FEEDING AND GROWTH VARIATIONS AFFECT δ13C AND δ15N BUDGETS DURING ONTOGENY IN A LEPIDOPTERAN LARVA

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ABSTRACT

Isotopes are widely used in ecology to study food webs and physiology. The fractionation observed between trophic levels in nitrogen and carbon isotopes, explained by isotopes biochemical selectivity, is subject to important within-trophic level variations, leading to imprecision in trophic levels estimation. Understanding the drivers of these variations is thus important to improve the study of food webs. In this study, we characterized this variation by submitting *Spodoptera littoralis* larvae to a gradient of starvation levels, a factor that we hypothesized would change the trophic fractionation between individuals. The various growth rates that resulted from these starvation levels resulted in a ∼2.5‰within-trophic level variation of the trophic fractionation in both carbon and nitrogen, which is substantial compared to the 3-4‰classically associated with between-trophic levels variations. Hence starved animals sampled *in natura* may be ranked at a higher trophic level than they really are if food availability is not estimated. We were able to gain an understanding of the effect of growth rate on isotopes fluxes between three easy-to-measure biological materials, food, organism and its wastes (frass), giving insight into physiological processes at play but also conveying helpful information to the sampling framework of field studies.

Keywords starvation · trophic fractionation · isotopic ecology

Introduction

Stable isotopes are frequently used to understand fluxes of nutrients in ecosystems as well as trophic position and animal body condition (Post, 2002). The systematic differences in stable isotopes levels between a consumer's tissues and its resource - the trophic fractionation, here denoted Δ13C and Δ15N - are used to

estimate the trophic level of consumers. It occurs because isotopes of different masses have slightly different kinetics during biochemical processes (i.e. respiration or absorption, see Fry, 2006). The consumer's 15N level is usually increased by 3-4‰relative to its resource because animals retain 15N preferentially over 14N (Martınez del Rio et al., 2009). Carbon fractionation, on the other hand, might vary due to differences in the content in *de novo* synthesized lipids (Melzer and Schmidt, 1987). However, the within-trophic level variability of trophic fractionation sometimes impedes trophic level accurate estimation (Martınez del Rio et al., 2009). Understanding the drivers of these variations is crucial to improve our estimations.

Most of the proposed mechanisms to explain Δ15N variation involve diet protein quality and metabolism (Starck, Wang, et al., 2005). An overlooked mechanism is the nutritional status, determined by the resource availability in the environment (Doi et al., 2017, Trochine et al., 2019). Physiological responses to nutritional stress involve adjustments in digestion, reserve utilization and metabolic rate. As these processes change in rate (see fig.1.a. and c.) biochemical processes that determine absorption, respiration and excretion, also change, therefore impacting Δ15N and Δ13C. Nutritional stress (food limitation) which causes wieght loss has the overall tendency to increase heavy isotopes content, leading to an overestimation of the trophic level (corresponding to negative growth rates in fig.1.b. and d., see Martınez del Rio et al., 2009 and Haubert et al., 2005). To improve the estimation of trophic levels by including these mechanisms, we need a detailed understanding of the relationship between variation in nutritional status and trophic fractionation.

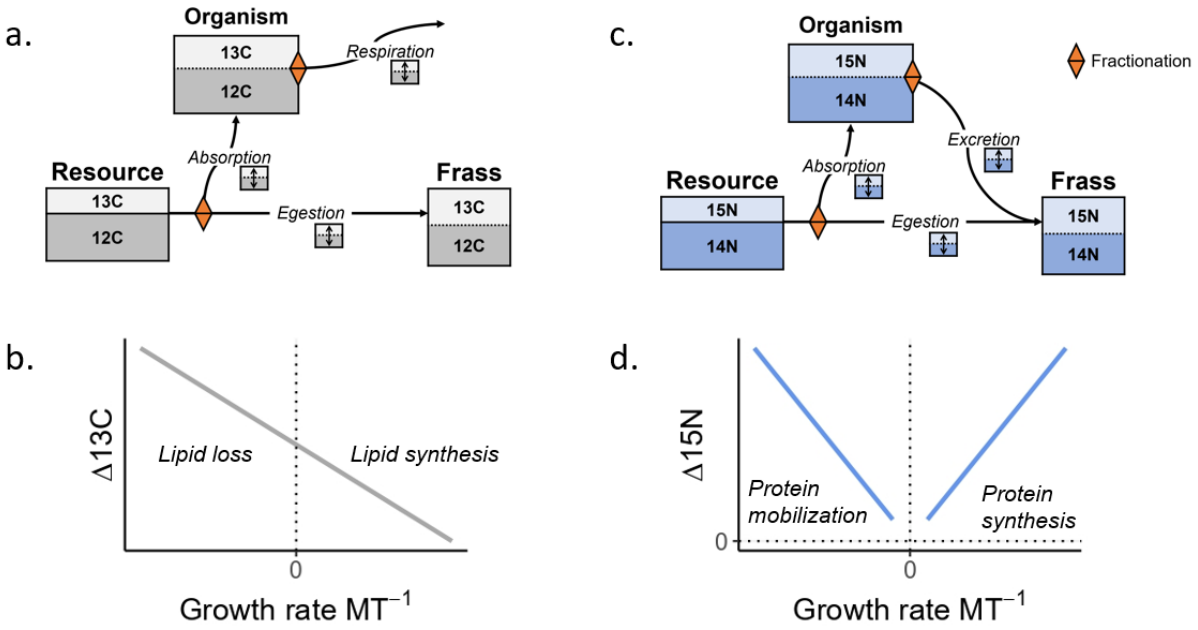

Figure 1: Isotopes routing and main hypotheses. a. & c. The three analyzed matrices, their relative (not-to-scale) content of isotopes, fluxes between them, as well as nodes where fractionation can occur (diamonds). The exact proportion of isotopes is not intended to represent reality faithfully but rather to illustrate the dynamical aspect of trophic fractionation. b. & d. Hypothesized relationship between trophic fractionation and growth rate for nitrogen and carbon. a. Most carbon is lost through either respiration or egestion and marginally through excretion. c. On the contrary, nitrogen is solely dropped through either egestion or excretion, with the impossibility of distinguishing their contribution only based on frass analysis. b. The hypothesized relationship between Δ13C and growth rate measured as mass gained per unit of time MT^{-1}. We expect a negative relationship because of the increasing proportion of 13C-poor *de novo* synthesized lipids, thus modulating the respiration fractionation. d. Hypothesized relationship between Δ15N and growth rate. High growth rates should increase protein synthesis and breakdown rates which retain preferentially 15N, and very low intake rates (weight loss) should increase protein catabolism also increasing Δ15N, both playing on the excretion fractionation.

Along this gradient in nutritional status, an important threshold is the maintenance feeding level (zero growth rate in fig.1.b. and d.). Below the feeding level required for maintenance, body mass decreases, adaptations in lipids and proteins metabolism are triggered. Lipids typically contain proportionally less 13C than proteins and carbohydrates (DeNiro and Epstein, 1977; McConnaughey and McRoy, 1979). A shrinkage of body lipid content should thus result in an increase in Δ13C compared to high feeding levels (Gaye-Siessegger et al., 2004), where the organism might be able to accumulate 13C-poor lipid reserves, therefore decreasing Δ13C (fig.1.a.).

Regarding nitrogen, low feeding levels are classically associated with an increase in Δ15N due to asymmetrical isotopic routing during protein mobilization for energetic catabolism (Hatch, 2012, see fig.1.c.). But high feeding levels, which are often accompanied by high growth rates, can also be accompanied by increase in Δ15N (Sick et al., 1997, Focken, 2001). Indeed, due to increase in protein synthesis and breakdown rates when the animal is growing fast, removal of 14N is intensified, thus enriching the consumer in 15N and increasing Δ15N (fig.1.c.). As a result, both very low and very high intake rates might increase Δ15N, but due to different processes, protein mobilization at low intake rates in a weight loss context and protein synthesis and breakdown rates at high intake rates in a growth context.

Moreover, as gut filling level varies with intake level, the food passage time and the biochemical conditions in the gut change. This change in temporal and chemical conditions might alter the isotopic fractionation right from the absorption stage (Schmidt et al., 2015). The relative increase in concentration of food in near-empty gut might increase the absorption of heavy isotopes. As a whole, trophic fractionation should depend both on the nutritional status and on body mass dynamics (Sears et al., 2009, Williams et al., 2007; see also Hatch, 2012 for a review), but these effects remain poorly investigated, especially with varying feeding levels (see Gaye-Siessegger et al., 2007).

Elucidating how the nutritional status modifies isotopic fractionation in a growing organism could shed light on the within-trophic level variability of estimated trophic level and should of interest for field studies as well. We conducted a feeding level experiment during the larval development of the cotton leaf worm *Spodoptera littoralis*, including severe food restriction, three intermediate restriction levels, and an *ad libitum* level, which corresponded to a range of growth rates. We assessed δ15N and δ13C budgets, measuring isotopic fractionations between food, body and frass (excretion + egestion).

More specifically, we tested the hypotheses that:

1. Δ15N should increase at negative growth rates due to protein catabolism during weight loss and increase at positive growth rates due to faster protein synthesis and oxidation. At maintenance level, as these two processes slow, Δ15N should decrease. Overall we should thus obtain a V-shaped relationship between Δ15N and growth rate (fig.1.d.).
2. Δ13C should decrease with growth rate because of the accumulation of 13C-poor lipid stores (fig.1.c.).
3. The relative absorption of 13C might increase at low feeding levels as both gut passage time and digestion efficiency increase.

Material and methods

Study system

S. littoralis larvae from a laboratory strain were reared on a semi-artificial diet at 23 °C, 60–70% relative humidity, and a 16:8 light/dark cycle (Hinks and Byers, 1976). In these rearing conditions and continuous access to food, the larvae go through 7 instars before entering metamorphosis (chrysalid stage). We isolated

400 6th instar larvae in individual 30 mL circular polypropylene boxes and provided them *ad libitum* food until 6th moult completion (start of the 7th instar).

Experimental design

We randomly assigned each of the 400 7th instar larvae to one of five food provision levels for the duration of the experiment. The food intake level was fixed to either 120, 240, 360, 480 or 900 mg of food per day (fw) depending on the larva. We had beforehand estimated the average maximal individual intake rate for 7th instar larvae and obtained 595 ± 43 mg/day. There were 80 individuals for each tested food intake level. We conducted this study over 10 weeks (10 temporal blocks), performing the experiment with 40 individuals each week, 8 for each food intake level. Individual measurements and sample collections took place over two or three days depending on whether the larva pre-pupation occurred on the third day of the 7th instar (in which case measures were taken during 2 days) or later (in which case measures were taken during 3 days).

Experimental workflow

During the experiment, each larva was fed the defined amount of freshly prepared food and weighed every day. Food subsamples were taken at every food preparation for subsequent chemical analysis. We provide the detailed food composition in Appendix 1, table 1. We collected and weighed everyday food leftover and frass produced by each larva to assess the actual intake and egestion rates. Food leftovers and frass were quickly stored at -20 °C, and later dried for 72 hours at 60 °C in an oven, to measure their dry mass. On the third day, half the larvae were quickly stored at -20 °C, dried for 72 hours at 60 °C in an oven, and their dry mass was measured. The other half of the individuals was left in the rearing chambers to later investigate the effect of food restriction on mortality, emergence success and body mass (not analyzed here).

Chemical analyses

Chemical analyses required that we pooled samples to obtain enough analyzable material. Hence groups of 4 caterpillars reared over the same week and on the same feeding level were composed, 2 that were pooled together for chemical analysis, and 2 that were left alive until emergence. The analyzed frass was a pooled sample of all 4 individuals.

All samples - food, larvae, and frass - were ground to a fine powder using a mixer mill (Retsch MM 200). Total carbon, total nitrogen, as well as δ13C and δ15N were measured using an elemental analyser coupled to a mass-spectrometer (Flash HT - Delta V Advantage, ThermoFisher). We checked for measurement errors using aromatic polyimide (EMA-P2) as standard.

Starvation proxy and isotopic data

Intake rate alone does not accurately represent the nutritional status. Rather, it depends on the balance between intake and requirements, which largely depend on body mass. We therefore used mass-specific ingestion rate (MSIR) as an indicator of the nutritional status. Low values of mass-specific ingestion rate define starvation, whereas high values of mass-specific ingestion rate represent sufficient intake.

$$MSIR_j = \frac{\sum_{i \in j} I_i}{\left(\frac{1}{4}\sum_{i \in j} d_i\right)\left(\frac{\sum_{i \in j} ib_i + \sum_{i \in j} fb_i}{2}\right)}$$

with I_i the total fresh mass of food ingested by the individual i of the group j over the course of the 7th instar, d_i the number of days spent in 7th instar by individual i, ib_i the initial body mass of individual i, and fb_i the final body mass of individual i.

PDB (Pee Dee Belemnite) and atmospheric nitrogen were used as standards for δ13C and δ15N, respectively. Isotopic data for sample s are reported using delta notation:

$$\delta 13C_s = 1000\left[\frac{13C_s/12C_s}{13C_{PDB}/12C_{PDB}} - 1\right]$$

and

$$\delta 15N_s = 1000\left[\frac{15N_s/14N_s}{15N_{air}/14N_{air}} - 1\right]$$

The trophic fractionation, *i.e.* the difference in δ13C or δ15N between larvae and food, was computed as follows:

$$\Delta 13C = \delta 13C_{larvae} - \delta 13C_{food}$$

and

$$\Delta 15N = \delta 15N_{larvae} - \delta 15N_{food}$$

We computed the ratio of absorption efficiencies between the two carbon isotopes (thereafter C IAER) to characterize how isotopes are differentially absorbed. This metric characterizes the absorption process, which is one of the two fluxes, along with respiration, determining carbon trophic fractionation (fig.1.a.). We did not compute this metric for nitrogen because unlike carbon, nitrogen excretion products also end up in insect frass, and it is therefore impossible to disentangle absorption from excretion effects using this metric (fig.1.c.). Moreover, as samples are heated during drying, some ammonium might volatilize, biasing the mass balance (Harrison, 1995).

$$C\ IAER = 1000\left(\frac{AE_{13C}}{AE_{12C}} - 1\right)$$

with AE_i the proportion of ingested isotope which is assimilated, and not egested/excreted, over the 7th instar, given in $\%$ dry weight:

$$AE_{jk} = 1 - \frac{C_{Ejk}E_j}{C_{Ijk}I_j}$$

with C_{Ejk} the proportion of isotope k in the frass of the group j, E_j the summed mass of frass produced by the four larvae of the group j and with C_{Ijk} the proportion of isotope k in the food of the group j, I_j the summed mass of food ingested by the four larvae of the group j . Please refer to Appendix 2 for detailed calculation of C_{Ejk} and C_{Ijk} .

Statistics

To test the effect of starvation and subsequent variation in growth rate (GR) on the trophic fractionation and relative carbon isotope absorptions, we used linear regressions. We chose to test the effect of growth rates on Δ15N and Δ13C, and the effect of mass-specific intake rate on C IAER. Details on modeling choices are provided in Appendix 3.

Results

Equation	n	R^2	F	p-value
$\Delta 13C = -0.0032 \times GR + -1.7$	92	0.35	48	p<0.01
$\Delta 15N = 0.0054 \times GR + 0.32$	92	0.53	100	p<0.01
C IAER $= -0.65 \times MSIR - 0.97$	100	0.28	38	p<0.01

Table 1: Summary of linear models describing the influence of growth rate and mass-specific ingestion rate (MSIR) on the trophic fractionation (Δ), and carbon isotope absorption efficiencies ratio (C IAER) respectively.

Despite strong starvation conditions, we were not able to force negative growth rate (fig.2.a.) and were therefore unable to test the relationships between trophic fractionation - Δ13C and Δ15N - and growth rates for negative growth rates. Here, we describe these relationships for positive growth rates only.

Trophic fractionation

As expected, larvae were always richer in 15N than the food they ate (Δ15N>0 for all larvae, see fig.2.d.). There was a clear positive correlation between Δ15N and (positive) growth rate ($F = 100$, $p < 0.01$, $R^2 = 0.53$; table 1.) in accordance with the right side of the graph in fig.1.d. As for carbon, larvae were always poorer in 13C than their food ($\Delta 13C < 0$ for all larvae, fig.2.c.), and this difference was exacerbated by a growth rate increase ($F = 48$, $p < 0.01$, $R^2 = 0.35$; table 1.), also in accordance with our hypothesis (fig.1.c.). In both cases, the Δ13C and Δ15N spanned over a range of $\sim$ 2.5 ‰, an important variation vis-à-vis the one classically attributed to one trophic level (3-4 ‰).

Isotope absorption efficiencies ratio (IAER)

The relative absorption of 12C and 13C depended on the mass-specific intake rate. 12C was systematically better absorbed than 13C, and this effect increased with feeding level ($R^2 = 0.28$, $F = 38$, $p < 0.01$, fig.2.b.).

Discussion

In agreement with our prediction, Δ15N showed an increase with growth rate of 2-2.5 ‰, which is comparable to differences typically associated with trophic fractionation (3-4 ‰). Our results agree with previous work showing that Δ15N is sensible to growth, at least in some tissues, as highlighted by Sick et al., 1997. We show that food limitation does not always increase Δ15N but rather depends on the starvation's intensity and whether starvation is concurrent with growth. This contrasts with the classic view that Δ15N should increase in starved individuals owing to protein depletion for energetic requirements. At least two studies suggested that this increase in Δ15N at high growth rates could be due to higher rates of deamination and protein synthesis at higher intake rates (Sick et al., 1997, Focken, 2001). Combining both predictions leads to a more comprehensive view of the effect of feeding level on nitrogen trophic fractionation. Despite very low intake rates, down to 10% of *ad libitum* levels, no weight loss was observed in our experiment. We can expect

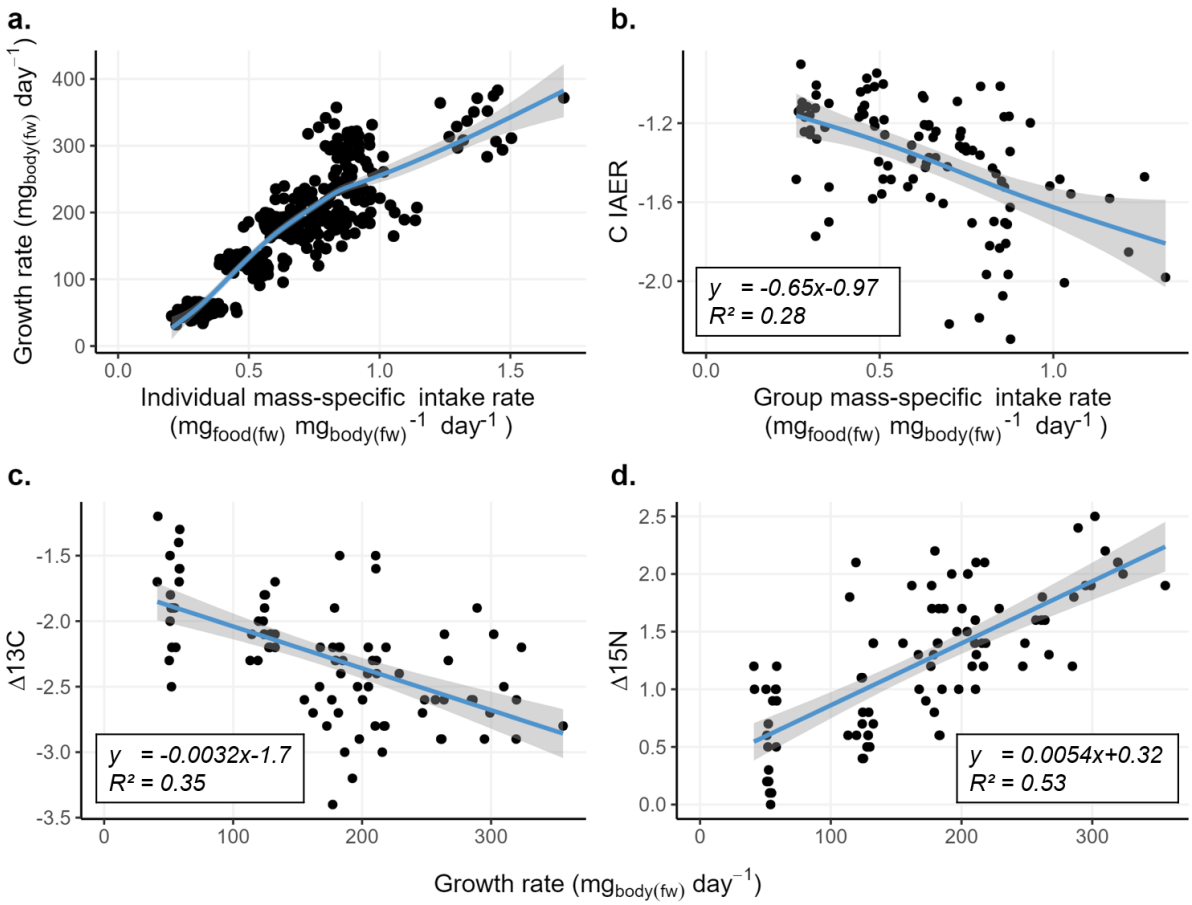

Figure 2: Growth and isotopic analyses. a. Individual growth rate as a function of mass-specific intake rate. The fitted curve is a generalized additive model. b. Carbon isotope absorption efficiencies ratio (IAER) as a function of mass-specific intake rate measured at the level of a group of 4 caterpillars, hence the term "group". c. Carbon trophic (Δ13C) fractionation as a function of growth rate. d. Nitrogen trophic fractionation (Δ15N) as a function of growth rate.

that reproducing this experiment with below-maintenance feeding levels, hence a decrease in body mass, could lead to a V-shaped relationship between Δ15N and feeding level.

On the other hand, Δ13C decreased with growth rate and intake level, which is consistent with previous findings (Doi et al., 2017). This is likely due to the possibility of constituting 13C-poor lipidic reserves at high growth rates (DeNiro and Epstein, 1977, McConnaughey and McRoy, 1979). To conclude, both Δ13C and Δ15N were affected by feeding level and growth rate. This shows that when assessing trophic levels using isotopic data, the nutritional status of individuals can hardly be ignored.

The mass budget of heavy and light isotopes revealed that 12C was more easily absorbed than 13C, which is consistent with the observation of a negative Δ13C. But as intake rate decreases, 13C is better absorbed compared to well-fed animals. This indicates that the biochemical environment of the gut varies with intake level, with effects on the processes of digestion and absorption. Moreover, we can also conclude that the respiration fractionation either is negligible compared to the one associated with absorption or that id further decreases the amount of 13C in the organism. But the biochemical origin of this modulation of 13C absorption is unclear. It could be due to longer gut passage time, or to increased food enzymatic availability at low gut filling levels.

Our results reveal that the within-trophic level differences in trophic fractionation imputable to nutriotional status (2-2.5 ‰) are comparable to differences typically associated with trophic level changes (3-4 ‰). Hence assessing trophic levels *in natura* using isotopic analysis requires caution, especially if the community is perturbed and might be subject to nutritional stress. We show that until starvation does not provoke body mass reduction, reducing feeding level might decrease body 15N compared to well-fed animals. However starvation reaches or exceeds the point where body mass starts to decrease, further decreasing the feeding level might increase body 15N compared to bodymass-stable individuals (Hatch, 2012). With the changes in frequency and intensity of drought episodes, one should be cautious to these potential biases in isotopic trophic ecology.

Contribution

S.C., A.M., J.M., D.S. and I.G. designed the study. S.C. ran the experiment, did the analyses, and wrote the first draft of that manuscript. All authors contributed to the final version of the manuscript.

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Appendix

1 Food ingredients

Ingredient	**% m/m**
Deionized water	76.7
Soja meal	6.79
Corn flour	6.79
Germalyne	3.40
Yeast	2.55
Agar	1.20
Casein	7.19E-01
D-Glucose	6.01E-01
Ascorbic acid	5.10E-01
Benzoic acid	2.69E-01
Linseed oil	1.92E-01
Nipagin	1.16E-01
Choline chloride	5.41E-02
Formaldehyde	3.60E-02
Alpha-Tocopheryl acetate	1.59E-02
Actitetra (Oxytetracycline 50%)	9.59E-03
Ampicillin sodium salt	7.19E-03
Myo-inositol	3.61E-03
Nicotinic acid	3.21E-03
Menadione	1.62E-03
Retinyl acetate	1.30E-03
Riboflavin	7.21E-04
Pyridoxine	7.21E-04
Thiamine hydrochloride	7.21E-04
Ergocalciferol	9.02E-05
Folic acid	6.49E-05
Biotin	1.44E-05
Cobalamin	9.74E-07

Table 1: Composition of the feed distributed to larvae, expressed as % mass/mass .

2 Isotope absorption efficiencies ratio calculation

Mass spectrometer usually directly gives isotopes ratio rather than isotopic content because usually, one of the isotopes has a low concentration.

Still, it is possible to compute the isotope content.

For carbon, ignoring the very low concentration in unstable isotopes, we have that the total carbon content is equal to the sum of the content of each stable isotope. So that, for sample s:

$$C_s = 13C_s + 12C_s$$

On the other hand the isotopic data are usually given in delta notation:

$$\delta 13C_s = 1000\left[\frac{13C_s/12C_s}{13C_{PDB}/12C_{PDB}} - 1\right]$$

We have thus two unknowns, $13C_s$ and $12C_s$, as well as two equations, enabling us to solve for the two isotopes content:

$$\frac{13C_{PDB}}{12C_{PDB}}\left(\frac{\delta 13C_s}{1000} + 1\right) = \frac{13C_s}{12C_s}$$

$$\frac{13C_{PDB}}{12C_{PDB}}\left(\frac{\delta 13C_s}{1000} + 1\right)(C_s - 13C_s) = 13C_s$$

$$13C_s = Cs\frac{\frac{13C_{PDB}}{12C_{PDB}}\left(\frac{\delta 13C_s}{1000} + 1\right)}{1 + \frac{13C_{PDB}}{12C_{PDB}}\left(\frac{\delta 13C_s}{1000} + 1\right)}$$

$$12C_s = \frac{C_s}{1 + \frac{13C_{PDB}}{12C_{PDB}}\left(\frac{\delta 13C_s}{1000} + 1\right)}$$

We have that $\frac{13C_{PDB}}{12C_{PDB}} \approx 0.0112372$, so, finally:

$$12C_s = \frac{C_s}{1 + 0.0112372\left(\frac{\delta 13C_s}{1000} + 1\right)}$$

Using the isotopic content, we can compute the absorption efficiency of each isotope.

3 Justification of the choice of linear models

We predicted that growth experienced during a given period at a certain rate would affect the isotopic content of the organism, that is, taking the example of carbon, which also holds for nitrogen:

$$13C_l = a.GR + b$$

with $13C_l$ the 13C content in the larva, GR the growth rate, a and b some constant, we should have $\delta 13C$ expressed as a function of growth rate GR as follows:

$$\delta 13C_l = 1000\left(\frac{c.a.GR + b}{1 - a.GR - b} - 1\right)$$

which is a hyperbolic function GR (c here is the standard isotopes ratio constant). We should thus expect non-linearity. However, as $13C_l$ is very low, and making the approximation that for $x << 1$, $\frac{x}{x-1} \approx x$ we can model this relation using a linear approach. We nevertheless tested for non-linearity by performing generalized additive models and examining the effective degree of freedom (edf).

GAM formula	n	EDF	p-value	R^2
$\Delta 13C \sim$ s(GR)	92	2.03	<2e-16	0.368
$\Delta 15N \sim$ s(GR)	92	1	<2e-16	0.524
C IAER $\sim$ s(MSIR)	100	1	<2e-16	0.27

Table 2: Generalized additive models results, with the sample size n, the effective degree of freedom (edf), p-value of the smooth term and the R^2.

For the two trophic fractionations and C IAER, the EDF indicate a linear dependence, with EDF roughly between 1 and 2 (2). We therefore chose to use linear models.