

1 **Amino acid quality modifies the quantitative availability of protein for reproduction in**

2 ***Drosophila melanogaster***

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12

13 **Abstract**

14 Diet composition, especially the relative abundance of key macronutrients, is well known to
15 affect animal wellbeing by changing reproductive output, metabolism and length of life.
16 However, less attention has been paid to the ways the quality of these nutrients modify
17 these macronutrient interactions. Nutritional Geometry can be used to model the effects of
18 multiple dietary components on life-history traits and to compare these responses when
19 diet quality is varied. Previous studies have shown that dietary protein quality can be
20 increased for egg production in *Drosophila melanogaster* by matching the dietary amino
21 acid proportions to the balance of amino acids used by the sum of proteins in the fly's *in*
22 *silico* translated exome. Here, we show that dietary protein quality dramatically alters the
23 effect of protein quantity on female reproduction across a broad range of diets varying in
24 both protein and carbohydrate concentrations. These data show that when sources of
25 ingredients vary, their relative value to the consumer can vastly differ and yield very
26 different physiological outcomes. Such variations could be particularly important for meta
27 analyses that look to draw generalisable conclusions from diverse studies.

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31 Introduction

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33 To optimise fitness, organisms must consume a sufficient quantity and quality of nutrients
34 to suit their needs (Hall et al., 2008, Lee et al., 2008, Bong et al., 2014). This is demonstrated
35 by the dramatic changes in reproductive function seen when flies or rodents are subjected
36 to food restriction or changes in diet balance, particularly when the relative proportion of
37 protein to carbohydrates is changed (Widdowson and Cowen, 1972, Good and Tatar, 2001,
38 Carey et al., 2002, Liang and Zhang, 2006, Lee et al., 2008, Skorupa et al., 2008, Simpson and
39 Raubenheimer, 2012, Solon-Biet et al., 2015, Camus et al., 2019).

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41 Food is comprised of dozens of nutrients that interact to modify animal physiology.
42 Understanding how nutritional interactions affect the consumer is complex but can be
43 facilitated using a structured approach such as Nutritional Geometry (Raubenheimer and
44 Simpson, 1997). Nutritional Geometry maps the responses of life history traits to
45 quantitative variations of two or more macronutrients. This is typically performed by
46 exposing animals to one of many diets that vary across a range of calorie compositions and
47 protein to carbohydrate ratios. A nutrient space is defined by axes (generally two) that
48 represent the quantity of nutrients that an organism has eaten – thus any point in space can
49 represent the status of an organism according to its nutritional history. By mapping an array
50 of organisms with different dietary histories into this space, their collective phenotypic
51 responses can be fitted by an overlaid surface represented by a heat map (z- axis). This
52 allows for the modelling of the interactive effects of nutrients on phenotypes of interest.
53 Although conceptually simple, assessing phenotypic responses to nutrition through the
54 perspective of Nutritional Geometry has revealed new understanding of biology and, in
55 some cases, has unified apparently conflicting interpretations about the way organisms
56 respond to diet change (Solon-Biet et al., 2016).

57 Nutritional Geometry experiments have shown that variation in two of the energy-yielding
58 macronutrients, protein and carbohydrate, affect the expression of many traits. In particular
59 the lifespan and reproduction of adult fruit flies (*Drosophila melanogaster*) are shaped by

60 the interactive effects of dietary protein and carbohydrate (Lee et al., 2008, Skorupa et al.,
61 2008, Jensen et al., 2015). In the case of protein, more recent work has shown that the
62 proportion of its constituent amino acids has an important role to play in protein's effects
63 on these traits (Grandison et al., 2009, Piper et al., 2017).

64 The dietary amino acid requirements of female flies for optimal egg production can be
65 determined from its genome by a process termed exome matching (Piper et al., 2017). To
66 exome match a diet, we *in silico* translate the exome of the consumer, sum the abundance
67 of each amino acid across all proteins, and find the relative proportion of each amino acid.
68 We then use this proportion as the basis for the abundance of each amino acid in the food.
69 By matching the dietary protein quality to the fly exome in this way, we found that for a
70 fixed mass of amino acids, exome matched diets supported higher levels of reproduction
71 than diets that were mismatched (Piper et al., 2017). Together, these data show that dietary
72 amino acid balance is important for determining fitness outcomes.

73 Although dietary amino acid balance is important, Nutritional Geometry experiments that
74 measure fitness responses to diets invariably treat protein as a single nutrient dimension
75 with a fixed proportion of all 20 amino acids. This is reasonable for experiments in which the
76 protein source is held constant across all diets. However, proteins vary in quality when
77 attained from different sources and, like many natural ingredients, the same type of protein
78 may vary in quality between locations and seasons. Given this, it may be difficult to directly
79 compare the effects of consumed "protein" on a trait when the data are from different
80 studies. To examine these effects, we designed an experiment using a Nutritional Geometry
81 design to assess how changing dietary protein quality modifies the interactive effects of the
82 amino acid (protein; P) to carbohydrate (C) ratio on egg laying. We designed an array of
83 diets varying in P:C ratio for each of two different amino acid (protein) mixtures. These
84 mixtures varied in the relative proportion of each amino acid, as well as the identity of the
85 most limiting essential amino acid and the degree to which it is predicted to be limiting.

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88 Method

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90 *Animal husbandry*

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92 We used the Dahomey outbred population of *Drosophila melanogaster* (Mair et al., 2005).

93 Routine rearing and maintenance of flies employed the techniques and sugar/yeast (SY) diet

94 described in Bass et al. (2007). All flies were maintained at 25°C with a 12 hr: 12 hr light dark

95 photoperiod. For the experiment, a population of flies was age synchronised as in Piper and

96 Partridge (2016).

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98 *Experimental diets*

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100 Completely defined synthetic (holidic) diets were made according to Piper et al. (2014), in

101 which free amino acids are used to make up protein equivalents. To convert amino acids to

102 protein equivalents, we used the molar quantities of nitrogen and the assumption that N

103 makes up 16% of whole proteins (Sosulski and Imafidon, 1990). Two protein qualities,

104 defined by their amino acid ratios, were compared: FLYaa (matched to the amino acid ratio

105 of the exome of adult flies), and MMAa (a ratio considered mis-matched to the flies'

106 requirements). The relative proportion of each amino acid within each amino acid ratios are

107 displayed in Table 1. From this point on, the concentration of total amino acids is referred to

108 as protein (P), calculated according to the above method.

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110 For both amino acid ratios, we generated diets that were one of five P:C ratios (1:3.6, 1:1.8,

111 1:1.1, 1:0.8, 1:0.6) and one of four caloric densities (66.8 kcal/L, 111.3 kcal/L, 155.8 kcal/L,

112 200.3 kcal/L), where dietary energy densities were estimated by calculation, using a value of

113 4 kcal/g for both protein and carbohydrates (Table 2). Thus, 20 diets were employed to test

114 the effect on egg laying of each amino acid ratio.

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119 **Table 1. The relative proportions of each amino acid in the two amino acid ratios tested, FLYaa and MMaa.**

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Amino acids			Ratio	
			MMaa	FLYaa
Essential amino acids	Phenylalanine	F	0.027	0.037
	Histidine	H	0.022	0.026
	Isoleucine	I	0.077	0.052
	Lysine	K	0.044	0.057
	Leucine	L	0.052	0.094
	Methionine	M	0.018	0.025
	Arginine	R	0.016	0.057
	Threonine	T	0.057	0.056
	Valine	V	0.081	0.062
	Tryptophan	W	0.008	0.010
Non-essential amino acids	Alanine	A	0.133	0.075
	Cysteine	C	0.001	0.017
	Aspartate	D	0.043	0.053
	Glutamate	E	0.058	0.063
	Glycine	G	0.144	0.062
	Asparagine	N	0.044	0.047
	Proline	P	0.044	0.052
	Glutamine	Q	0.058	0.046
	Serine	S	0.061	0.079
	Tyrosine	Y	0.013	0.031

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135 **Table 2.** The equivalent protein: carbohydrate (P:C) ratio, displayed with the nutrient densities.

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P:C equivalent	Sum mass of amino acids (g/L)	Equivalent protein (g/L)	Equivalent carbohydrate (g/L) ¹	Estimated caloric content (kcal/L)
1:3.6	3.92	3.54	12.77	66.8
1:3.6	6.53	5.91	21.29	111.3
1:3.6	9.14	8.27	29.81	155.8
1:3.6	11.75	10.63	38.32	200.3
1:1.8	6.42	5.81	10.27	66.8
1:1.8	10.7	9.68	17.12	111.3
1:1.8	14.98	13.55	23.97	155.8
1:1.8	19.26	17.42	30.82	200.3
1:1.1	8.32	7.52	8.38	66.8
1:1.1	13.86	12.54	13.96	111.3
1:1.1	19.41	17.55	19.54	155.8
1:1.1	24.95	22.57	25.13	200.3
1:0.8	9.57	8.66	7.12	66.8
1:0.8	15.95	14.43	11.87	111.3
1:0.8	22.33	20.20	16.62	155.8
1:0.8	28.71	25.97	21.37	200.3
1:0.6	10.99	9.94	5.70	66.8
1:0.6	18.32	16.57	9.50	111.3
1:0.6	25.64	23.20	13.30	155.8
1:0.6	32.97	29.65	17.10	200.3

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139 *Experimental set-up*

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141 Flies were housed in devices called dFlats, which are made up of a block of Perspex with 12-
142 wells drilled into them, such that the drilled wells have a volume the same as standard fly
143 vials (FS32, Pathtech) and their arrangement is such that the 12 openings match the position
144 of wells in a 12-well plate (based on the design: <https://www.flidea.tech/projects>). 3ml food
145 was dispensed into each of the wells in a 12-well plate. Each dFlat well contained 10 mated
146 female flies and we maintained one dFlat for each of the 20 holidic experimental diets (12
147 replicate wells x 10 flies = 120 flies per diet).

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¹ Carbohydrate is added to the diet as sucrose.

149 *Reproduction experiment*

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151 Once a week for 3 weeks the number of eggs laid on the media over an 18-hour period was
152 counted and recorded. Measuring reproductive output during the first weeks of egg laying
153 has shown to be representative of life-long reproduction of flies (Chapman and Partridge,
154 1996, Muller et al., 2001). For each well in each dFlat device, the number of eggs laid per
155 female on each experimental day (day 8, 15 and 22) was calculated. The value for eggs laid
156 by an average female in a well was summed across days and used to calculate the
157 cumulative egg laying of an average female in a food type. We call this value the index of
158 reproduction. The number of eggs in each well and food type was obtained by taking images
159 using a web camera attached to a stereomicroscope. The images were then processed in
160 Image J to acquire the correct image size, which were then automatically counted in the
161 software called QuantiFly (Waithe et al., 2015).

162

163 *Statistical analyses*

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165 All analyses were conducted using R (version 3.3.0, available from [http://www.R-](http://www.R-project.org/)
166 [project.org/](http://www.R-project.org/)). To analyse the relationship between our index of reproduction and the
167 protein (P) and carbohydrate (C) concentration, we generated separate response surfaces
168 for each of the two amino acid ratios. Each surface was estimated using multivariate
169 second-order polynomial regression, whereby the linear, quadratic and cross-product terms
170 from this model capture the linear and non-linear effects of P and C concentration on fly
171 reproduction. For each amino acid ratio, the minimum adequate model for each linear
172 model was found by determining if eliminating the most complex parameter significantly
173 reduced the model fit. We visualised the response surface of each amino acid ratio using
174 predictions derived from thin-plate splines from the fields package (see
175 www.github.com/NCAR/Fields) and subsequently visualised via the ggplot package.

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178 Results

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180 To capture the effects of differences in protein quality on egg production of *D.*
181 *melanogaster*, we made media containing two different amino acid ratios, one known to be
182 mismatched to fly requirements for egg laying (MMaa) and the other known to be matched
183 precisely to the fly's exome (FLYaa) (Piper et al., 2017). For each amino acid ratio, an array
184 of 5 different P:C ratios, each at 4 different nutrient densities, was tested. Thin-plate splines
185 were used to visualise egg laying response surfaces of flies maintained on the different diets
186 (Figure 1A and 1B).

187

188 Across all food types, flies maintained on the MMaa diets laid fewer eggs per female per day
189 than those on the FLYaa diets (MMaa diet, 24.07 ± 1.42 ; FLYaa 31.69 ± 2.14 ; one-way
190 ANOVA: $F_{1,33} = 11.205$, $p < 0.001$). For flies on both the MMaa and the FLYaa diet, there was
191 a significant effect of the linear component of protein, such that egg laying increased with
192 increasing dietary protein concentration (Table 2.). For FLYaa, we also found the quadratic
193 term for protein concentration to be significant, which is shown in the plots as the peak of
194 egg laying in FLYaa occurred at intermediate concentrations of protein (P:C of 1:0.8) (Figure
195 1a; Table 2). From this point, egg laying dropped away as the protein concentration either
196 increased or decreased (Figure 1a; Table 2). In contrast, peak egg laying on MMaa occurred
197 on the food with the highest nutrient density with P:C of 1:0.6. There was no detectable
198 effect of carbohydrate concentration on egg production across foods of both amino acid
199 ratio and there was also no significant interaction between carbohydrate and protein
200 concentration detected (Table 2). These results show that for the range of diets we tested,
201 protein was the principle determinant of egg production, and that per gram of amino acids
202 supplied, FLYaa supported higher levels of egg laying than MMaa.

203

204

205 **Table 2.** Egg production on each amino acid ratio (FLYaa and MMaa) was modelled using linear predictors for
206 protein (P) and carbohydrate (C) and their interaction (P x C). Because the data appear non-linear, we also
207 assessed the quadratic terms of protein (P^2) and carbohydrates (C^2). Minimum adequate models are reported.
208 β , indicates the the slope estimate for each variable, and; SE, the standard error. Highlighted bold signifies
209 significance, * $p < 0.05$; ** $p < 0.001$.

210

Amino acid ratio		P	C	P^2	C^2	P x C
FLYaa	β	3.250	-0.206	-0.127	-0.007	0.042
	SE	1.393	1.203	0.037	0.024	0.050
	t-value	2.332*	-0.171	-3.464**	-0.301	0.846
MMaa	β	1.186	0.129	N/A ²	N/A ²	-0.032
	SE	0.502	0.341			0.027
	t-value	2.365*	0.377			-1.184

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^{2 2} N/A, not applicable. For the MMaa model, P^2 and C^2 were not significant in the model and so were removed during model reduction.

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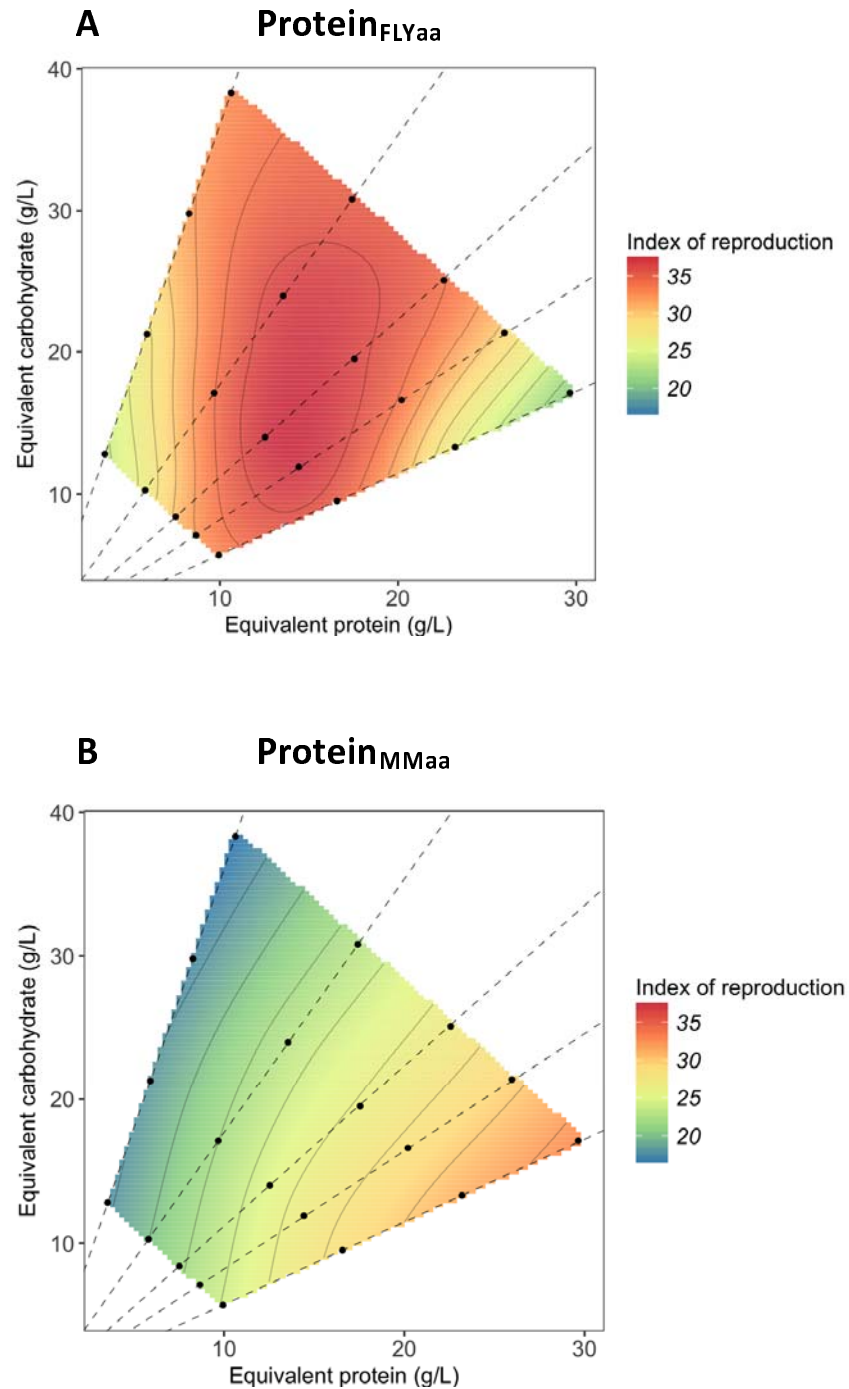


Figure 1. Egg laying varies with the quantity of carbohydrates and the quantity and quality of protein. Flies were maintained on diets containing amino acids in the ratio of either **(A)** FLYaa (fly exome matched amino acid ratio) or **(B)** MMaa (mis-matched amino acid ratio). The black dots represent the individual 20 diets from which the index of reproduction was assessed and the dashed lines represent nutritional rails of fixed P:C.

245 Discussion

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247 The ratio of dietary protein to carbohydrate in the diet has an important role in determining
248 reproduction in *Drosophila melanogaster*: higher density diets with greater P:C ratios
249 support higher female egg laying (Mair et al., 2005, Lee et al., 2008, Skorupa et al., 2008,
250 Jensen et al., 2015). Here we show that varying the protein quality of a diet can also
251 dramatically alter its bioavailability for reproduction of female *D. melanogaster*. These data
252 demonstrate how diverse outcomes in important fitness traits can occur when the quality of
253 food ingredients vary.

254

255 Protein is often a limiting component of the diet for terrestrial animals (White, 1993), which
256 means that changes in the quality of dietary protein consumed should have observable
257 effects on fitness for many animals in diverse settings. Indeed, supplementing the diet of
258 wild cotton rats and cottontail rabbits with an essential amino acid (methionine) can
259 improve their reproductive success and increase population size (Lochmiller et al., 1995,
260 Webb et al., 2005), while maintaining blue tits on a diet supplemented with amino acids
261 matched for egg protein formation laid more eggs per clutch than those that received a
262 mismatched balance of amino acids (Ramsay and Houston, 1998). Similarly, female
263 copepods fed on a diet containing essential amino acid profiles that were closely matched
264 to female body composition had higher reproductive success (Guisande et al., 1999) and,
265 supplementing amino acids in the diet of livestock, like boars and chickens, can increase
266 reproductive output and yield more lean muscle (Dong et al., 2016, Cerrate et al., 2019).
267 Finally, recent work in the lab on flies and mice has shown that differences in protein
268 quality, specified at the level of essential amino acid balance, can have dramatic effects on
269 animal reproduction and feeding behaviour (Leitao-Goncalves et al., 2017, Piper et al., 2017,
270 Solon-Biet et al., 2019). Thus, changing the quality of dietary protein by altering the
271 proportion of amino acids can have important effects on fitness traits of both laboratory-
272 reared and wild animals.

273

274 In our experimental diets, we found large changes in egg output because the amino acid
275 ratio was altered so that the bioavailability of the nitrogen source varied 2.5-fold (Piper et
276 al., 2017). In other words, the level of FLYaa that was required to support maximal egg
277 output was ~15g/L, which is 2.5-times less than the amount of MMaa (37 g/L) that would be
278 required to achieve the same output. Thus, smaller amounts of high-quality food are
279 required for optimal egg laying. With defined diets, this large difference in protein quality is
280 easily generated because each amino acid can be independently manipulated over a wide
281 range of concentrations. Interestingly, similarly large differences in the relative abundance
282 of amino acids (i.e. changes in protein quality) can also be found between natural proteins
283 in whole foods. For example, when comparing the average amino acid profiles across food
284 groups published in an FAO report (Food Policy and Food Science Service, 1970), we found
285 that for a fixed quantity of protein, the average amino acid proportions in meat represent
286 ~2-fold higher quality than the average legume-based protein. This is because the limiting
287 essential amino acid in legume protein, methionine, is more deficient compared to the
288 limiting essential amino acid, leucine, in meat protein. Thus, we predict that using different
289 dietary components to feed animals or humans will result in dramatic changes to physiology
290 and behaviour. Furthermore, without explicit knowledge of the source of ingredients and
291 their quality, it will be difficult to extract generalisable conclusions from meta-analyses that
292 incorporate diverse studies.

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294 It is important to note that protein is not the only compound axis in most published
295 Nutritional Geometry experiments. For example, the other energy yielding macronutrients,
296 carbohydrate and fat, also exist as a diverse array of molecules in food. This can be even
297 more complicated since in typical studies with flies, sucrose and yeast are often used the
298 sources of carbon and protein, respectively (Bass et al., 2007, Min et al., 2007, Lee et al.,
299 2008), and yeast contains various types of carbohydrates as well as other nutritional
300 components, such as sterols, nucleic acids, vitamins, and minerals (Lange and Heijnen,
301 2001). In addition to our findings about protein quality, other work has shown that varying
302 the identity of the carbohydrate component of the diet from sucrose to fructose can alter
303 fly physiology (Lushchak et al., 2014). Thus, it will be important in future studies to

304 understand the relative contribution of different carbohydrates, fats and other nutrients in
305 modifying fitness traits.

306

307 Conclusion

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309 By showing that the precise amino acid composition of dietary protein is key for dictating
310 female fecundity, we demonstrate the need for more information to be provided when
311 labelling composite nutritional axes in Nutritional Geometry experiments. In particular,
312 protein should be labelled with its source to indicate its quality – a metric that would be
313 further improved if the most limiting essential amino acid were identified by referencing the
314 dietary protein amino acid profile to the *in silico* translated exome of the consumer (Piper et
315 al., 2017). We anticipate this will facilitate the comparability of data between studies.

316

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318

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