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Revisiting macronutrient regulation in the polyphagous herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): New insights via nutritional geometry

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ABSTRACT

Insect herbivores that ingest protein and carbohydrates in physiologically-optimal proportions and concentrations show superior performance and fitness. The first-ever study of protein–carbohydrate regulation in an insect herbivore was performed using the polyphagous agricultural pest *Helicoverpa zea*. In that study, experimental final instar caterpillars were presented two diets – one containing protein but no carbohydrates, the other containing carbohydrates but no protein – and allowed to self-select their protein–carbohydrate intake. The results showed that *H. zea* selected a diet with a protein-to-carbohydrate (p:c) ratio of 4:1. At about this same time, the geometric framework (GF) for the study of nutrition was introduced. The GF is now established as the most rigorous means to study nutrient regulation (in any animal). It has been used to study protein–carbohydrate regulation in several lepidopteran species, which exhibit a range of self-selected p:c ratios between 0.8 and 1.5. Given the economic importance of *H. zea*, and it is extremely protein-biased p:c ratio of 4:1 relative to those reported for other lepidopterans, we decided to revisit its protein–carbohydrate regulation. Our results, using the experimental approach of the GF, show that *H. zea* larvae self-select a p:c ratio of 1.6:1. This p:c ratio strongly matches that of its close relative, *Heliothis virescens*, and is more consistent with self-selected p:c ratios reported for other lepidopterans. Having accurate protein and carbohydrate regulation information for an insect herbivore pest such as *H. zea* is valuable for two reasons. First, it can be used to better understand feeding patterns in the field, which might lead to enhanced management. Second, it will allow researchers to develop rearing diets that more accurately reflect larval nutritional needs, which has important implications for resistance bioassays and other measures of physiological stress.

1. Introduction

The ability of insect herbivores to acquire an optimal mixture of dietary nutrients has profound effects on their performance and fitness (Bernays and Bright, 1993; Bernays and Minkenberg, 1997; Raubenheimer and Jones, 2006; Unsicker et al., 2008; Behmer, 2009; Simpson et al., 2015). In general, plant nutrient content is highly variable, both spatially and temporally (Elser et al., 2000; McGroddy et al., 2003; Deans, 2014), indicating that the majority of herbivores forage in a highly heterogeneous nutritional landscape. To deal with this variability, insect herbivores assess the nutrients present in different plant tissues and regulate the intake of specific nutrients to meet their physiological demands (Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 1999; Behmer, 2009; Simpson et al., 2015). The process of acquiring the optimal balance of key nutrients to fuel growth and reproduction strongly impacts insect performance, with consequences for the evolution of plant–insect interactions and host–plant associations (Bernays and Chapman, 1994; Bernays and Bright, 2005), dispersal and movement patterns (Simpson et al., 2006, 2010; Bazazi et al., 2008; Srygley et al., 2009; Hansen et al., 2011) and even the evolution of higher order social interactions (Guttal et al., 2012; Lihoreau et al., 2014, 2015). For these reasons, delineating the nutritional requirements of an insect species is integral to understanding its feeding ecology, life history strategies, and physiological capabilities.

The cotton bollworm, *Helicoverpa zea*, is a highly polyphagous agricultural crop pest that feeds on over 100 different host plants in North America (Fitt, 1989). *H. zea* was also the first species to

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be used for exploring nutrient regulation in herbivorous insects (Waldbauer et al., 1984). In this study, a choice test was performed to determine the extent to which *H. zea* larvae regulated their protein (p) and carbohydrate (c) intake. To do this, larvae were offered one of two artificial diet pairings over the course of their final instar, either a diet with a protein-to-carbohydrate ratio (p:c) of 100:0 (all protein) and one with a p:c of 0:100 (all carbohydrates), or two diets both with a 50:50 ratio. The consumption results indicated that, when allowed to self-select, larvae ingested a diet with an average p:c ratio of 80:20, or 4:1; in the experimental approach of the geometric framework geometric framework (GF), this self-selected p:c ratio is referred to as an intake target (IT) (Simpson and Raubenheimer, 1995).

Since Waldbauer et al. (1984), protein and carbohydrate regulation has been tested in several lepidopteran species using the GF, including *Heliothis virescens* (Lee et al., 2006; Telang et al., 2001; Roeder and Behmer, 2014), *Heliothis subflexa* (Lee et al., 2006), *Manduca sexta* (Thompson and Redak, 2005), *Malacasoma disseria* (Despland and Noseworthy, 2006), *Plutella xyostella* (Warbrick-Smith et al., 2009), *Spodoptera exigua* (Merkx-Jacques et al., 2008), *Spodoptera exempta* (Lee et al., 2004b), *Spodoptera littoralis* (Lee et al., 2004a), and *Spodoptera litura* (Lee, 2010). Across these species, the ITs range from slightly carbohydrate-biased ratio for *S. exempta* (0.8:1) to slightly protein-biased ratio for *H. virescens* (1.5:1) and *S. littoralis* (1.3:1), with several species selecting for a balanced 1:1 p:c ratio. Comparatively, the 4:1 IT for *H. zea* as determined in Waldbauer et al. (1984) stands apart from these other caterpillar species because it is extremely protein-biased. It is also much more protein-biased than the 1.5:1 IT reported for *H. virescens* (Lee et al., 2006), which is a close relative to *H. zea*, and which shows similar feeding biology to *H. zea* (both are extreme generalists) (Mitter et al., 1993).

Given the economic significance of *H. zea* and the major discrepancy between Waldbauer et al. (1984) and other lepidopteran studies employing the GF, we wanted to reassess protein–carbohydrate regulation in *H. zea*. We had two objectives. The first was to determine the IT for *H. zea* using the experimental approach of the GF. To do this, a choice-experiment was performed in which individuals were offered pairings of two diets that differed in their p:c ratios; for each treatment newly-molted final instar caterpillars were maintained individually and consumption of each food was measured over the final instar. The total amount of protein and carbohydrates consumed over the study was then used to calculate the IT. The second objective was to understand how diet p:c impacts performance when larvae cannot choose. This was done with a no-choice experiment by rearing larvae from neonate to pupation on diets with a specific p:c ratio and then measuring growth rate, developmental time, and pupal mass. Given the IT results for other caterpillar species, especially the IT reported for the closely related *H. virescens* (Lee et al., 2006), we expected the IT for *H. zea* to be only slightly protein-biased, approximating the upper range found in these other studies. We also hypothesized that performance in the no-choice study would be best on the diet treatment that most closely matched the IT calculated from the choice experiments, given that ITs have been shown to be functionally optimal (Behmer and Joern, 2008; Roeder and Behmer, 2014).

2. Methods

2.1. Insects

H. zea eggs were purchased from Benzon Research (Carlisle, PA, USA). Upon hatching, neonates were individually placed, using a fine-tipped paint brush, into 1 oz. clear condiment cups with paper lids. Each cup also contained one or two blocks of experimental food that differed in soluble protein and digestible carbohydrate content (see below). All individuals were kept in a growth chamber (Model I-66VL; Percival Scientific, Perry, IA, USA) set at 25 °C with a 14:10 L:D cycle for the duration of each experiment.

2.2. Artificial diet

The synthetic diet used in this study was originally developed by Ritter and Nes (1981), and then later modified as described by Jing et al. (2013). The key ingredients were vitamin-free casein, sucrose, cellulose, Wesson's salt mix, Torula yeast, lipids (cholesterol, linoleic and linolenic acid) and vitamins. In total, 11 different diets were made that had unique protein and digestible carbohydrate profiles by altering the proportion of casein and sucrose in the diets. All other ingredients, except for cellulose, were held constant between the different diets; the amount of cellulose in a diet varied inversely with total macronutrient content. Although the nutritional components of this artificial diet may differ from plant tissues in important ways, larvae readily feed on this diet and show high survival and successful development to adulthood. Despite being an animal-based protein, casein (the primary protein source in this diet) is considered a high quality protein source for lepidopterans (Lee et al., 2008) and is commonly used in insect artificial diets (Cohen, 2003). The original diet from Ritter and Nes (1981) contained 34% protein (p) and 12% sucrose (digestible carbohydrate (c)). This diet (p34:c12), plus three others (p12:c34, p17:c29 and p23:c23) had the same total macronutrient content (p + c) of 46%, but varied in p:c ratio from 0.35 to 2.8 (see Fig. 1a). Collectively, these four diets were used in various combinations in a choice experiment (described below).

The remaining seven diets (see Fig. 1b) were used in a no-choice experiment (see below). To maintain ecological relevance to natural conditions, these ratios and concentrations were selected to mimic the empirically-determined range of macronutrient content found in different cotton tissues under different growing conditions (Deans, 2014). Cotton is a common resource for *H. zea*, and as a result, larvae are likely to encounter resources of this quality in a natural setting. Table 1 shows the relationship between our experimental diets and the nutrient values for different cotton tissues. Three of these diets had total macronutrient content of 21%, but varied in p:c ratio from 0.4 to 2.5 (p6:c15, p12:c9 and p15:c6). The next three had a higher total macronutrient content of 42% with the same p:c ratios (p12:c30, p24:c18 and p30:c12), and the final diet had total macronutrient content of 68% and a p:c ratio of 1.6 (p42:c26). This resulted in the same three ratios being tested at two different total macronutrient concentrations: 0.4 (p15:c6 and p12:c30), 1.3 (p12:c9 and p24:c18), and 2.5 (p15:c6 and p30:c12) (see Fig. 1b).

All of the experimental diets were mixed as dry ingredients with a slightly warm 1% agar solution. After cooling, the diets were cut into blocks and presented to the experimental caterpillars. In this way caterpillars received both nutrients and water.

2.3. Experimental protocol

2.3.1. Choice experiment

All caterpillars were reared on the original Ritter and Nes (1981) diet (p34:c12, 46% total macronutrients) from hatching through to the start of the final instar. Upon molting to the final instar, larvae were weighed, then transferred to a petri-dish (with holes in the lid for ventilation) and offered two foods that differed in p:c ratio. There were three unique treatments: (1) p12:c34 paired with p34:c12; *n* = 15, (2) p17:c29 paired with p34:c12; *n* = 15, and (3) p23:c23 paired with p34:c12; *n* = 14. Diet cubes were individually weighed and placed at opposites ends of the petri-dish (100 mm diameter). Both diet cubes were replaced every 1–2 days so that