

Reviewer 1

The authors present methodological study investigating the effect of nutritional conditions on ^{13}C and ^{15}N isotopic fractionation in Lepidoptera larvae. By exploring fractionation under different feeding intensity regimes, the authors provide support for the hypotheses that (1) ^{15}N is accumulated under fast growth rates (probably due to intensive protein synthesis) and (2) ^{13}C accumulated under low growth rates and starvation due to depletion of ^{13}C -poor fat tissues.

The experiment is nicely designed and analysed. The study is nicely and clearly written.

Answer: Thank you for your time reviewing the manuscript and for your helpful suggestions. We answer your points below.

However, one has to admit that the topic of starvation in isotopic ecology is not novel. Some more literature here can be mentioned. Eight studies exploring starvation (mostly in invertebrates) are compared in Fig. 5 in Martinez del Rio et al. (2009), few more are listed in Potapov et al. (2019), Table 1.

- Martinez del Rio, Carlos, Nathan Wolf, Scott A. Carleton, and Leonard Z. Gannes. 'Isotopic Ecology Ten Years after a Call for More Laboratory Experiments'. *Biological Reviews* 84, 1 (2009): 91–111. <https://doi.org/10.1111/j.1469-185X.2008.00064.x>
- Potapov, Anton M., Alexei V. Tiunov, and Stefan Scheu. 'Uncovering Trophic Positions and Food Resources of Soil Animals Using Bulk Natural Stable Isotope Composition'. *Biological Reviews* 94, 1 (2019): 37–59. <https://doi.org/10.1111/brv.12434>.

Nevertheless, the study has a nice replication and looks closer into the mechanisms behind the patterns. Thus, it reports some interesting and rather novel relationships.

Answer: Thank you for pointing out that these references were lacking. We added them to the introduction, along with a paragraph defining more precisely how the study inserts into the literature (line 18-25).

“Total food restriction, which causes weight loss (corresponding to negative growth rates in fig.1.b. and d.), has the overall tendency to increase heavy isotopes content (^{15}N and ^{13}C), leading to an overestimation of the trophic level (see Adams and Sterner, 2000; Boag et al., 2006; Gorokhova and Hansson, 1999; Haubert et al., 2005; McCue, 2008; Oelbermann and Scheu, 2002; Olive et al., 2003; O. Schmidt et al., 1999). But more rarely have the effect of various feeding levels been considered, with no convincing conclusion to this day (Hertz et al., 2015). 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.”

As a note of caution, the study overstating the observed difference and I think the conclusions and the abstract should be rephrased. The difference between (extreme!) feeding categories is on average 1.5‰ only (Fig. 2d). 2.5‰ is the total range of individual variation, not fully related

to the growth/food intake (individual variation of isotopic composition is typically high, e.g.). As such the difference is smaller than a trophic level difference, and much smaller if we do not consider extreme nutritional situations, but non-neglectable.

Answer: We nuanced our conclusions by using the 1.5‰ as advised at all the previous instances of the number 2.5‰.

Revealed correlation explained 35-50% of individual variation in the laboratory conditions. I agree that this is a good argument for “when assessing trophic levels using isotopic data, the nutritional status of individuals can hardly be ignored”. But at the moment I am missing the realistic application perspective of this knowledge. In most cases it is not feasible to evaluate nutritional status or growth rates of the entire population or even community in the field. For now, it is just ‘one should be cautious’ statement. Or maybe authors can propose something more specific?

Answer: Indeed, correcting for nutritional status is not an easy task. A more realistic procedure could be to check at least if the animal is under extreme nutritional stress and consider this fact in the error evaluation. We propose two methods to do so (line 168-172).

“Despite being hard to estimate without destructive measurements, at least severe starvation and underfeeding might be detectable through environmental conditions. Physiologically, the nutritional state at which an individual grows can be assessed through age-size comparison, sclerochronology if applicable (Castanet, 1994), or biochemical indicators (e.g. ketone bodies, Chowdhury et al., 2014; Shah and Bailey, 1976).”

We can only hypothesize the ‘V’ shape of $\delta^{15}\text{N}$ – growth rate relationship. It is still to be tested for the negative growth rates, unfortunately. Therefore, I’m not sure if study anyhow contributed to revealing the ‘V’ shape. And I don’t think it is fair to give this hypothesis again in the middle of your conclusions.

Answer: We emphasized the hypothetical and speculative aspects of this idea in our discussion. Still, we think that it could hardly not be the case given that most studies give an increase in $\Delta^{15}\text{N}$ with starvation intensity (in a weight loss context), whereas we find the contrary for positive growth rates. We detail this view in the discussion (line 158-163)

“Despite very low intake rates, down to 10% of ad libitum levels, no weight loss was observed in our experiment, leaving the complete shape of the relationship between $\Delta^{15}\text{N}$ and growth rate only speculative, although the fact that most studies show an increase of $\Delta^{15}\text{N}$ with starvation intensity or fasting duration in a negative growth context, whereas we find the contrary for positive growth rates, suggest such a relationship (Del Rio and Wolf, 2005). But whether a V-shaped relation can arise or not requires further investigation.”

As a methodological note, it is not perfectly clear if there was diet switch in the experiment or all animals fed on the same diet as before the experiment. It would be good to clearly state this.

Answer: thank you for pointing this out. The diet was indeed kept the same for the whole duration of the breeding and experiments, and we added this detail in the methods section (line 74)

“Food was kept the same as before the start of the feeding level experiment.”

Reviewer 2

In this paper the authors investigate the assimilation of carbon and nitrogen isotopes by larvae of *Spodoptera littoralis* under different conditions of starvation. They show that starvation does have an effect on isotope incorporation and that the magnitude of the effect is appreciable in the context of isotope ratio differences that are used to infer trophic level.

Overall, the work seems sound and the conclusions that the authors draw are supported by the results.

Answer: Thank you for your time reviewing the manuscript and for your helpful suggestions. We answer your points below.

There are a few additional points the the authors may wish to consider.

1. It is my understanding that *Spodoptera littoralis* larvae, like many Noctuids can be cannibalistic (the congener *Spodoptera frugiperda* is notorious for this). If I understand the methods correctly there was no opportunity for cannibalism in these experiments because larvae were kept as isolated individuals. Nevertheless, the authors may want to make this more explicit. They may also wish to consider in the discussion how cannibalism may affect the inference of trophic level from isotope data.

Answer: Thank you for this suggestion. We emphasized the impossibility of cannibalism in the methods (line 69-70)

“To enable proper mass balance calculation and prevent cannibalism, we isolated the 400 larvae intended for the experiments at the 6th instar in individual 30 mL circular polypropylene boxes and provided them ad libitum food until 6th moult completion (start of the 7th instar).”

2. The authors' focus is on the effect that starvation may have on C and N isotope ratios in the context of inferring trophic level. Stable isotopes are also used to study other aspects of Lepidopteran biology. Ratios of C and N isotopes are used to study the geographical origins of migrating moths. It would be nice to see the authors discuss the significance of their results to studies of this nature. Plants that use C3 and C4 carbon fixation mechanisms differ in their incorporation of stable carbon isotopes. Consequently, carbon isotope ratios have been used to infer the larval host plants of adult moths. Again, it would be interesting if the authors considered the implications of their results to the inference of larval diet. In this context it is noteworthy that the diet the authors used in their experiments includes both maize (C4) and soy (C3).

Answer: This discussion suggestion is very interesting. We added a paragraph on the topic in the discussion with a rough estimate of the error that starvation could lead to in the evaluation of C3 fraction in diets (line 173-180).

“Diet indicators are also prone to estimate error owing to variable nutritional status. The carbon isotopic signature of herbivores is sometimes used to estimate if their diet is composed primarily of C4 plants, rich in ^{13}C (12 to 20‰), or of the ^{13}C -poorer C3 plants (25 to 32‰), O’Leary, 1981). Elevated $\delta^{13}\text{C}$ values in the consumer can hence indicate a predominance of C4 plants in the diet. The proportion of C3 in the diet of insects has sometimes been inferred through this tool. It is not clear whether the intensity of isotopic fractionation due to starvation could change as a result of the difference between C4 or C3-based diets, the present case being an example of an artificial diet containing both. Still, starvation is likely to lead to overestimates of the C4 fraction, although not by much (around 10% based on fig. 3 in Fry et al., 1978).

3. Assorted minor points:

- Page 2, line 17. Typing error, should be "weight"
- Page 3, line 39. I do not understand this. A near-empty gut has a low food concentration by definition, no? Do the authors mean that food moves more slowly through a near-empty gut?

Answer: Indeed, it should have been written “decreased” instead of “increased” food concentration.

- Page 6, line 144. Replace "sensible" with "sensitive". Note: this appears to be a case of "false friends". "Sensible" (French) = "sensitive" (English). "Sensible" (English) = "sérieux" (French).
- Page 7, line 163. Typing error, replace "id" with "is".
- Page 8, lines 169 - 174. I found these last few sentences to be confusing. I suggest a re-write for clarity.

Answer: We discarded the speculative part on negative growth rates, which should not have been part of the factual conclusions, and hope this makes the paragraph much clearer.

Answer: Thank you for taking the time to identify these typos.