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Balancing of specific nutrients and subsequent growth and body composition in the slug *Arion lusitanicus*

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HIGHLIGHTS

- Slugs were restricted to nutritionally variable diets or given a choice.
- Food intake and nutrient utilization were affected by diet nutrient balance.
- Slugs given a choice were able to maximize growth.
- High dietary carbohydrate to protein ratio limited growth but increased fat mass.

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ABSTRACT

Feeding generalists typically occupy broad ecological niches and so are potentially pre-adapted to a range of novel food objects. In northern Europe, the slug *Arion lusitanicus* has spread rapidly as an invasive species and a serious horticultural and agricultural pest. We used nutritional geometry to analyze nutrient balancing capabilities and consequences for performance in *A. lusitanicus* when provided with one of three nutritionally fixed diets or when given dietary choice. The slugs over-ingested high amounts of the most abundant nutrient in order to get more of the limited nutrient. However, they regulated protein intake more tightly than carbohydrate intake resulting in a very high food intake when fed a protein-poor diet. Growth and body composition were highly affected by the nutrient balance of their diet. When given the choice to feed from two nutritionally different diets, the slugs selected an intake balance of protein and carbohydrate with sufficient precision to maximize growth. Nutrient utilization efficiency increased with increasing deficiency of the specific nutrient in the diet. Ingested carbohydrate was more efficiently stored as lipid in slugs fed more carbohydrate-poor diets, and ingested nitrogen was more efficiently incorporated into slug bodies in slugs fed more protein-poor diets. Our experiments suggest that the evolved behavioral and physiological regulatory capacities of *A. lusitanicus* may explain some of the success that this slug experiences as an invasive species. We furthermore propose that invasive species might be more dependent on high protein availability in the environment than non-invasive species.

1. Introduction

Optimal foraging theory predicts that animals have evolved foraging mechanisms that maximize their fitness [1]. The traditional approach to optimal foraging has centered around finding sufficient food, how long to reside in one patch before moving on to search for a more lucrative one, and trading off foraging behavior against the risk of predation. These studies have typically focussed on the acquisition of a single currency, often energy [1]. However, an increasing number of studies have shown that animals will actively regulate their intake of specific nutrients and that the composition of nutrients rather than the total amount determines consumption and performance [2].

The Geometric Framework for Nutrition has over the years proven to be a powerful tool to disentangle the interactive effects of specific nutrients on animal feeding behavior [2,3]. It has also been applied to study physiological regulation of specific nutrients after ingestion [4,5], and the most recent trend has been to link specific nutrient intake to performance [6,7]. Whereas nutritional geometry has now been applied in different areas of research including conservation [8,9], obesity [10,11], aging and lifespan [12,13], and immune function [14], the framework has not been applied in the context of describing the nutritional requirements and balancing capacities of invasive species. This

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would provide insight into the behavioral and physiological mechanisms that these animals possess, which might explain some of the reason why invasive species thrive in novel environments where food objects are often novel as well.

The slug *Arion lusitanicus* Mabille 1868 (synonym: *Arion vulgaris* Moquin-Tandon 1955) has spread rapidly over the last two decades and now occurs as an invasive species throughout northern Europe [15,16]. The slugs are considered a major pest, causing damage to agricultural and horticultural plants with reductions of crop yields by up to 50% [17,18], and are listed as one of the most important invasive animals in Europe [19,20]. The species was recently described to be robust and have a high phenotypic plasticity as it continued reproducing under environmentally challenging conditions [21]. It furthermore has high impact on ecosystem dynamics by consuming and out-competing other species [22,23]. In addition to the common notion that *A. lusitanicus* deter large amounts of plant material, the slugs are observed to feed selectively when provided with a buffet of different species of plants [16,24–27], indicating either nutrient balancing behavior or detergent avoidance. Using artificial diets, another common pest slug, *Deroceras reticulatum*, was shown to forage selectively for nutritionally complementary food after nutritional restriction [28]. They did so regardless of added detergent content in the diets, supporting the view that slugs forage to balance their intake of nutrients.

We used nutritional geometry to analyze the nutrient balancing behavior and the consequences of specific nutrient intake for growth and body composition in juvenile *A. lusitanicus*. We then investigated post-ingestive nutrient regulation by analyzing the efficiency of incorporating specific ingested nutrients into body tissue. Our study provides basic information on the nutrient balancing behavior and physiology of *A. lusitanicus* and thereby on the nutritional requirements and plasticity of this invasive species.

2. Materials and methods

2.1. Animals and housing

Adult *A. lusitanicus* were collected at a single site in Aarhus, Denmark, in late August and allowed to lay eggs in the laboratory. All hatchlings emerged within a span of a few days. They were maintained in an incubator at 5 °C and a 12 h light:12 h dark regime and provided with carrots, cucumber and dog food for 1 month until the start of experiments. Three days before experiment start, all food was removed and the temperature was raised to 18 °C where it remained during the experiments. At setup, all slugs were weighed to the nearest μg and distributed at random to experimental treatments. During experiments the slugs were housed individually in transparent plastic boxes (15 cm length, 10 cm width, 5 cm height) containing a water saturated sponge to maintain 100% humidity and a piece of white chalk to ensure coverage of calcium requirements.

2.2. Experimental design

A no-choice and a choice experiment were conducted simultaneously over eight weeks using three semi-synthetic diets (Table 1) manufactured in pellet form by Altromin GmbH (Germany). A full ingredient list for the diets is presented in Table S1. In the no-choice experiment, a total of 45 slugs each received only one of the three diets throughout the experiment, while in the choice experiment a total of 30 slugs were each given the choice to feed from two of the three diets (three dietary combinations) throughout the experiment. Since we found no effect of diet on survival (no-choice: $\chi^2 = 0.56$, $df = 2$, $P = 0.76$; choice: $\chi^2 = 1.40$, $df = 2$, $P = 0.50$), slugs that died during experiments were excluded from the analysis. After mortality that occurred during the 8 week feeding period, our final sample sizes were 7 slugs on the 9%P diet, 11 slugs on the 31%P diet and 12 slugs on the 48%P diet in the no-choice experiment and 8 slugs on the 9%P vs. 31%P diet pair, 10 slugs on the 9%P vs. 48%P diet pair and 7 slugs on the 31%P vs. 48%P diet pair in the choice experiment.

Table 1
Nutritional compositions (mass based) of the three artificial diets used in the two feeding experiments. Ingredient compositions are presented in Table S1.

Components	9%P	31%P	48%P
Crude protein (%)	9.0	31.4	47.7
Carbohydrate (%)	71.8	49.5	33.1
Crude lipid (%)	7.8	7.6	7.7
Crude fiber (%)	6.0	6.0	6.0
Ash (%)	5.4	5.5	5.4
Carbohydrate:protein	8:1	1.6:1	0.7:1
Energy content (kJ/g)	16.0	16.0	16.0

The diets also contained mineral mix (K_2CO_3, Coal. Chalk, NaCl, $MgCl_2$, K_2HPO_4, Fe_2O_3, $FeSO_4$, $MnSO_4$, NaF, KI, Na_2SeO_3, Na_2MoO_4, $CoCl_2$, $ZnCO_3$, $CuSO_4$), and a vitamin mix (C 1000, Altromin).

2.3. Experimental procedures

Diet was provided by the start of the experiment and renewed every seventh day. Diet was provided on a glass dish (19 mm diameter, 5 mm height). In the choice experiment we placed each glass dish with diet inside an inverted Petri dish lid (35 mm diameter, 5 mm height) to prevent diet mixing. Diets were kept in a drying oven at 60 °C for at least three days before weighing and were weighed to the nearest milligram on the glass dish before and after the seven day feeding period. Any feces were removed from the glass dishes before weighing. Diet intake was calculated as the difference in diet dry mass before and after feeding, and specific nutrient intakes were calculated using the known proportions of protein and carbohydrate in the diets. All slugs were weighed by the end of the experiments. They were then left for two days without food to discard gut contents before they were again weighed and killed by freezing at −20 °C.

2.4. Body nutrient analysis

Each slug was dried at 60 °C over four days and weighed to the nearest milligram. Lipids were then extracted during five 24-h washes in 10 ml petroleum ether. After again drying the slugs at 60 °C over four days and reweighing, the lipid mass of each slug was calculated by subtracting the lipid extracted, lean dry mass from the dry mass. The slugs were then pulverized using mortar and pestle and the mass based proportion of nitrogen in a subsample of 4–6 mg pulverized slug was analyzed in a dry combustion analyzer (Na 2000, Carlo Erba, Italy). The total nitrogen content of each slug was calculated by multiplying the proportion of nitrogen in the subsample with the slug lean dry mass. The crude protein mass of each slug was then calculated by multiplying body nitrogen mass by 6.25 [29].

2.5. Statistical analyses

2.5.1. No-choice experiment

We examined differences in the dry mass intake and the growth of slugs across the different diets using a multivariate analysis of variance (MANOVA) for repeated-measures [30]. In this model, we included diet (fixed effect), time (repeated measure) and their interaction. As there were three diet treatments, we ran pair-wise comparisons between the dietary treatments to determine which of them that contributed significantly to overall significant effects. One-way analysis of variance (ANOVA) with Tukey's HSD post-hoc tests were also conducted at each time period to show over which feeding periods the main differences in dietary intake and growth occurred. For each feeding period, we estimated the slope of the cumulative protein vs. carbohydrate intake array across diets (β_a) using linear regression and tested this against a hypothetical slope (β_h) of −1 using a *t*-test where $(\beta_a - \beta_h) / (SE_{\beta a})$