	− .360	− .845
Pro	− .793	− 1.604
Tyr	− .035	− 1.490
Arg	− 1.310	− 1.558
Gly	− .698	2.139

Classification based on life stage

We also attempted to use $\delta^{13}C$ values of the 15 amino acids to classify the different life stages regardless of the carrion type. Canonical discriminant analysis plot also showed very distinct clustering for each of the life stages (Fig. 6a). We also included a variable called "tissue," which was the combination of all carrion meat. The first function was responsible for explaining ~ 81% of the variance. Using original rules, 100% of groups were correctly classified using the original rules, and 71% of the samples were correctly classified to tissue type after leave-one-out cross-validation (Fig. 6b). Of course, standard evidence collection procedures imply that it is unlikely that a sample would be analyzed without knowing the nature of the tissue sample or life stage of blow fly that was used. Still, this approach indicates that, regardless of diet, the fractionation of amino acids by the different life stages of the blow flies is sufficiently reproducible between the different carrion sources to permit life-stage determination. In the future, we will be interested to examine the correlation between fractionation of amino acids and the age of the larvae/blow flies. To date, the data are collected at discrete times during major developmental processes. It is possible that the changes in $\delta^{13}C$ values of certain amino acids could change quite reproducibly with time, which would then provide an objective, chemical method for establishing the age of a blow fly larvae. Variables with large absolute coefficients have a bigger impact on the discrimination scores. Highlighted variables are essential amino acids.



b

Classification Results[a,c]

		Predicted Group Membership				
	Life	.0	1.0	2.0	3.0	Total
Original Count	.0	8	0	0	0	8
	1.0	0	6	0	0	6
	2.0	0	0	8	0	8
	3.0	0	0	0	9	9
Original %	.0	100.0	.0	.0	.0	100.0
	1.0	.0	100.0	.0	.0	100.0
	2.0	.0	.0	100.0	.0	100.0
	3.0	.0	.0	.0	100.0	100.0
Cross-validated[b] Count	.0	7	1	0	0	8
	1.0	1	2	2	1	6
	2.0	0	3	5	0	8
	3.0	0	1	0	8	9
Cross-validated %	.0	87.5	12.5	.0	.0	100.0
	1.0	16.7	33.3	33.3	16.7	100.0
	2.0	.0	37.5	62.5	.0	100.0
	3.0	.0	11.1	.0	88.9	100.0

a. 100.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 71.0% of cross-validated grouped cases correctly classified.

Fig. 6 **a** Canonical discriminant analysis (CDA) plot not averaged carbon isotope ratios of 15 amino acids using *C. vicina* life stages (larvae, pupae, and adult fly) as the classification factor. Tissue corresponds to the combined diet signature (all carrions). The absolute and percent variance explained by the first two functions is shown in the axis titles. **b** Assignment to life stage groups based on CDA original rules and leave-one-out cross-validation. 0 = tissue, 1 = larvae, 2 = pupae, 3 = adult fly

The underlying separation of groups based on life stage stems from the reproducible extent of fractionation for the different amino acids from the diet to the fly stages. The largest absolute values in discriminant functions 1 and 2 tended to be the essential amino acids (His, Lys, and Val for DF1; His, Lys, Thr, and Arg for DF2) (Table 3). Arg is considered an essential AA for insects because insects lost the ability to synthesize Arg during the evolutionary transition to uricotelism (excretion of uric acid) [66].

Table 3 Standardized canonical discriminant function coefficients table for life stage as discrimination factor

Standardized canonical discriminant function coefficients			
	Function		
	1	2	3
His	4.429	−2.757	5.136
Ile/Leu	−1.607	.884	3.082
Lys	−4.762	−9.445	−6.982
Phe	−.937	.683	2.715
Thr	−.339	2.282	1.740
Val	2.005	.762	−.789
Asp	.172	.482	.382
Glu	1.271	.963	1.727
Ser	−.100	.454	−1.450
Ala	−2.487	.763	−.088
Pro	1.482	2.149	1.794
Tyr	−.338	−.865	−2.507
Arg	.109	4.788	−3.876
Gly	1.552	−.328	−.718

Conclusions

Bulk isotope ratio analyses were sufficient to identify blow flies fed on beef, chicken or pork. However, bulk isotope ration analyses could not reliably distinguish blow flies raised on the latter two carrions. Amino-acid specific $\delta^{13}C$ analysis provided insight into the different types of metabolism at each of the major life stages. Essential amino acids tended not to undergo any fractionation because their structures and carbon-atom composition are unaffected by blow fly metabolic processes. However, the non-essential amino acids were enriched or depleted—depending on life stage—by as much as ~ 8‰.

Additional compound-specific isotope experiments are necessary to better understand the fractionation patterns between the life stages of *C. vicina*. These studies could also assess any correlation between fractionation and time, which might then be useful for forensic PMI estimations, as well as for a better understanding of the biochemistry of amino acids during insect development. Because of the inter-individual isotopic variation in cattle, pigs, or chickens, it is difficult to draw any conclusions about how well this method might predict the species that a questioned fly has fed upon. However, this proof-of-principle study does indicates that amino-acid-specific isotope ratio analysis can be used to exclude the possibility that a particular food served as the primary diet for a blow fly in any of its life stages. More optimistically, these results indicate that if the within-species variance in isotope ratios is smaller than the between-species variance, then species-level determinations of carrion sources might be possible. Further research is being conducted to include human tissues in the pool of samples.

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Compliance with ethical standards

No human subjects were used in this research, and no live animals were used. Animal flesh (meat) was purchased from grocery stores.

Conflict of interest The authors declare that they have no conflict of interest.

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