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Solitary bee larvae prioritize carbohydrate over protein in parentally provided pollen

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Abstract

1. Most organisms must regulate their nutritional intake in an environment full of complex food choices. While this process is well understood for self-sufficient organisms, dependent offspring, such as bee larvae, in practice have limited food choices because food is provided by parents. Nutrient balancing may therefore be achieved by offspring, by parents on offspring's behalf, or by both, whether cooperatively or in conflict.
2. We used the Geometric Framework to investigate the capacity of dependent larval mason bees (*Osmia bicornis*) to regulate their intake of protein and carbohydrate. Female *Osmia* seal eggs individually inside cells they have provisioned with pollen, and have no contact with developing offspring, allowing offspring choices to be studied in isolation. Herbivorous insect larvae are typically expected to balance protein and carbohydrate to maximise growth and reproduction.
3. Contrary to prediction, carbohydrate and not protein mediated both growth and survival to pupation. Accordingly, larvae prioritised maintaining a constant intake of carbohydrate and self-selected a relatively carbohydrate biased diet compared to other hymenopterans, while tolerating wide excesses and deficiencies of protein, rendering them potentially vulnerable to dietary change or manipulation. Reasons for prioritising carbohydrate may include (1) the relative abundance of protein in their normal pollen diet, (2) the relative paucity of nectar in parental provisions making carbohydrate a scarce resource, or (3) the requirement for diapause for all *O. bicornis* larvae. Larvae were intolerant of moderate dietary dilution, likely reflecting an evolutionary history of nutrient-dense food.
4. Our results demonstrate that dependent offspring can remain active participants in balancing their own nutrients even when sedentary, and, moreover, even in mass provisioning systems where parents and offspring have no physical contact. Research should now focus on whether and how evolutionary interests of parent and dependent offspring coincide or conflict with respect to food composition, and the implications for species' resilience to changing environments.

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

Introduction

Most animals manage their nutrient intake by combining nutritionally different foods (Simpson & Raubenheimer 2012). However, the importance of this ability depends upon the nutritional variability of the animals' typical food (Despland & Noseworthy 2006; Raubenheimer, Simpson & Mayntz 2009). Extreme specialists, for example, can lose the capacity to regulate nutrition (Warbrick-Smith *et al.* 2009; Poissonnier *et al.* 2018). One way in which organisms can experience limited nutritional choice is if they are dependent upon others for nutrition, or "alloregulation" (Lihoreau *et al.* 2014), such as dependent offspring of altricial birds, human toddlers, and many larval insects. Under these circumstances, by what rules offspring regulate their own consumption should depend upon provisioning rules of parents. On the one hand, offspring tend to have different requirements from parents (Harper & Turner 2000; Michaelsen *et al.* 2003) - particularly for protein, given their elevated rates of somatic growth and development. Accordingly, parents often make different nutritional choices for their offspring versus when foraging for themselves (Royama 1970; Dussutour & Simpson 2009; Burt & Amin 2014). For example, granivorous birds usually provision young with insects, rather than the seed diets of adults, to fulfil protein requirements (Wiens & Johnston 2012). If parents alloregulate offspring nutrition tightly, then offspring should have no need for self regulation, like extreme specialists (Poissonnier *et al.* 2018). On the other hand, parents may provide suboptimal nutrition for offspring - either through inefficiency (e.g. Seidelmann 2006), or if parents' and offspring's evolutionary interests do not coincide (Trivers 1974). Here, offspring may be able to use nutritional regulation to mitigate costs arising from their parents' nutritional choices. While there has been much research into evolutionary compromises involving offspring solicitation and corresponding parental responses (e.g. Smiseth, Wright & Kölliker 2008), less is known about whether or how offspring may exert control by discriminating among parental provisions.

The Geometric Framework for Nutrition (GF) allows us to investigate foraging decisions made by animals in multi-dimensional "nutrient space" (Simpson & Raubenheimer 1993). The GF can be used to determine animals' nutritional choices relative to their "intake target" - the optimal amount and balance of multiple macronutrients - as well as their "rule of compromise" that governs their choices when restricted to suboptimal food (Raubenheimer & Simpson 1999b). The GF has provided insights into the nutritional ecology of a broad range of taxa (reviewed in

Simpson & Raubenheimer 2012). Its application to dependent offspring, though, has typically been as part of studies of social insect systems (e.g. Helm *et al.* 2017) and studies have often inferred offspring requirements indirectly from patterns of alloparental feeding in studies more broadly focused on adult foraging (see Dussutour & Simpson 2009; Cook *et al.* 2010; Vaudo *et al.* 2016). In such systems, multiple adults normally contact offspring, progressively feeding and adjusting nutrition in response to feedback (Field 2005; Schmickl & Karsai 2017), making the responses of individual larvae difficult both to follow and interpret.

In solitary bees, by contrast, typically females provision offspring individually with a pollen ball before sealing the cell and leaving. This behaviour makes solitary bees an ideal, manipulable model for directly studying the nutrition of dependent larvae (Strohm *et al.* 2002) independently of provisioning decisions made by parents. Larvae of bees, like most aculeate hymenopterans, rely on parents or alloparents for nutrition (Field 2005). Nutritional requirements for bee adults and offspring differ, often radically (Weeks *et al.* 2004; Filipiak 2019); adults primarily feed on carbohydrate-rich nectar (although see Cane 2016) while larvae feed mostly on protein-rich pollen (Filipiak 2019). Solitary bees, along with most other hymenopterans and many other parental insects, typically have a simple one-to-one parent-offspring relationship whereby parents “mass provision” their young, providing a finite, fixed-mass food provision, and have no contact with their young during development (Costa 2006). Such systems are almost unstudied in a rigorous nutritional context (but see e.g. Roulston & Cane 2002). In these species, there is no opportunity for parents to adjust nutrition according to offspring feedback, and the larva must therefore make the best of what it is given. It may be that offspring regulate their own nutrition to compensate for variation, as in more independent insect larvae (Lee *et al.* 2002), or possibly to mitigate costs imposed by parents. Alternatively, they may have lost this capacity, like extreme specialists (Warbrick-Smith *et al.* 2009; Poissonnier *et al.* 2018). We know very little about how bee larvae deal with variable nutrition (but see Helm *et al.* 2017) - a knowledge gap with potentially important consequences, considering the proposed link between nutritional stress and bee declines (Roulston & Goodell 2011; Goulson *et al.* 2015).

In this study, we used a commercially important solitary bee species, *Osmia bicornis*, to investigate how dependent larvae cope with varying nutrition, and whether they can regulate their own intake. *O. bicornis* are pollen generalists (Falk 2015) and the solid, roughly spherical pollen balls that parents provide to offspring are variable in species composition (Haider *et al.*

2014). Although *O.bicornis* larvae are sedentary, they are capable of limited movement, in principle allowing them to preferentially consume specific parts of a fixed provision (note that other invertebrates are also capable of extracting and consuming preferred nutrients from nutritionally complex food items (Mayntz *et al.* 2005). The species is entirely solitary with no known tendency for offspring to "help at the nest" as in other bees (Hogendoorn & Velthuis 1993; Rehan *et al.* 2014) so there is no reason to believe mothers would alter offspring nutrition to force them to help, as in other systems (Lawson, Helmreich & Rehan 2017) and therefore no obvious potential for parent-offspring conflict over offspring nutrition. Natural variation in pollen ball nutrient content is largely unquantified (although see Budde & Lunau 2007), so there is no prior expectation about the capacity of larvae to regulate their consumption. We used a classic GF design (e.g. Lee *et al.* 2008), focusing on protein and carbohydrate, with two experimental phases. In the first "no-choice" phase we raised *O. bicornis* larvae on fixed diets of differing protein to carbohydrate ratios 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 protein:carbohydrate ratios to determine whether larvae defend an intake target. Given the central role of protein in growth of insect larvae (Scriber & Slansky 1981; Behmer 2009), and following Hunt & Nalepa's (1994) exhortation to "follow the protein", we predicted that (1) protein would be a key driver of fitness in larval *O. bicornis*, (2) larvae would accordingly aim for a relatively protein-biased intake target, and (3) larvae would prioritize regulating intake of protein over carbohydrate.

Methods

Study organism

Osmia bicornis is a common, cavity-nesting solitary bee (Falk 2015), and a commercially important pollinator (Jauker *et al.* 2012). *O. bicornis* larvae were obtained as diapausing adults in cocoons (Mauerbienen®). These were released at the nesting site at the University of Hull in April 2017, and emerging adults allowed to breed. 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. Details of nesting apparatus and monitoring protocols are available in the supplementary methods.

Diet Formulation

Existing artificial diet protocols for solitary bees have met with limited success in terms of larval survival (Nelson, Roberts & Stephen 1972; Fichter, Stephen & Vandenberg 1981). We used six diets, consisting of three different protein:carbohydrate (P:C) ratios (Diet A = 1:1.2, Diet B = 1:2.3 & Diet C = 1:3.4) and two total macronutrient concentrations (concentration 1 = 90% nutrients, 10% diluent, or concentration 2 = 70% nutrients, 30% diluent; see table S1). Diet ratios were chosen based on a combination of the nutrient ratios in honeybee-collected pollen loads and published data for protein content of *O. bicornis* pollen balls (Budde & Lunau 2007). Diets were diluted with sporopollenin, the primary constituent of the exine of pollen (Mackenzie *et al.* 2015), an extraordinarily stable natural polymer. Sporopollenin is a novel dietary diluent for bees; its suitability has been demonstrated in a separate study (Tainsh *et al.* 2020). For a more detailed description of sporopollenin and its preparation, see supplementary methods.

Experiment 1: No-choice phase

Two-day-old larvae, randomized by parentage, were allocated to one of 6 treatments corresponding to our 6 artificial diets (n = 20/treatment). Provisions were made to resemble the size of natural provisions (mean initial artificial provision weight = 0.323g +/- 0.034g). 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, ensuring the diet was always available in excess. Weight of provision consumed was recorded upon provision replacement. A “water control” group, containing artificial diets 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 for mortality. Cocoon weight was recorded at the completion of pupation.

Experiment 2: Choice phase

In the choice experiment, 32 two-day-old larvae of mixed parentage were randomly divided among four treatments. Treatments consisted of strategic pairwise combinations (see Fig

1; Table 1) of four possible diets: A1 (1P:1.2C, 90%), A2 (1P:1.2C, 70%), C1 (1P:3.4C, 90%), C2 (1P:3.4C, 70%). Because *O. bicornis* larvae are sedentary and receive a single provision, it is not biologically appropriate to present choices between two diets simultaneously. Therefore, choices were offered temporally by swapping the provision every other day, presenting one diet at a time. This required the larvae to differentially feed over time to compensate for temporal imbalance, in order to converge on an intake target (see e.g. Raubenheimer & Jones 2006). All larvae were kept on the same treatment from two days post-hatching up to pupation, whereupon diet replenishment ceased. The diet that the larvae would be fed first was randomly assigned via coin toss prior to the experiment.

Statistical Analysis

All analyses were conducted in R version 3.4.2 (R Core Team, 2017). For the no-choice experiment, we calculated total nutrients consumed (protein and carbohydrate) from raw diet consumption data for each swap, adjusted for water loss and dilution. Values were then summed for each larva.

To investigate consumption rules, including rules of compromise, we first asked whether diet ratio and concentration affected consumption of (a) the total provision, (b) protein, or (c) carbohydrate, using models of each respective variable with “ratio” and “concentration” as predictors. Rules of compromise can include nonlinear effects, particularly curves around the intake target (Simpson & Raubenheimer 1993). To account for potentially curvilinear relationships we also added ratio^2 as a predictor, as well as two-way interactions between all predictors.

To assess fitness consequences of macronutrient consumption, we analysed cocoon weight at pupation and survival to pupation. For both analyses, to analyse potentially nonlinear effects of nutrient consumption upon fitness, we used polynomial regression, fitting both first- and second-order polynomial terms for “protein consumed [P]” and “carbohydrate consumed [C]”. We analysed cocoon weight using a linear model with “cocoon weight” as a response. The full model contained linear (P and C) and quadratic effects for both nutrients (P^2 and C^2) and their interaction ($P \times C$), as well as diet concentration (high or low), and two-way interactions between concentration and nutrients (conc × P, conc × P^2, conc × C, conc × C^2). We used standard diagnostics to check the fit of models, and used a reverse stepwise process to determine the minimal model, at each step dropping the least significant term until the model contained only significant terms. To analyse survival, we used parametric survival analysis in the *survival* package in R and fitted the same full model as described above. We assessed model fit graphically by inspecting the Kaplan-Meier estimates of the residuals against the assumed Weibull distribution. Again we used reverse stepwise selection to determine the minimal model, comparing models with likelihood ratio tests against a chi squared distribution. To visualise these fitness effects, we calculated response surfaces for cocoon weight and survival, and visualised them using non-parametric thin-plate splines.

In the choice experiment, the mean final consumption of each nutrient was investigated using linear models with diet combination, dilution and their interaction as predictors, and using Tukey's post hoc tests to compare individual treatments against each other. Under a null expectation we would expect larvae to eat randomly from each diet (Fig 1). Thus, for each larva we calculated the deviation from this null expectation. We then tested whether these values systematically departed from zero for protein and carbohydrate, and whether these departures from random consumption differed by treatment group. We used a linear model with "deviation from random consumption" as the response variable and "treatment group" as a predictor.

Larvae that died pre-pupation were not used in the calculation of the mean protein and carbohydrate consumption for diets in either experiment, or for cocoon weight, but were used in analyses involving survival.

Results

No-choice phase

Dietary P:C ratio had a significant effect on the overall amount of provision consumed, with larvae consuming more provision on high P:C ratio diets ($F_{1,78}$=21.55, p<0.0001). Total consumption was also affected by diet concentration ($F_{1,78}$=14.03, p<0.001); larvae on less concentrated diets consumed more provision, indicating compensatory feeding. The quadratic term was not significant (Fig. 2a; Table S2a).

Dietary P:C ratio had a strong effect on the total amount of P eaten ($F_{1,79}$=146.93, p<0.0001); more protein was eaten by larvae raised on the higher P:C diets (Fig. 3). Diet concentration had no effect on the amount of P consumed; neither was there a ratio:concentration interaction, nor a quadratic effect of ratio (Table S2b).

In contrast, larvae consumed similar amounts of C across all diets, with neither concentration nor dietary P:C ratio (linear or quadratic) having an influence on the amount of C consumed (Table S2c). A mean of 0.23 ± 0.01 g of C was consumed by (surviving) larvae across all diet treatments (Fig. 3).

Cocoon weight varied differently with macronutrient intake depending on the overall concentration of the diet (carbohydrate × conc interaction, $F_{1,72}$=6.50, p=0.01; protein × conc interaction, $F_{1,72}$=4.82, p=0.03). At 90% nutrient density, cocoon weight was correlated positively with the amount of carbohydrate consumed, and negatively with protein (Fig. 4a). For our range of diets, the greatest weights were obtained by larvae that ate above approx. 0.3g C and below 0.15g P. In contrast, at 70% nutrient density, cocoons were lower in weight than on the 90% diets, and were fairly uniform in weight irrespective of macronutrient intake (Fig 4b). No quadratic effects were observed, nor interactions involving quadratic effects, meaning that we did not identify an optimal amount of P or C that maximised cocoon weight within the range of diets we used (Table S3a).

The relationship between survival and nutrition similarly depended upon dietary concentration (carbohydrate × conc interaction: χ_1=6.50, p=0.01). Survival of larvae fed diets at 90% concentration depended primarily upon carbohydrate consumption (Fig 5a). Those larvae that consumed high amounts of carbohydrate saw the highest survival irrespective of how much protein was consumed. At lower levels of carbohydrate, interestingly, protein weakly mediated survival (protein × carbohydrate interaction: χ_1=-4.88, p=0.046). Survival of larvae raised on the more dilute diets was much lower, and was not substantially affected by intake of P or C (Fig 5b). Again, there were no significant quadratic terms, whether as main effects or as part of interactions (Table S3b).

Choice phase

We found no evidence of larvae defending a common intake target *sensu stricto (Raubenheimer & Simpson 1993, 1999b)*, i.e. a common ratio *and* amount of nutrients consumed, which would have been evident as all groups clustering at a common point in nutrient space in Fig 6a. Nevertheless, consumption deviated from random so as to converge upon a target P:C ratio (see e.g. Deans, Sword & Behmer 2019) represented by a common line, or "nutritional rail", of approx. 1:1.8 (Fig. 6a). The amount of protein consumed by larvae was significantly affected by diet combination: more protein was consumed by individuals offered diet combinations that were overall more concentrated ($F_{3,23}$=7.43, p<0.01, Fig 6a; Table S4a). Similarly, carbohydrate consumption was significantly affected by diet combination ($F_{3,23}$=4.58, p=0.01, Fig 6a; Table S4b). Unlike with protein, though, this pattern appeared to be driven by the diets at the extreme; only the most concentrated diet pair (C1A1) differed from the least concentrated pair (C2A2; Fig 6a); other pairwise comparisons were not significant (Table S4).

Despite the lack of a common intake target, larvae were not consuming diets at random (Fig 6b, Table S4c, d). 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 (protein, $F_{4,20}$=19.67, p<0.001; carbohydrate, $F_{4,20}$=51.65, p<0.001). When visualised as the amounts of protein and carbohydrate consumed during each 48h treatment period (Fig. 7), it is clear that larvae were achieving a degree of homeostasis in carbohydrate consumption (Fig 7b) compared to what would be expected under random consumption of each diet choice (Fig 7a), whereas their consumption of protein (Fig 7d) aligned closely with what would be expected under random consumption (Fig 7c).

Discussion

We found that carbohydrate was positively associated with both body size and survival in *Osmia bicornis* larvae (Figs. 4a, 5a), although within our range of dietary ratios we did not specifically identify an optimum (fitness-maximising peak) in intake for either carbohydrate or protein. Accordingly, given a choice, larvae converged on a relatively carbohydrate-biased protein:carbohydrate ratio of 1:1.8 (Fig. 6a). Moreover, larvae prioritised carbohydrate over protein intake, showing tighter control over carbohydrate consumption than over protein consumption (Fig 7), and they pupated after eating about 0.23 g carbohydrate irrespective of protein and of dietary dilution (Fig 3). Yet this carbohydrate target fell short of the amount of carbohydrate that maximised cocoon weight or survival to pupation (Fig 4a, 5a). Dietary dilution imposed costs upon larvae regardless of nutritional intake, in the form of greater mortality and lower cocoon weights (Figs 4b, 5b). Taken together, these results show that (1) larval *O. bicornis* are at least partially responsible for their own nutritional regulation, and (2) their performance and consumption rules suggest adaptation to a pattern of carbohydrate-limited growth and survival. In what follows, we suggest how and why these patterns in *O. bicornis* depart from expected results based on studies of related organisms, and more generally what these findings suggest about nutritional cooperation and/or conflict between parents and offspring in (mass) provisioning species.

Larvae grew and survived best on our highest carbohydrate (i.e. lowest protein:carbohydrate) diets. Accordingly, across our range of diets, larvae maintained a constant carbohydrate intake while tolerating excesses or deficiencies of protein (a “no-interaction” rule of compromise; Raubenheimer & Simpson 1999b) - although it is conceivable that alternative rules of compromise, such as the “equal distance rule” more typically seen in generalist herbivores (Raubenheimer & Simpson 1999b; Behmer 2009), might have been evident over a broader array of diets. Both these patterns are unusual because insect herbivores are generally considered to be limited by protein (e.g. Bernays & Chapman 2007; although see Le Gall & Behmer 2014). In the few existing studies involving larval bees, e.g. honeybees (Helm *et al.* 2017), bumblebees (Kraus *et al.* 2019) and sweat bees (Roulston & Cane 2002), protein and not carbohydrate mediated larval growth and/or survival. More broadly, insect larvae often grow and survive best on balanced or moderately high protein:carbohydrate ratios (Roeder & Behmer 2014; Rodrigues *et al.* 2015), although low protein:carbohydrate ratios are associated with longevity in adults (e.g. Lee *et al.* 2008). Moreover, animals generally prioritise regulation of the nutrient that is typically limiting in their normal diet, and tolerate variation in nutrients that are abundant (Raubenheimer & Simpson 1999a). Tolerance of wide variation in protein is thus usually seen in predators (e.g. Raubenheimer *et al.* 2007; Kohl, Coogan & Raubenheimer 2015). In contrast, herbivores often regulate protein more tightly than carbohydrate (Lee *et al.* 2002; Le Gall & Behmer 2014; VanOverbeke, Thompson & Redak 2017). It is worth noting that protein *did* weakly mediate survival in our larvae to some extent, although only at low carbohydrate - possibly as a result of switching to protein as an energy source.

During the choice phase, when allowed to self-select diets, *O. bicornis* larvae converged on a protein:carbohydrate ratio of 1:1.8. This ratio is considerably more carbohydrate-biased than that preferred by bumblebees foraging on behalf of microcolonies (1:0.25, Vaudo *et al.* 2018; 1:0.08, Kraus *et al.* 2019), and (to a lesser extent) than ants foraging for colonies with offspring

(1:1.5, Dussutour & Simpson 2009). It is also more carbohydrate-biased than that selected by reproductive, solitary phytophages such as grasshopper adults and lepidopteran larvae (1:0.25 - 1:1.4, reviewed in Behmer 2009) and is closer to diets selected by *Drosophila* larvae (1:2, Rodrigues *et al.* 2015). Notably, though, 1:1.8 was more protein-biased than the ratio that we found maximised both cocoon weight and survival (1:3.4), suggesting *O. bicornis* larvae may choose diets that favour other fitness-related quantities (such as reproduction and/or developmental time) over body size/survival, as in *Drosophila* (Lee *et al.* 2008; Rodrigues *et al.* 2015). As a cautionary note, the specific source of nutrients may also affect the preferred ratio: for example, adult honeybees exhibited different target P:C ratios when fed different protein sources (Altaye *et al.* 2010). Whether larvae are similarly sensitive is still unknown.

Two main features of *O. bicornis'* ecology may help to explain their prioritization of carbohydrate, and their relative preference for this macronutrient, compared to what we know of related taxa. First, the relative paucity of carbohydrate in *O. bicornis'* larval diet may help to explain these findings. Despite being herbivorous, *Osmia* larvae are unlikely to be protein-limited, because pollen is among the most protein-rich of plant tissues (Mattson 1980). Moreover, in *Osmia* specifically, nectar constitutes only a tiny fraction of the pollen ball, less than 4% (Maddocks & Paulus 1987; see Radmacher & Strohm 2010), in contrast to many other bees where nectar is a principal source of carbohydrate for larvae (e.g. Kraus *et al.* 2019). *O. bicornis* larvae may therefore be limited more by the amount of digestible carbohydrate within pollen than by dietary protein (see Roulston & Cane 2000). Second, *O. bicornis* is (to our knowledge) the first truly solitary hymenopteran studied under the GF; other studies have concerned individuals likely to become workers of social species. Unlike social hymenopterans, *O. bicornis* offspring are all reproductive and undergo diapause (Fliszkiewicz *et al.* 2012) - both activities dependent on the fat body, where carbohydrate-derived fat is stored (Kawooya & Law 1988; Ziegler & Van Antwerpen 2006; Hahn & Denlinger 2007; Wasielewski *et al.* 2013). Thus, *O. bicornis* larvae may have additional requirements for carbohydrate over and above those of developing nonreproductive, nondiapausing hymenopteran workers. These contrasting findings reinforce the idea that bees' nutritional needs may be just as diverse as their ecologies.

Although larvae retained the ability to regulate carbohydrate by over- or under-eating protein, they nevertheless coped very poorly with dietary dilution (Fig 4b, 5b), despite displaying compensatory feeding behaviour (Fig 2) that suggests they both detected and responded to such dilution. The dilution was not excessive (70% nutrient density) compared to similar studies offering very highly dilute diets (14%, Raubenheimer & Simpson 1993; 16.8%, Lee, Raubenheimer & Simpson 2004). The locusts and caterpillars in those studies, though, are adapted for diets that vary greatly in nutrient density, beginning dilute and becoming even more dilute over the season (Scriber & Slansky 1981). By contrast, pollen is among the most consistently nutrient-rich parts of a plant (Roulston & Cane 2000) and does not broadly vary in composition over a season (DeGrandi-Hoffman *et al.* 2018). With a normal diet of unadulterated pollen and very little nectar, *Osmia* larvae may have had no need to evolve mechanisms to cope with dilution. In comparison, caterpillars reared on an invariant diet for generations lost the ability both to regulate intake and to cope with dilution (Warbrick-Smith *et al.* 2009). *Osmia* larvae appear to have retained the former capacity, but lost the latter, suggesting a normal diet that is dense in nutrients, but variable in composition.

In systems where parents gather food for offspring from the environment, both parents and offspring can be active participants in nutritional regulation. The lack of protein regulation shown by *O. bicornis* larvae highlights the importance of understanding (a) whether mother *O. bicornis* adjust protein content of provisions in response to imbalances in the landscape, and (b) whether larvae have physiological adaptations (e.g. post-ingestive processing) for tolerating protein imbalance. Budde & Lunau (2007) found that *O. bicornis* provisions contained about 19% protein regardless of pollen species used, suggesting a degree of homeostasis by parents. Yet human activity is reducing floral diversity and quality (Ziska *et al.* 2016; Papanikolaou *et al.* 2017). Evidence is mixed concerning whether, in practice, parent bees assess pollen nutrients at the flower (reviewed by Nicholls & Hempel de Ibarra 2016). Both bumblebees and ants balance nutrition on behalf of colonies (Dussutour & Simpson 2009; Vaudo *et al.* 2018), regulating more tightly when foraging for offspring - protein in the case of both taxa (Dussutour & Simpson 2009; Kraus *et al.* 2019) and carbohydrate in ants (Dussutour & Simpson 2008; Cook *et al.* 2010). On the other hand, protein gathered by honeybees varies passively with landscape usage (while maintaining carbohydrate and lipid; Donkersley *et al.* 2014). Which regulatory strategy *Osmia* parents and larvae collectively pursue may have important implications for their vulnerability to human-induced landscape change, and so should now be a focus for research.

Additionally, the ability to discriminate among nutrients provided by parents may be one tool offspring can use to exert some control over their nutrition, even in the absence of contact with parents. *Osmia* parents may provide suboptimal resources simply because of inefficiency in gathering pollen: efficiency drops across the season (Seidelmann 2006) and is lower in smaller-bodied parents (Seidelmann, Ulbrich & Mielenz 2010). Moreover, less efficient parents actively switch to producing male offspring (Seidelmann *et al.* 2010), so male and female offspring may experience different selection for regulation. This is well documented in other groups (e.g. Maklakov *et al.* 2008) and sex differences in larval regulation should now be a focus for research. But it is also well known that the evolutionary interests of parents and offspring frequently differ over how resources should be allocated (Trivers 1974; Crespi & Semeniuk 2004; Kilner & Drummond 2007; Haig 2010). The potential for offspring to use nutritional regulation to mitigate parentally imposed costs has been relatively overlooked, as most research to date has focused on parent-offspring conflict over *amount* of parental provisions, despite clear potential for conflict over composition (e.g. in discus fish, Buckley *et al.* 2010). Among primitively social Hymenoptera, some parents actively stunt offspring by restricting provisions (Lawson *et al.* 2017), securing their help by forcing them to become workers (Craig 1983). But the composition of food provided by parents is also critical to offspring fitness (e.g. Roulston & Cane 2002) and in extreme cases caste-determining (Anderson 1984). *O. bicornis* are solitary and lack castes, but this does not preclude parent-offspring disagreement over the optimal balance of offspring nutrition, as in e.g. *Drosophila* (Rodrigues *et al.* 2015).

More broadly, understanding the relative roles of offspring (intake regulation and post-ingestive processing) versus parents in nutrient balancing, as well as their evolutionary interests, will be key to understanding the nutritional ecology of species with parental provisioning. Such species include not just bees and other Hymenoptera, but other important ecosystem service providers such as dung beetles (Frank *et al.* 2017) and burying beetles (Hopwood, Moore & Royle 2013), as well as altricial birds (Wiens & Johnston 2012) and even humans (Burt & Amin 2014). Alloregulation by parents is not a given; the relative roles and interests of parent and offspring in these groups are likely to reflect species' ecologies. Recent studies have found nutritional mismatches between oviposition sites selected by parents and the nutritional requirements of the offspring that will develop in those sites (Rodrigues *et al.* 2015; Lihoreau *et al.* 2016). Parent sweat bees (*Lasioglossum zephyrum*) appear not to regulate protein in larval provisions, despite protein mediating offspring performance (Roulston & Cane 2002). In *O. bicornis*, we have shown that offspring retain the ability to regulate their nutritional intake despite all food selection being done by parents whom they never meet. Larvae appeared to pay closest attention to regulating dietary carbohydrate, consistent with this nutrient mediating both growth and survival. Yet protein remains a key requirement for development; key now is to (a) establish the nutritional rules used by parents when provisioning offspring, and whether these coincide with or depart from those employed by larvae, and (b) establish specifically how protein balance is achieved, and whether parents or larvae carry that responsibility.

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Tables & Figures

Table 1. 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, determined by coin toss. Surviving larvae are in parentheses.

	Order 1	Order 2	Total
A1C1	1 (1)	6 (5)	7 (6)
A1C2	5 (5)	4 (3)	9 (8)
A2C1	3 (2)	5 (5)	8 (7)
A2C2	5 (2)	3 (1)	8 (3)

Figure captions

Figure 1. (a) Expected protein and carbohydrate consumption if larvae ate indiscriminately between two diets. Diet choices are pairwise combinations of diets A1, A2, C1 and C2, which each contain protein and carbohydrate at different ratios and concentrations. Solid lines represent P:C ratios; black points represent actual nutrient content of each diet, which depends upon dilution as well as P:C ratio. Red points represent expected consumption if larvae eat randomly (i.e. equally) from each of a choice of two diets (choices denoted by the red point labels). **(b)** schematic describing how larvae were assigned to each diet grouping. Coloured arrows show the period in days that each larva was fed a particular diet.

Figure 2. Amount of provision in grams consumed by larvae raised on the 3 different P:C ratio artificial diets at the 2 different macronutrient concentrations (90% and 70% macronutrient content).

Figure 3. Mean total (+/- 1 SE) amount of P and C consumed in grams by larvae on each diet before pupation. Solid lines and letters represent three P:C ratios (A = 1:1.2, B = 1:2.3, C = 1:3.4). Numbers following letters denote diet concentration (1 = 90%, 2 = 70%). Dotted lines show global mean consumption of each nutrient.

Figure 4. Effects of P and C consumption upon cocoon weight (g) in larvae fed diets at (a) 90% and (b) 70% nutrient density. Transition from blue to red indicates heavier cocoons. For context, mean total consumption of P and C for each diet is plotted (white points; data as in Fig. 2) alongside raw data (grey points). Solid lines and letters represent three P:C ratios (A = 1:1.2, B = 1:2.3, C = 1:3.4).

Figure 5. Effects of P and C consumption upon estimated survival time (colour) in larvae fed diets at (a) 90% and (b) 70% nutrient density. Transition from blue to red indicates longer survival. Black points, dead larvae; white points, larvae surviving to pupation. For context, mean total consumption of P and C for each diet is plotted (large white points; 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).

Figure 6. (a) The mean (+/- 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 consumption. Letters represent diet P:C ratio (A = 1:1.2, C = 1:3.4); numbers represent diet concentration (1 = 90%, 2 = 70%), hence, for example, "A2C1" represents the pairing of diet A2 with diet C1. Solid lines represent dietary P:C ratios (Top line = Diet C, Bottom line = Diet A). Dashed red line shows expected average P:C ratio based on random consumption. Dashed black line shows average P:C ratio of observed intake across larvae. (b) Deviation from random intake of protein and carbohydrate for larvae in different treatment groups during the choice phase. Treatment groups are given in order of overall diet concentration. Bars with similar letters displayed above or below are not statistically significantly different (Tukey's post-hoc comparisons).

Figure 7. (a, c) Mean expected intake over successive diet swaps assuming random consumption of diets (+/- 1 SE, inner ribbon, and SD, outer ribbon) of protein (red, lower ribbons) and carbohydrate (blue, upper ribbons), irrespective of the concentrations of the diet choices, for larvae starting on (a) diet A or (c) diet C. (b, d) Nutrient intake actually observed for larvae starting on (b) diet A or (d) diet C (+/- 1 SE, inner ribbon, and SD, outer ribbon). For details of calculations of expected consumption, see text. Swap 11 lacks confidence intervals because only one larva in each group reached this stage.

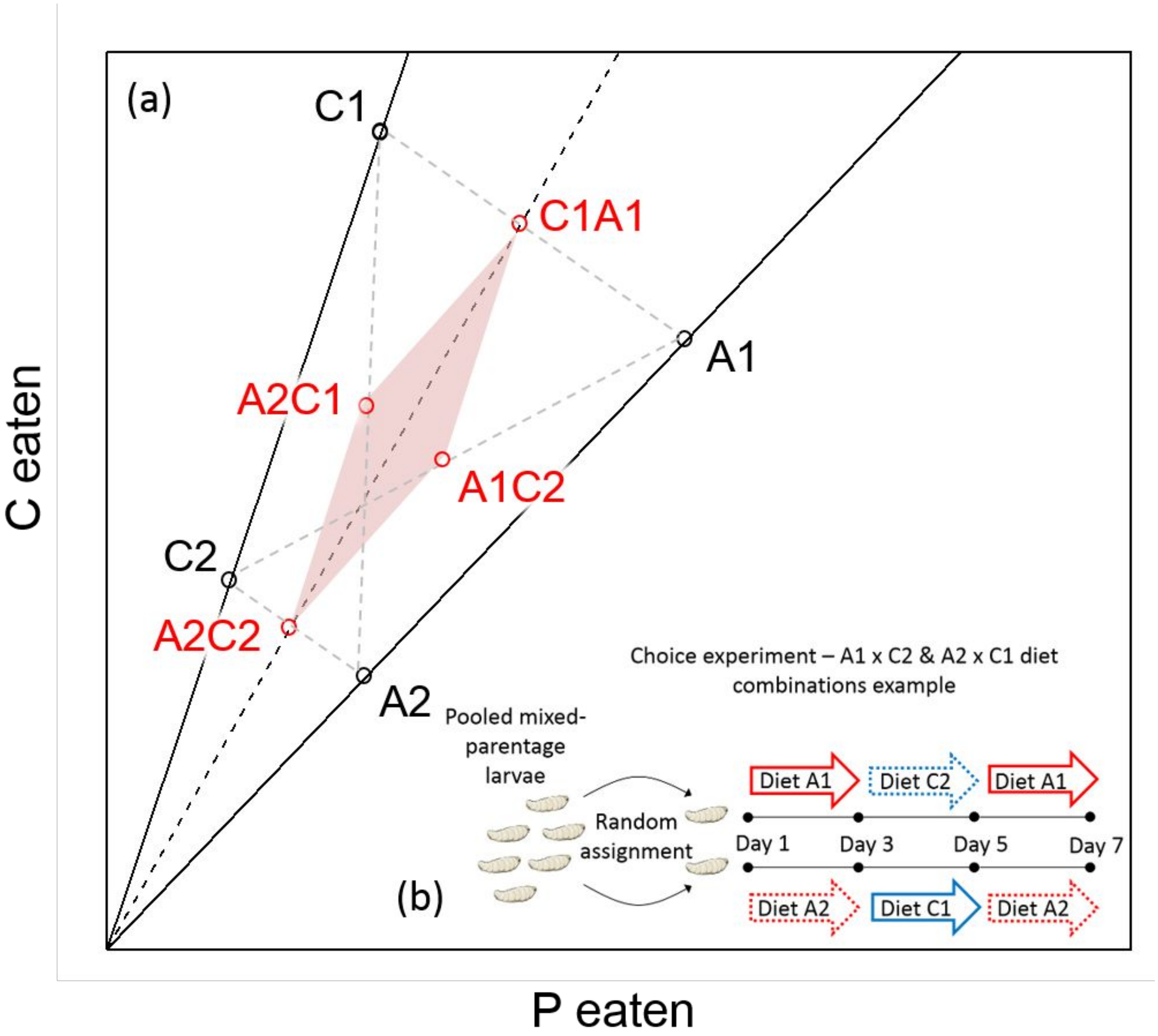

(a)
C1
C1A1
A1
A2C1
A1C2
C2
A2C2
A2
C eaten
P eaten
Choice experiment – A1 x C2 & A2 x C1 diet combinations example
Pooled mixed-parentage larvae
Random assignment
(b)
Diet A1
Diet C2
Diet A1
Day 1
Day 3
Day 5
Day 7
Diet A2
Diet C1
Diet A2

Fig. 1.

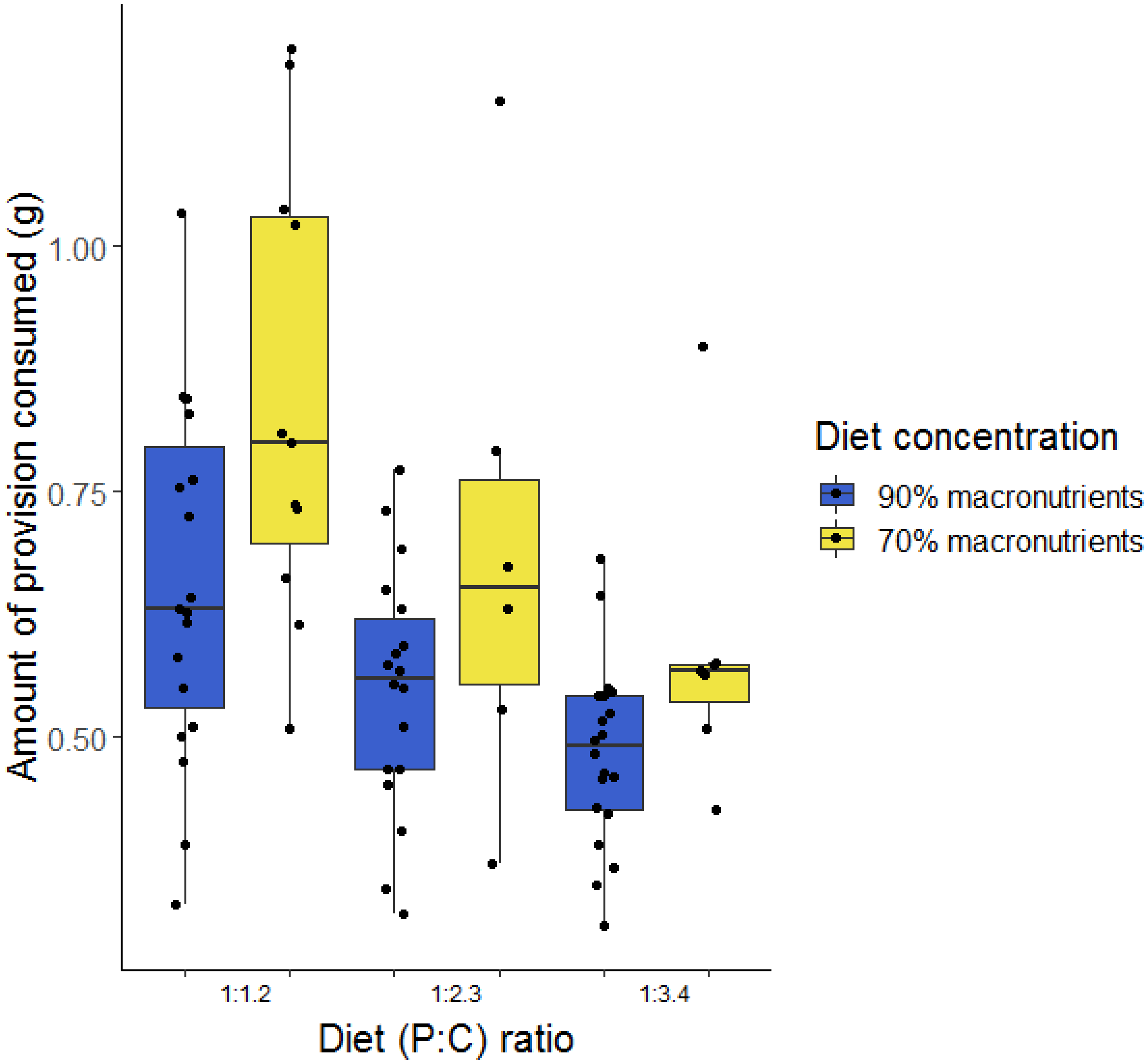

Amount of provision consumed (g)
1.00
0.75
0.50
1:1.2
1:2.3
1:3.4
Diet (P:C) ratio
Diet concentration
90% macronutrients
70% macronutrients

Fig. 2.

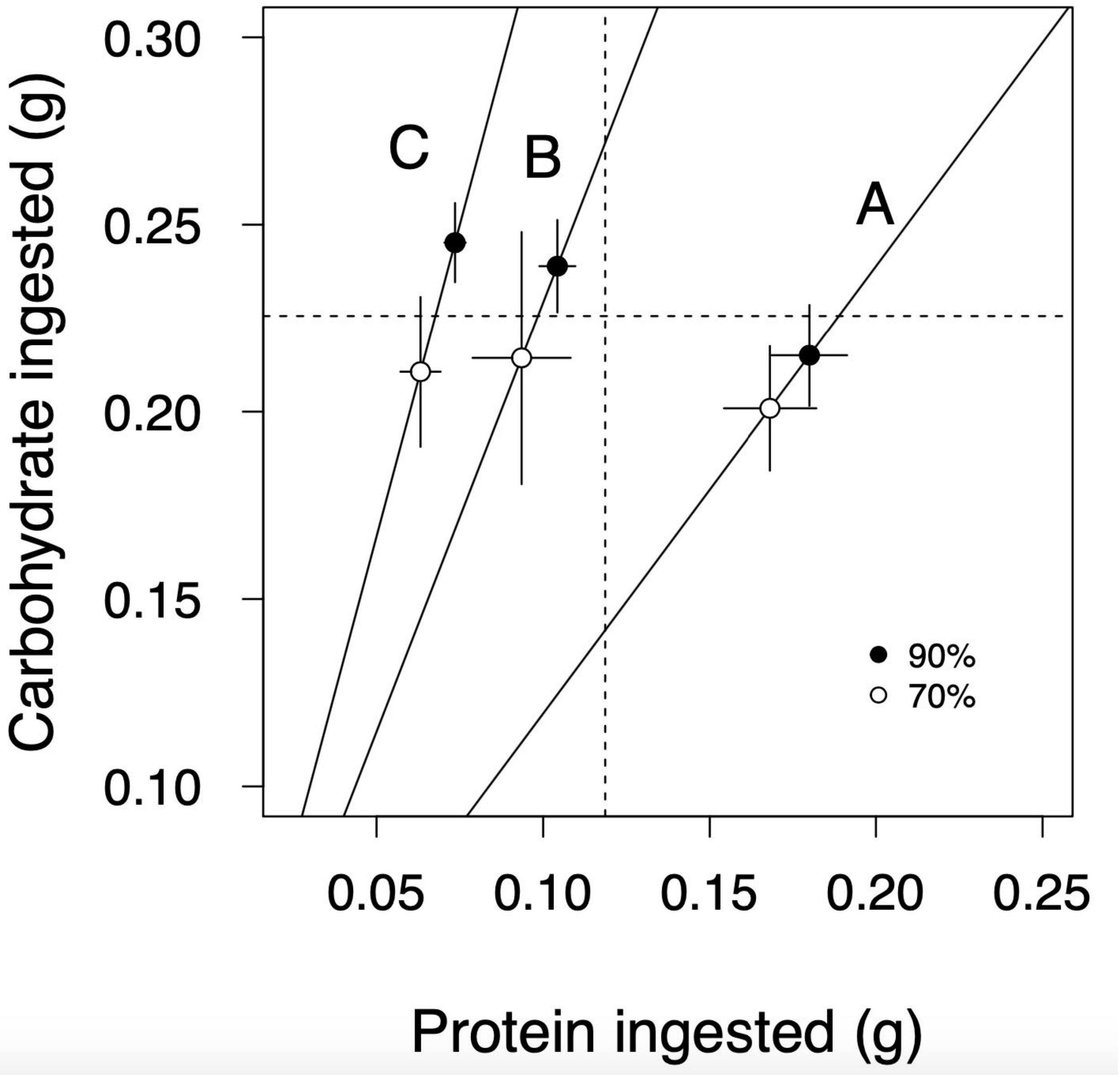

Carbohydrate ingested (g)
0.30
0.25
0.20
0.15
0.10
Protein ingested (g)
0.05
0.10
0.15
0.20
0.25
C
B
A
● 90%
○ 70%

Fig. 3.

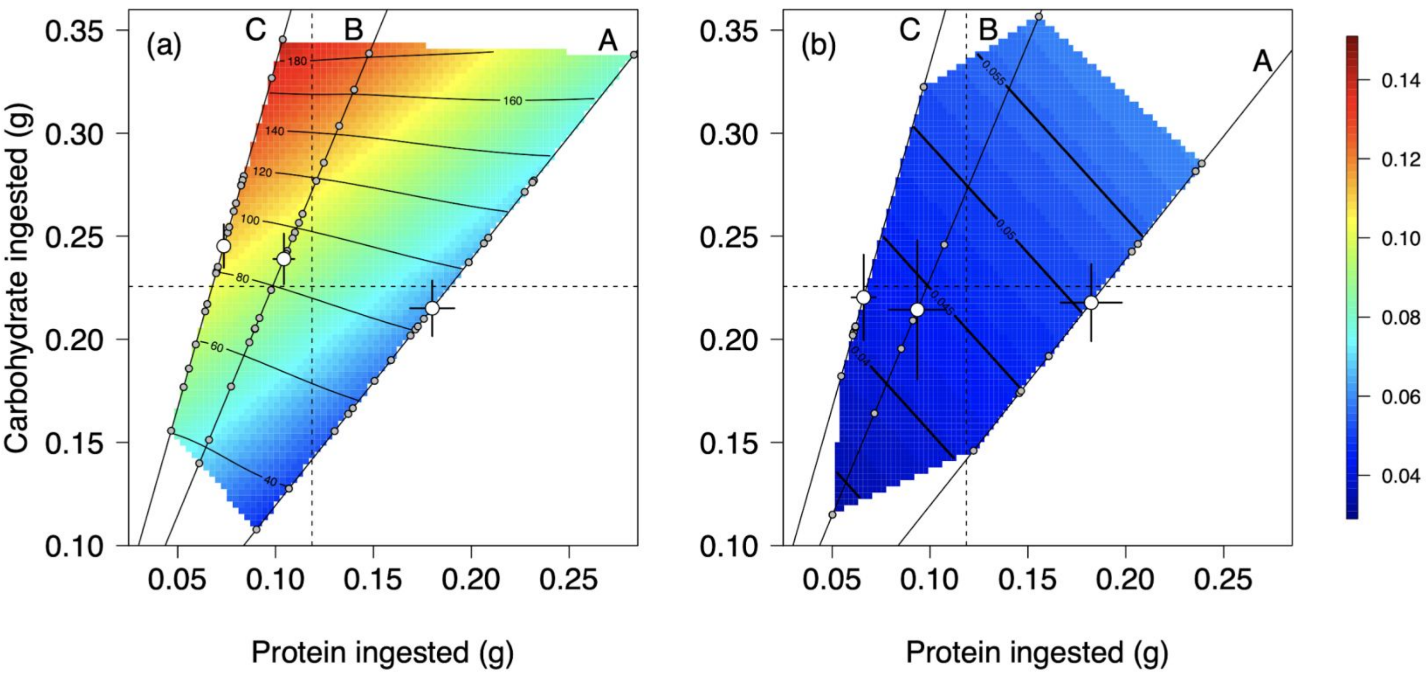
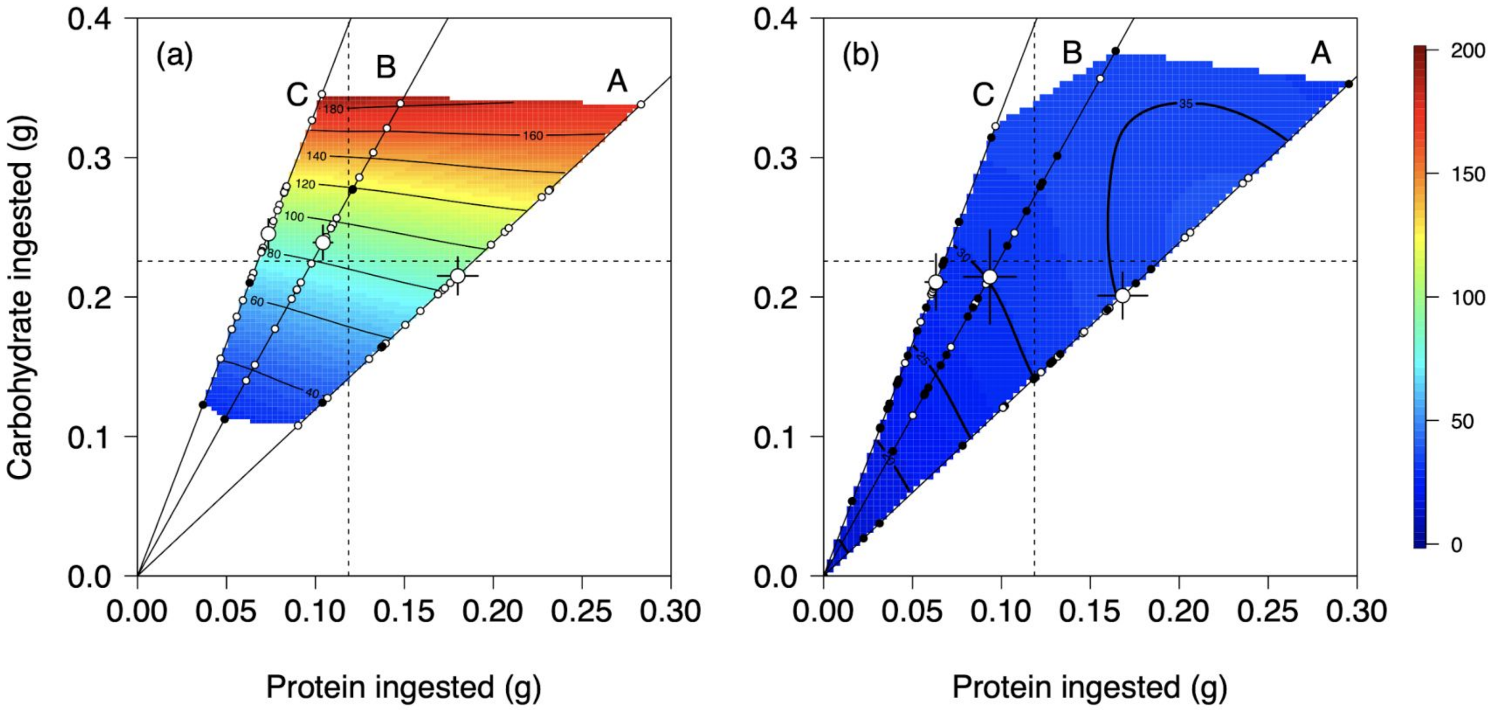

(a)
C
B
A
180
160
140
120
100
80
60
40
Carbohydrate ingested (g)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.10
0.15
0.20
0.25
Protein ingested (g)
(b)
C
B
A
0.055
0.05
0.045
0.04
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.10
0.15
0.20
0.25
Protein ingested (g)
0.14
0.12
0.10
0.08
0.06
0.04

Fig. 4.


(a)
(b)
A
B
C
180
160
140
120
100
80
60
40
35
30
25
Carbohydrate ingested (g)
Protein ingested (g)
0.0
0.1
0.2
0.3
0.4
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0
50
100
150
200

723 Fig. 5.

724

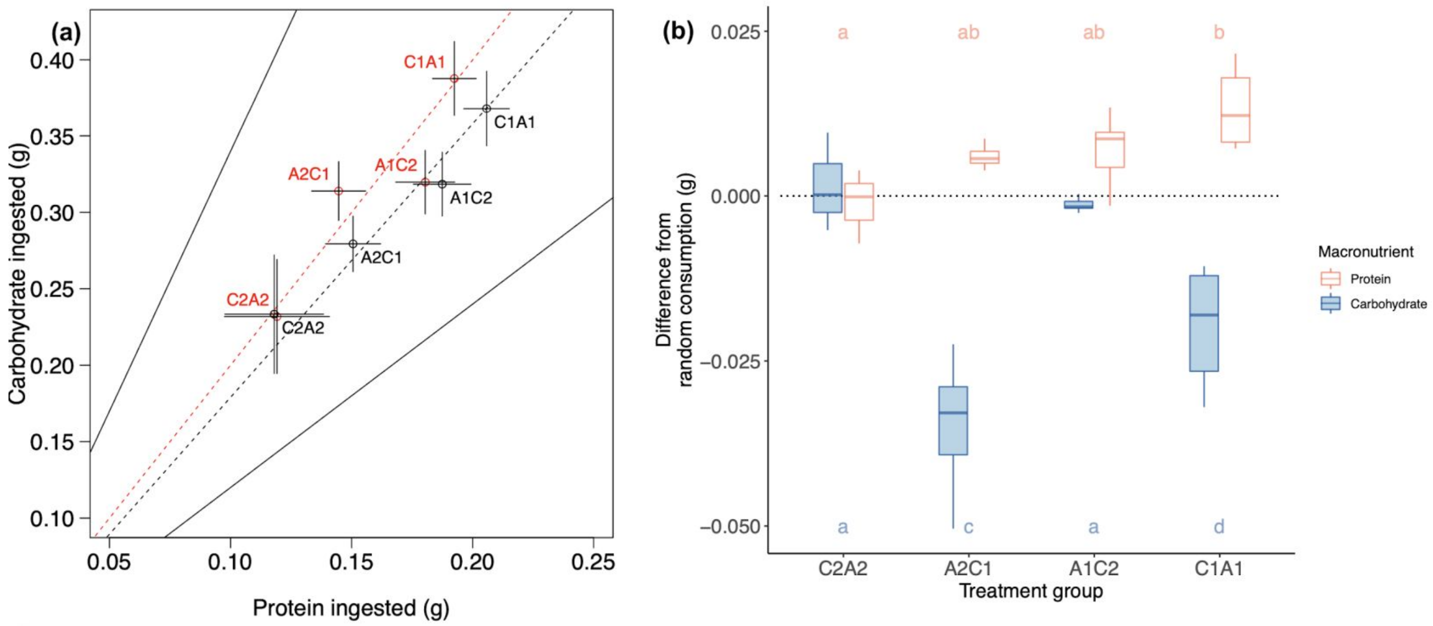

(a)
Carbohydrate ingested (g)
Protein ingested (g)
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.05
0.10
0.15
0.20
0.25
C1A1
C1A1
A2C1
A1C2
A1C2
A2C1
C2A2
C2A2
(b)
Difference from random consumption (g)
0.025
0.000
−0.025
−0.050
a
ab
ab
b
a
c
a
d
C2A2
A2C1
A1C2
C1A1
Treatment group
Macronutrient
Protein
Carbohydrate

Fig. 6.

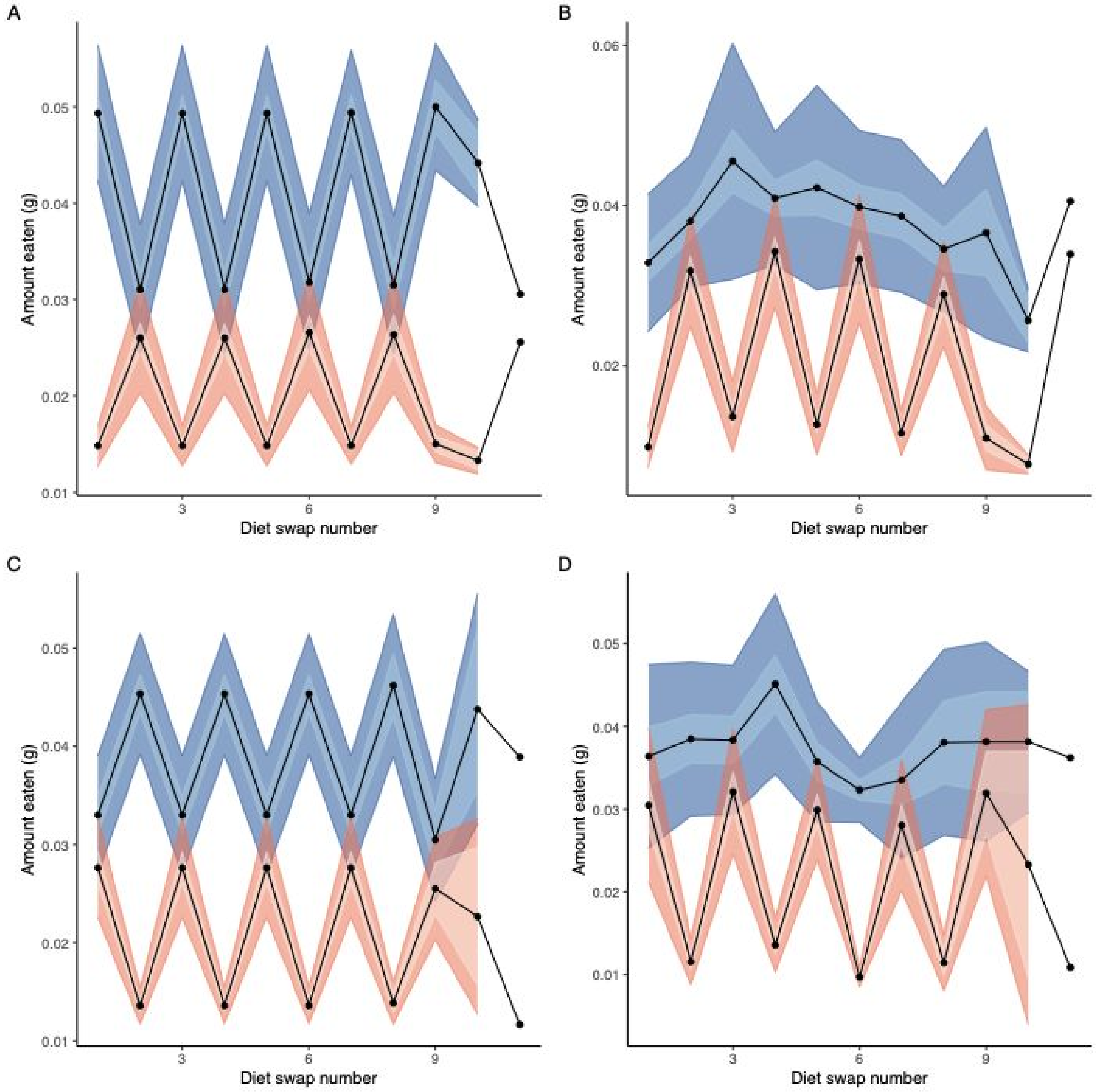

A
B
C
D
Amount eaten (g)
Diet swap number
0.01
0.02
0.03
0.04
0.05
0.06
3
6
9

Fig. 7.

Dear Editor,

Thank you for reconsidering our MS, FE-2020-00442, and for the many helpful comments which have helped us substantially improve it. Please find enclosed our revision, now entitled “Solitary bee larvae prioritize carbohydrate over protein in parentally provided pollen” as requested. We have now revised the MS to reflect comments from all reviewers and editors. Most notably, we have

(1) incorporated throughout all sections of the MS the excellent suggestions by the reviewers to frame the finding in terms of potential conflict of interest between parents and offspring, or of parental inefficiency, and the consequent utility to the offspring of discriminating among parental provisions. We feel this has really strengthened the angle.
(2) Re-run all our statistical models incorporating nonlinear terms to demonstrate that fitting nonlinear effects did not improve the explanatory power of the models.

A detailed breakdown of responses to the reviewers’ and editors’ comments is appended below, in which our responses are prefaced with a # character.

We would be delighted to be involved in a podcast or video to accompany the piece.

Thank you once again for your attention; we look forward to hearing from you.

Alex Austin and James Gilbert

05-Aug-2020

FE-2020-00442
The geometry of dependence: solitary bee larvae prioritize carbohydrate over protein in parentally provided pollen
Austin, Alexander; Gilbert, James

Dear Dr James Gilbert,

Your manuscript has now been assessed by two reviewers and one of our editors. The reviewers are very positive about your paper. They do make quite a few suggestions for improvement, but these are mostly minor points that you should be able to address without difficulty. I refer you to the editor's and reviewers' comments, appended below my signature, for details of the requested revisions.

Plus, here are some of my own comments:

- I agree with the editor regarding the title, but suggest using his first suggestion (which references your solitary bee) rather than the shorter one that drops reference to the study organism.

We have amended the title as requested.

- Your abstract ends (point 4) very focused on your study organism. Please delete the current final sentence ("Research should now ...") and instead add a sentence that places this into broader context. What is the take-home message for someone who does not work on or care about bees solitary bees?

We have revised this sentence to reflect more general implications of the work.

- You have four fairly large analysis tables, each of which will have quite a lot of wasted space. Please condense these or move them to supplemental material.

We have moved all these tables to ESM and instead quote the relevant statistics inline in the text. As a rule we would prefer model tables in the main document for transparency, but appreciate the need to avoid bloating the MS here.

- Please increase the size of the fonts on Figure 2. It will be published in one column of the journal page and the fonts will be very small after the figure is compressed.

We have increased the font size as requested.

- For some figures, such as 2, 6 and 7, you use different colors that may be indistinguishable when the paper is printed in greyscale or for color blind readers. Please use different symbols and/or shading in addition to color differences.

We have updated the colour schemes for the relevant figures and we hope they are now more colour-blind and print friendly.

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Please remember to provide an explanation of how you have addressed all the comments made. If you are interested in producing a podcast or video for your paper, please do mention this in your revision cover letter.

As above, we'd be very much interested in this.

If a revised manuscript is not received within one month then it will be treated as if it were a fresh submission. Please contact the Editorial Office at the earliest opportunity if you need extra time to revise your paper.

Yours sincerely,

Dr Charles Fox
Senior Editor

ASSOCIATE EDITOR'S COMMENTS TO THE AUTHORS
I have now received two reviews on your manuscript, FE-2020-00442, The geometry of dependence: solitary bee larvae prioritize carbohydrate over protein in parentally provided pollen. Both of of the reviews (and my own reading of the manuscript) indicate that the work makes important contributions. As such I am happy to recommend "minor revisions" to the EIC.

The reviewers report minor concern about reporting clarity of specific results and methodological details (see below). Each of these should be addressed in a revised manuscript and in a rebuttal letter. More importantly, I feel that more to be done in the text (and perhaps the title) to highlight the central public interest finding of the study: that larvae discriminate between nutrients provided by parents. This issue is highlighted by general comments by Reviews 2 and a specific comments by Reviewer 1 (See Reviewer 1, Comment 11). The question about how children respond to nutrients provided by parents relates to a broad field of research on parent-offspring conflict. A lot has been written on child elicitation and parent response and parent/offspring trade-offs, but much less on child discrimination of parental responses. For example, one interpretation of larvae discrimination of parental nutrients, is potential for offspring to mitigate against costs imposed by their parents.

This is a superb point and we thank the associate editor for the suggestion. We have now added text incorporating this angle into the abstract, introduction and discussion which we feel has strengthened the argument substantially; we hope you agree.

My suggestions are as follows:

1. Shorten the title.

Perhaps this: Larvae prioritize carbohydrate over protein in parentally provided pollen in a solatary bee.

"Larvae prioritize carbohydrate over protein in parentally provided pollen" would be even better.

The title has now changed to "Solitary bee larvae prioritize carbohydrate over protein in parentally provided pollen."

2. Add a lines in the abstract/introduction/discussion on parent/offspring conflict.

The intro, in particular, would need referencing on parent/offspring conflicts over nutrition, stressing the lack of studies on how offspring discriminate parentally provided nutrition.

*Note, these are suggestions for improving the clarity of message to a broad audience, not requirements for publication.

We have incorporated both these suggestions (line 6, lines 50-7)

Thank you for your submission for Functional Ecology. Good luck!

Nick Priest

See comments of Reviewers 1 and 2 below (also attached):

Reviewer 1:
In the manuscript submission "The geometry of dependence: solitary bee larvae prioritize carbohydrate over protein in parentally provided pollen", Austin & Gilbert use the techniques of nutritional geometry to study nutrient regulation in the mason bee Osmia bicornis. The authors find that despite females provisioning each individual offspring with a care package in the form of a pollen ball, larvae are able to balance their own macronutrient intake. The authors further find that - contrary to prior expectation - larvae prioritize the regulation of carbohydrate intake over that of protein intake.

Overall I found the results of this paper to be informative and well presented. I believe that the application of the Nutritional Geometric Framework and its theory to a solitary hymenoptera with no continual parental contact represents a novel and important contribution to the field. In particular, the mason bee study system chosen allows for the choices that offspring make after their parents have provided complete larval nutrition to be elegantly analysed independent of parental decisions.

Comments/concerns that I believe need to be addressed follow below:

1. Line 212 "We found no evidence of larvae defending a common intake target at the choice phase". I disagree that there is no evidence. I accept that intake target is properly defined as a target of both ratio and intake of macronutrients, but I feel that the evidence you provide for a target ratio (approx 1:1.8) constitutes some evidence of an intake target. Indeed,

many authors tend to use intake target as meaning simply a ratio. I would suggest clarifying this point.

Thank you for this suggestion - we have endeavoured to clarify. As you suggest we use intake target sensu Raubenheimer & Simpson 1993, i.e. where data cluster at a common point rather than just a common ratio. We were not previously aware of authors using “intake target” to mean a ratio, but have now found some examples and have clarified as follows: “We found no evidence of larvae defending a common intake target sensu stricto (Raubenheimer and Simpson 1993; Raubenheimer and Simpson 1999), i.e. a common ratio and amount of nutrients consumed, which would have been evident as all groups clustering at a common point in nutrient space in Fig 6a. Nevertheless, consumption deviated from random so as to converge upon a target P:C ratio (see e.g. Deans et al. 2019) represented by a common line, or “nutritional rail”, of approx. 1:1.8 (Fig. 6a).” (lines 244-9)

2. Line 207: “Survival of larvae raised on the more dilute diets was greater, and was not substantially affected by intake of P or C (Fig 5). Again, all linear models showed a good fit, so we did not look for non-linear patterns in larval survival.”. I understand what you have done here, but I don’t feel that a ‘good fit’ in a linear model is a good reason to not look for non-linear patterns. I would like to see a reason why we should be satisfied by a linear model fit (e.g. non linear isn’t significantly better / loses simplicity / less explanatory power.

On reflection we agree, and have now added quadratic predictor terms to all our full models, confirming that the form of the minimal models was unchanged. To avoid bloating the tables in the main document, and also as requested by the editor, we have moved the full model tables to the ESM and instead cited the relevant statistics inline.

3. Table 1: What is the reason for the imbalance in sample size for order 1 vs order 2? Would it not make better sense to randomly allocate individual larvae to order 1 or order 2 but keep the balance between the two? The sample size of n=1 for A1C1 order 1 vs n=6 for order 2 seems odd.

We agree, and in hindsight would probably have approached this differently. We allocated larvae to treatment orders using a coin toss with the resulting sample sizes shown in the MS, and stopped allocating when no more suitable larvae were available.

4. Figure 3: In general I found this figure to be a little cluttered. It might benefit from the use of color to distinguish better between the groups. Also, the B1 and B2 points don’t sit on the line, the horizontal dotted line isn’t referenced in the legend, and the lack of a vertical dotted line (I assume for median protein intake) should be explained

We have now aligned the lines with the points, added a vertical dotted line, and explained the lines in the figure. We have also cleaned up the figure by using colour to represent concentration.

5. Figure 4: I would like to see the consumption by individual larvae plotted as points on these plots, either in addition to or instead of means (in line with what you do in Fig. 5). This would provide confidence in the interpolation between points that has to be done for the surface fitting (and would follow the precedent of many papers in this field).

We have now added individual consumption to these figures.

6. In Figure 3 (no choice experiment) standard deviation is chosen as the measure of variation in P and C intake, whereas in Figure 6 (choice experiment) standard error is used instead. What is the reason for this difference?

We agree that SE would be the more appropriate error estimate to give. We have changed the SD bars to SE in Figures 2, 3 and 4, and have added SE ribbons as well as SD to Figure 7.

7. Figure 6: I find the bars showing significance from post-hoc tests to be a step too far in this plot, and that it adds too much complexity to a single figure. I would consider either separating it out into a seperate plot (with e.g. bar charts) or just stick to it being explained clearly in the text somewhere line (which you do on lines 217-220). You may also want to consider showing the p-values somewhere.

We have removed the significance bars from this figure. We have also zoomed in on the points slightly for clarity and have moved the barchart (previously Figure 7a) showing deviation from random consumption to be next to this plot (it is now Figure 6b).

8. Figure 7: Why is there no error / confidence interval around the final point in panels B-D? Also, why is one standard deviation used as the confidence interval (this calls back to my previous comment on lack of clarity on why SD vs SE is used in different scenarios). Further, stylistically this plot may look better if you make the confidence intervals slightly transparent so that one isn’t sometimes hidden behind the other.

We have implemented these changes: we have added ribbons for the SE and the colours are now transparent and printer-friendly. We have also now explained the lack of CI for the final point in the figure caption (this is because at this point in the experiment only one larva from each group remained alive).

9. Line 231: “Although we did not identify an optimal (fitness-maximising) intake for either carbohydrate or protein, carbohydrate was positively associated with both body size and survival in Osmia bicornis larvae (Figs. 4a, 5a)”. Similar to my comment earlier about intake targets, I feel that you did find some evidence of an optima of sorts in that both traits measured (weight and survival) were maximised at the same ratio of around 1:3.4 (P:C). Many studies using nutritional geometry to look at fitness maximisation refer to optimal intake purely in terms of ratios rather than specific quantities of each macronutrient.

In this case what we meant was that there was no maximum (i.e. peak) in the relationship, whether this be a ratio or a specific point. Rather, fitness increased smoothly up to the boundary without decreasing. Thus, any real optimum could have been outside of the range of diets we offered to the bees. We have clarified this point in our discussion.

10. Line 250: " it is conceivable that this [constant carb intake] represented a narrow window on a more curved intake array that would be visible over a broader diet range." Could you first clarify and second perhaps explain this more? Do you mean that if e.g. intermediate nutritional rails were used, carbohydrate intake might be regulated to a different amount? Is there any knowledge that can be used to suggest what might happen if more nutritional rails were used.

Yes, this is broadly what we meant - we have clarified thus: "it is conceivable that alternative rules of compromise, such as the "equal distance rule" more typically seen in generalist herbivores (Raubenheimer and Simpson 1999; Behmer 2009), might have been evident over a broader array of diets." (lines 288-90).

11. One area that I would like to see addressed more in the discussion is that of parent-offspring conflict. There is a vast literature on parent-offspring conflict over provisioning in situations where parents provide continual care, and I think one of the strengths of this paper is in its implications for how these conflicts can play out in situations where we might not have expected them to exist (because we might have expected that parents provide the food and offspring are stuck with their parents choices).

Thank you for this perceptive and insightful comment - we have taken this on board and added material to the abstract, introduction and discussion to reflect this possibility.

12. A second area that I would have like to have seen addressed is any possible sex differences in results. There is evidence in O. bicornis that larger females can provide larger pollen allocations. As such, larger females may address more in females than males (as males require fewer food provisions). There is also evidence that older females may make different allocations due to their lower efficiency increasing risk of open cell parasitism. The possibility for open cell parasitism presumably represents a time constraint on parents, and may contribute to an explanation for why offspring retain the ability to regulate their nutritional balancing (e.g. parents rush to provide a pollen load and seal the cell, and let the offspring do the optimal nutrient regulating safe from parasitism). There may be reasons why sex differences are not relevant in this study, but given the interesting ecology of the species and the known sex-differences in nutritional needs from many other nutritional geometry studies (Lee 2008 Maklakov 2008 to name a few) I feel that this could be addressed in some way. O. bicornis References for this comment: Seidelmann 2006 [doi:10.1093/beheco/arl017]; Seidelmann et al. 2010 [doi:10.1007/s00265-009-0850-2]

This is an good point which we very much appreciate - in this study, we did not sex the larvae, but this is a clear future direction and more targeted experiments looking at sex effects are planned for future work. NB. We did attempt to account for sex retrospectively in this study

using a combination of the position in the nest and cocoon mass to predict offspring sex, but this ended up bloating the study and reducing statistical power, partly because we had to exclude ambiguous individuals.

We absolutely take the excellent suggestion about the potential importance of parental inefficiency (and its knockon effects for sex differences) in offspring nutritional balancing, and have added that to our introduction (line 51) and discussion (lines 372-8).

Reviewer 2:
I really like this paper by Austin and Gilbert, where the authors asked whether Osmia bicornis, a solitary species, regulated their intake of protein and carbohydrates in the larval stage. The question of how nutrients are utilized by pollinators is interesting from a basic science perspective, but it also has implications for pollinator and landscape conservation. I agree with the authors that much of the research on nutrition and how organisms regulate their dietary intake has been done on adults, not larvae, so this adds an additional level of interest to the paper. I also like that the work was done on a solitary bee, as this provides further context toward understanding how different life histories influence nutrition.

The authors collected larvae from the outdoors and then brought them in the lab to assay consumption of diets with different protein and carbohydrate ratios and nutrient concentrations. Importantly, they also looked at traits like survival and cocoon weight. The authors found that the larvae tended to optimize carbohydrate intake over protein intake. As they discuss in the discussion section, this is not common to all insects, so, again, it provides some context to the idea that life history influences nutrition.

I don’t have any major criticisms of this paper. The introduction is well written and clear, and the authors show a good handle on the literature. The The discussion is thought through well and they do not over-interpret their results. They provide good interpretation of their data and provide support for this interpretation. I do have some minor questions and points that could use some clarification. I list these here:

1) Study organism section – can you describe the pollen ball, how would larvae select certain aspects of the ball that are higher in carbs? Would they just eat the sugar and leave the pollen? Do you see evidence of this preference in observations of bees in the field, such as when they are preparing for winter?

To our knowledge there is no work on whether the pollen ball is heterogeneous in composition, nor of any evidence that larvae differentially reject parts of their provisions. Larvae do have limited mobility that might theoretically allow them to access different parts of the pollen ball. However, they remain in control of *how much* they consume - allowing them at least to regulate one nutrient while tolerating excesses or deficiencies of the other, as we demonstrate here. This is why we presented our choices sequentially rather than simultaneously, as we explain elsewhere. For clarity, we have added a sentence to the relevant section here: “Although O.bicornis larvae are sedentary, they are capable of limited movement,

in principle allowing them to preferentially consume specific parts of a fixed provision (note that other invertebrates are also capable of extracting and consuming preferred nutrients from nutritionally complex food items (Mayntz et al. 2005))" (lines 94-8)

2) Line 175 – just a question, but does the source of protein or carbs affect your result? Would you expect it to?

This is a good point and we have added a line to this effect - the source of protein has been found to affect preferred intake ratios in adult bees. Our line reads as follows: "As a cautionary note, the specific source of nutrients may also affect the preferred ratio: for example, adult honeybees exhibited different target P:C ratios when fed different protein sources (Altaye et al. 2010). Whether larvae are similarly sensitive is still unknown." (Lines 317-9)

3) Line 220 – So you have 9 bee larvae per group? That seems a bit low, but perhaps the limitations of the system necessitate this? Or did you alternate among all 4 of these groups for all 36 bees? Or did you have the 1:1.2 vs 1:3.4 P:C diet at 70% for 18 bees and then 1:1.2 vs 1:3.4 P:C diet at 90% for another 18 bees? It's a bit confusing how the experiment was laid out at this point, so some clarification is warranted. Tables 1 and 2 help, but I wonder if an additional figure would help even more. Perhaps something showing a sequence of what was fed when.

You are right: sample sizes were slightly low because of the study system; each larva was assigned to a single group; each group was given a unique pairwise combination of diets (A1-C1, A1-C2, A2-C1, A2-C2). We have clarified this with a "Total" column in Table 1 and a schematic as requested (Figure 1b). We hope this helps make the design clearer.

Figure 7 is not very helpful and it's not clear what you are trying to show, although it does relate to the choice experiment.

We have now rearranged our figures into what we think is a clearer arrangement: what was Figure 7a has been moved to become Figure 6b. In the new arrangement, Figures 7a,c now depict expected consumption of P and C if provisions are consumed randomly (for each order or presentation), whereas Figures 7b, d now depict what was actually observed.

4) Line 257 – And especially since this is honey-bee collected pollen that you used in your experiments – if bees choose what they forage on, it's possible that this choice results in a different pollen profile across bee species.

You are correct that honeybee pollen is not necessarily an optimal diet for Osmia - a better, although more experimentally intensive, alternative would be to use Osmia's natural provisions as a base. However, it would be difficult to harvest the required number of pollen balls, and difficult to ensure nutritional homogeneity across experimental samples. Moreover, we have demonstrated elsewhere that honeybee pollen results in successful survival and development of Osmia larvae (Tainsh et al, in press, see reference list - and M. Filipiak, pers. comm.).

5) Line 272 – Can you define this “good fit” better?

We have now removed this line because we have now formally tested models with nonlinear terms against the linear models we originally presented. The goodness of fit of all models was tested using standard diagnostic plots.

6) Line 270, 277, 278, 279 – this is important to show, so it was nice to see this.

Thank you!

7) Line 315-316 – That survival became dependent on protein at low carbohydrate levels – this possibly reflects the fact that it takes more energy to break down protein as an energy source. Carbohydrates and lipids are easier to access. I also buy the diapause hypothesis (line 357).

Thank you for this suggestion - we have now incorporated it into the discussion: “It is worth noting that protein did weakly mediate survival in our larvae to some extent, although only at low carbohydrate - possibly as a result of switching to protein as an energy source.” (Lines 303-5)

8) Line 341 – The reference is not formatted correctly.

We have double-checked the format of all references, so this should be cleared up.

9) Line 462 – This sentence seems clunky. Can it be revised?

Unfortunately we were not able to check this because the line number quoted is in the reference section of our copy of the MS, and we were therefore unsure what sentence was being referred to. We’ll be happy to revise if we are able to pinpoint the relevant sentence.

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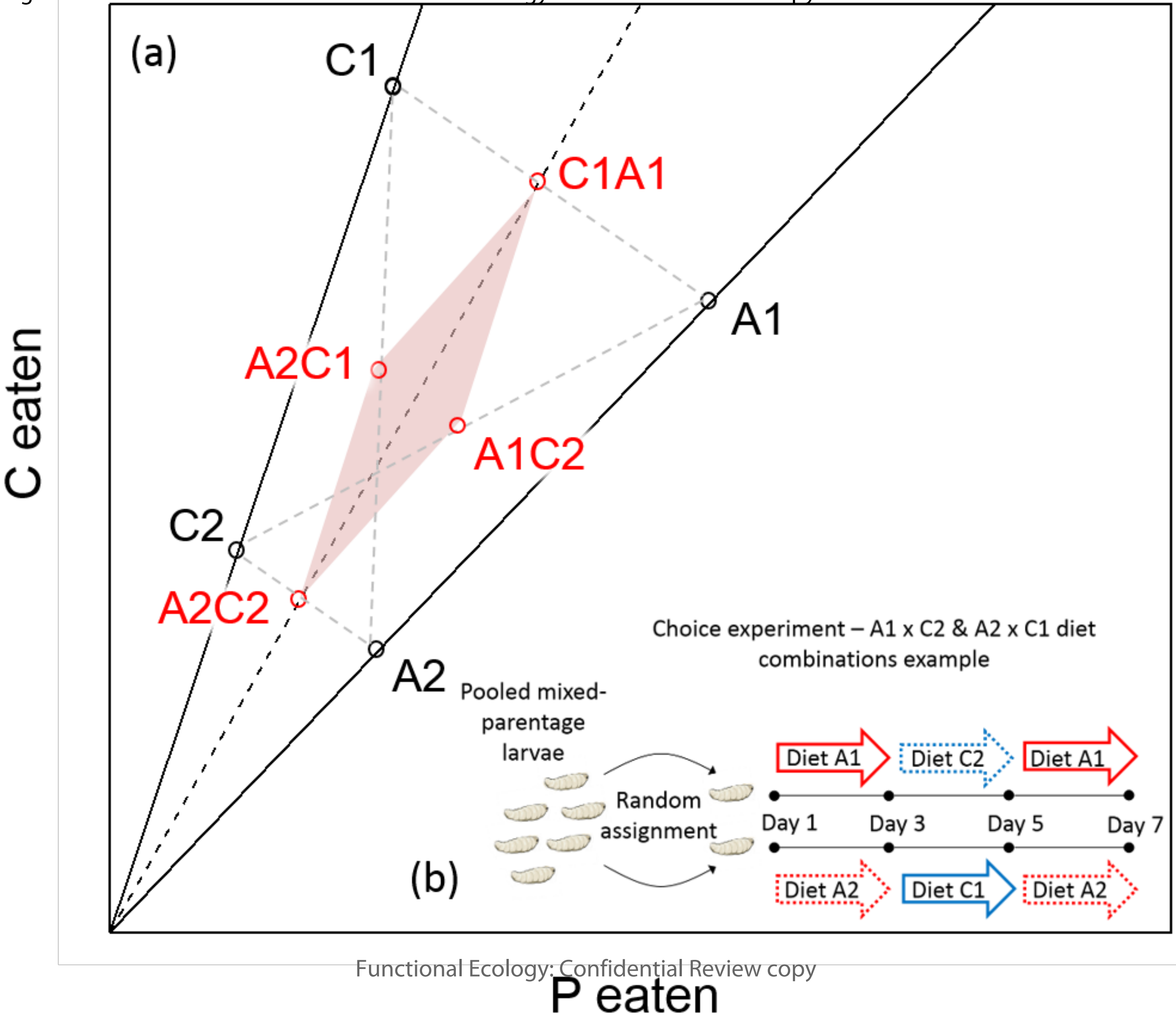
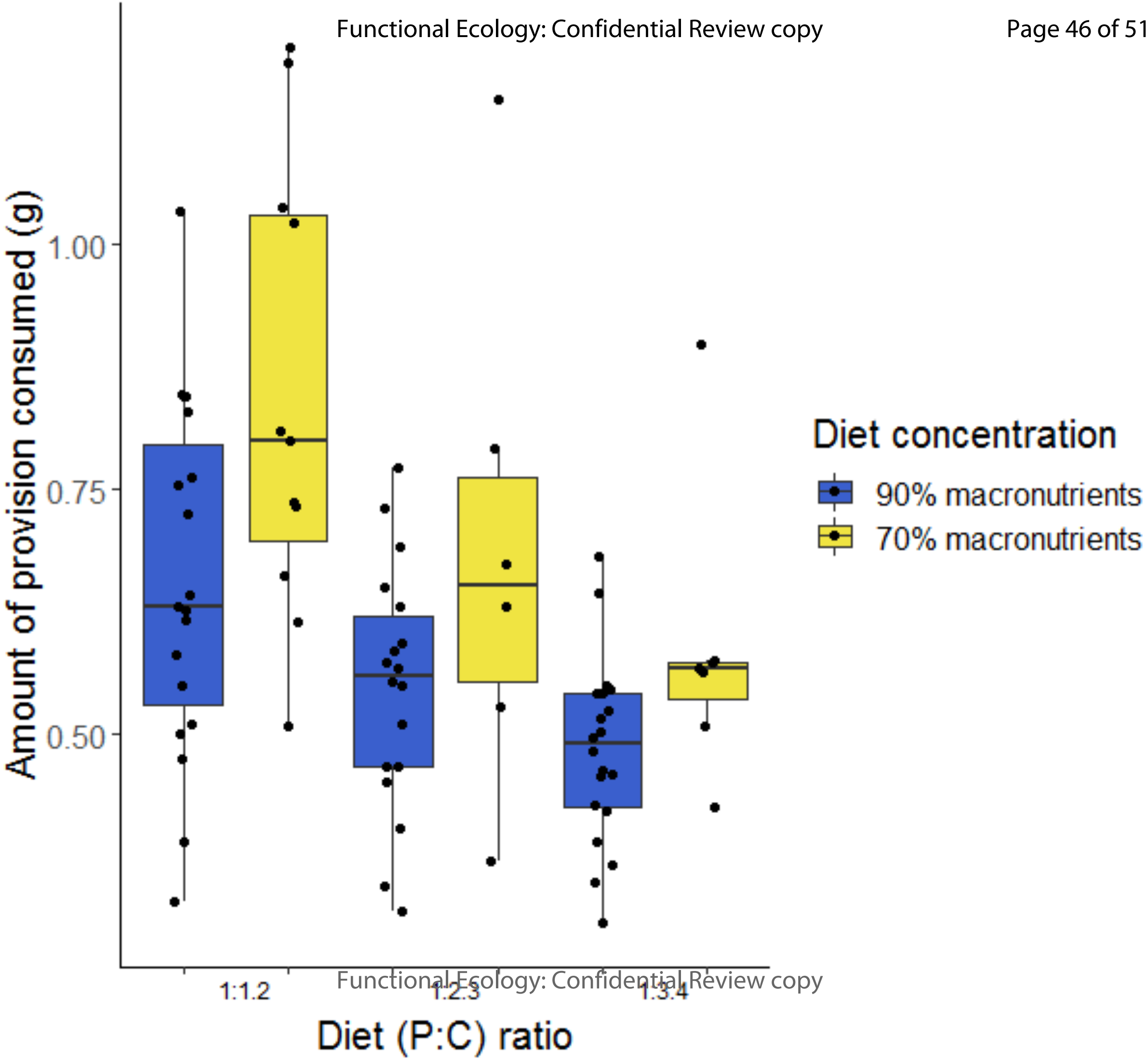
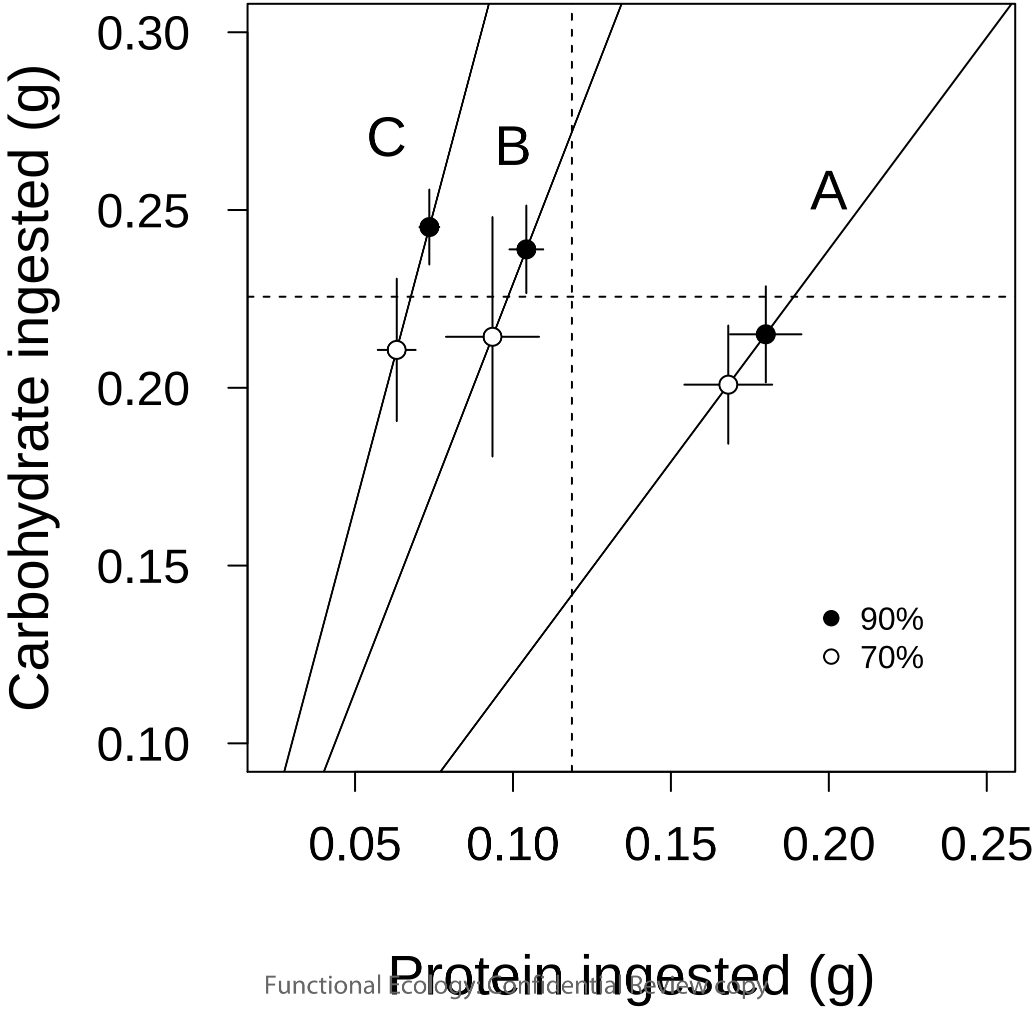

(a)
C eaten
P eaten
C1
C1A1
A1
A2C1
A1C2
C2
A2C2
A2
(b)
Choice experiment – A1 x C2 & A2 x C1 diet combinations example
Pooled mixed-parentage larvae
Random assignment
Diet A1
Diet C2
Diet A1
Day 1
Day 3
Day 5
Day 7
Diet A2
Diet C1
Diet A2


Amount of provision consumed (g)
1.00
0.75
0.50
1:1.2
1:2.3
1:3.4
Diet (P:C) ratio
Diet concentration
90% macronutrients
70% macronutrients


Carbohydrate ingested (g)
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0.25
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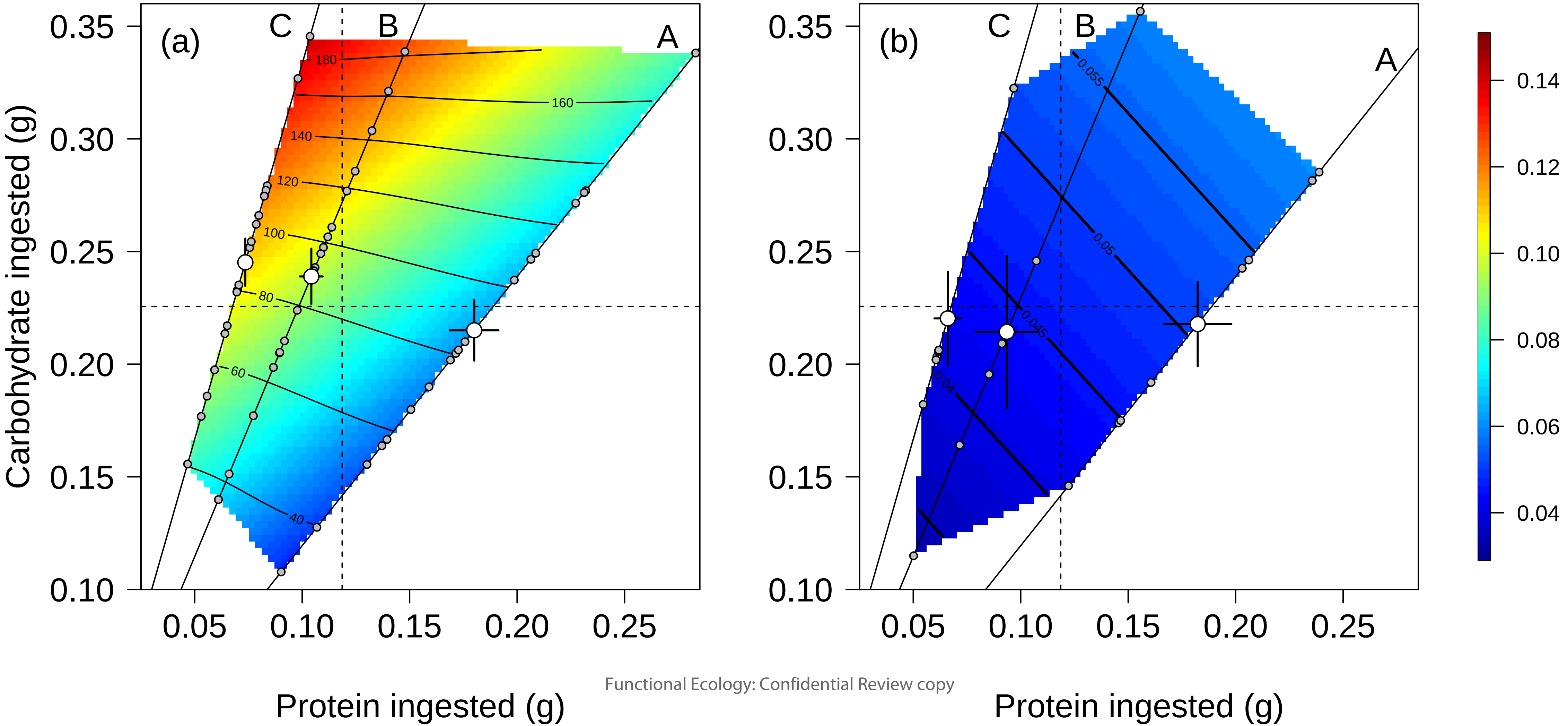
○ 70%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(b)
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Carbohydrate ingested (g)
Protein ingested (g)
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0.15
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160
140
120
100
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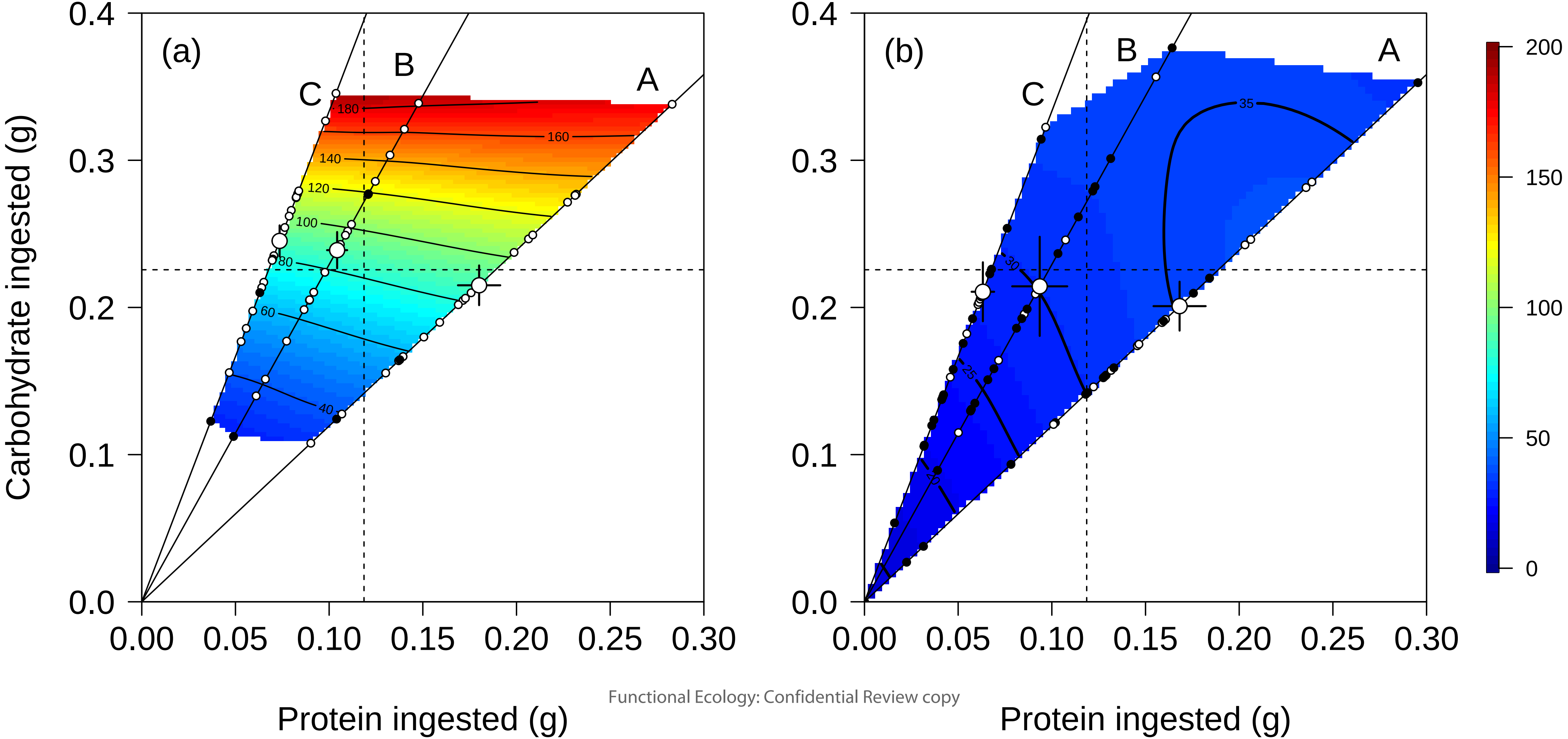
(a)
(b)
A
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C
Carbohydrate ingested (g)
Protein ingested (g)
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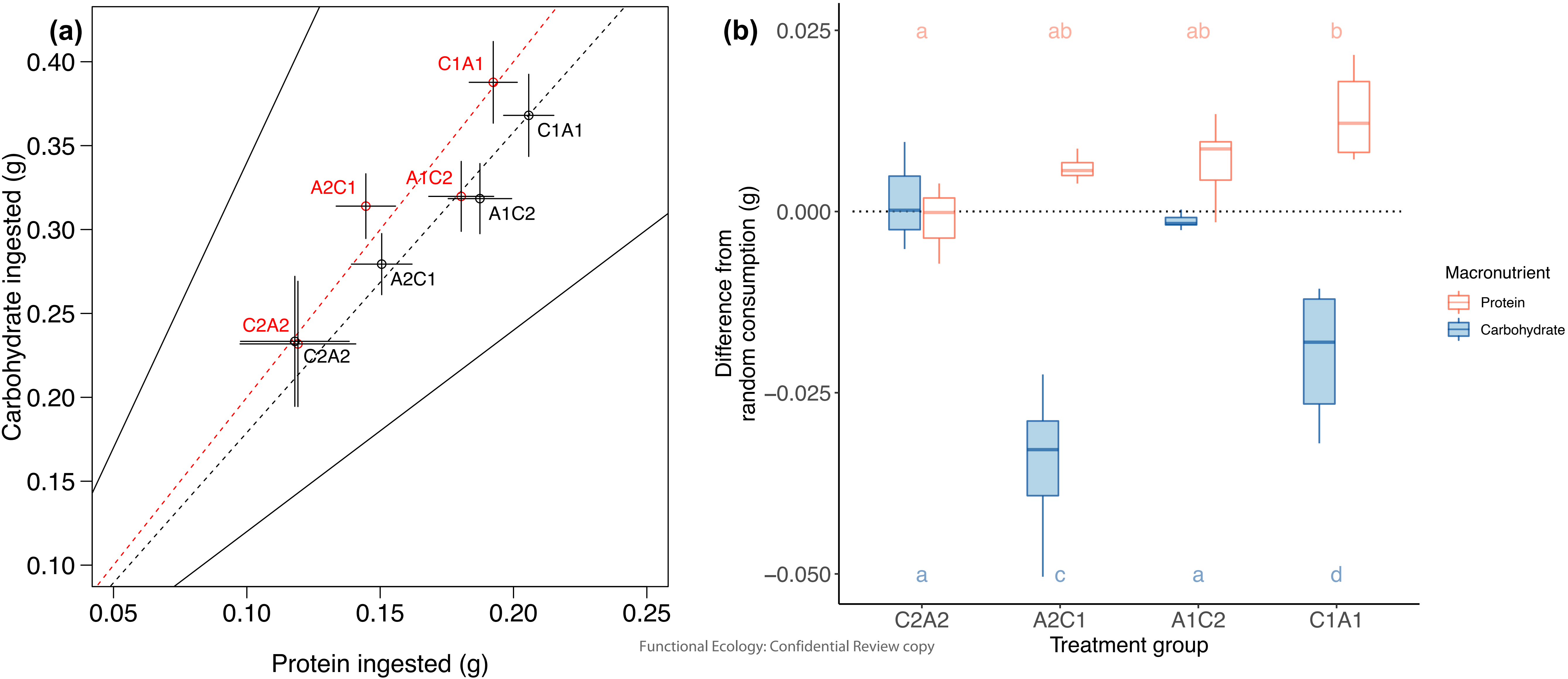
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0.30
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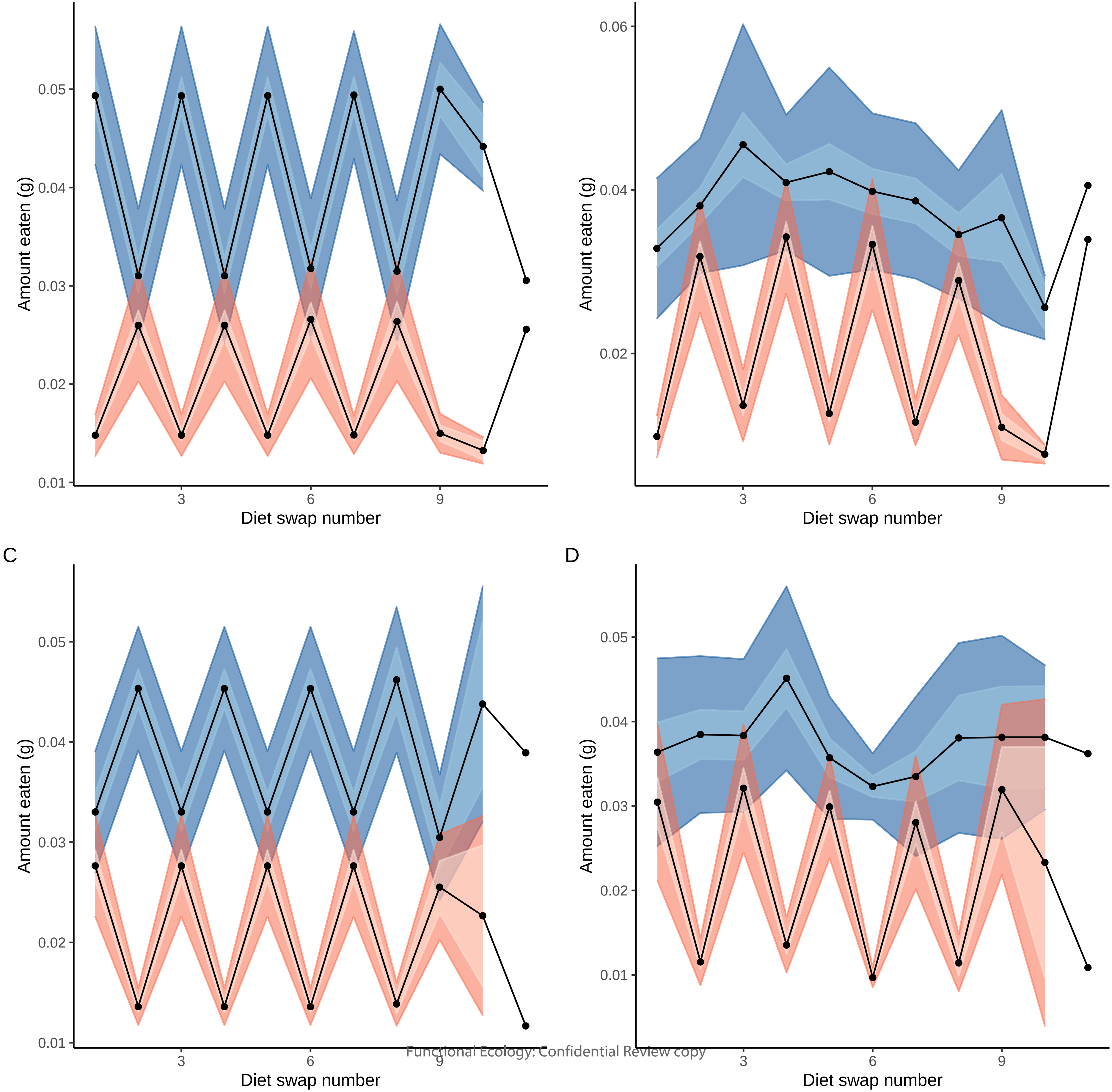
0.20
0.15
0.10
Carbohydrate ingested (g)
0.05
0.10
0.15
0.20
0.25
Protein ingested (g)
C1A1
C1A1
A2C1
A1C2
A1C2
A2C1
C2A2
C2A2
(b)
0.025
0.000
−0.025
−0.050
Difference from random consumption (g)
a
ab
ab
b
a
c
a
d
C2A2
A2C1
A1C2
C1A1
Treatment group
Macronutrient
Protein
Carbohydrate


A
Amount eaten (g)
0.01
0.02
0.03
0.04
0.05
Diet swap number
3
6
9
B
Amount eaten (g)
0.02
0.04
0.06
Diet swap number
3
6
9
C
Amount eaten (g)
0.01
0.02
0.03
0.04
0.05
Diet swap number
3
6
9
D
Amount eaten (g)
0.01
0.02
0.03
0.04
0.05
Diet swap number
3
6
9