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The geometry of dependence: solitary bee larvae prioritize carbohydrate over protein in parentally provided pollen

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Abstract

1. Bees, important pollinators, have declined significantly in recent decades, and human-induced changes to nutritional landscapes are partly responsible. Changes to nutritional quality rather than quantity have been overlooked as a threat to bee health. Yet knowledge of bee nutrition is currently largely restricted to adults of social species. Larval stages, where most growth occurs, are relatively understudied - perhaps because most social bees provision progressively and collectively, making nutrition difficult to trace.
2. In mass-provisioning solitary bees (*Osmia bicornis* L.), we can manipulate and follow larval nutrition, and thereby determine effects of changes in diet quality. Under the Geometric Framework for Nutrition, we restricted larvae to 6 diets: 3 protein:carbohydrate ratios and 2 nutrient concentrations. We asked: (a) which diets maximise body size and survival, (b) what consumption rules do larvae follow when nutrients are imbalanced? Finally, (c) given a choice of complementary diets, are larvae able to select a dietary balance?
3. Larvae pupated after consuming a fixed carbohydrate amount, but tolerated a wide range of protein. Body size and survival were maximised on our lowest P:C ratio diet, and having consumed the most carbohydrate. When eating freely from two diets, larvae converged on a P:C ratio of 1:1.8, but not an overall nutrient intake target. Nevertheless, larvae maintained stable carbohydrate intake, while protein intake varied with the available diet.
4. Our results suggest solitary bee larvae regulate carbohydrate most closely, but that excessive indigestible material may limit their actual nutrient intake. Carbohydrate may

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be critical to overwinter survival, and/or may be more limiting than protein. The large variation in protein tolerated, despite its importance, suggests bee larvae may be vulnerable to landscape changes - and therefore reliant on parents to regulate protein. Given the mixed evidence on whether parents can sense pollen protein content, our results highlight bees' potential vulnerability to a "nutritional trap", i.e. where rapid changes in their nutritional environment outstrip their evolved capacity to detect those changes, impairing their fitness.

Keywords: pollination, foraging ecology, agriculture, nutritional geometry, limiting nutrient, diapause, ecological trap, environmental change, bee health

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Introduction

Bees are critical not only to global ecological stability but also to humans' food security, as major pollinators for 90% of the world's food crops (Klein et al., 2018, 2007; Potts et al., 2016) and many wildflowers (Ollerton et al., 2011). Many wild and domesticated bees have seen marked declines in recent decades, with both significant range contractions and extinctions (Ollerton et al., 2014). Honeybees have suffered huge losses across Europe and North America (Hayes et al., 2008; Potts et al., 2010) and wild bees have similarly struggled (Wood et al., 2016). These declines have been driven by a suite of reasons including, amongst others, nutritional stress (Goulson et al., 2015; Roulston and Goodell, 2011). Nutritional stress suffered by bees is mainly driven by human induced changes (Robertson et al., 2013), causing rapid changes to floral diversity, quantity and quality (Goulson et al., 2015; Robinson and Sutherland, 2002; Ziska et al., 2016). In particular, agriculture, and the spread of intensive practices specifically, has severely altered the floral landscape, with farmland often supporting lower floral diversity (Letourneau et al., 2011; Poggio et al., 2013). Agriculture also affects temporal availability, with mass-flowering crops creating brief, monotonous gluts of food followed by periods of resource scarcity (Goulson and Nicholls, 2016). This change in the quantity of nutrition is a common attribute of human-altered systems; however, a largely under-recognised risk for global ecosystems is the wholesale change in *quality* of nutrition, rather than just quantity (Ziska et al., 2016). A change in nutritional quality could be of particular concern for bee larvae, as their diet, pollen, varies widely in nutrient content (Roulston and Cane, 2000a), with recent studies also showing changes in pollen nutrient content within plant species due to environmental change (Ziska et al., 2016). Wholesale changes to the nutritional quality of landscapes are of critical concern, as nutrition mediates animals' ability to grow, reproduce, and

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maintain themselves (Simpson and Raubenheimer, 2012a). Understanding how animals cope in a changing nutritional environment requires us to understand not only how animals gather the correct balance of nutrients they need, but also how they adjust their foraging when resources are imbalanced (Simpson and Raubenheimer, 2012b).

In bees, the larval stage is where almost all growth occurs (Nijhout et al., 2014) as well as resource accumulation for diapause (Giejdasz and Wasielewski, 2017) - the way larval bees behave in the face of variable nutrition may be critical for bee health generally. Thus, bee nutrition research should focus on larvae at least as much as on adults. Unfortunately, we know relatively little about the nutritional ecology of most bee species, whether as larvae or adults (Roulston and Cane, 2002; Vanderplanck et al., 2014), with findings generally restricted to the latter. Findings in adult bees cannot necessarily be applied to their larvae; larval bees have a distinctly different diet to adults, adults primarily feeding on nectar (although see Cane, 2016) and larvae feeding almost solely on pollen (Muth et al., 2016).

The Geometric Framework for Nutrition (GF) allows us to investigate foraging decisions made by animals in multi-dimensional "nutrient space" (Simpson and Raubenheimer, 2012b, 1993). The GF can be used to determine an organism's intake target (the optimal amount and balance of macronutrients that an animal should aim to consume in order to achieve maximal fitness (Simpson and Raubenheimer, 1993) as well as how that target is achieved. Additionally, we can use the GF to investigate the *rule of compromise* - that is, the rules governing consumption that an animal uses when it is unable to reach its intake target with the nutritional options available (e.g. Lee et al., 2004b; Simpson and Raubenheimer, 2001).

The GF has provided profound insights into broad topics from ant agriculture (Shik et al., 2016) to human obesity (Simpson and Raubenheimer, 2005). While the GF has been used to

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investigate the nutrition of some highly social hymenopterans (Arganda et al., 2014; Dussutour and Simpson, 2009, 2008), including bees (Altaye et al., 2010; Archer et al., 2014; Paoli et al., 2014a; Stabler et al., 2015; Vaudo et al., 2016), those studies have focused almost invariably on adults rather than larvae (although see Helm et al., 2017). It is extremely difficult to investigate larval nutrition in social species, principally because it is rarely possible to accurately track nutrition within a colony - food brought in by workers is often shared and/or modified within the nest, and is then continually fed to the larvae (Field, 2005). This means that nutritional insights from GF studies into the parent-offspring relationship are currently limited.

In solitary bees, by contrast, typically each reproductive female provisions each of her offspring individually with a single, independent "pollen ball" before sealing the cell and leaving. This pollen ball contains all the resources that the larva will need to grow to adulthood. This behaviour makes solitary bees a far better model for larval nutritional studies, and studies of parental provisioning, than social species - once the female has left, both larva and pollen ball can be manipulated, and larval development monitored.

In this study, we used a commercially important solitary bee species, *Osmia bicornis*, to investigate how larval bees cope with varying nutrition: different diets, and different diet choices. Bee larvae are typically entirely sedentary and parents supply all their nutritional demands (Field, 2005). Yet we have little knowledge about whether parent bees consistently provide offspring with a ready-balanced diet, whether pollen ball composition varies passively with the flowers available to foraging adults in the landscape, or somewhere in between. Adults forage in a heterogenous nutritional environment, but we know that, despite this, honeybee foragers are nevertheless able to collect food that balances out deficiencies in colony nutrients

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(Hendriksma and Shafir, 2016). In general, though, evidence is scarce and mixed on whether adult bees can directly detect pollen quality at the flower (Nicholls and Hempel de Ibarra, 2016).

Given that parents may bring pollen of variable quality, the question of whether offspring are able to regulate their own nutrition to compensate for deficiencies in their provisions is a fundamental, but overlooked, component of bee nutrition. Even if parents cannot provide consistently balanced nutrition, larvae may still be able to eat selectively in order to achieve a nutritional target. Such regulation has been demonstrated in other insect larvae that develop independently of parents (Lee et al., 2002; Merkx-Jacques et al., 2008) but is unstudied in bees.

We used a classic GF design (Jensen et al., 2012) with two experimental phases: in the first "no-choice" phase we raised *O. bicornis* larvae on fixed diets of differing protein to carbohydrate (P:C) ratios (two macronutrients regarded as critical to insects; Behmer, 2009; Clissold and Simpson, 2015; Huang, 2012; Scriber and Slansky, 1981) in order to determine their rules of compromise and the diet composition that maximised fitness. In a second "choice" phase, we then provided larvae with targeted choices between sets of two imbalanced diets that differed in their P:C ratios to determine whether larvae consistently aim for an intake target. Sterile adult workers of some social insect species have been shown to have carbohydrate-biased intake targets (Paoli et al., 2014a; Stabler et al., 2015); however, we focus here on the growing larvae of *O. bicornis*, whose adults are all reproductive. Given the traditionally assumed importance of protein for growth and reproduction in insects and animals generally (Chapman and Chapman, 1998; Simpson and Raubenheimer, 2012a), we predicted (1) that protein would be a key driver of fitness in larval *O. bicornis*, (2) that larvae would accordingly aim for a relatively protein-biased intake target, and (3) that larvae would prioritize protein intake over carbohydrate in their rule of compromise. Alternatively, among bees such as *Osmia* that undergo diapause

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over winter, carbohydrate may also be important in building up sufficient fat reserves for overwinter survival (Arrese and Soulages, 2010; Giejdasz and Wasielewski, 2017).

Methods

Study organism

Osmia bicornis is a common, univoltine, cavity-nesting solitary bee native to Europe (Falk, 2015), and a commercially important pollinator of multiple crops (Jauker et al., 2012; Schulze et al., 2012). It is polylectic, feeding from a wide variety of flowers, and flies from March to July with males emerging a few weeks prior to females (Falk, 2015). Females nest in a variety of pre-existing cavities but can also be encouraged to nest in artificial constructs (Strohm et al., 2002).

All brood care in bees is performed by the female (Field, 2005) and *O. bicornis* larvae are entirely dependent on the food supply provided by their mother (Seidelmann et al., 2010) who builds a ball of pollen upon which she lays an egg. These provisions are stored in linear mud-lined cells each containing a single larva with each larva receiving a pollen provision directly from the mother.

Study population

O. bicornis larvae were obtained as diapausing adults in cocoons (Mauerbienen®, Germany), and released at the nesting site at the University of Hull in April 2017. Nesting material consisted of Styrofoam blocks (Styrodur 3035 CS), with a 9x9mm furrow and polycarbonate lid, housed within a wooden frame (modified from Strohm et al., 2002); Fig.

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S1a,b). Completed nests, signified by a mud plug at the entrance, were then brought into the laboratory. Early trials revealed that fresh eggs and newly emerged larvae were too fragile for manipulation. Therefore, newly emerged larvae were left alone for two days before we transferred them to a single-occupancy nest and assigned each to an experimental treatment.

The majority of nests were filled before the larvae hatched. When this was not the case, any larva reaching the two-day-post-hatching stage was removed at the nest site and placed into a single-occupancy nest.

Diet Formulation & Treatments

We used the Geometric Framework of Nutrition (Simpson and Raubenheimer, 2012a), as described above, to investigate the intake target and rule of compromise employed by *O.bicornis* larvae. In the no-choice treatment, larvae were restricted to one of six separate diets at three different protein:carbohydrate (P:C) ratios (Diet A = 1:1.2, Diet B = 1:2.3 & Diet C = 1:3.4) at two concentrations (90% or 70%, see table S1 for amounts of macronutrients). To our knowledge there is no precedent for the composition of artificial pollen diets for larval solitary bees, so these diet ratios were chosen based on a combination of the nutrient ratios in honeybee-collected pollen loads and published data for protein in *O. bicornis* pollen balls (Budde and Lunau, 2007). All diets contained an equal amount of honeybee-collected pollen and honey to which was added specific amounts of protein (micellar casein) and carbohydrate (trehalose), creating differing P:C ratios. The two diet concentrations were achieved by adding sporopollenin (see S1 for protocol), a major component of the outer wall of pollen considered largely indigestible by bees (Nepi et al., 2005; Roulston and Cane, 2000a; Suárez-Cervera et al., 1994). Sporopollenin was chosen rather than the more commonly used α-cellulose (Lee et al., 2004a;

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Muth et al., 2016; Pernal and Currie, 2002) as (1) initial trials showed high larval mortality when fed α-cellulose, and (2) sporopollenin more closely resembled the natural fibre found in larval bees' diet, and is indigestible (Roulston and Cane, 2000a).

In the choice treatment, larvae were provided with two alternating diets (A [1:1.2] and C [1:3.4]) each of which was at one of two possible concentrations (90% or 70%; see Fig. 1), together forming 4 separate treatment groups. Because *O. bicornis* larvae are sedentary, it is biologically inappropriate to allow access to both diets simultaneously (Chambers et al., 1995; Shik et al., 2014; VanOverbeke et al., 2017). Therefore, choice was offered temporally by swapping the provision every other day, allowing the larvae to differentially feed over the course of the experiment. All larvae were kept on the same treatment from two days post-hatching up to pupation, whereupon diet replenishment ceased and cocoons were weighed..

Experiment 1: No-choice phase

Two day old larvae, of mixed parentage, were randomly allocated to one of 6 treatments, corresponding to our 6 artificial diets (n = 20/group). The larva was removed from its natal nest onto a scoop within a single-occupancy nest containing one of the diets. The scoop was used to facilitate removal of food material and prevent the food from soiling the nest block. The single-occupancy nest, scoop and provision were weighed prior to use. The nest was then weighed (OHAUS Pioneer, PA-213) when containing the scoop, with the scoop and the provision, and then finally with the larva added to the provision. This ensured that the weights of all individual components could be separated, allowing for the monitoring of provision consumption. Initial provision weight was not tightly controlled as the diets were provided in excess (i.e. regularly replenished), but were made to resemble the size of natural provisions.

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Once provisioned, larvae were placed in an incubation chamber (Gallenkamp, IH-270) at 23°C and 80% RH. Provisions were replaced weekly, to avoid desiccation and mould formation, or when fully consumed by larvae. Weight of provision consumed was recorded upon provision replacement. Control larvae underwent the same manipulation but were supplied with natural provisions, i.e. a pollen ball from a cell containing an experimental larva whose pollen ball had been removed. Should they finish this provision, it was replenished with a fresh pollen ball, making the simplifying assumption that all parentally provided provisions were of equal composition. A "water control" group, containing pollen provisions but no larvae, was used to track water loss from the diets, going through the same weighing regime as above with weight loss recorded at each swap.

Nests were checked daily to ensure the health of the larvae. Final provision consumption was calculated once larvae had pupated by summing consumption across diet changes. Protein and carbohydrate consumed by each larva across the course of the experiment was then back-calculated from the final provision consumption. Cocoon weight was also recorded at the completion of pupation.

Experiment 2: Choice phase

Larvae received two diets, presented one at a time, in alternating order. 36 two-day-old larvae of mixed parentage were randomly divided among four complementary diet pairings consisting of the 1:1.2 and 1:3.4 P:C diets at the 70% and 90% concentration. Larvae were randomly assigned which diet within their pair they would be fed first. The paired diets were designed so that larvae would need to differentially feed from each in order to converge on an intake target. An additional group were assigned as controls and underwent the same

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manipulation protocol but were fed natural provisions. Performance criteria were recorded as in Experiment 1. Total provision consumption per larva was calculated by summing the consumption of each of the paired diets across the duration of the experiment, and macronutrient consumption was back-calculated as for Experiment 1.

Statistical Analysis

All analyses were conducted in R version 3.4.2 (R Core Team, 2017). For the no-choice experiment, raw diet consumption data were first adjusted for water loss and dilution, and then total nutrient content (P and C) calculated from adjusted figures based on the known nutrient percentages in the dry diets. Values were then summed for each larva and plotted onto nutritional space. Response surfaces were calculated for cocoon weight and visualised using non-parametric thin-plate splines. Larvae that died pre-pupation were not used in the calculation of the mean P and C consumption for diets in either experiment, but were used in analyses involving survival.

In the choice experiment, mean final consumption of each nutrient was investigated using linear models with diet combination, dilution and their interaction as predictors. The minimal model was determined using reverse stepwise model selection and pairwise differences among groups were examined using Tukey's Post Hoc tests. We additionally tested whether larvae were exercising a choice at all, i.e. whether they were consuming the available diets non-randomly. We calculated the expected protein and carbohydrate that would be consumed under random consumption of each diet by assigning exactly half the total amount of food consumed by each larva to each of the two choices offered to that larva. We then re-ran our models, using "random vs. observed consumption" as a predictor variable.

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Results

No-choice phase

Dietary P:C ratio had a strong effect on the total amount of P eaten (linear model using P:C ratio and diet concentration as predictors, dropping the main effect of ratio, $F=74.16$, $p<0.0001$, $df=2, 81$). P was eaten approximately in accordance with its proportion in the diet (Fig. 2a). Controls, on average, ate similar amounts of P as the C1 & C2 treatments (i.e. the lowest P:C ratio diets) (table S2), although this is tentative as the provisions were not directly analysed for their nutrient content, having instead been calculated using published values for *O. bicornis* pollen ball protein levels (Budde and Lunau, 2007). Diet concentration had no effect on amount of P consumed (linear model, dropping main effect of concentration, $F=1.85$, $p=0.18$, $df=1, 79$) and neither was there a diet:ratio interaction ($F=<0.01$, $p=0.99$, $df=2, 78$). In contrast, neither concentration nor dietary P:C ratio had an influence on the amount of C consumed (linear model dropping interactions and main effects of P:C ratio and concentration, all NS; minimal model contained no terms). C was consumed to similar levels across all diets (Fig. 1). When we included the controls into the analysis, controls ate very slightly less C than the experimental treatments (linear model, $F=4.07$, $p=0.01$, $df=6, 94$; Fig. 1). All ANOVA models showed a good fit.

Both the amount of protein and the amount of carbohydrate consumed were associated with cocoon weight (linear model using protein and carbohydrate as predictors, dropping the main effect of protein, $F= 12.44$, $p<0.001$, $df=1, 76$, and the main effect of carbohydrate, $F = 29.28$, $p<0.001$, $df=1, 76$). Higher amounts of protein resulted in lower cocoon weights whereas higher amounts of carbohydrate resulted in higher cocoon weights (Fig. 3). The linear model

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showed a good fit. Note that no non-linear effects were observed, that is, we did not identify an optimal amount of protein or carbohydrate that maximised cocoon weight.

Diet also influenced the survival of larvae, with those held on the more dilute diets suffering greater mortality (parametric survival regression, χ^2=72.7, df=6, p<0.0001, Fig. 4). When analysed according to amounts of carbohydrate and protein actually consumed, survival depended upon the interaction of carbohydrate with protein (χ^2=4.88, df=1, p=0.02). Those larvae that consumed high amounts of carbohydrate saw the lowest mortality irrespective of how much protein was consumed. At lower levels of carbohydrate however, mortality increased with decreasing amounts of protein (Fig. 5).

Choice phase

If, as predicted, larvae regulate their nutrient consumption, we would expect to see no difference among diet treatments in the total P and C eaten. This was not the case (Fig. 6a) and so no single identifiable intake target could be detected. The amount of protein consumed by larvae during the choice experiment was significantly affected by diet combination (linear model using diet combination as a predictor, dropping the main effect of diet combination, F= 7.43, p=0.0016, df=3, 23) with more protein consumed in those diet combinations that contained the more concentrated diets (Tukey's Post Hoc tests; A2C2-A1C2, p=0.015; A1C1-A2C1, p=0.019; A2C2-A1C1, p=0.0031). Similar results were seen for carbohydrate, with consumption being significantly affected by diet combination (F= 4.58, p=0.013, df=3, 23). However, unlike with protein, this appeared to be driven solely by the diets at the extreme, with significantly more carbohydrate being eaten only by those in the most concentrated diet pair compared to the least

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concentrated pair (Tukey's Post Hoc test; C2A2-C1A1, p=0.016). Other pairwise comparisons of diet treatments were not significant.

Despite not converging upon an intake target in nutritional space, larvae were nevertheless not consuming the diets at random, instead seeming to align on a P:C ratio of about 1:1.8 (Fig. 6a). For both carbohydrate and protein we saw differences in consumption from what would have been expected for each larva based on random consumption, and this effect was dependent on the specific set of diet choices (carbohydrate: linear mixed models with ID as a random effect and "diet combination" and "random or observed" as predictor variables; dropping the interaction, χ^2=42.76, df=3, p<0.0001; protein: dropping the interaction, χ^2=16.91, df=3, p<0.001, Fig. 7).

Moreover, when visualised as the amounts of P and C consumed during each 48h treatment period (Fig. 7a, b), it is clear that larvae were achieving a degree of homeostasis in C consumption compared to what would be expected under random consumption of each diet choice (Fig. 7c, Fig. 7d). In contrast, their consumption of P aligned very closely with what would be expected under random consumption (Fig. 7).

Discussion

When fed a diet with a fixed protein:carbohydrate ratio, larval *Osmia bicornis* pupated after eating a particular amount of carbohydrate, irrespective of whether they over- or under-ate protein, i.e. they exhibited a no-interaction rule (de Carvalho and Mirth, 2017). Although we did not identify an optimal intake amount for either nutrient, carbohydrate was positively associated with both cocoon weight and survival, and at low carbohydrate levels, survival instead became dependent on protein. When larvae were given a choice of complementary foods, they partially

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adjusted their intake of each food to compensate for the variation in nutrient content. In this choice phase, although consumption of both protein and carbohydrate differed significantly from expected based on random consumption of each diet choice, larvae did not converge on an intake target as expected. Nevertheless, larvae converged on a common P:C ratio of approx. 1:1.8, and showed tighter control over carbohydrate consumption than over protein consumption, adding weight to the findings from the no-choice phase that suggest that *O.bicornis* larvae prioritise carbohydrate over protein consumption. Here we argue that overwintering and the regulation of the typically limiting nutrient may explain these findings.

Within the range of diets studied, cocoon weight was positively related to carbohydrate consumed, with little influence of protein (Fig. 3). Increased body size is related to the size of nutrient stores in *Osmia* (Bosch et al., 2010) and other insects (Briegel, 1990; Hahn, 2005; Strohm, 2000) - and may reflect the size of the fat body, where carbohydrate-derived fat is stored in insects (Arrese and Soulages, 2010). The fat body is critically important to species such as *O. bicornis* that undergo diapause - not only during diapause (Giejdasz and Wasielewski, 2017; Wasielewski et al., 2013) but also afterwards (Hahn and Denlinger, 2007). It would now be interesting to determine the specific relationships between larval nutrition and fat body size, overwintering success and subsequent fitness in *O. bicornis*. It is also important to note that increased body size may have several other benefits, e.g. larger females may be more robust to changeable weather conditions (Bosch, 2008; Bosch and Vicens, 2005). Whilst carbohydrate-biased (and protein-poor) intake targets have traditionally been seen as detrimental to female fitness (Lee et al., 2008), lipid is a key component in insect oocytes, comprising 30-40% of the dry weight (Kawooya and Law, 1988; Ziegler and Van Antwerpen, 2006) of which the vast majority comes from the fat body reserves of the female (Arrese and Soulages, 2010). Female *O.*

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bicornis may therefore prioritise carbohydrate intake in order to provide adequate lipid stores to meet energy demands of their developing eggs (Beenakkers et al., 1985). Nevertheless, (Bosch and Vicens, 2005) found little correlation between body size in *O.cornuta* and fecundity, which was instead more related to longevity and provisioning rate. It is also worth noting that although we found that high carbohydrate increased cocoon weight we did not identify a fitness peak at which cocoon weight was maximised. Further work could employ a wider range of diet ratios in order to locate this.

Dietary macronutrients also had noticeable effects on survival to pupation within our range of diets (Figs. 5, 6): the larvae consuming most carbohydrate had the greatest survival rates. Among *adult* insects, high carbohydrate (or more specifically, low P:C ratio) has often been associated with increased lifespan (Fanson et al., 2009; Le Couteur et al., 2016; Lee et al., 2008), including in both honeybee and bumblebee adults (Paoli et al., 2014a; Pirk et al., 2010; Stabler et al., 2015). Conversely, high P:C ratios have been linked to reduced lifespan in the adults of many insects (Dussutour and Simpson, 2012; Lee, 2015; Lee et al., 2008). Focusing on larvae, we saw somewhat different survival patterns with respect to protein consumption. Although high-carbohydrate consumption increased survival to pupation, among those larvae that ended up eating low quantities of carbohydrate, *protein* consumption mediated survival, with those on higher protein diets (higher P:C) experiencing greater survival (Fig. 5). This pattern may be driven by alternative mechanisms for surviving diapause. Well-fed larvae that have eaten sufficient carbohydrate may survive the winter via lipogenesis of carbohydrate-derived fat body reserves (Arrese and Soulages, 2010). However, if carbohydrates are limited, larvae may instead utilise dietary protein in order to provide lipid stores via deamination and gluconeogenesis of amino acids, as in some caterpillars (Lee et al., 2003, 2002; Merkx-Jacques et al., 2008).

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Interestingly, in the only other study to have used the GF to investigate nutrition in larval bees, in this case honeybees (Helm et al., 2017), precisely the opposite effect was found. *In vitro* feeding revealed that, at high carbohydrate, larval survival was dependent on the amount of dietary protein, but at low carbohydrate survival was independent of protein. Larvae showed significantly reduced survival on high carbohydrate, low protein diets and the highest growth rates on diets that contained a medium level of protein but low carbohydrate - suggesting that worker recruitment could suffer in situations where protein is limited (Helm et al., 2017). The reasons behind this stark difference are unclear but it is worth noting that honeybee workers have a different role as adults from individuals of *O.bicornis*; all female *O.bicornis* are destined to be reproductives, unlike the honeybee larvae in Helm et al's study. Additionally, the honeybee larvae used in Helm et al's study were 'summer bees' (Steinmann et al., 2015) and therefore would not need to accrue nutrient reserves in order to enter diapause over the winter. If nothing else, these contrasting findings highlight not only the importance of understanding larval *as well as* adult nutrition for our general understanding of bee health, but also that knowledge of the nutritional ecology of more commonly studied social species cannot necessarily be applied to the more numerous, mass-provisioning solitary bees that collectively provide the bulk of our pollination services (Winfree et al., 2007).

Interestingly, when allowed to self-select their diets, *O.bicornis* larvae did not cluster in nutritional space but were instead spread out along a P:C ratio of 1:1.8 (Fig. 6a), approximately according to overall diet concentration. Notwithstanding the variable amounts actually eaten, assuming at least that this 1:1.8 *ratio* reflects the ratio of the larvae's true intake target, this would differ markedly from that reported for adult honeybees (1:12; Altaye et al., 2010), and would lie closer to that of ants foraging for offspring (1:1.5; Dussutour and Simpson, 2009).

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Unlike sterile adult honeybees, all *O.bicornis* larvae are destined to be reproductive, and do not require fuel for immediate flight, so might be expected to require more protein. Unlike larval ants, however, *O.bicornis* larvae need to compile enough stores to survive winter diapause (Fliszkiewicz et al., 2012) so may require higher amounts of carbohydrate. The fact that we did not observe the expected clustering in nutrient space could potentially be explained by the fact that the more dilute diets contained more indigestible pollen husks (Roulston and Cane, 2000a), increasing the proportion of indigestible material passing through the gut. Some insects may be volumetrically limited when consuming dilute foods (Lee et al., 2008), so the additional fibrous material may have effectively limited the ability of bee larvae to regulate their diet by compensatory feeding. This may also explain why, although on average larvae ate more of the dilute diets on no-choice treatments (Fig. 2b), they were clearly unable to compensate for the reduced nutrient concentration, consuming less of each macronutrient than larvae fed more concentrated diets (Fig. 2a). Despite the fact that total food consumption was similar across treatments in the choice phase (Fig. 6b), larvae on less concentrated diets may have been unable to reach the same point in nutritional space due to consumption rate limitations.

The apparent lack of protein regulation shown by *O.bicornis* larvae is perhaps surprising given that, (1) the opposite is seen in larvae of other insects e.g. *Drosophila* flies (de Carvalho and Mirth, 2017) and *Helio virescens* caterpillars (Telang et al., 2001), (2) protein is important for somatic growth and survival (Lee, 2007; Povey et al., 2009; Roulston and Cane, 2002; Tasei and Aupinel, 2008), (3) bees primarily receive their protein (and lipid) requirements from pollen (Vaudo et al., 2016), and (4) larval bees feed primarily on pollen (Muth et al., 2016). Although adult workers of social bees have also been shown to prioritise carbohydrate over protein, their need for protein is relatively low (Paoli et al., 2014b; Stabler et al., 2015), requiring large

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amounts of carbohydrate to fuel flight (Darveau et al., 2014) and their high metabolism (Harrison and Roberts, 2000). Considering that growth is concentrated in the larval stage, it would seem reasonable to expect that protein acquisition would drive nutritional decisions in larval bees, even if not adults.

Tolerance of wide variation in dietary protein, such as we saw in *O. bicornis* larvae, is typically seen in predators - both vertebrates,, e.g. cats (Hewson-Hughes et al., 2011) and mink (Mayntz et al., 2009), and invertebrates (Kohl et al., 2015; Raubenheimer et al., 2007). However, bees are considered herbivorous (Larkin et al., 2008); herbivores tend to have protein-based decision rules, including pollen-foraging adult bumblebees (Vaudo et al., 2016) and many other herbivores [e.g. caterpillars (Lee et al., 2002; VanOverbeke et al., 2017)] and also omnivores [e.g. humans (Simpson and Raubenheimer, 2005)]. While clearly not predators, bees do share a common ancestor with predatory social wasps and ants (Johnson et al., 2013; Peters et al., 2011), and ant workers have been shown to tolerate varying protein levels in favour of a carbohydrate target (Dussutour and Simpson, 2009). Furthermore, “nutrient-generalist” species (i.e. those which tolerate wide ranges of dietary compositions) tend to be more able to tolerate swings in the particular nutrient which is least limiting (Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 2012b). Larval bees feed on pollen, which is amongst the most protein-rich of plant tissues (Mattson, 1980), and, as such, carbohydrate may be the more limiting nutrient for larvae. It is likely that starch, the standard form of digestible carbohydrate storage in pollen (Pacini, 1996), rarely exceeds the protein content of pollen; (Roulston and Buchmann, 2000) found that average starch content of pollen ranged from 0-22%, considerably less than the range for protein (2-60%; Roulston and Cane, 2000a). Thus, the relative abundance of protein in pollen

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may help to explain why *O.bicornis* larvae appeared to eat to a carbohydrate target whilst tolerating varying levels of protein.

The fact that larvae consumed very different amounts of protein, despite its importance to larval insects, suggests that bee larvae may be vulnerable to environmental variations in the protein:carbohydrate ratio of pollen - in a similar but opposite manner to humans, who will consume excesses of carbohydrate in order to eat enough protein, rendering them vulnerable to variation in protein:carbohydrate ratio of food (Simpson and Raubenheimer, 2005). Since bee larvae appear to be able to regulate carbohydrate, they may therefore rely on parents to regulate protein on their behalf. Given the natural variation in pollen protein content in the environment, bee parents may be able to sense the macronutrient composition of pollen directly at the flower and thus actively regulate the composition of pollen provisions they provide to offspring. Some studies support this idea (Muth et al., 2016; Ruedenauer et al., 2016, 2015), yet many studies have found otherwise (Konzmann and Lunau, 2014; Roulston and Cane, 2002; Roulston and Cane, 2000b; for review, see Nicholls and Hempel de Ibarra, 2016). Conceivably, though, adults may instead simply collect pollen for young indiscriminately, relying on (1) larval tolerance of varying protein (this study), which may carry costs unmeasured here, and (2) variation in pollen quality balancing out owing to the historically rich diversity of the floral environment (Bukovinszky et al., 2017). In modern agricultural landscapes, where floral diversity is reduced in favour of brief gluts of monotonous crops, this strategy may no longer be effective and may indeed be deleterious. Under this scenario, the ways larvae deal with excesses and deficiencies of protein would be of paramount importance for bee health. With few exceptions (see Helm et al., 2017), we know very little about larval nutrition in bees as most studies focus on adults, whether foraging for nectar (Altaye et al., 2010; Kriesell et al., 2017; Paoli et al., 2014a; Ruedenauer et

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al., 2015; Vaudo et al., 2016) or pollen (Altaye et al., 2010; Kriesell et al., 2017; Paoli et al., 2014a; Ruedenauer et al., 2015; Vaudo et al., 2016). Considering that (1) all the nutrients required to reach adulthood are accrued during the larval stage, and (2) larvae and adults have different nutritional requirements (Cridge et al., 2017), the way larval bees deal with macronutrient imbalances is a considerable knowledge gap. In particular, further studies that assess whether *O.bicornis* larvae employ post-ingestive processing to regulate protein intake, as shown in some other insects (Lee et al., 2004a; Raubenheimer and Simpson, 2003; Rho and Lee, 2017; Telang et al., 2001), may help to explain the large variation of protein tolerated by larvae.

That (1) larvae seem not to regulate protein, (2) parents are likely unable to assess protein content for their young, and (3) human activity, particularly intensive agriculture, is reducing floral diversity, potentially make for a toxic combination for bees. Changes to the composition of available nutrition, driven by loss of floral diversity (Goulson et al., 2015), or potentially through changes in the nutritional composition of pollen *within* a plant species in response to human activity (Ziska et al., 2016), may cause mother bees to unwittingly feed their offspring nutrient-deficient diets. This would mean that, despite otherwise favourable environments, *O.bicornis* and possibly bees more generally, would become caught in a “nutritional trap”, gathering food that no longer provides offspring with appropriate nutrition. More research is needed into the nutrition of larval bees, especially solitary species where knowledge is sparse, in order to inform conservation management and stewardship schemes. Further studies should focus on whether larval bees have physiological adaptations to overcome nutrient imbalances, e.g. post-ingestive processing, and whether mother bees are able to adjust the provisions they provide their offspring in order to counter changing nutritional composition of pollen available in the environment.

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Tables & Figures

Table 1. Diet combinations used for choice phase.

	Concentration 1 (90%)	Concentration 2 (70%)
P:C Ratio:		
A (1P:1.2C)	A1	A2
C (1P:3.3C)	C1	C2

Table 2. Sample sizes for each diet combination used for choice phase (allocated by random coin toss). "Order" refers to diet order - e.g. for A1C1, Order 1 would receive A1 first whereas Order 2 would receive C1 first. Surviving larvae are in parentheses.

	Order 1	Order 2
A1C1	1 (1)	6 (5)
A1C2	5 (5)	4 (3)
A2C1	3 (2)	5 (5)
A2C2	5 (2)	3 (1)

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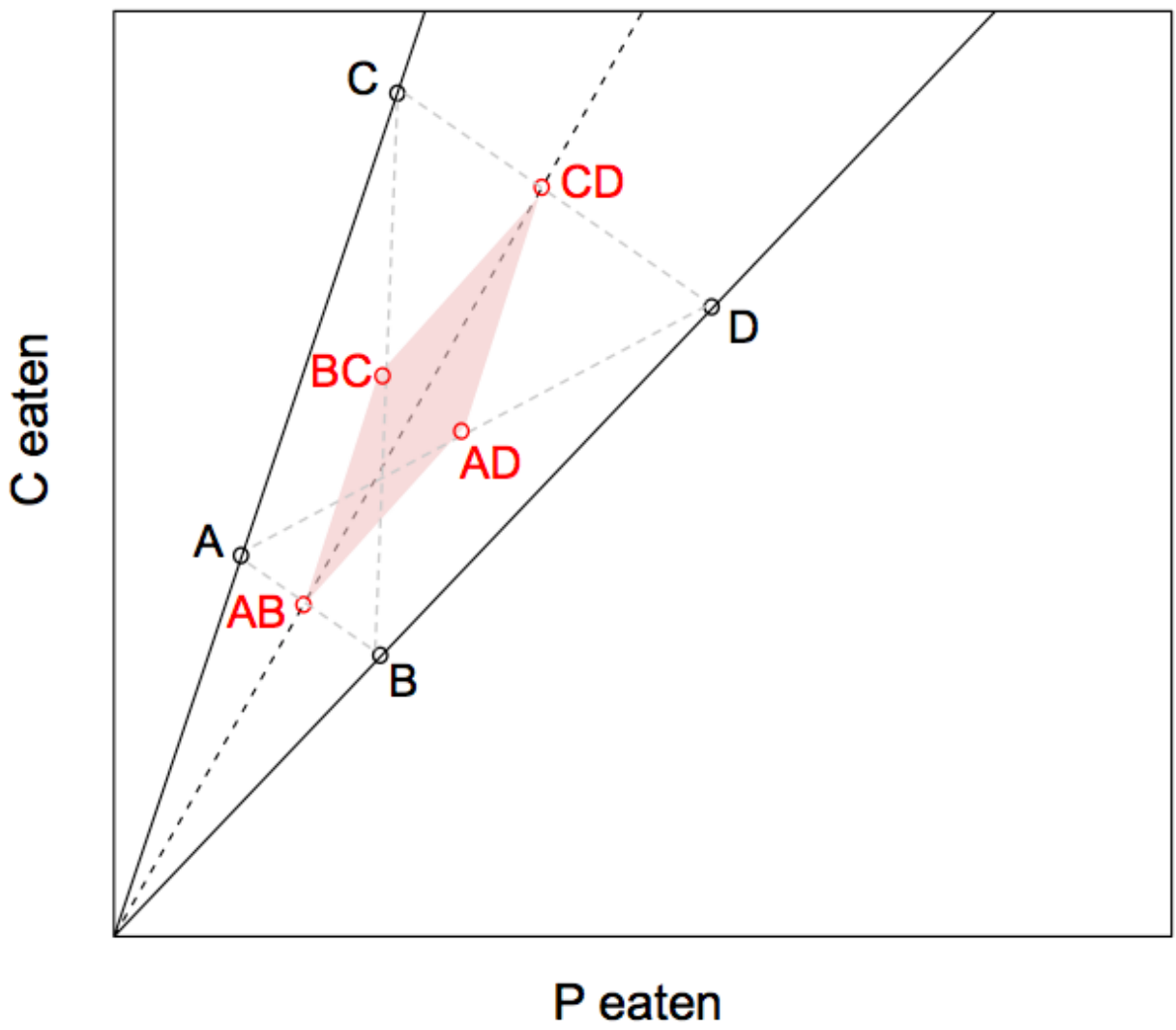

Fig 1. The expected amounts of protein and carbohydrate consumed if larvae hypothetically eat indiscriminately between two diets. Diet choices are pairwise combinations of diets A, B, C and D, which each contain protein and carbohydrate at different ratios and concentrations. Solid lines represent the P:C ratios of the individual diets; black points represent actual nutrient content of each diet, which depends upon dilution as well as P:C ratio. Red points represent the expected nutrient consumption if larvae eat randomly (i.e. equally) from each of a choice of two diets (choices denoted by the

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red point labels). Note that random consumption patterns resemble a diamond shape surrounding the line that bisects the two rails.

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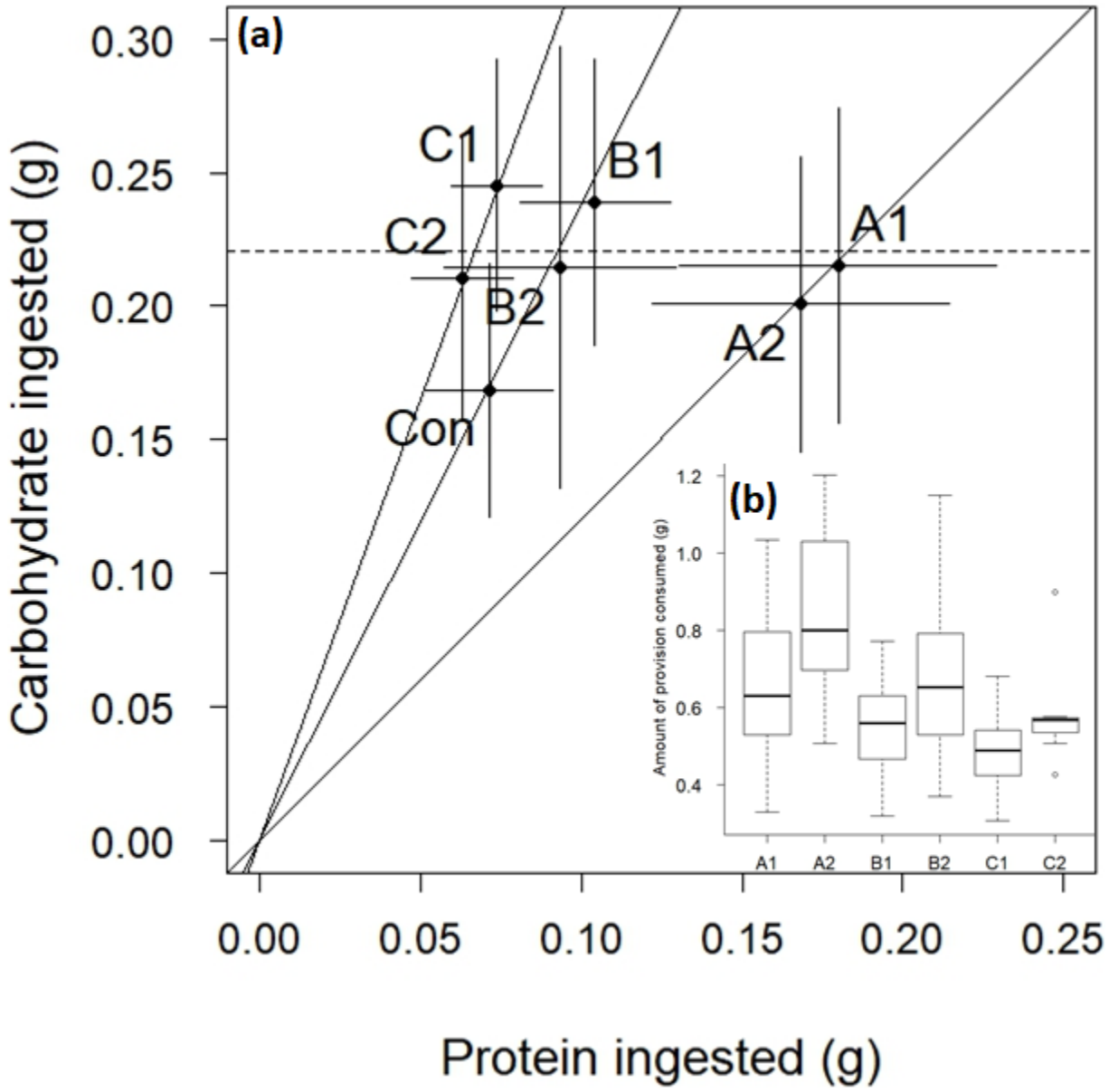

Fig. 2. (a) Mean total (+/- 1 SD) amount of P and C consumed in grams by larvae on each diet across the duration of development. Solid lines and letters represent three P:C ratios (A = 1:1.2, B = 1:2.3, C = 1:3.4), 'Con' represents control group. Numbers following letters denote diet concentration (1 = 90%, 2 = 70%). (b) the total amount of raw provision consumed by each group, after water correction, in grams.

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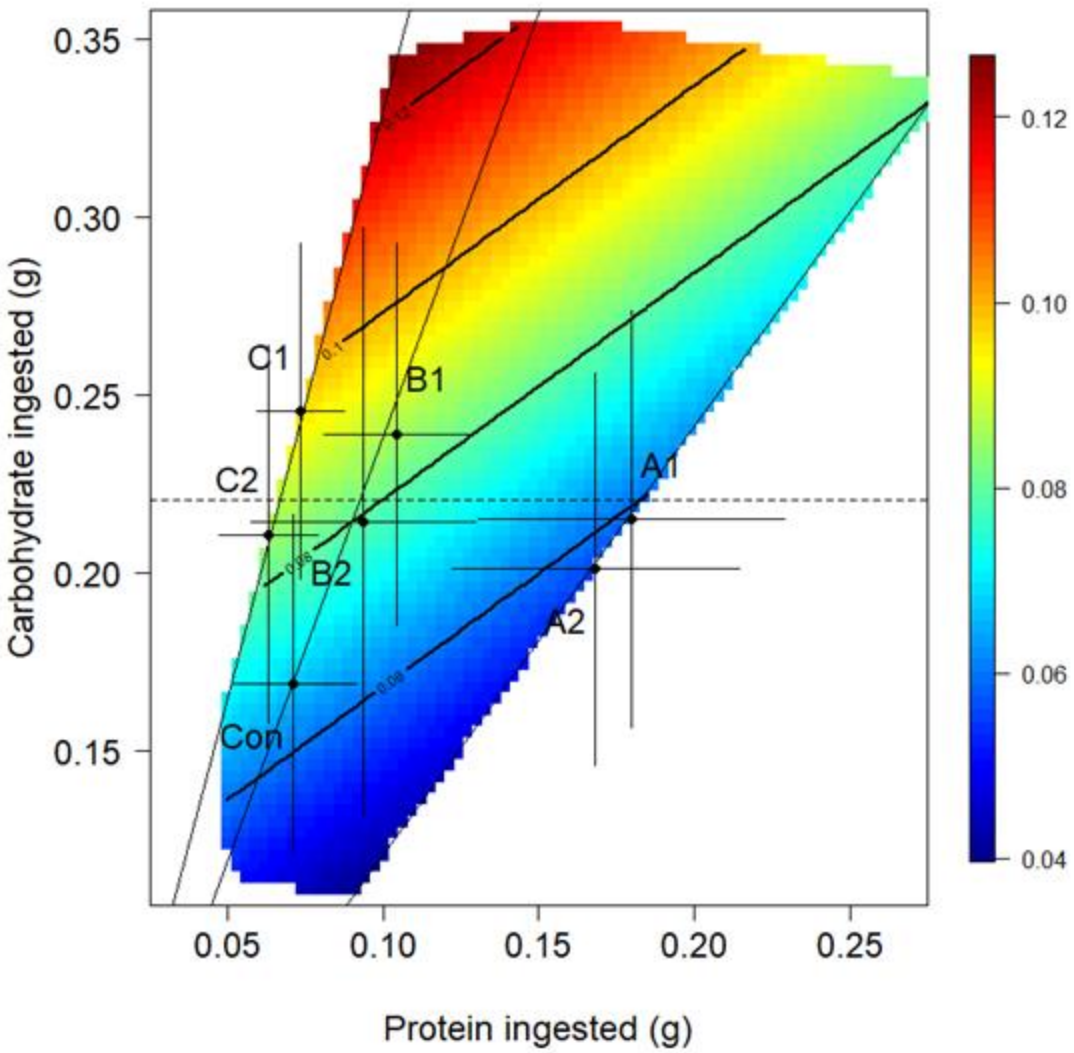

Fig. 3. Response surface showing the effects of the amount of P and C consumed on Cocoon weight (g). Transition from blue to red indicates heavier cocoons. For context, mean total consumption of P and C for each diet is plotted (data as in Fig. 2). Solid lines and letters represent three P:C ratios (A = 1:1.2, B = 1:2.3, C = 1:3.4), 'Con' represents control group. Numbers following letters denote diet concentration (1 = 90%, 2 = 70%).

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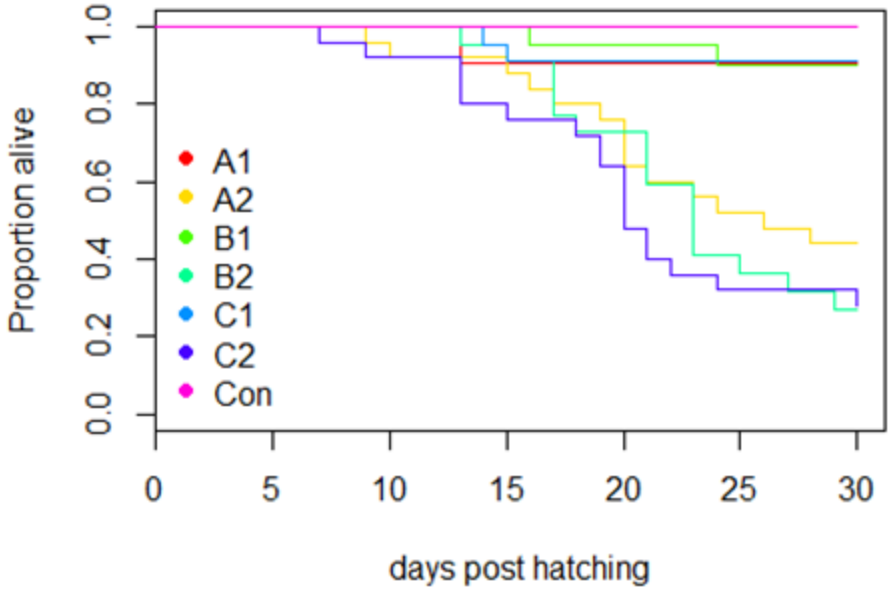

Fig. 4. Survival of larvae on fixed diet treatments according to the diet they were fed Letters represent three P:C ratios (A = 1:1.2, B = 1:2.3, C = 1:3.4), 'Con' represents control group. Lines represent the proportion of larvae still alive at a given time point. Numbers following letters denote diet concentration (1 = 90%, 2 = 70%).

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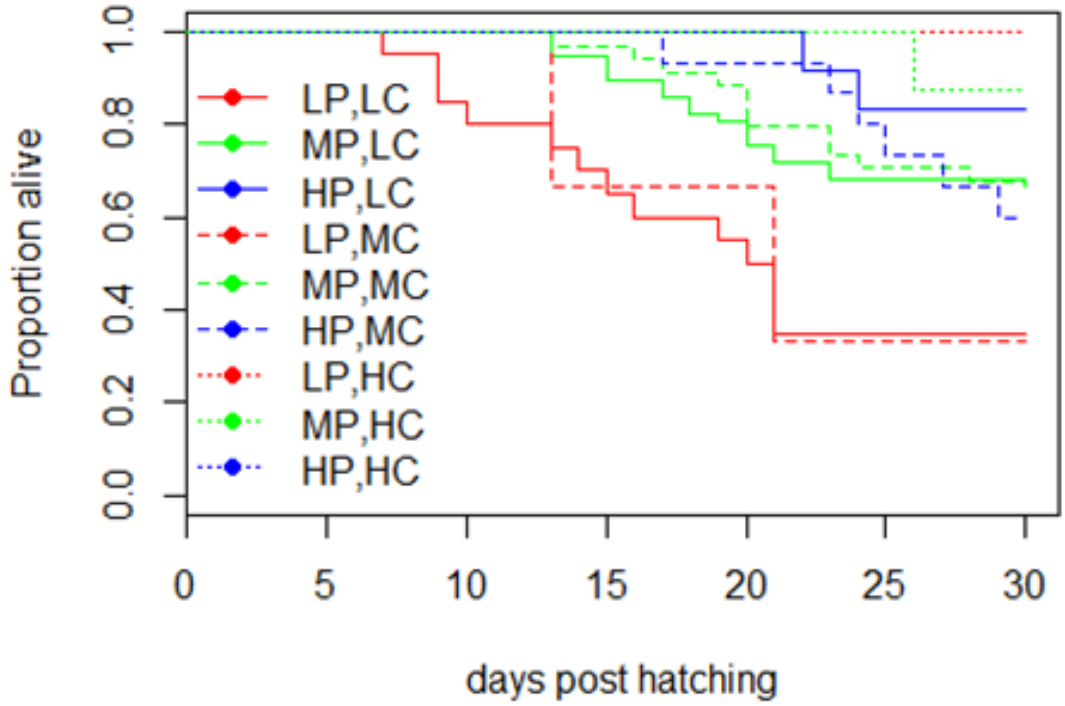

Fig. 5. Survival of fixed-diet groups broken down by amounts of macronutrients actually consumed. Lines represent the proportion of larvae alive at a given time point. Key: P = protein, C = carbohydrate; H = high, M = medium, L = low consumption (top, middle and bottom 33% of consumption, respectively)

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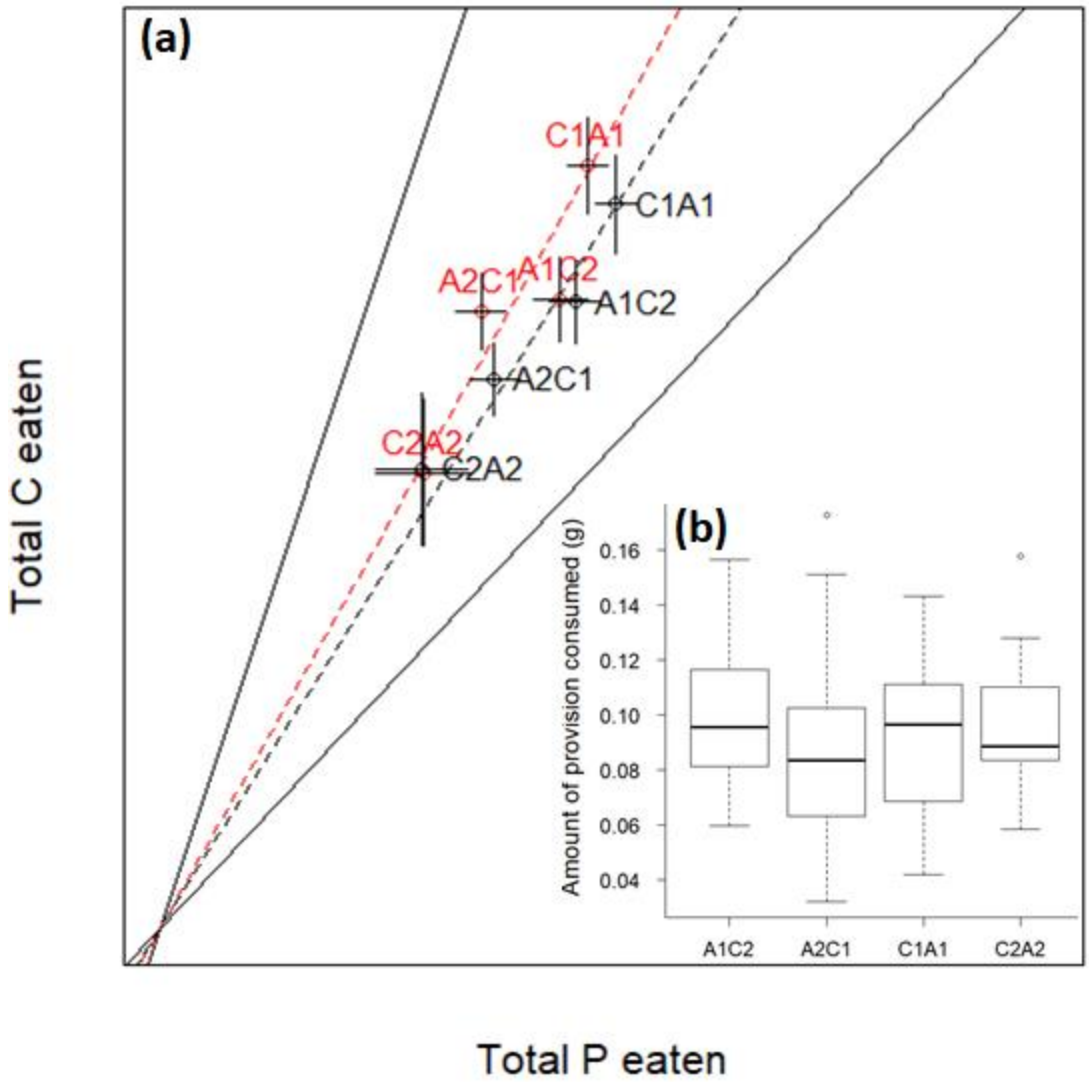

Fig. 6. (a) The mean total (+/- 1 SE) amount of protein (P) and carbohydrate (C) eaten by larvae in the choice experiment. Each point label denotes a choice of two diets, one A and one C; black labels show observed intake, red labels show expected intake under random (i.e. equal) consumption. Letters in diet names represent two P:C ratios (A = 1:1.2, C = 1:3.4). Numbers in diet names (e.g. A1, A2) represent the concentration of each individual diet within a pairing (1 = 90%, 2 = 70%). Solid lines represent the P:C ratio of the individual diets within the pairings (Top line = Diet A, Bottom line = Diet C). Dashed red line shows the expected average P:C ratio for all

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larvae, if the larvae eat randomly between the diets within their pairing (random). Dashed black line shows the P:C ratio to which the larvae conformed based on their observed intake. (b) The amount of raw provision consumed by each diet pairing, after water correction.

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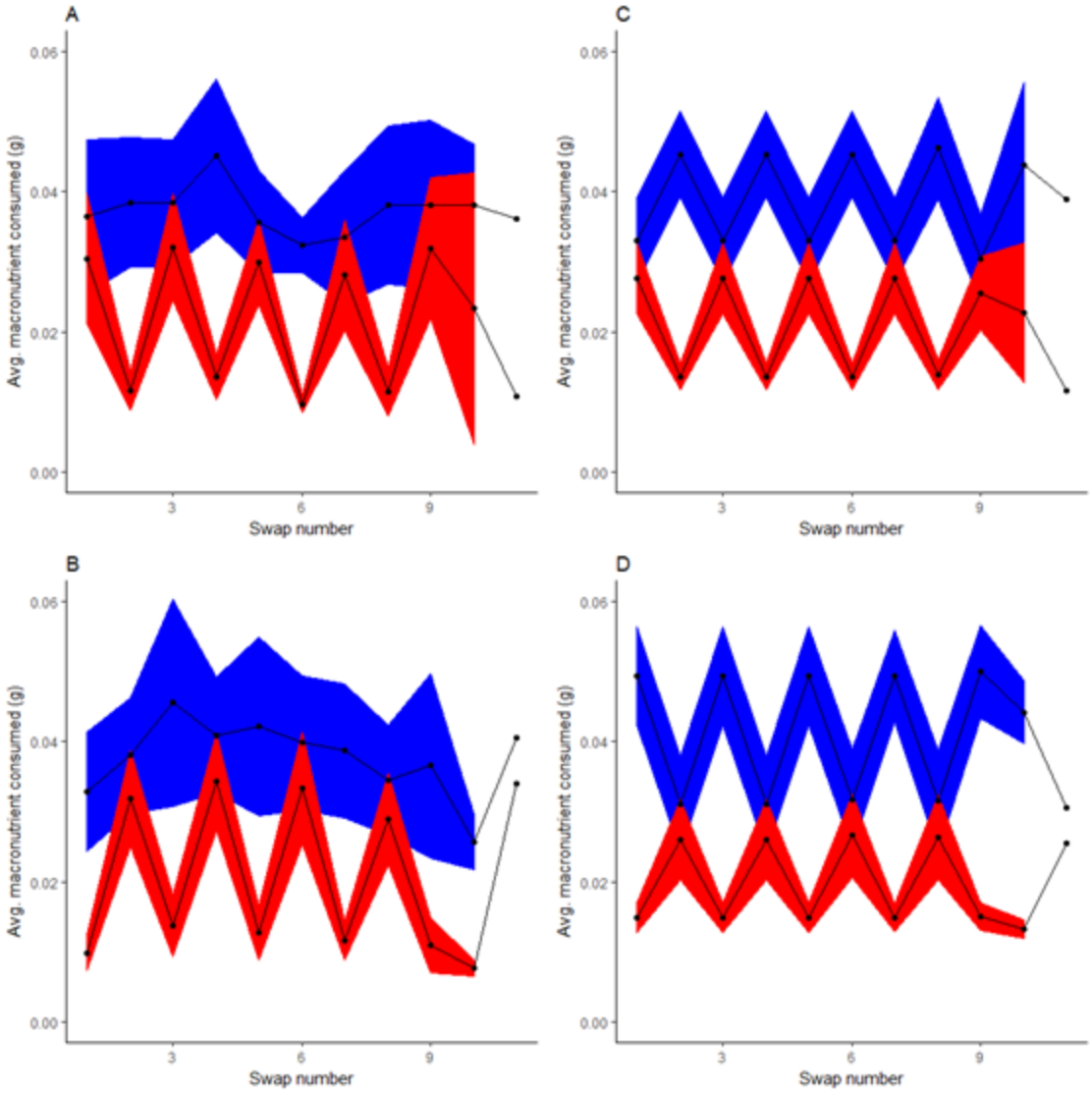

Fig 7. Mean (+/- 1SD) intake of protein (red) and carbohydrate (blue) over successive diet swaps for observed larval consumption (A & B), versus the expected nutrient intake under random consumption of diets (C & D), irrespective of the concentrations of the diet choices (for calculations of expected consumption, see text). A & C show larvae starting on diet A, B & D show larvae starting on diet C.