THE UNIVERSITY OF HULL

"Moving Targets:

Nutritional geometry of development in solitary bee, *Osmia bicornis* "

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ABSTRACT

Eating the correct amount, and balance, of nutrients is key for fitness at the individual, and population level. Understanding dietary nutrient requirements and feeding behaviour is integral to addressing important questions, such as how an organism is able to respond and adapt to its nutritional environment, and the fitness consequences associated. We know that the relationships between nutrient intake and fitness can vary across different life stages, such as adults versus young. To date, the majority of studies have focused on single points in time or looked at overall patterns across whole life stages, and few have focused on age related shifts in nutritional requirements within a single life stage. In holometabolous insects, such as bees, the nutrients eaten during the larval stage have persistent effects on fitness. The majority of existing research in bee nutrition is limited to adults of social species, despite knowledge that most bee species are solitary. In this study, building on the work by Austin and Gilbert (2018), I re-focused the analysis onto weekly changes during the larval developmental period of important pollinator species, *Osmia bicornis*, to examine the changes over time in the relationship between nutrient consumption and fitness. I used data from nutritional geometry feeding experiments with six artificial protein-carbohydrate diets, two major macronutrients considered critical to insect development. I found that rules governing consumption changed over the developmental stage, and accordingly, so did the patterns of growth. I showed an age-related behavioural shift towards carbohydrate intake regulation. Macronutrient dilution of the diet carried costs to all measured traits. This study highlights that the rules governing feeding behaviour can be complex and

dynamic, and could have important implications for the design of habitats for conservation of wild and domestic bees.

INTRODUCTION

The amount and balance of nutrients eaten by an organism are key determinants of multiple fitness-related traits (Simpson and Raubenheimer, 2012) such as longevity (Runagall-McNaull et al., 2015; Hosking et al., 2019; Papanastasiou et al., 2019), fecundity (Hosking et al., 2019), body size (Koyama and Mirth, 2018; Poças et al., 2020), and immune defence (Ponton et al., 2011). Understanding dietary nutrient requirements and feeding behaviour is integral to addressing important questions such as how an organism is able to respond and adapt to its nutritional environment, and the fitness consequences associated (Simpson and Raubenheimer, 2012). We know that the relationships between nutrient intake and fitness can vary across different life stages, such as adults versus young (Runagall-McNaull et al., 2015; Kraus et al., 2019b). To date, the majority of studies have focused on single points in time or looked at overall patterns across whole life stages (Helm et al., 2017b), and few have focused on age related shifts in nutritional requirements *within* a life stage (but see Paoli et al., 2014; Al Shareefi and Cotter, 2018): Yet, these could have important implications for understanding the effects of diet on organismal fitness, and could reveal the true complexity of these relationships. The nutritional 'geometric framework' is a modelling approach where nutrients and fitness measures are represented by axes of a multi-dimensional 'nutrient space' used to reveal which nutrients an

organism will over- or under-eat when given nutritionally imbalanced diets, and the fitness consequences of what they consume (Simpson and Raubenheimer, 2012). In these models, the optimal nutrient requirement of an animal is represented as a point (or a trajectory over time) in the nutrient space, called an 'intake target'. This framework can reveal the 'rules of compromise' governing an organism's nutrient intake when restricted to a nutritionally imbalanced food, for example, it can over- or under-eat some food components.

With climate warming and human-induced landscape change altering natural nutritional landscapes, this framework is particularly important for investigating how animals are able to adapt to a changing nutritional environment (Simpson and Raubenheimer, 1993; Raubenheimer et al., 2012; Simpson and Raubenheimer, 2012; Kutz et al., 2019). In recent years, economically and ecologically important pollinator species, such as bees (Goulson et al., 2015), have suffered significant declines, extinctions, and range contractions (Ollerton et al., 2014), in part, due to nutritional stress (Goulson et al., 2015; Klein et al., 2017; Tosi et al., 2017). Human induced landscape change has reduced the quality, quantity, diversity, and temporal availability of food resources for bees (Goulson et al., 2015; Ziska et al., 2016). For example, intensification of agriculture has led to increasingly patchy natural resources, and climate change has been linked to shifting phenology in flowers and bee emergence, leading to mismatches between bee activity and availability of floral resources (Miller-Struttmann et al., 2015; Schenk et al., 2018). In addition, climate change is also influencing the nutritional quality of pollen resources (Ziska et al., 2016). Nutrition has persistent effects on bee fitness and colony health (Brodschneider and Crailsheim, 2010; Di Pasquale et al., 2013; DeGrandi-Hoffman et al., 2016). The majority of bee species are solitary, their life history differing significantly from that of social bee species, such as honeybees and bumblebees, who live in groups (Wood et al., 2016; Falk, 2019). Solitary bees

are the most efficient pollinators (Winfree et al., 2008). The majority of existing research in bee nutrition is limited to adults of social bee species (Brodschneider and Crailsheim, 2010; Altaye et al., 2010; Paoli et al., 2014; Vanderplanck et al., 2014; Stabler et al., 2015; Kraus et al., 2019a), fewer for larvae (Helm et al., 2017b), despite knowledge that almost all growth occurs at the larval stage (Nijhout et al., 2014). In holometabolous insects (which undergo a complete metamorphosis), larval nutrition has persistent effects on adult lifespan (Runagall-McNaull et al., 2015; Klepsatel et al., 2020), reproductive output (Klepsatel et al., 2020), and is crucial to adult body size (Helm et al., 2017a; Helm et al., 2017b; Poças et al., 2020). The nutritional needs of adults can not necessarily be compared to developing insects since their nutritional requirements can vary a great deal (Boggs, 2009), especially in holometabolous insects with their complex life cycle (Nestel et al., 2016). Adult bees feed on carbohydrate-rich nectar for energy (Nicolson and Thornburg, 2007; Brodschneider and Crailsheim, 2010; Vaudo et al., 2015; Filipiak, 2019), whereas larval bees primarily require nutrients for growth, and feed almost entirely on protein-rich pollen (Filipiak, 2019). Pollen generalist, *Osmia bicornis*, is a commercially important solitary bee species, vital for pollination of orchards and oil-seed rape (Conrad et al., 2017). During development, all food is given to their offspring as a single provision by the mother in the form of a ball of pollen (Nicholls et al., 2017). It is uncertain whether solitary bees can detect pollen quality and provide offspring with nutritionally balanced provisions (Nicholls and Hempel de Ibarra, 2017).

In this study, I used data from nutritional geometry feeding experiments with six artificial protein-carbohydrate diets, two major macronutrients considered critical to insect development (Scriber and Slansky Jr, 1981; Behmer, 2009; Kraus et al., 2019b). Building on the work by Austin and Gilbert (2018), I re-focused the analysis onto weekly changes during the larval

developmental period. I examined the changes over time in the relationship between the nutrient content of the larval diet with consumption, growth, cocoon mass, development time, and likelihood of survival to pupation. I suspected that, as is true for the diapause stage (Wasielewski et al., 2013; Wasielewski et al., 2014), there would be changes in the intake target and feeding behaviour of *O. bicornis* during their developmental stage.

METHODS

Contribution

Artificial diet creation and feeding experiments were carried out by Dr Alexander Austin for a study by Austin and Gilbert (2018). Statistical analysis for the current study was carried out by Charlotte Howard for an MSc by thesis.

Study Population

Diapausing Adult *O. bicornis* cocoons were commercially obtained (Mauerbienen®, Germany). All cocoons were stored at 4°C and 70% RH to overwinter until they were released. The cocoons were released at the nesting site at the University of Hull in April 2017, and allowed to hatch naturally, collect pollen, and lay their eggs in artificial nesting constructs. These consisted of Styrofoam blocks (Styrodur 3035 CS) with a 9mm hollowed-trough and a polycarbonate lid inside a wooden box (Austin and Gilbert, 2018, see appendices). After laying their eggs, *O. bicornis* mothers plug the entrance of a nest using mud, signifying nest completion. Completed nests, containing eggs and pollen balls, were brought into the lab. Each

larva was transferred to a single occupancy nest and randomly assigned to one of seven treatment groups. Early trials revealed that fresh eggs and newly emerged larvae were too fragile to be transferred, hence transference was delayed two days post emergence. During this time, larvae were allowed to feed on their natural provisions.

Diet treatments

Feeding experiments were designed using the Geometric framework for nutrition (Simpson and Raubenheimer). Growth, final cocoon mass, development speed, and survival to pupation were used as fitness proxies for the larvae. Each two-day old larva was removed from the natal nest and placed onto a scoop in a single-occupancy nest within an incubation chamber to maintain constant conditions (Gallenkamp, IH- 270 at 23°C and 80% RH). Larvae were randomly assigned to one of six artificial diet treatment groups (n=134). The protein:carbohydrate (P:C) content of the artificial diets was experimentally manipulated (Diet A = 1:1.2, Diet B = 1:2.3, and Diet C = 1:3.4) (Table 1). There was no precedent for composing artificial pollen diets for solitary bee larvae, so these diet ratios were chosen based on a combination of the nutrient ratios in honeybee-collected pollen and published data for protein in *O. bicornis* pollen balls (Budde and Lunau, 2007). Each of these diets were given at one of two total macronutrient concentrations (concentration 1 = 90% nutrients, 10% diluent, or concentration 2 = 70% nutrients, 30% diluent; [see Austin and Gilbert (2018) for raw amounts of macronutrients] to reveal whether the larvae compensated for a reduced nutrient concentration through feeding behaviour. All diets consisted of a base made from a fixed amount of honey and honeybee-collected pollen (Budde and Lunau, 2007). To the diet base, measured amounts of carbohydrate (trehalose), and protein (micellar casein) were added to create each diet ratio. The

two distinct diet concentrations were made by diluting with sporopollenin, the major component of pollen outer-walls, considered to be largely indigestible to bees (Nepi et al., 2005; Roulston and Cane, 2000; Suárez-Cervera et al., 1994; Tainsh et al., 2020) made from exines from sunflower pollen (Henan Shengchoa Apiculture Co., China) [see S.1 of Austin and Gilbert (2018) for details]. This was chosen over the more commonly used α-cellulose (Lee et al., 2004; Muth et al., 2016; Pernal and Currie, 2002) because (1) initial trials showed high larval mortality when fed α- cellulose, and (2) sporopollenin more closely mimics the natural indigestible fibre of a bees diet (Roulston and Cane, 2000; Tainsh et al., 2020). The diet mixture was made to resemble the size of natural *O. bicornis* pollen balls. To prevent the provision from running out or spoiling, it was swapped for a fresh provision every seven days and weighed. The nest construct was weighed, along with each individual component of the nest, to facilitate monitoring of provision consumption. The change in mass of the provision was used to back-calculate the amount of each macronutrient eaten by the larvae between each swap. Additional provisions of each diet were left in an empty nest without a larva (n=36), and underwent the same weighing regime at each swap to account for any mass lost due to evaporation of the water from the provision. The experiment ran until pupation of the surviving larvae. Nests were checked daily for any larval deaths.

Table 1. Number of bee larvae assigned to each diet treatment group (n=134), and the protein:carbohydrate content of each diet.

Diet	P:C	Concentration	sample size
A1	1:1.2	90	21
A2	1:1.2	70	25
B1	1:2.3	90	21
B2	1:2.3	70	22
C1	1:3.4	90	22
C2	1:3.4	70	23

Statistical Analysis

Sample size for the experiment was 134 larvae. Measurements were taken every week, each time the dietary provision was swapped for a fresh one. All analyses were conducted in R version 4.0.0 (R Core Team, 2020) (and can be found online at https://osf.io/3hm2q/?view_only=7f6e3c6b2b2447589ee52cc86282e4ca). Consumption data were first adjusted for diet dilution and water loss. The nutrient intake for each larva (n=134) was then back-calculated from the known diet constituents and the volume of food consumed. Data were analysed “by swap”, i.e each time food provisions were swapped for fresh ones (every seven days), building on the results from Austin and Gilbert (2018), to look for changes in intake target over time.

For growth data, I used reverse-stepwise merging of adjacent swaps to determine the distinct breakpoints in the relationship. This required the use of generalised additive mixed models (GAMMs) with a smooth function, for which I used the ‘mgcv’ package (Wood, 2017). A mixed model was required to account for multiple measurements of growth for each individual larva. Multiple fitted GAMMs were compared using AIC to determine whether data for any swaps showed the same pattern and should be amalgamated. This began with fitting a GAMM with all eight swaps left separate. The swaps were then merged, beginning with the final two adjacent swaps and working backwards. Growth over time was modelled using a GAMM containing weight change per swap as the response variable and swap number as a predictor with the random effect of sample ID. Amalgamation of swaps using this model showed a similar pattern for swaps 4 to 8, hence a single surface was modelled for this grouping, revealing four distinct 'growth periods'. To model differential use of nutrients for growth, we used a GAMM containing weight change per swap as the response variable and swap number as a predictor, with a bivariate smoother fitted to protein and carbohydrate consumption per swap. Again, this included the random effect of sample ID. This revealed the same four distinct growth periods as did the growth model.

For cocoon mass, development, and survival to pupation, there was only one measurement for each larva, hence mixed models were not required when modelling these data. For modelling cocoon mass and development time, only data for those larvae which survived until the end of the experiment could be used (n=82). Final cocoon mass upon pupation was modelled using generalised additive models (GAMs) with final cocoon mass as the response, diet concentration

as a predictor and a bivariate smoother fitted to protein and carbohydrate consumption per swap. Similarly, development time (from hatching to pupation) was modelled using GAMs with development time as the response, diet concentration as a predictor and a bivariate smoother fitted to protein and carbohydrate consumption per swap. Survival analysis over the developmental period (up to pupation) was conducted by fitting linear parametric survival regression models. Predictor variables included consumption of protein and carbohydrate per swap, their interaction, and diet concentration. This was done using the 'survreg' function from the 'survival' package (Therneau, 2020). All model selection was based on AIC since competing models were non-nested. Response surfaces for larval fitness measures over time were all calculated and visualised using non-parametric thin-plate splines with the 'fields' package (Nychka et al., 2017).

RESULTS

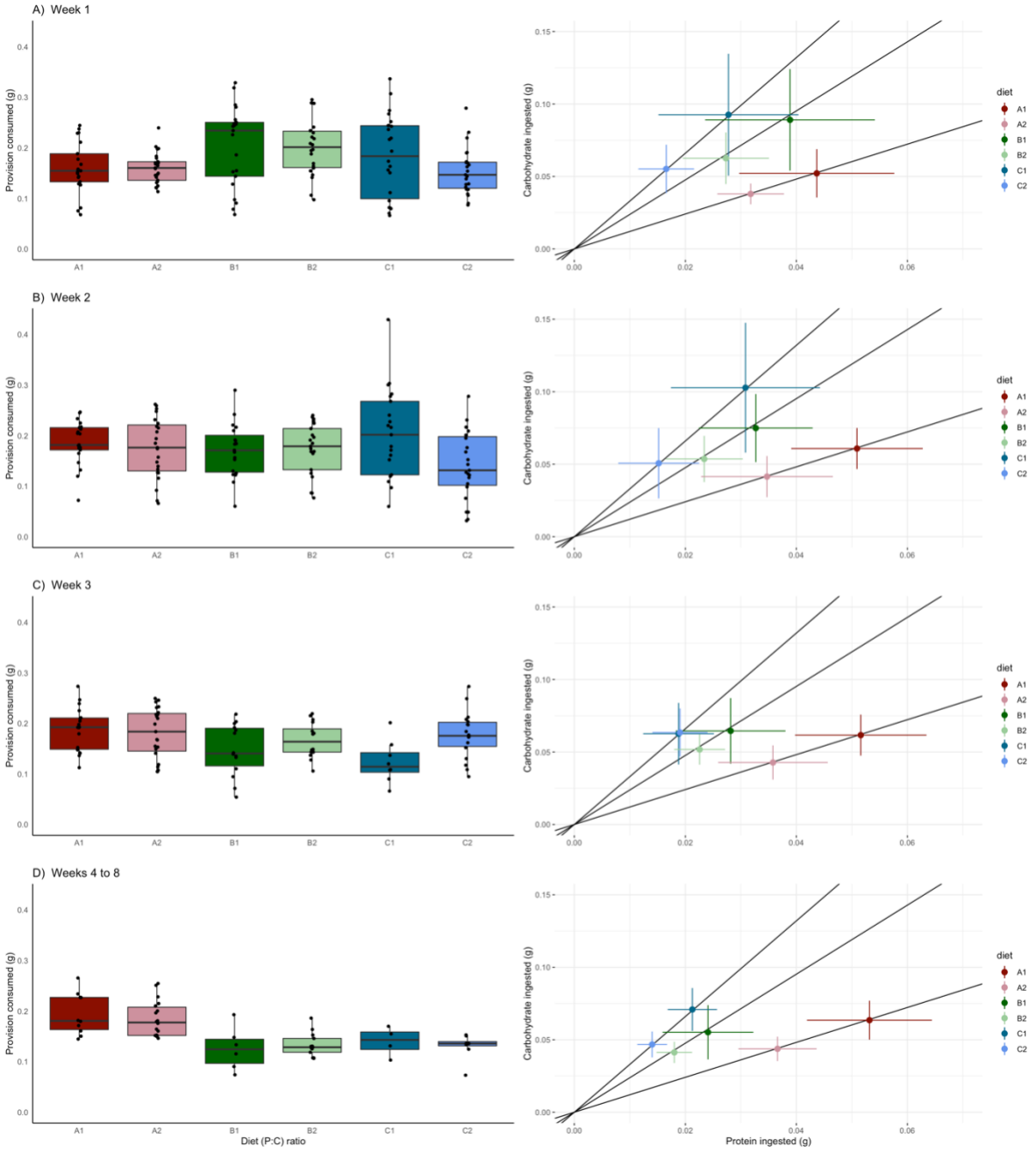

Figure 1: Total and mean (±SD) consumption (left) of dietary provision (grams), and average macronutrient intake (right) for 134 *O. bicornis* larvae on 6 artificial diets that varied in P:C ratio (diet A, B and C, 1:1.2 1:2.3 1:3.4 respectively) and P+C concentration (1, 90% and 2, 70%). Development has been split into the 4 distinct growth periods identified in the analysis where weeks 4 to 8 show similar patterns and thus have been amalgamated. Sample sizes for plots for each distinct growth period were 64, 62, 40, and 20 fed concentration 1, and 70, 67, 57, and 39 fed concentration 2. For boxplots (left), the lower and upper hinges show the first and third quartiles, and the black line represents the median. The whiskers extend from the hinge to the largest and smallest value, no further than 1.5 * interquartile range from the hinge. Points are represented by filled black circles, and were added to the boxplot using jitter (Wickham, 2016). The solid black lines (right) represent the ratio of protein to carbohydrate available to the larvae on each of the 3 diets, A, B and C.

Provision Consumption

Stepwise amalgamation of swaps using the model for growth over time revealed four distinct ‘growth periods’; week 1, week 2, week 3, and weeks 4 to 8. (S.1, a-b) (Fig. 1). The pattern in the total amount of provision consumed at each growth period by larvae on different diets changed throughout development (Fig. 1). Larvae from all diet treatment groups appeared to eat similar total amounts of their food provision at the beginning of the development period, and gradually, larvae on different diets began to eat different amounts of food, where those on lower-carb diets ate more total provision on average than those on the highest-carb diet, diet C (Fig. 1, left panels). In terms of nutrient intake, compensatory feeding behaviour shifted across development, becoming more apparent in the final growth periods, where larvae on the low-carb diets ate more protein in order to converge at a similar level of carbohydrate ingestion (~0.06g) (Fig. 1, right panels). During week 3, larvae on the more dilute version of diet C ate more of the provision, resulting in the same average amount of protein and carbohydrate being eaten by both groups on diet C (Fig. 1c). Compensatory feeding behaviour shifted across development, becoming more apparent in the final growth periods, where larvae on the low-carb diets tolerated more protein ingestion in order to converge at a similar level of carbohydrate ingestion (~0.06g) (Fig. 1, right panels). In broad terms, larvae appeared to begin by eating less discriminately following an ‘equal distance‘ rule of compromise. Then, they gradually started to more closely regulate carbohydrate intake where they appeared to be following a ‘no-interaction‘ rule of compromise, consuming enough food to bring them as close as possible to a carbohydrate target, resulting in average consumption points for all diets gradually falling in a roughly horizontal line (Fig. 1) (Simpson and Raubenheimer, 1993; Lihoreau et al., 2015). In order to do so, those larvae

on the low-concentration diets ate a larger total amount of their food provision (Fig. 1, left panels). A wider array of diets would confirm the rules of compromise being followed.

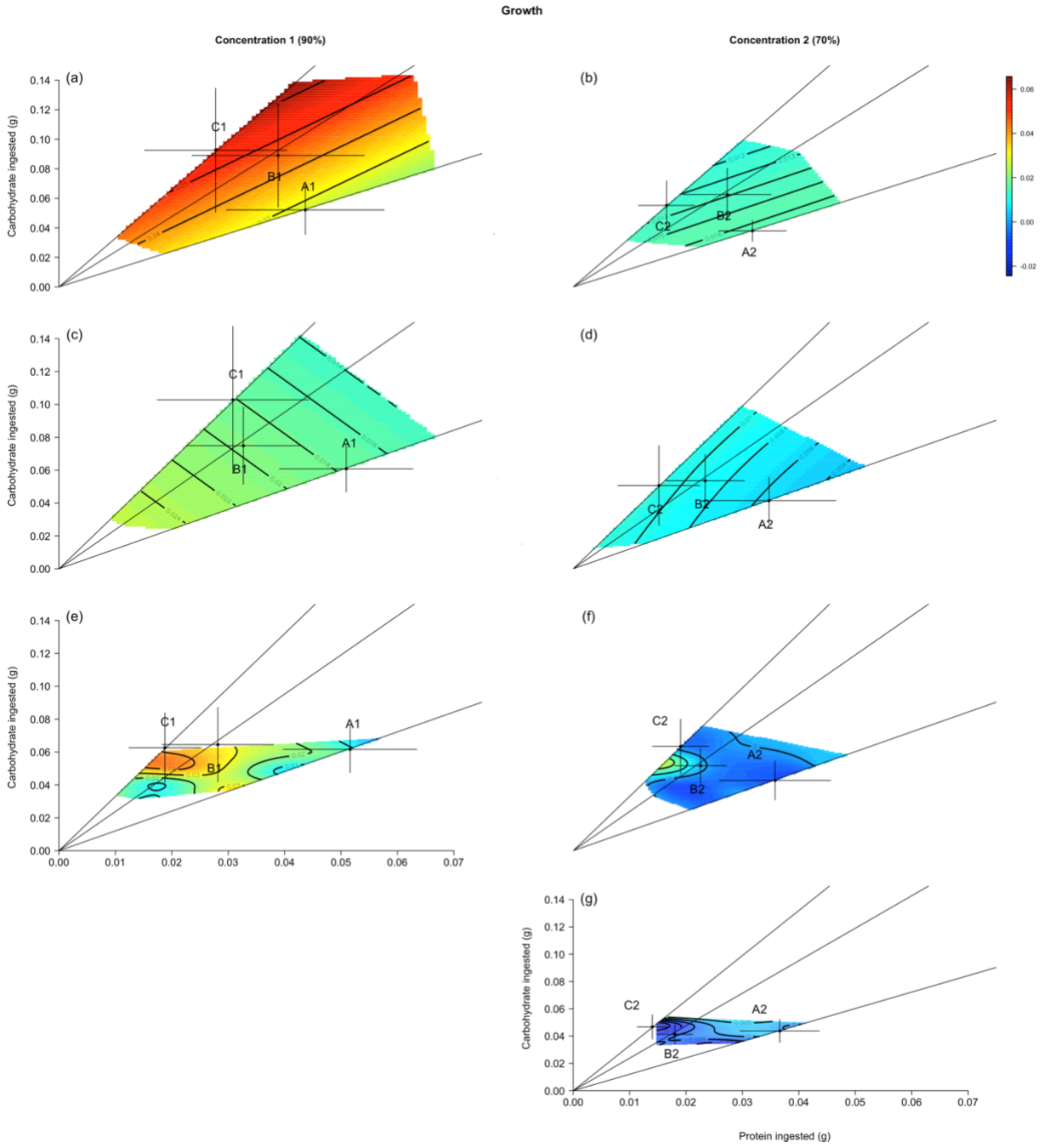

Figure 2: Weight change of *O. bicornis* larvae during the feeding experiment in weeks 1 (a&b), 2 (c&d), 3 (e&f), and 4-8 (g) projected over 3 diets (straight lines) that varied in P:C ratio (diet A, B and C, 1:1.2 1:2.3 1:3.4 respectively) and P+C concentration (1, 90% and 2, 70%). For each response surface, the regions where weight change was expressed at the highest and lowest level are represented by dark red and blue, respectively. Axes are matched for comparability among plots. Contours are indicated by solid lines and the values are written along the contours. Mean total (+/- SD) amount of P and C consumed in grams is represented by a cross. Response surface was modelled on actual amounts eaten. Development has been split into the 4 distinct growth periods identified in the analysis where weeks 4 to 8 show similar patterns and thus have been amalgamated. Sample sizes for plots for each distinct growth period were 51, 28, 13 and 4 fed concentration 1, and 65, 48, 33, and 21 fed concentration 2. There is no plot for weeks 4-8, concentration 1, because too few larvae survived past this period to be weighed. The solid straight black lines represent the ratio of protein to carbohydrate available to the larvae on each of the 3 diets, A, B and C.

Growth

Amalgamation of the model for differential use of nutrients for growth revealed the same distinct growth patterns as identified using the model for growth over time (S.1, c). Patterns in the relationship between growth and weekly nutritional intake changed across the development period (Table 2&3a) (Fig. 2). For larvae fed diets at 90% concentration there were interactive effects of carbohydrate and protein on growth in week 1. During this time, the highest growth was achieved by larvae on diet C, the food with the highest amount of carbohydrate with respect to protein at approximately 0.12g of protein and 0.04 of carbohydrate ingested (P:C 1:3.4). However, growth was the same irrespective of nutritional intake in week 2 (Fig. 2a-d). During week 3, growth was optimised when larvae ate a specific amount of protein and carbohydrate (0.018, and 0.06 respectively), which fell on the high carbohydrate diet (diet C (1:3.4)) (Fig 2e-f). Larvae fed the less concentrated diets grew the least during every growth period (Fig. 2). For these larvae, nutrient intake seemed to have little effect on weight change, except for during period 3 where the optimum intake of nutrients matched that for the larvae on the concentrated diet, although growth at that optimum was much reduced in comparison (Fig. 2). During weeks 4 to 8, larvae mostly lost weight regardless of nutritional intake (Fig 2g). For all larvae, on both nutrient concentrations, most growth occurred during the week 1 (Fig. 2a-b). The average amount of provision consumed in week 3 is very close to the carbohydrate growth optimum regardless of diet concentration treatment (Fig. 2c).

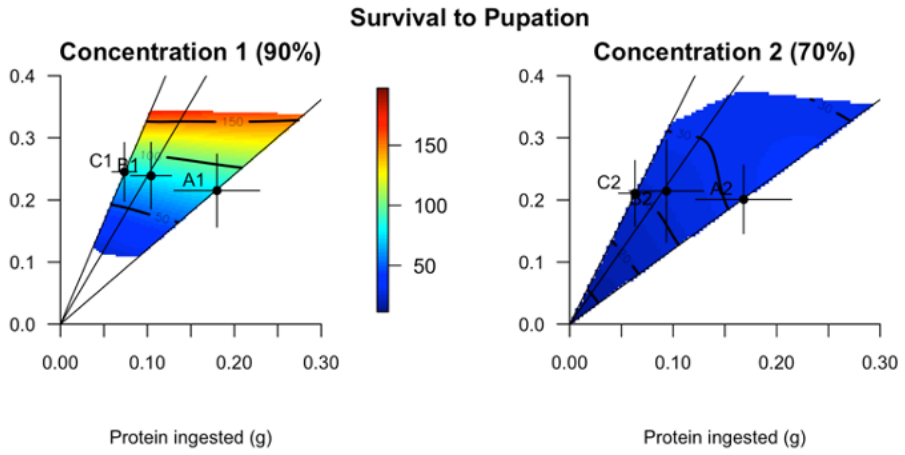

Figure 3: Predicted time to death (days) of *O. bicornis* larvae projected over 3 diets (straight lines) that varied in P:C ratio (diet A, B and C, 1:1.2 1:2.3 1:3.4 respectively) and P+C concentration (1, 90% and 2, 70%) from a Weibull model of the data . For each response surface, the regions where predicted time to death (days) was expressed at the highest and lowest level are represented by dark red and blue, respectively. Axes are matched for comparability among plots. Contours are indicated by thick solid lines and the values are written along the contours. Response surface was modelled on actual amounts eaten. Mean total (+/- 1 SD) amount of P and C consumed in grams is represented by a cross. Sample size was 134 larvae, 64 and 70 fed concentration 1 and 2 respectively. The straight solid black lines represent the ratio of protein to carbohydrate available to the larvae on each of the 3 diets, A, B and C.

Survival to Pupation

Patterns in the relationship between predicted time to death and weekly nutritional intake did not change across development (Table 2&3b). Survival of larvae fed the concentrated diets depended primarily upon carbohydrate consumption (Fig. 3) and those larvae that consumed the highest total amounts of carbohydrate saw the lowest mortality irrespective of how much protein was consumed. Survival of larvae raised on the dilute diets was reduced compared to those on the concentrated diets, and was not substantially affected by macronutrient intake (Fig. 3).

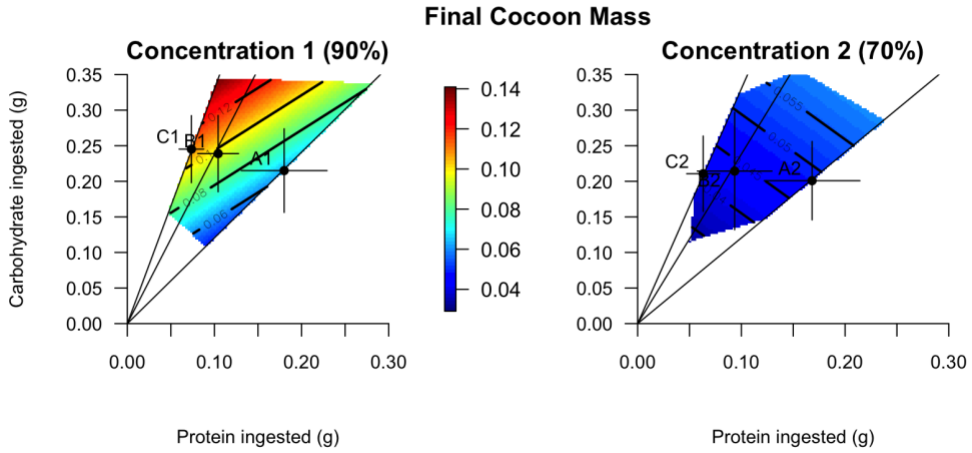

Figure 4: Final cocoon mass at pupation of *O. bicornis* larvae projected over 3 diets (straight lines) that varied in P:C ratio (diet A, B and C, 1:1.2 1:2.3 1:3.4 respectively) and P+C concentration (1, 90% and 2, 70%). For each response surface, the regions where cocoon mass was expressed at the highest and lowest level are represented by dark red and blue, respectively. Axes are matched for comparability between plots. Contours are indicated by thick solid lines and the values are written along the contours. Mean total (+/- 1 SD) amount of P and C consumed in grams is represented by a cross. Response surface was modelled on actual amounts eaten. Larvae which did not survive to pupation were not included for this plot. Sample sizes were 58 and 24 for concentration 1 and 2 respectively. The straight solid black lines represent the ratio of protein to carbohydrate available to the larvae on each of the 3 diets, A, B and C.

Cocoon Mass

Patterns in the relationship between final mass of the cocoon at pupation and weekly nutritional intake did not change across development (Table 2&3c). Larvae fed more concentrated diets had a final cocoon mass correlated positively with the total amount of carbohydrate consumed, and negatively with protein (Fig .4). For our range of diets, the greatest weights were obtained by larvae that ate above ~0.3g of carbohydrate and below ~0.15g of protein on the concentrated diets (Fig. 4). In contrast, those fed the low concentration diets, cocoons were lower in weight than those fed the concentrated diets, the mass of their cocoons was not substantially affected by intake of protein or carbohydrate (Fig. 4).

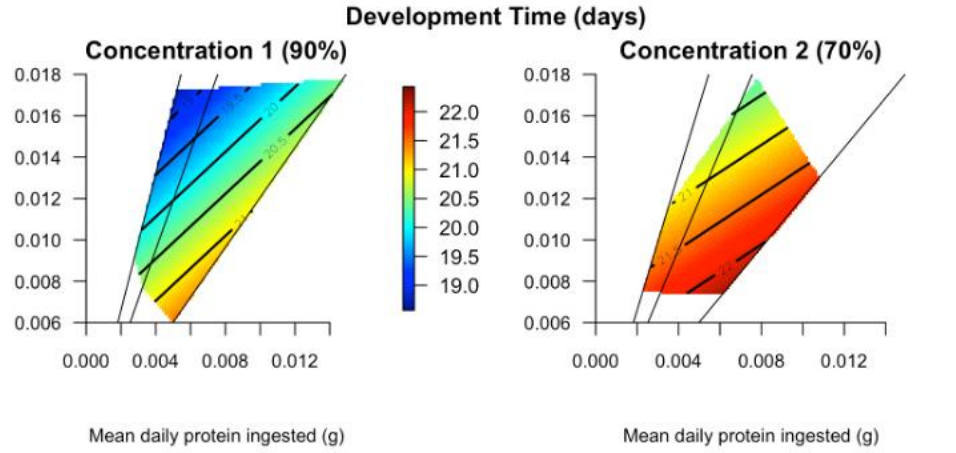

Figure 5: Time taken to pupation for *O. bicornis* larvae projected over mean daily consumption of 3 diets (straight lines) that varied in P:C ratio (diet A, B and C, 1:1.2 1:2.3 1:3.4 respectively) and P+C concentration (1, 90% and 2, 70%).. For each response surface, the regions where development time was expressed at the slowest and fastest speed are represented by dark red and blue, respectively. Axes are matched for comparability between plots. Contours are indicated by thick solid lines and the values are written along the contours. Response surface was modelled on actual amounts eaten. Larvae which did not survive to pupation were not included for this plot. Sample sizes were 58 and 24 for concentration 1 and 2 respectively. The straight solid black lines represent the ratio of protein to carbohydrate available to the larvae on each of the 3 diets, A, B and C. Mean total (+/- 1 SD) amount of P and C consumed in grams is not included since it is off the scale for this graph.

Development Time

Patterns in the relationship between time taken to pupate after hatching and weekly nutritional intake did not change across development (Table 2&3d). Development time was quickest for those fed a lower amount of protein relative to carbohydrate, and those fed the more diluted food developed more slowly on all three diets, with a narrower range of development times (Fig. 5).

Table 2: Tables for models of fitness correlates. Key; conc, overall nutrient concentration (1=90%, 2=70%); swap, week number; d.carb, amount of carbohydrate ingested (g); d. protein, amount of protein ingested (g), s(), a spline (repeating functions, each with its own coefficient) called a 'smoother' which makes a smooth line through the data to best show the details in the trend without over-fitting due to noise in the data. LogLik, log-likelihood; AICc, Akaike's Information Criterion corrected for small sample size; delta, Difference in AICc between model X and the top model; weight,

(a) Weight change (generalised additive mixed model)

	Model	K	logLik	AICc	delta	weight
gammw1.68$lme	conc + swapnum1 + s(d.protein, d.carb) |sample	12	763.01	-1500.82	0.000	0.999
gammw2.68$lme	conc + swapnum1 + s(d.protein, d.carb, by=swapnum1) |sample	27	773.43	-1486.71	14.110	0.001
gammw3.68$lme	conc + s(d.protein, d.carb) |sample	7	727.63	-1440.84	59.985	0.000

(b) Survival to pupation (parametric survival regression)

	Model	K	logLik	AICc	delta	weight
s4	d.protein + d.carb + conc + d.protein:d.carb + d.carb:conc	7	-810.37	1634.98	0.000	0.825
s1	d.protein + d.carb + conc + d.protein:d.carb	6	-813.72	1639.62	4.644	0.081
s5	d.protein + d.carb + conc + d.protein:d.carb + d.protein:conc	7	-813.16	1640.56	5.579	0.051
s6	swap.num + d.protein + d.carb + conc + d.protein:d.carb + d.carb:conc	14	-806.18	1641.28	6.304	0.035
s0	swap.num + d.protein + d.carb + conc + d.protein:d.carb	13	-809.25	1645.29	10.307	0.005
s7	swap.num + d.protein + d.carb + conc + d.protein:d.carb + d.protein:conc	14	-808.81	1646.53	11.550	0.003
s8	swap.num + d.protein + d.carb + conc + d.protein:d.carb:swap.num + d.carb:conc	21	-803.84	1651.72	16.741	0.000
s3	d.protein + d.carb	4	-862.30	1732.68	97.700	0.000
s2	d.protein + d.carb + d.protein:d.carb	5	-862.22	1734.57	99.594	0.000

Akaike weight (relative likelihood of model X)/(sum of relative likelihoods of all models).

(c) Cocoon mass (generalised additive model)

	Model	K	logLik	AICc	delta	weight
gamc5	conc + s(d.protein, d.carb, by=conc)	10	527.72	-1033.85	0.000	0.953
gamc3	conc + s(d.protein, d.carb)	5	519.04	-1027.83	6.025	0.047
gamc1	conc + swapnum1 + s(d.protein, d.carb)	11	519.65	-1016.18	17.675	0.000
gamc2	conc + swapnum1 + s(d.protein, d.carb, by=swapnum1)	21	524.33	-1002.60	31.259	0.000
gamc4	swapnum1 + s(d.protein, d.carb)	14	502.79	-974.39	59.468	0.000

(d) Development time (generalised additive model)

	Model	K	logLik	AICc	delta	weight
gamd3	conc + s(d.protein, d.carb)	5	-600.51	1211.28	0.000	0.989
gamd1	conc + swapnum1 + s(d.protein, d.carb)	11	-599.11	1221.36	10.083	0.006
gamd5	swapnum1 + s(d.protein, d.carb)	13	-597.18	1221.94	10.659	0.005
gamd4	conc + swapnum1 + s(d.protein, d.carb, by=conc)	16	-596.48	1228.32	17.040	0.000
gamd2	conc + swapnum1 + s(d.protein, d.carb, by=swapnum1)	21	-593.98	1234.11	22.835	0.000

Table 3. Coefficient tables for minimal models of fitness correlates. Key; conc, overall nutrient concentration (1=90%, 2=70%); swap, week number; d.carb, amount of carbohydrate ingested (g), d.protein = amount of protein ingested (g); s() = a spline (repeating functions, each with its own coefficient) called a 'smoother' which makes a smooth line through the data to best show the details in the trend without over-fitting due to noise in the data; estimate, estimated parameter coefficient; std.error, estimated standard error of coefficient; edf, array of estimated degrees of freedom for the model terms; ref.df, reference degrees of freedom; R^2, variance explained.

(a) Weight change (generalised additive mixed model) parametric coefficients of minimal model

term	estimate	std.error	Chi-squared	p.value
Intercept (conc1)	0.038	0.002	16.662	<0.0001
conc2	-0.022	0.003	-7.827	<0.0001
swapnum12	-0.010	0.002	-5.300	<0.0001
swapnum13	-0.015	0.002	-6.339	<0.0001
swapnum14	-0.020	0.003	-6.596	<0.0001
swapnum15	-0.025	0.005	-4.977	<0.0001
swapnum16	-0.027	0.007	-3.867	0.00014

Approximate significance of smooth terms

term	edf	ref.df	Chi-squared	p.value
s(d.protein,d.carb)	2	2	4.151	0.017

Adjusted R^2=0.42

(b) Survival to pupation (parametric survival regression) parametric coefficients of minimal model

term	estimate	std.error	Chi-squared	p.value
Intercept (conc1)	2.692	0.224	12.040	<0.0001
d.protein	29.770	5.339	5.576	<0.0001
d.carb	20.110	5.173	3.887	0.0001
conc2	-0.174	0.166	-1.045	0.29586
d.protein:d.carb	-356.933	101.287	-3.524	0.00043
d.carb:conc2	-8.756	3.491	-2.508	0.01213
Log(scale)	-1.211	0.062	-19.633	<0.0001

(c) Cocoon mass (generalised additive model) parametric coefficients of minimal model

term	estimate	std.error	Chi-squared	p.value
Intercept (conc1)	0.086	0.002	38.446	<0.0001
conc2	-0.033	0.006	-5.767	<0.0001

Approximate significance of smooth terms

term	edf	ref.df	Chi-squared	p.value
s(d.protein,d.carb):conc1	3.961	5.306	11.095	<0.0001
s(d.protein,d.carb):conc2	2.000	2.000	0.558	0.57

Adjusted R^2=0.357

(d) Development time (generalised additive model) parametric coefficients of minimal model

term	estimate	std.error	Chi-squared	p.value
Intercept (conc1)	20.728	0.217	95.330	<0.0001
conc2	1.609	0.423	3.807	0.00018

Approximate significance of smooth terms

term	edf	ref.df	Chi-squared	p.value
s(d.protein,d.carb)	2.001	2.001	9.513	1e-04

Adjusted R^2=0.0138

DISCUSSION

In summary, I found that rules governing consumption of provisioned food changed over the developmental stage, and accordingly, so did the patterns of growth. Despite these changes, we found that weekly nutrient consumption had uniform effects for final cocoon mass, development time, and likelihood of survival to pupation (Fig. 3-5). Dietary dilution carried costs to all measured traits (Fig. 2-5). In what follows I explain why developmental physiology may help to explain these patterns.

Austin and Gilbert (2019) found that *O. bicornis* larvae tightly regulated the amount of carbohydrate they ingested, converging on approximately 0.22g of carbohydrate in total before pupation, irrespective of protein consumption. In the current study we revealed that larvae began to tighten their regulation of carbohydrate over protein as they developed. Austin and Gilbert (2019) suggested that carbohydrate may be regulated more closely than protein due to it's importance for overwinter survival, and/or may be more limiting than protein in their natural diet of pollen. The larvae feed almost exclusively on pollen (Strohm et al., 2002), the most protein-rich part of a plant (Mattson, 1980), and *O. bicornis* mothers uniquely add only small amounts of carbohydrate-rich nectar, roughly 4% of total provisions (Maddocks and Paulus, 1987; Strohm et al., 2002), whereas for some species it can be up to 50% (Nicolson, 2011). Thus, it is conceivable that they would encounter excess dietary protein and limited carbohydrate in their natural diet of mostly pollen. However, the protein content of plant species varies between 2-

60% (Roulston et al. 2000), and it is likely that the carbohydrate content also varies widely. Bees are living in a changing nutritional environment where human induced landscape change has reduced the quantity, diversity, and temporal availability of food resources for bees (Goulson et al., 2015; Ziska et al., 2016). Additionally, increases in atmospheric carbon dioxide are leading to declines in the nitrogen (protein) content of many species of plants (Taub et al., 2008), and in pollen consumed by bees (Ziska et al. 2016). It is uncertain whether solitary bees can detect pollen quality and provide offspring with nutritionally balanced provisions (Nicholls and Hempel de Ibarra, 2017). Their diet could potentially vary widely with location or with increased anthropogenic environmental change. Alternatively, perhaps it is more costly for *O. bicornis* larvae to ingest excess carbohydrate than it is to over-ingest protein. While excess fat storage from a carbohydrate rich diet can be an advantage in some circumstances (Dussutour et al., 2016), it can result in reduced fitness in animals, including insects (Simpson et al., 2004; Raubenheimer et al., 2005; Warbrick-Smith et al., 2006; Simpson and Raubenheimer, 2012; Pooraiiouby et al., 2018; Raubenheimer and Simpson, 2019), as can also be true for excess protein (Dussutour and Simpson, 2012: Runagall-McNaull et al., 2015; Arganda et al., 2017; Raubenheimer and Simpson, 2019) and excess lipids (Haddad et al. 2007; Manning et al. 2007). An example can be seen in a recent study with holometobolous beetles, *Nicrophorus vespilloides,* a species which regulates nutrients differentially at different life stages (Al Shareefi and Cotter, 2018). Al Shareefi and Cotter (2018) found that whilst the beetles are regulating protein, the low protein, high fat diets resulted in “obese” beetles with rapid and excessive growth (‘Protein leverage behaviour’ (Raubenheimer and Simpson, 2019)), whereas individuals regulating fat intake maintained a relatively constant weight on all diets (Al Shareefi and Cotter, 2018). Perhaps *O. bicornis* larvae have more efficient post-ingestive mechanisms for dealing

with excess protein than they do for dealing with excess carbohydrate, such as storage of the excess for later use in egg production, as in caterpillars (Telang et al., 2003), or excretion as nitrogenous waste, as in locusts (Simpson and Raubenheimer, 2001; Behmer, 2009). This is expected since carbohydrate is likely the limiting nutrient in their natural diet. We know that adult social bees regulate carbohydrate over protein (Altaye et al., 2010; Pirk et al., 2010; Paoli et al., 2014; Stabler et al., 2015; Vaudo et al., 2016), yet since there can be a great deal of variation in diet and nutritional requirements of holometabolous adults and their larvae, (Boggs, 2009; Nestel et al., 2016), the same may also be true for bees and their larvae. While few studies have been carried out on solitary bees or larval bees (Austin and Gilbert, 2018), a nutritional geometry study found that a low-protein, high-carb diet has a negative effect on honeybee larval growth and survival (Helm et al., 2017b).

By splitting the data into the distinct growth periods, I found that the relationship between growth and nutrient intake at each growth period changed across the development stage (Fig. 2). The average amount of carbohydrate eaten does, to some extent, reflect the carbohydrate intake for optimising larval growth (Fig. 2). For example, at swap 3, the growth optimum occurs at a point reachable for those larvae given the most carbohydrate-biased diet (diet C, P:C 1:3.4), and all larvae, on high and low concentration diets, ate an average amount of food which took them very close to their carbohydrate optima. In order to do so, larvae on the low concentration diet ate a considerable amount more, and the larvae on the other diets tolerated the consumption of excess protein (Fig. 1-2). To our knowledge this is the first time that changes in the rules governing consumption have been shown in solitary bee larvae. Perhaps this shift reflects changes in the nutritional needs of the *O. bicornis* larvae (Boggs, 2009; Simpson and

Raubenheimer, 2012), as we know is true during their overwintering phase (Wasielewski et al., 2013; Wasielewski et al., 2014). The roles particular nutritional components play in overall growth and development are well characterised in many insect species (e.g Runagall-McNaull et al., 2015; Boggs and Niitepõld, 2016; Wright et al., 2018; Ma et al., 2020). There have been multiple studies looking at the role of dietary protein in larval development and reproduction in social bee species; honeybees, bumblebees and sweat bees, however it must be noted that these often do not take into consideration the potential variation in non-protein constituents of the pollen species they use (Génissel et al., 2002; T'ai and Cane, 2002; Human et al., 2007; Tasei and Aupinel, 2008; Li et al., 2012; Vanderplanck et al., 2014). Knowledge in resource allocation is lacking for solitary bee species and their larvae (Praz et al., 2008; Vaudo et al., 2015). We know that different dietary nutrients are required for different physiological functions in insects; energy, growth, reproduction, and maintenance (Kraus et al., 2019b). For example, proteins are a source of nitrogen and amino acids, vital for somatic growth and enzymes, and can be stored for later use in egg maturation (Arrese and Soulages, 2010; Kraus et al., 2019b). Sugars for energy can be synthesised from carbohydrate, or lipids, and can be stored as fat or glycogen (Kraus et al., 2019b). Lipids are required for pheromone and hormone production (Stanley, 2006). Insects can also use protein for energy synthesis, but this carries substantial metabolic costs (Kraus et al., 2019b). All nutrient storage and allocation is controlled by the fat body, a regulatory organ specific to insects, which detects and responds to an insect's nutritional state (Arrese and Soulages, 2010; Koyama and Mirth, 2018; Kraus et al., 2019b). Shifts in the rules governing nutrient consumption to reflect changes in nutritional needs during a single life-stage have previously been found in other holometabolous insects. For example, the royal jelly produced by nurse bees for newly hatched honeybee queens varies significantly in nutritional composition

across each day over the first four days post-hatching (Zheng et al., 2011: Wang et al., 2017) to the extent that jelly produced on different days has varying potential to rear queen castes (Zheng et al., 2011). In young adult honeybees, the intake target shifts to a more carbohydrate biased diet over a two-week period, when their behavioural caste within a colony shifts to a forager caste (Paoli et al., 2014), involving substantial physiological changes, such as ovarian atrophy and fat body reduction (Seehuus et al., 2007; Ament et al., 2008). The most striking example of changes in behavioural nutrient regulation in response to changing physiological needs can be seen in a recent study with carnivorous beetle, *Nicrophorus vespilloides* (Al Shareefi and Cotter, 2018). This species has a clearly defined growth stage leading up to pupation, followed by a distinct post-pupation stage for maturation of the reproductive organs (Al Shareefi and Cotter, 2018). This allows for comparison between the behavioural nutrient regulation for growth, and the nutrient regulation for maturation feeding. The results of the study revealed a shift in the intake array of the beetles as they aged. During the ten days of maturation feeding, the beetles regulated protein consumption and ate more food, whereas afterwards, they regulated fat consumption and ate less (Al Shareefi and Cotter, 2018). The intake target in species of carnivorous beetles has been shown to shift to a slightly more protein biased diet once the reproductive system has matured (Raubenheimer et al., 2007; Al Shareefi and Cotter, 2018).

This concept, that the changes in a nutritional intake target may reflect an animals' changes in physiological needs, is similar to the widely accepted life-history trait trade-offs, whereby species show adaptive allocation of limited nutritional resources between multiple competing metabolic activities, such as somatic repair versus reproductive effort (Roff and Fairbairn, 2007; Boggs, 2009; Creighton et al., 2009; Simpson and Raubenheimer, 2012; Hosking et al., 2019;

Farchmin et al., 2020). Further to this, differences in the costs of dietary nutrient imbalance to insects of different ages is suggested to be evidence of the same nutrient being used for different physiological processes between age groups (Runagall-McNaull et al., 2015). Interestingly, in a foraging experiment where *O. bicornis* larvae were given a pollen ball from a single plant species, offspring mortality occurred at different times during the development stage for individuals fed from different species: Those on one species suffering early mortality, and those on another suffering an inability to reach the late stages or successful enclosure into viable adults (Bukovinszky et al., 2017). The variation in nutritional content or digestibility between the different pollen species affects *O. bicornis* larvae at different times in their developmental stage (Bukovinszky et al., 2017) which perhaps could suggest interference with, or failure to meet the requirements of, different physiological processes of different larval stadia by each of the pollen species (Boggs, 2009).

Despite the changes in the rules governing provision consumption and patterns of growth over the developmental stage, I found uniform effects of nutrient consumption on final cocoon mass, development time and likelihood of survival to pupation across all weeks (Fig. 3,4&5). A larger sample size may have made any changes in these patterns easier to distinguish, especially towards the end of development where some larvae had died. However, this is not to say that there was no stadium-specific effect of nutrient consumption on eventual adult fitness. Deficiencies in the developmental diet can incur costs in later life stages (Boggs, 2009) (the silver spoon concept (Klepsatel et al., 2020)). For example, in holometabolous insects, larval nutrition affects adult lifespan (Runagall-McNaull et al., 2015; Klepsatel et al., 2020) and reproductive output (Klepsatel et al., 2020). Future studies could investigate the effects of

weekly nutrient intake during development on fitness traits into adulthood (Runagall-McNaull et al., 2015). Since the results showed that most growth occurred during week 1, for future studies, it could also be beneficial to find a way to manipulate the fragile eggs before they hatch in order to start the feeding experiments from day one post-hatching.

Dilution carried fitness costs to all measured traits. We can see that, at least in the later growth periods, the larvae compensated for the dilution effect in terms of nutrient consumption, the average macronutrient consumption being very similar for different groups, and in some cases, the same (Fig. 1). Pilot tests revealed that sunflower pollen exines (the outer husk) used in the artificial diets are non-toxic and carry no nutritional value for *O. bicornis* larvae, making it an ideal dilution agent (Tainsh et al., 2020). Thus the fitness costs could be linked with the metabolic processing costs of consuming indigestible material, for example, interference with nutrient absorbance in the gut (Slansky and Wheeler, 1989; Lee et al., 2002; Tainsh et al., 2020). Plant defence of pollen from palynivores includes mechanical and chemical defences which can interfere with digestibility, and can affect fitness in bees (Brochu et al., 2020; Rivest and Forrest, 2020). A striking example of the complex effects of dilution on insect nutrition can be seen in a nutritional geometry study with caterpillars (Lee et al., 2002). Final instar caterpillars were provided with a choice of a protein-biased food, and an equal-ratio protein-carbohydrate food. All caterpillars were able to regulate to an intake target (approx 60:40 P:C), except for those given the equal-ratio food at a low-nutrient concentration with the protein biased diet at a high-nutrient concentration (Lee et al., 2002). These larvae progressively abandoned regulation, ate more of the protein biased food, and showed higher protein ingestion than the other caterpillars. Yet, in spite of this change, they showed no differences in survivorship, growth, or development

(Lee et al., 2002). This is suggestive that the cost of eating the indigestible element of their diet (cellulose) became higher than the cost of consuming excess of a dietary macronutrient when the diet was dilute enough (Lee et al., 2002). As we can see from the results, fitness of larvae on the more dilute diets is greatly reduced in many ways (slower growth, longer development, increased mortality, and smaller cocoon size), even when the larvae achieve the same nutrient intake as those on the concentrated diets (Fig. 2-5). Moreover, I believe this is one of the first studies to quantify nutrient consumption in bee larvae (Austin and Gilbert, 2018). The only other nutritional geometry study to date to look at the relationship between diet and development time in bees is for honeybee larvae, and found no effect of artificial P:C diets on development time (Helm et al., 2017b). Dietary dilution is an increasingly realistic threat to bees due to the decreasing protein content of pollen (Ziska et al., 2016).

The results of this study suggest that the nutritional needs of developing bees are dynamic and complex, and this could have important implications for farmers, landowners, conservationists, and producers of 'bee-friendly' seed-mixes when providing floral food resources for bees. Currently, the plant species used to create bee-friendly forage, including wildflower strips and hedgerows in agroecosystems, are often chosen based on the frequency of visits by adult social bee species to each plant species (Burkle et al., 2020; McMinn-Sauder et al., 2020), despite the fact that most species are solitary, and the fact that it is uncertain whether bee species can detect the quality of pollen (T'ai and Cane, 2002; Ruedenauer et al., 2015; Muth et al., 2016; Ruedenauer et al., 2016; Nicholls and Hempel de Ibarra, 2017; Corby-Harris et al., 2018; Ruedenauer et al., 2020). Another common method is to choose plants which are rich in pollen and nectar (Pywell et al., 2006; Carvell et al., 2007; Haaland et al., 2011; Filipiak, 2019),

or those that provide resources throughout the agricultural growing season (Williams et al., 2015). Even for studies in which pollen quality is considered, rather than just considering pollen quantity, the focus is often only on protein, and the balance of other nutrients is not considered (Génissel et al., 2002; T'ai and Cane, 2002; Human et al., 2007; Tasei and Aupinel, 2008; Li et al., 2012; Vanderplanck et al., 2014). The results of the current study suggest that food quality, not just quantity, is crucial for larval bee development. Moreover, plant species with more readily-available pollen could potentially have increased plant defences to protect their pollen (Haider et al., 2014), such as indigestible compounds (Brochu et al., 2020; Rivest and Forrest, 2020), of which we have shown some of the potential serious detrimental effects on the fitness of a developing bee (Austin and Gilbert, 2018). In fact, many species with freely accessible pollen have been found to exhibit pollen properties that negatively affect bee larval survival (Levin and Haydak, 1957; Williams, 2003; Pywell et al., 2006; Praz et al., 2008; Sedivy et al., 2011; Haider et al., 2014). When selecting floral resources for bee conservation, perhaps plant species should be selected to match the nutritional requirements of the bee species, (based on their nutritional ecology and environment), and we should consider the balance of nutrients, rather than nutrients in isolation (Vaudo et al., 2015; Filipiak et al., 2017; Moerman et al., 2017; Filipiak, 2018; Filipiak, 2019). This can be achieved, with studies such as this one, by improving understanding of the nutritional needs and feeding behaviour of bee species.

Conclusion

In conclusion, I found that the rules governing provision consumption changed over the development period of a solitary bee, and accordingly, so did the patterns of growth. This has revealed the underlying patterns of consumption and fitness in relation to nutrient intake.

Building on the work by Austin and Gilbert (2018), I showed an age-related behavioural shift towards carbohydrate intake regulation. Dietary requirements of bee larvae is relatively poorly understood, particularly for solitary species (Filipiak, 2018; Austin and Gilbert, 2018). To date, the majority of studies have focused on single points in time or looked at overall patterns across whole life stages (Helm et al., 2017b), and few have focused on age related shifts in nutritional requirements (Paoli et al., 2014; Al Shareefi and Cotter, 2018). This study highlights that the rules governing feeding behaviour can be complex and dynamic, and could have important implications for the design of habitats for conservation of wild and domestic bees.

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SUPPLEMENTAL

Supplementary Table 1: Model tables for models of weight change per swap (a-b) and differential use of nutrients for growth (c). Key; LogLik, log-likelihood; AICc, Akaike's Information Criterion corrected for small sample size; delta, Difference in AICc between model X and the top model; Weight, Akaike weight (relative likelihood of model X) / (sum of relative likelihoods of all models).

(a) Weight change per swap (generalised additive mixed model) parametric coefficients of the minimal model

term	estimate	std.error	Chi-squared	p.value
(Intercept)	0.025	0.002	13.291	<0.0001
swapnum2	-0.011	0.002	-5.720	<0.0001
swapnum3	-0.016	0.002	-6.950	<0.0001
swapnum4	-0.023	0.003	-8.698	<0.0001

(b) Weight change per swap, amalgamated models (generalised additive mixed model)

Model	K	logLik	AICc	delta	weight
gamm1-2-3-4:8$lme	6	737.81	-1463.30	0.000	0.372
gamm1-2-3-4-5:8$lme	7	738.44	-1462.46	0.839	0.244
gamm1-2-3:4-5:8$lme	6	736.67	-1461.02	2.276	0.119
gamm1-2:3-4:8$lme	5	735.36	-1460.49	2.808	0.091
gamm1-2-3-4-5-6:8$lme	8	738.45	-1460.37	2.934	0.086
gamm1-2-3-4-5-6-7:8$lme	9	738.85	-1459.01	4.289	0.044
gamm1-2-3:8$lme	5	734.61	-1458.99	4.309	0.043
gamm1-24-5:8$lme	5	731.26	-1452.30	11.002	0.002
gamm1-2:8$lme	4	727.64	-1447.13	16.174	0.000
gamm13-4:8$lme	4	711.11	-1414.07	49.231	0.000
gamm1:4-5:8$lme	4	702.31	-1396.47	66.834	0.000

(c) Differential use of nutrients for growth (generalised additive mixed model)

Model	K	logLik	AICc	delta	weight
gamm1-2-3-4:8$lme	10	762.26	-1503.68	0.000	0.304
gamm1-2-3-4-5:8$lme	11	762.96	-1502.91	0.768	0.207
gamm1-2-3:4-5:8$lme	10	761.62	-1502.40	1.279	0.160
gamm1-2:3-4:8$lme	9	760.43	-1502.17	1.512	0.143
gamm1-2-3-4-5-6:8$lme	12	763.01	-1500.82	2.858	0.073
gamm1-2-3:8$lme	9	759.59	-1500.49	3.192	0.062
gamm1-2-3-4-5-6-7:8$lme	13	763.54	-1499.69	3.996	0.041
gamm1-2:4-5:8$lme	9	757.64	-1496.61	7.077	0.009
gamm1-2:8$lme	8	754.24	-1491.93	11.748	0.001
gamm1:3-4:8$lme	8	739.51	-1462.48	41.200	0.000
gamm1:4-5:8$lme	8	732.79	-1449.04	54.640	0.000

APPENDICES

Appendix 1: A note on work not included in this thesis due to mitigating circumstances

Experiments and lab work were halted due to closure of the university and a UK Lockdown from March 2020 in response to the COVID-19 pandemic. This changed the course of the work in March 2020 (project start date and deadline were September 2019- September 2020).

Pre-March 2020, the focus of the MSc was post-ingestive macronutrient processing in solitary bee larvae. The aim was to determine whether *Osmia bicornis* larvae use excretion as a post-ingestive mechanism to regulate and process macronutrients (Protein, carbohydrate, and lipid): Particularly to regulate the excess protein which they are willing to ingest to hit a carbohydrate target when given a nutritionally-unbalanced diet (Austin and Gilbert 2018). I predicted that between groups of *O. bicornis* larvae given diets of varying rations of protein and carbohydrate, there would be little variation in the amount of carbohydrate excreted. However, the amount of protein in the excreta (frass) would likely vary a large amount between treatment groups. If the bees were regulating to reach a nutrient target, then there would be a positive correlation between amount of nutrient consumed and amount of nutrient excreted after the intake target had been reached. Larvae with a low protein:carbohydrate diet may have also had a smaller body size (protein is used for growth) since they stopped eating before their protein intake target was met. Due to the 2020 COVID related lockdowns and restrictions, this work was never finished (see Appendices 5-9).

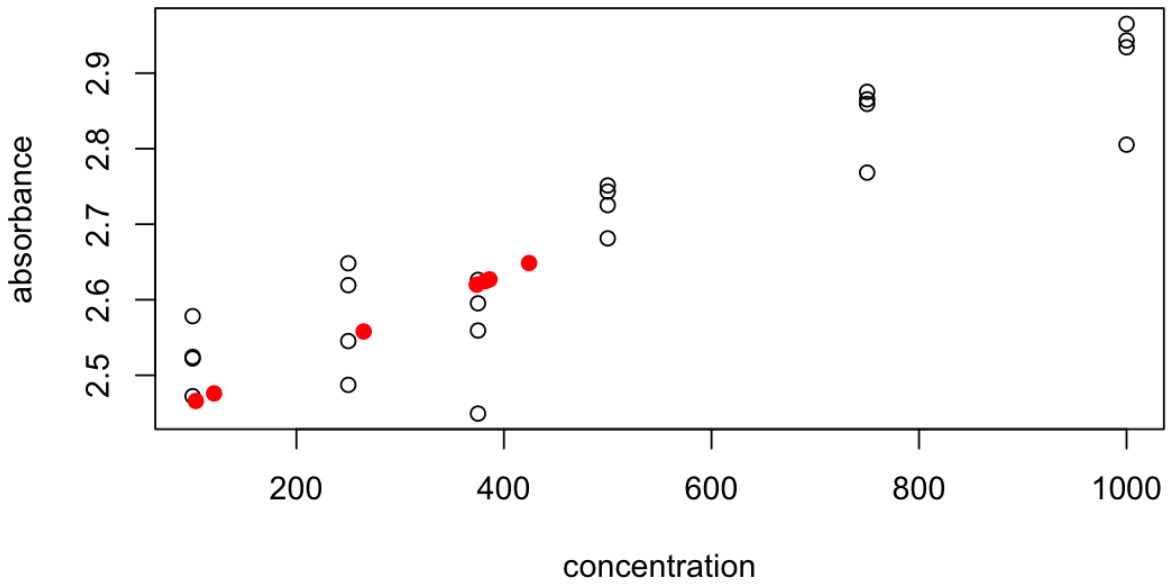
Cancelled Spring 2020 work

From the feeding experiments from which the analyses of this thesis were based, all excreta (frass) from the larvae were collected on a weekly basis. I investigated the possibility of using Fourier Transform Infrared Spectroscopy to determine chemical composition of the frass, which, after consultations and demonstrations, was deemed not to be worth the initial investment of time in training. Eventually, I planned a colorimetric analysis of the larval frass for protein, lipid and sugar content using the Van Handel (1985a,b) and Van Handel and Day (1988) method: a popular method for analysing the macronutrient content of insect bodies, by building on an adapted quick and cost-effective version designed by Foray at al. (2012). I piloted the methods using 'spare' frass samples from a previous *O. bicornis* feeding experiment (see Appendix 2). Frass samples were lyophilised (freeze-dried) and re-weighed in order to obtain a dehydrated mass for scaling the absorbance readings by size. To my knowledge, this would have been the first time this method would have been used for very small samples of near-dry insect frass; this being the case, I invested considerable time adapting the standard methods for use in this context (Appendix 3), and wrote the COSHH forms from scratch (Appendix 4). If the colorimetric method had not been successful for frass analysis, I would have instead used a CHN analyser to obtain an elemental measure of the frass content. This would have given an indication of the amount of protein excreted, which in insects is excreted as nitrogenous waste.

The second aim of the project was to identify the intake target of *O.bicornis* larvae by building on the nutritional experiments done by Austin and Gilbert 2018. We had 1000 *O. bicornis* cocoons ready to be released to our on-site beehives. Feeding experiments were planned to start in the spring once the *O. bicornis* eggs were laid. This would have included a range of diet ratios and concentrations to further expand the response graphs. If time permitted, I also wanted to

investigate the role of storage as a mechanism for post-ingestive processing by adapting the frass analysis protocol for analysis of the larval bodies to calculate the proportion of macronutrients stored in the body. These plans were never realised as a result of the 2020 COVID related lockdowns and restrictions.

Appendix 2: Example pilot data for a Bradford colorimetric test for protein concentration.



A fitted linear model for absorbance values of standards with known protein concentration (black) was used to predict the protein concentration of practise frass samples (red) based on their absorbance values. Made using R with the predict function.

Appendix 3: Example of adapted methods for pilot tests using methods from (Foray et al. (2012), Muller et al. (2017) and Cook (2019).

Step	**Aim**	**Notes**
defrost the samples		
check plate reader/PC is booked and switched on		
	PROTEIN ASSAY	
Remove the bradford reagent from the fridge, invert, incubate at room temperature.	only works at room temperature.	keep in fume hood.
Prepare standards	calibration curve	
Add 5ul of each standard concentration to 3 separate wells (triplicate).		Dilute them with same buffer as the samples
in triplicate, add 5ul of each sample		
check that the bradford reagent is room temperature or above.		
Pippette 180uL aqueous lysis buffer solution into sample tube + standards	cell lysis	need to adjust amount of buffer
and crush for 30s with a pestle (25Hz).	protein solubilisation	
centrifuge 180G at 4degrees **if** there is any cuticle/debris that wont dissolve	sedimentation of cuticle	but avoid centrifuging if you can
Pippette 2.5uL of supernatant into microplate		avoid the surface slick if present
Pippette 250uL bradford reagent into microplate		can use a plastic microplate
incubate at room temperature for 15-20mins		stable for up to 50-60mins
gently shake plate 10Hz 3s	to disrupt dye aggregates	
read protein concentration at 595nm		Absorbance can be measured at 580–610 nm (BioRad)
potential causes for problem with previous pilot test:	***to address this:***	
the bradford reagent was not up to room temperature so slow colour change reaction?	*check temperature with thermometer*	
the buffer is not working because the pH is too low?	*made new buffer, pH 7.4*	
the buffer is not working because the EDTA is really old?	*made new buffer with different source of EDTA*	

the standards were too concentrated leading to a standard curve which is too high?	*use lower concentrations*	
the homogenisation step did not mash up the samples enough?	*spend longer homogenising and vortexing*	
sample too dilute	*Change volume of buffer used at homogenising step*	
buffer interfering	*do a sample and a standard curve in deionised water in stead of buffer along side buffer ones*	

prepare standards of D-glucose	WATER SOLUBLE CARBS ASSAY	
prepare fresh anthrone	must be prepared 2-3days prior to use	
Pippette 20uL of 20% sodium sulfate solution to homogenate	to dissolve all carbohydrates	
Pippette 2.5uL of the buffer to the homogenate		
Pippette 1500uL of 2:1 chloroform:methanol	solubilise lipids and water soluble carbs	
Vigorously vortex each sample	mix	
centrifuge for 15mins at 180G at 4degrees.	remove glycogen from supernatant	
Next you will pippette supernatant to a new tube:		keep pellet for later
Pippette 150uL of the supernatant into glass microplate		glass microplate required
evaporate at room temp for approx 30min till volume is approx 10uL		
pippette 240uL anthrone reagent to each well		
incubate at room temp for 15mins		
cover plate and heat in water bath at 75degrees for 15-20mins		90 is too high for glass plate
read absorbance at 625nm		
make series of dilitions with D-glucose standard	GLYCOGEN ASSAY	
wash the pellet with 400uL 80% methanol		
centrifuge 16000G for 5mins at 4degrees		
pippette out the supernatant	removes sugar from pellet	
wash the pellet with 400uL 80% methanol	repeat washing step	
centrifuge 16000G for 5mins at 4degrees		
pippette out the supernatant		
pippette 1mL anthrone reagent to add to pellet		
incubate 15-20mins at 75degrees max in water bath		90 is too high for glass plate
cool samples on ice	stop the reaction	
filter samples		
read absorbance at 625nm		
make some vanillin reagent and wrap bottle in foil		stable for several weeks in darkness
prepare a series dilution of triolein	TOTAL LIPID ASSAY	
pipette 100uL of supernatant into glass plate		
heat at 75degrees until complete solvent evaporation		90degrees too hot for glass plate
pipette 10uL of 98% sulfuric acid into each well		

incubate at 75degrees 2-3mins in a water bath		90degrees is too hot for plate
cool the plate on ice		
pipette 190uL vanillin reagent to each well		
shake the plate		
incubate plate at room temp for 15mins		
read ansorbance at 525nm		520-540nm

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Appendix 4:Approved COSHH form for colorimetric assays

https://docs.google.com/document/d/1MPnvAbXnhBKgfhhICOkuzATX8RsO0KP3/edit?usp=sharing&ouid=104389765816490063086&rtpof=true&sd=true

Appendix 5: Timeline of work completed from September to March

September

- Reading about nutritional geometry.
- Reviewing the literature for frass analysis methods (see bibloography).
    - Chose the microplate method = high-throughput, small volume, low waste approach.
    - The Foray et al., (2012) method is a validated, fast, reproducible, and cheap method and was the most commonly used/adapted for similar work to mine involving small chitinous samples.
    - Investigated the option of FTIR and decided the time taken for training plus using an oversubscribed machine was not a good option.

October

Planned a lab protocol for my own assays using the literature.

- Made some adaptations to the foray method using more recent, simplified versions (Muller et al., 2017; Cook, 2019).
- Wrote COSHH form.

November

- Claculation of how much of each chemical is needed to analyse the samples (with some to spare) and ordered chemicals and lab equipment.
- Enquiries about a platereader.
- Sorted the samples from the freezer.
- Lab induction and learned how to use the freeze dryer (Appendix 7).

December

- Could not start assays yet - waiting for the ordered chemicals and kit to arrive.
- Ordered the cocoons for the feeding experiment.

January

- Plate reader in Hardy cannot measure at all the necessary wavelengths. Enquired in other departments if they have a platereader which I could use.
- Made the reagents needed once the chemicals arrived.
- Training on a new platereader (Appendix 8).
- Tested all pipettes and machines prior to pilot testing.
- Practised making the standard curves.
- Did 1st pilot tests and plotted in R (Appendix 6).
- Made the buffer with checks to ensure the correct pH.
- Presented a poster at the hull post grad conference.
- Researched scaling the assay results by mass of the samples (Tochen et al., 2016; Cook, 2019; Lee, 2019).
- Lyophilising real samples (samples to be weighted 1st and then dried and weighed again) – needed dry mass since I was going to be scalling the results of the assays by mass of the frass sample.
- 1st Vanillin and Anthrone assays unsuccessful (evaporation issues, time issues, etc) so decided to focus on the Bradford assays for February.

February

- Bradford assay pilot tests (Rsquared of standards curve was too low, suspected buffer not working, samples giving zero protein reading which seemed odd (issues outlined in lab book below).
- Lab busy due to talk of university closure (machines booked up, issues with machine being turned off which ruined experiments).

- Learned how to make the bee food ready for feeding experiments.
- Arranging CHN analysis of samples since the colorimetric analysis is taking a long time and feeding experiments expected to start soon.

Early March

- Project planning regarding potential closure of the university – getting prepared and setting up for remote working.

Appendix 6: Bradford assay pilots lab book

https://docs.google.com/spreadsheets/d/154QqPzsV_S54L2VF93ldOWrDfjJ_vrYaahsvg-W5bDs/edit?usp=sharing

Appendix 7: Lyophiliser protocol

https://docs.google.com/document/d/13LwkSxh1I6yAhrJzBD9lICdNK1p2SHNaH25pfQXq_FM/edit?usp=sharing

Appendix 8: Plate reader protocol

https://docs.google.com/document/d/1LJkuIydgHaeBkteKkaOO1YyhDpGfEGzOnPwVfAtUKRk/edit?usp=sharing

Appendix 9: Bibliography for frass analysis research

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