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The development of a synthetic diet for investigating the effects of macronutrients on the development of *Plodia interpunctella*

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Abstract

The use of chemically defined artificial diets has allowed researchers to examine questions within nutritional ecology about how macronutrients affect life-history traits and resource-based trade-offs. Using a chemically defined diet, it is possible to manipulate both the total nutritional content and the ratio of macronutrients (i.e., proteins, carbohydrates, or lipids) within the diet. Studies using the geometric framework have made use of these diets to examine lifespan, fecundity, and immune responses. Here, we develop an artificial diet suitable for rearing lepidopteran larvae. We created diets with three proportions of non-nutritive material (30, 50, and 70% indigestible cellulose) relative to protein and carbohydrate macronutrients, and compared these to standard wheat bran laboratory diet. We then examined the effects of variable nutrient content on lifespan and development time in *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae). The artificial diets supported development (almost) as well as bran-based laboratory diets. Total nutrient content affected development time: females that fed on the diet with the highest nutrient content took the longest time to reach eclosion. We also found evidence to support dietary restriction, with larvae receiving the fewest nutrients having the longest lifespan as adults. These findings are indicative of the usefulness of this diet as a tool to further investigate the effects of nutrient content and macronutrient imbalance on resource-based trade-offs and life-history traits.

Introduction

An essential part of many mass-rearing programmes, artificial diets maximize cost- and time-effectiveness by, for example, eliminating the need for the maintenance of multiple trophic levels (e.g., artificial diets can replace host plants and prey as a food source for herbivorous insects and predators, respectively). Commercial examples of artificial diets include their use in mass rearing insects for sterile insect technique or predatory insects for pest control (Robinson & Hendricks, 2005; Riddick, 2008). On a smaller scale, artificial diets can be used within an academic research setting to rear insects as model organisms to answer a number of evolutionary and ecological questions (Vanderzant, 1974). The development of these diets is often complex and time-consuming, however, because they must be tailored to the individual nutrient requirements or feeding behaviour of each species.

If diets are chemically defined, then the ratio of macronutrients (i.e., protein, carbohydrate, or lipid) is known and can be adjusted, which has facilitated recent research in nutritional ecology on the importance of various components of nutrition on different aspects of fitness such as lifespan, total egg production, or immune responses (Simpson & Abisgold, 1985; Lee et al., 2008; Povey et al., 2009; Cotter et al., 2011; Harrison et al., 2014). Known as the geometric framework, the work of Simpson and Raubenheimer in creating a visualization and space-state modelling tool has allowed us to model life-history traits over a response surface of different intake quantities of macronutrients (Simpson & Raubenheimer, 1995). This is only possible when using multiple artificial diets containing different ratios of macronutrients while also varying the total available nutrition. Using the geometric framework, it has been possible to visualize the

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relationship between different macronutrients and the performance consequences of consuming them, even locating the individual nutritional optima for traits such as reproduction or longevity, facilitating our knowledge of resource-based life-history trade-offs (Lee et al., 2004; Povey et al., 2009; Simpson & Raubenheimer, 2009).

Here, we describe a synthetic diet suitable for rearing larvae of Indian meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae). *Plodia interpunctella* is an economically important pest of stored products and is commonly found in post-harvest settings such as freight containers, warehouses, and grain mills. The larvae are dietary generalists, infesting various food sources including grain, dried products (e.g., fruit, nuts, and seeds), and animal feed. Major research topics using *P. interpunctella* as an insect model include sexual selection (Gage, 1998; Lewis et al., 2011), host-parasite dynamics (Sait et al., 1994; Knell et al., 1996), and their status as agricultural pests. The creation of this artificial diet, and the examination of its effects on development will facilitate the use of *P. interpunctella* in nutritional ecology. Artificial diets for *P. interpunctella* have been created in the past, as it is a well-studied insect model organism. However, these have not been chemically defined, and were predominantly created for ease of rearing *P. interpunctella* in laboratory conditions (Fraenkel & Blewett, 1946; Silhacek & Miller, 1972). We also determined how total nutrient availability of the new artificial diet affected length of the developmental stages of *P. interpunctella*, their longevity as adults, and whether this differs from the length of life stages in moths reared on standard wheat bran-based laboratory diet.

Material and methods

Plodia interpunctella culturing

A stock population of *P. interpunctella* has been maintained at Queen Mary University since December 2011, when it was started using animals from an outbred population at the University of Leeds. The population is maintained on ad-libitum standard lab food [organic wheat bran (Mount Pleasant Mill, Kirton-in-Lindsey, UK), brewers' yeast (MPBio, Cambridge, UK), and glycerol (Alfa Aesar, Heysham, UK) in a 10:1:1 ratio] at 27 °C and L12: D12 photoperiod. To create the next generation, over 200 mixed adults are placed in a funnel with both ends secured with net, and allowed to mate. The resulting eggs are collected and placed on standard laboratory food, and the larvae are allowed to grow until adulthood.

Creating artificial diet suitable for rearing *Plodia interpunctella*

The diet constructed is agar-based (Lee et al., 2004; Povey et al., 2009; Cotter et al., 2011), as multiple pilot experiments showed that powder-based diets are not suitable to support the growth of *P. interpunctella* (Simpson & Abisgold, 1985). Three artificial diets were tested with different amounts of non-nutritive material (indigestible cellulose) relative to protein and carbohydrate macronutrients (30, 50, and 70% cellulose). These percentages of cellulose were selected based on pilot experiments to give a broad range of total nutritional content while still being able to support development.

The cellulose (Sigma-Aldrich, New Road Gillingham, UK), sucrose (Sigma-Aldrich), cholesterol (Fisher Scientific, Loughborough, UK), linseed oil (MPBio), glycerol, and Wesson's salts (MPBio) (Table 1) were weighed and added to an autoclavable pot, totalling 75 g per diet. A 1% solution of agar was added in a 4:1 ratio to the dry ingredients (300 ml of agar per diet). The mixture was thoroughly blended with a magnetic stirrer and the solution was left to cool to 37 °C. The casein (Sigma-Aldrich), peptone (Sigma-Aldrich), albumen (Fisher Scientific), methyl hydroxybenzoate preservative (1 g l^{-1} diet dissolved in 2 ml ethanol; VWR, Lutterworth, UK) and Vanderzant vitamin mix (Sigma-Aldrich) were added. The solution was mixed again and set in the refrigerator at 4 °C overnight. Using this artificial diet formulation, both the total quantity of nutrients and the ratio of individual nutritional components can be varied.

Fresh standard laboratory diet was made by mixing organic wheat bran, brewer's yeast, and glycerol in a 10:1:1 ratio using a food mixer. Third instars were collected from the stock population, and each larva was added to an individual 55-mm Petri dish with 0.85 ± 0.05 g of one of the four diets (n = 50 per treatment). Third instars were used because, although the diet

Table 1 Formulation for the three final artificial diets for the rearing of *Plodia interpunctella*, based on the percentage cellulose (i.e., non-nutritive inert material). Quantities are given in percentages (wt/wt) and for convenience also in g (or ml, for glycerol and linseed oil)

	30% cellulose		50% cellulose		70% cellulose	
Ingredients	%	g	%	g	%	g
Cellulose	30	22.5	50	37.5	70	52.5
Casein	13.11	9.83	8.67	6.50	4.22	3.17
Peptone	13.11	9.83	8.67	6.50	4.22	3.17
Albumen	13.11	9.83	8.67	6.50	4.22	3.17
Sucrose	19.67	14.75	13	9.75	6.33	4.75
Cholesterol	1	0.75	1	0.75	1	0.75
Wesson's salts	1	0.75	1	0.75	1	0.75
Glycerol (ml)	4	3	4	3	4	3
Linseed oil (ml)	4	3	4	3	4	3
Vitamin mix	1	0.75	1	0.75	1	0.75

is capable of supporting *P. interpunctella* from egg to adulthood, very small larvae sometimes drown in the diet, making it difficult to obtain a large enough sample size to perform the experiment. The diet was changed every 2–3 days to prevent desiccation. Larvae were weighed as wandering fifth instars 23 days after egg laying, but as many of the larvae consuming the bran-based diet were already pupating, the sample sizes for this treatment are uneven (30% cellulose: n = 43; 50% cellulose: n = 44; 70% cellulose: n = 44; bran-based diet: n = 7). The larvae were checked daily, and time to pupation, eclosion, and death as adults was recorded.

Statistical analysis

Development time, adult lifespan, and fifth-instar weight data were analysed in R v.3.0.1 (R Development Core Team, 2013) using ANOVA and post hoc Tukey tests. Development time was defined as the amount of time until eclosion (i.e., as larvae and pupae), and any larvae that did not eclose were removed from this analysis. The percentage of larvae that survived until eclosion was analysed using a generalized linear model with binomial errors and a logit link. Likelihood-ratio tests were used to compare models with and without specific explanatory variables and non-significant terms were dropped until minimal adequate models were reached (Zuur et al., 2009). Following the examination of diagnostic plots from the fitted models, the data in the analysis of development time were square-root transformed to reduce heteroscedasticity.

Results

There was a significant interaction between sex and diet affecting development time (measured as number of days spent in the larval and pupal stages) (ANOVA: $F_{3,137} = 4.12$, $P = 0.0078$; Figure 1). The post hoc Tukey test indicated that females consuming the 30% cellulose diet took significantly longer than those given the other diets to reach eclosion, taking 41.9 days. In comparison, females consuming the 50% cellulose diet took 35.3 days, females consuming the 70% cellulose diet took 36.2 days, and females consuming the bran-based diet took 31.6 days. The variance in development time for females on the 30% cellulose diet was much greater than all of the other sex*diet combinations (Figure 1). In males, none of the diet treatments led to significant differences in development time.

Larval diet treatment had a significant effect on adult lifespan (ANOVA: $F_{3,137} = 16.99$, $P<0.0001$; Figure 2). Figure 2 indicates that larvae consuming the 30% cellulose diet had the shortest adult lifespan (7.27 days), and as the nutrient content of the diet decreased, adult lifespan increased (50% cellulose: 8.45 days, 70% cellulose: 9.43 days). Larvae consuming the bran-based diet had the longest adult lifespan (10.2 days). A post hoc Tukey test confirmed this, showing that the 30% cellulose diet was different from the 70% cellulose diet and the bran-based diet. The 50% cellulose diet was different from the bran-based diet. Also, sex had a significant main effect: females lived longer than males ($F_{1,137} = 7.01$, $P = 0.0090$). The interaction of sex and diet treatment was not significant ($F_{3,137} = 2.095$, $P = 0.10$). Diet had no effect on fifth instar weight ($F_{3,131} = 1.34$, $P = 0.27$). Only sex affected weight, with females weighing 21.8% more than males ($F_{1,134} = 77.9$, $P<0.0001$).

The diet consumed had a weakly significant effect on the percentage of larvae surviving to eclosion (i.e., dying in the larval or pupal stages) (likelihood ratio test

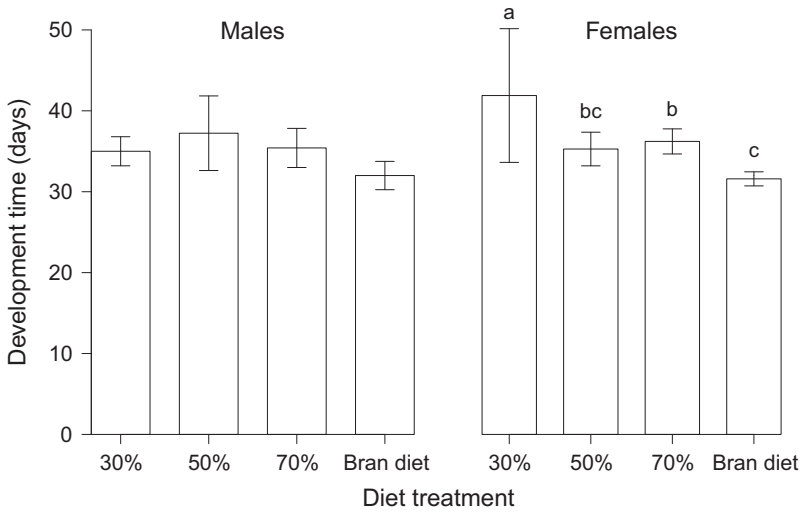

Figure 1 Development time (mean number of days spent in the larval and pupal stage + 95% confidence interval) of *Plodia interpunctella* males and females on diets containing 30, 50, or 70% cellulose, and a control bran-based diet. Means within a panel capped with different letters are significantly different (Tukey test: $P<0.05$). Comparisons between sexes indicated a significant difference only in the 30% cellulose treatment (Tukey test: $P<0.05$).

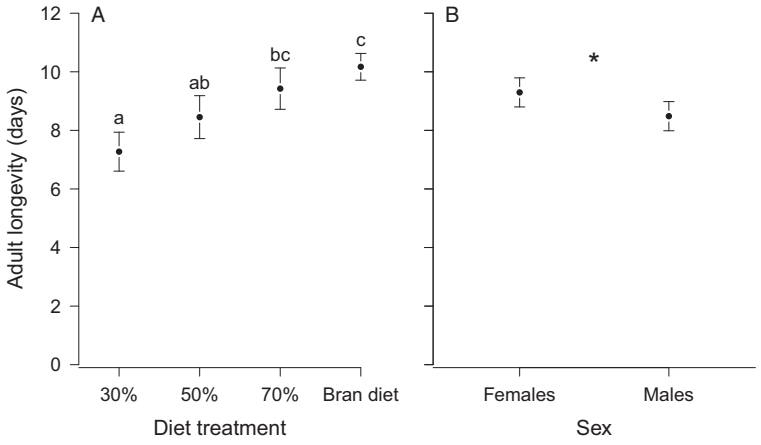

Figure 2 Lifespan (mean number of days as adults + 95% confidence interval) of *Plodia interpunctella* as influenced by (A) diets containing 30, 50, or 70% cellulose, and a control bran-based diet, and (B) sex. Means in A capped with different letters are significantly different (Tukey test: $P<0.05$). The asterisk indicates a significant difference between sexes (ANOVA: $P<0.05$).

statistic = 8.38, d.f. = 3,192, P = 0.039). Table 2 illustrates the survival for each diet treatment. Sex had no effect (likelihood ratio test statistic = 0.345, d.f. = 1,185, P = 0.56), nor any interaction between sex and diet (likelihood ratio test statistic = 1.30, d.f. = 3,185, P = 0.73).

Discussion

Using gel-based formulations, we successfully created artificial diets that produced similar survival, longevity, and development times when compared with the grain-based diet that *P. interpunctella* usually consume in the laboratory. The larval and pupal development time on the 50% cellulose diet was not significantly different from the bran diet in females. In males, none of the diets were significantly different from each other in terms of effects on development time. For both sexes, the 70% cellulose diet allowed *P. interpunctella* to achieve an adult lifespan comparable to the bran-based diet. Finally, diet did not affect the weight at fifth instar. Therefore, the artificial diets created are sufficiently similar to the grain-based diet, allowing us to infer realistic facts about the biology of *P. interpunctella* when we artificially manipulate their nutrition.

These similarities with the standard laboratory diet notwithstanding, there were several interesting effects produced by the range of artificial diets examined here, which bodes well for experiments using *P. interpunctella* as a model organism to examine the effects of macronutrient manipulation. For example, increasing the ratio of cellulose to macronutrients decreased the adult lifespan. Individuals reared on the bran-based diet had the longest adult lifespan, and the artificial diet with the least available nutrition (70% cellulose) was comparable to this. This is surprising, given that a greater ratio of nutritional to inert components would increase the amount of resources available for acquisition during the larval stage, and therefore could intensify investment in traits such as longevity during the adult stage, potentially leaving more time to find a mate.

Table 2 Survival (%) of *Plodia interpunctella* larvae until eclosion for each diet treatment (see Table 1 for diet description)

Larval diet treatment	% survival
30% cellulose	66a
50% cellulose	67.4a
70% cellulose	80ab
Bran-based diet	87.2b

Means followed by different letters are significantly different (Wald tests: $P<0.05$).

Several possible explanations for this are gaining traction within the nutritional ecology literature. Dietary restriction experiments have shown that limiting a component of nutrition can increase the organism's lifespan, sometimes with dramatic effects. This mechanism is highly conserved, with taxa as diverse as yeasts, rotifers, insects, rats, and primates showing similar effects of lifespan extension (Yu et al., 1982; Partridge et al., 2005; Weithoff, 2007; Colman et al., 2009). There is still debate as to the precise nature of the limitation needed, e.g., caloric restriction or limitation of one particular macronutrient or even amino acid (Mair et al., 2005; Masoro, 2005; Lee et al., 2008; Grandison et al., 2009; Piper et al., 2011). Although essential for life, certain macronutrients may in fact be toxic when consumed in excessive quantities. Current evidence suggests excessive amounts of protein can be detrimental to lifespan, possibly due to higher levels of reactive oxygen species or increased DNA damage (Simpson & Raubenheimer, 2009; Solon-Biet et al., 2014).

Another interesting effect of the high macronutrient diet is the dramatic extension of the development time required by females to reach eclosion, which is longer than on any other diet. This effect is particularly increased by

four individuals that took longer than 50 days in the development period, possibly entering a diapause state induced by suboptimal diet quality. *Plodia interpunctella* larvae are able to enter a facultative pre-pupal diapause which can be induced by photoperiod, temperature, strain of origin, or diet (Williams, 1964; Bell, 1994; Wijayaratne & Fields, 2012). Diapause seems to be an important life stage for most stored-product Lepidoptera (Bell, 1994), which are able to undergo diapause in crevices of warehouses undisturbed. Termination of diapause then occurs when more favourable conditions resume. Finally, a lower proportion of larvae survived to eclosion on the artificial diets with higher macronutrient content, although this was possibly because slightly more larvae drowned in the moist texture of these two diets.

To conclude, a synthetic artificial diet was created to support the development of *P. interpunctella* larvae. The ratio of available macronutrients within it can be manipulated, increasing the tools available to us to use *P. interpunctella* as a model organism for the study of resource-based life-history trade-offs and the effects of macronutrient imbalance.

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