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1 **Larval diet affects adult reproduction but not survival regardless of injury and**
2 **infection stress in *Drosophila melanogaster***

3

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8 **ABSTRACT:**

9 Early-life conditions have profound effects on many life-history traits. In particular,
10 early-life diet affects both juvenile development, and adult survival and reproduction. Early-
11 life diet also has consequences for the ability of adults to withstand stressors such as starvation,
12 temperature and desiccation. However, it is less well known how early-life diet influences the
13 ability of adults to respond to infection. Here we test whether varying the larval diet of female
14 *Drosophila melanogaster* (through altering protein to carbohydrate ratio, P:C) influences the
15 long-term response to injury and infection with the bacterial pathogen *Pseudomonas*
16 *entomophila*. Given previous work manipulating adult dietary P:C, we predicted that adults
17 from larvae raised on higher P:C diets would be more likely to survive infection and have
18 increased reproduction, but shorter lifespans and an increased rate of ageing. For larval
19 development, we predicted that low P:C would lead to a longer development time and lower
20 viability. We found that early-life and lifetime egg production were highest at intermediate to
21 high larval P:C diets, but there was no effect of larval P:C on adult survival. Larval diet had no
22 effect on survival or reproduction post-infection. Larval development was quickest on
23 intermediate P:C and egg-to-pupae and egg-to-adult viability were higher on higher P:C.
24 Overall, despite larval P:C affecting several traits measured in this study, we saw no evidence
25 that larval P:C altered the consequence of infection or injury for adult survival and early-life
26 and lifetime reproduction. Taken together, these data suggest that larval diets appear to have a
27 limited impact on adult response to infection.

28

29 **Key words:** larval diet, infection, injury, life-history trade-offs, Drosophila, *Pseudomonas*
30 *entomophila*

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31 **INTRODUCTION:**

32 Early-life conditions are important in determining many key life-history traits
33 (reviewed in Metcalfe and Monaghan, 2001, 2003). In particular, diet in early-life has been
34 shown to have profound effects on later life-history traits such as survival and reproduction,
35 and poor early nutrition can have costs associated with catch-up growth in adulthood (reviewed
36 in Metcalfe and Monaghan, 2001, 2003). Nutrition is also important for the ability of an
37 organism to respond to a number of key environmental stresses such as infection or temperature
38 stress, as has been demonstrated in both juveniles (e.g. Lee *et al.*, 2006; Venesky *et al.*, 2012;
39 Kutz, Sgrò and Mirth, 2019) and adults (e.g. Peck, Babcock and Alexander, 1992; Kim, Jang
40 and Lee, 2020; Ponton *et al.*, 2020). However, work on the effect of early-life diet on adult
41 responses to environmental stress is much more limited (but see e.g. Andersen et al. 2010;
42 Kelly and Tawes 2013; Knutie et al. 2017). To investigate the long-term effects of early-life
43 diet on adult traits and infection stress resistance, here we combine multiple larval diets, apply
44 injury and infection to the adults and measure both larval and adult life-history responses in
45 *Drosophila melanogaster.*

46 A vast literature exists using various approaches to manipulate diet and investigate the
47 consequences of these manipulations (reviewed in Simpson and Raubenheimer, 2012). A
48 particularly well-investigated manipulation is adult dietary restriction (DR), the restriction of
49 calories or a particular nutrient without malnutrition, which has been shown to increase
50 lifespan, delay ageing and reduce reproduction across a wide range of species (e.g. Mair and
51 Dillin, 2008; Simpson *et al.*, 2017). Recent evidence suggests that this effect is mostly driven
52 by changes in the protein to non-protein ratio of the diet, often protein to carbohydrate (P:C)
53 ratios, particularly in insects (e.g. Lee *et al.*, 2008; Simpson *et al.*, 2017, but see Speakman,
54 Mitchell and Mazidi, 2016). Regarding the effects of juvenile diet on juvenile and adult traits,
55 there have been many studies testing the effects of caloric content (e.g. May, Doroszuk and
56 Zwaan, 2015; Adler, Telford and Bonduriansky, 2016; House *et al.*, 2016; Littlefair and Knell,
57 2016; Hooper *et al.*, 2017; Krittika, Lenka and Yadav, 2019). As it has become clearer that
58 macronutrient content is more important than total caloric content, recent work has shifted to
59 testing how the macronutrient composition of the juvenile diet may affect both juvenile and
60 adult traits (reviewed in Nestel *et al.*, 2016). However, these studies often do not consider
61 additional stressors (but see e.g. Andersen *et al.*, 2010; Kelly and Tawes, 2013; Pascacio-
62 Villafán *et al.*, 2016).

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63 Changing juvenile diet has been shown to alter the rate and success of the
64 developmental period in both holometabolous (reviewed in Nestel *et al.*, 2016) and
65 hemimetabolous insects (e.g. Hunt *et al.*, 2004; Kelly and Tawes, 2013; Houslay *et al.*, 2015).
66 In general, juveniles on higher or intermediate P:C diets have a quicker development rate and
67 improved development success (e.g. Matavelli *et al.*, 2015; Rodrigues *et al.*, 2015; Silva-Soares
68 *et al.*, 2017, but see Cordes *et al.*, 2015; Houslay *et al.*, 2015; Davies *et al.*, 2018; Gray,
69 Simpson and Polak, 2018; Kim *et al.*, 2019). In holometabolous insects, larvae have to pass
70 several size assessment thresholds for successful pupation, and it has been suggested larvae
71 feed until they have enough resources for metamorphosis and to survive the non-feeding state
72 of pupation (reviewed in Mirth and Riddiford, 2007; Nestel *et al.*, 2016). As amino acids from
73 protein in the diet signal a cell cycle for growth of tissues (Britton and Edgar 1998; Colombani
74 et al. 2003), and larvae do not develop on diets lacking in essential amino acids (e.g. Chang,
75 2004), it seems that higher larval P:C diets facilitate quicker growth and accumulation of
76 essential resources that allow successful development into adulthood. There may be an upper
77 limit after which increasing P:C has detrimental effects, potentially due to toxic effects of
78 protein metabolism (Fanson et al. 2012), for example the accumulation of toxic wastes in food
79 (reviewed in Simpson and Raubenheimer, 2009) or the highest P:C diets being limiting in
80 carbohydrates, but the exact reasons are currently unknown.

81 Early-life diet has also been shown to have important consequences for many adult life-
82 history traits, including reproduction, lifespan and ageing (reviewed in Metcalfe and
83 Monaghan, 2001). In insects, measures of both early-life and lifetime egg production peak on
84 higher or intermediate larval P:C diets (e.g. Rodrigues *et al.*, 2015; Silva-Soares *et al.*, 2017;
85 Duxbury and Chapman, 2019, but see Matavelli *et al.*, 2015). For lifespan, results of early-life
86 dietary manipulation in insects are mixed, with lifespan being maximised at different P:C
87 levels, and even no effect of P:C depending on the study (e.g. Runagall-McNaull,
88 Bonduriansky and Crean, 2015; Stefana *et al.*, 2017; Davies *et al.*, 2018; Duxbury and
89 Chapman, 2019). Indeed, a recent meta-analysis showed no consistent effect of early-life diet
90 on adult lifespan across taxa (English and Uller 2016). The age-related decline in various traits
91 may also be altered by larval diet, however the direction of the effect is again unclear, with
92 higher P:C or calorie diets leading to quicker, slower or having no effect on ageing (Tu and
93 Tatar 2003; May et al. 2015; Adler et al. 2016; Hooper et al. 2017). The effect of larval diet on
94 adult reproduction may be a result of adults being able to use nutrient stores of, for example,
95 protein or lipids in body tissues, including the fat body and haemolymph (reviewed in Boggs,
96 2009; Nestel *et al.*, 2016). However, it is less clear how these stored resources could affect

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97 lifespan. Potential explanations for inconsistencies in results across studies and life-history
98 traits include that stored nutrients may trade-off between different adult life-history traits in an
99 environment or species-specific manner, that juvenile diet effects may be dependent on adult
100 food environment, or that storage of nutrients can be re-allocated in adulthood, for example by
101 reabsorption of flight muscles (reviewed in Boggs, 2009; Nestel *et al.*, 2016). Overall, it seems
102 that increasing P:C in the larval diet increases reproduction and juvenile diet often has effects
103 on adult lifespan, but the directionality of the effects are inconsistent.

104 Despite the wealth of information on how larval diet affects multiple adult traits, studies
105 focusing on adult stress resistance are rarer, despite the likelihood that stress resistance is a key
106 trait in natural populations (e.g. Hoffman and Hercus, 2000; Van Voorhies, Fuchs and Thomas,
107 2005; Kawasaki *et al.*, 2008; Adamo, 2020). Some data exist on a small number of
108 environmental stressors including temperature, desiccation and starvation (Andersen et al.
109 2010; Pascacio-Villafán et al. 2016; Davies et al. 2018), however the direction of effects are
110 often mixed and potentially stress-specific. Particularly poorly studied is the effect of larval
111 diet on adult infection response. In *Anopheles gambiae,* melanising ability decreased with
112 severity of larval calorie restriction (Suwanchaichinda and Paskewitz 1998). Female *Gryllus*
113 *texensis* crickets on lower P:C as nymphs and adults survive better over five days post-infection
114 (Kelly and Tawes 2013). As this study changed both adult and larval diet, it is not possible to
115 disentangle the effect of larval diet alone. Without a direct immune stress, there is evidence for
116 differential adult response to immune challenge due to larval diet. For example, the production
117 of antimicrobial peptides (AMPs) decreased with lower larval P:C in *D. melanogaster* (Fellous
118 and Lazzaro 2010). For other immune response measures, in *Lestes viridis* damselflies, lower
119 calories and starvation led to reduced phenoloxidase (PO) activity and haemocyte
120 numbers/levels in adults (Rolff et al. 2004; De Block and Stoks 2008). However, to our
121 knowledge, no study to date has tested the effect of larval dietary P:C on adult life-history traits
122 when exposed to infection or injury stress.

123 Several hypotheses have been put forward to explain the effect of larval diet on adult
124 survival post-infection. These include increased stress response capability due to overall better
125 body condition, and increased investment into immunity, either through the growth of specific
126 tissues, or through increased availability of limiting nutrients (Fellous and Lazzaro 2010). The
127 first suggestion is supported by studies where both immune response and body condition are
128 lower with starvation (Suwanchaichinda and Paskewitz 1998; Rolff et al. 2004), but the
129 independent effects are difficult to separate (Fellous and Lazzaro 2010). The second hypothesis

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130 is supported by studies where indicators of immune response increase in adults or pupae outside
131 of effects on general body condition with a diet higher in P:C in *D. melanogaster* (Fellous and
132 Lazzaro 2010) or plant diets of worse quality in *Epirrita autumnata* moths (Klemola et al.
133 2007). In general protein seems to be an important nutrient in relation to survival post-infection
134 (e.g. Lee *et al.*, 2006; Povey *et al.*, 2009, 2014; Cotter *et al.*, 2011, 2019; Savola *et al.*, 2020),
135 suggesting individuals developing on higher larval P:C diets should have improved resistance
136 to infection.

137 To test the effects of larval P:C on larval and adult life-history and adult survival post-
138 infection, we reared larvae on various P:C diets and exposed female adults to injury and
139 infection stress (with a bacterial pathogen, *Pseudomonas entomophila*). For the larvae, we
140 measured development time to adulthood and measures of viability (egg-to-pupae, egg-to-adult
141 and pupae-to-adult viability). For the adults, we measured the key life-history traits of survival
142 and reproduction. We predicted that low P:C larval food would lead to longer development
143 time and lower viability across all stages. If larval diet affects adult life-history traits
144 independent of adult food, we predicted a similar effect to that observed when P:C ratio is
145 manipulated in adults (e.g. Lee *et al.*, 2008; Jensen *et al.*, 2015; Savola *et al.*, 2020), with low
146 larval P:C extending lifespan, and reducing reproduction and the senescent decline in egg
147 laying. Conversely, if larval diet has no long-term effects on life-history traits, we would expect
148 to see similar survival and reproduction patterns across all diets. As low P:C diets have been
149 found to be especially detrimental for survival post-infection in our host-pathogen system
150 (Savola et al. 2020), we predicted that low larval P:C would reduce survival and reproduction
151 to a greater extent in injured and infected flies than in control flies.

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152 **METHODS:**

153 *LARVAL DIETS:*

154 Larval diets consisted of five diets varying in P:C composition from 1:16 to 2:1 P:C
155 (corresponding to 5 to 61% protein content, Table S1) based on a modified version of Lewis
156 food (Lewis, 1960, Table S1). These diets are a subset of ten diets used in an earlier study
157 (Savola et al. 2020).

158 *LARVAL EXPERIMENTAL METHODS:*

159 *D. melanogaster* experimental individuals were from an outcross DGRP population
160 established from 100 pairwise crosses of 113 lines from the *Drosophila* genetic reference panel
161 (DGRP) (Mackay et al. 2012) (see methods and supplementary material in Savola *et al.*, 2020).
162 From the 35th generation, we pipetted 5 µl of egg solution into each larval diet vial (following
163 Clancy and Kennington, 2001) to establish density controlled groups of eggs (on average 50
164 (± 19) eggs). The mean volume of food in vials was 7.66 (± 0.58) ml. 30 vials of each diet were
165 prepared in this way, and an additional 21 of the lowest P:C diet to ensure enough adults for
166 adult collection. Due to very low egg counts in a small number of vials, a further 5-10 µl was
167 added to these vials (17/171 vials, approximately 10%). For each vial, eggs were counted twice
168 under a microscope to get an average egg count. Starting from experimental day 1 (one day
169 after adding eggs to diets), vials were inspected daily for adult eclosion and the total number
170 of adults eclosed per vial was recorded. The number of pupae per vial was counted once all
171 adults had eclosed, based on pupal cases and undeveloped pupae.

172 *ADULT COLLECTION:*

173 Eclosion began on experimental day 8, from which point onwards adults were counted
174 and removed from vials twice a day, in the morning and evening. Pilot data suggested different
175 development times on the different diets, and therefore for each diet adults were collected
176 primarily across three days per diet, starting one day after adult eclosion began (Figure S1). In
177 this way, adult females were collected across a total of six days across all diet treatments to
178 create six blocks. Some diets with quicker development times required some adults to be
179 collected on the fourth day to achieve sufficient sample sizes (Figure S1 and Table S2; sample
180 sizes = 18 to 40 adult flies per larval diet and stress treatment).

181 After adult collection, all flies were placed singly in vials containing standard Lewis
182 medium in our laboratory, corresponding to the 1:6 P:C diet (Table S1, 14% protein diet), and

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183 were maintained on this diet for their remaining lifespan. Trays were rotated in the incubators
184 daily to minimise microclimate effects. The day following adult collection, each female was
185 provided with an age-matched male from the same outcross DGPR population. The female was
186 left with the male for 24 hours to allow mating, following which the male was removed.

187 *STRESS TREATMENTS:*

188 On the seventh day post-eclosion for each block, female flies were exposed to one of
189 three stress treatments: unstressed control, injury, and infection with a bacterial pathogen
190 *Pseudomonas entomophila*. Treatments for each block were done at the same time each day
191 (around 14:00) to minimise time-of-day effects on immunity (e.g. Lee and Edery, 2008). After
192 stress treatments, the fly was placed into a new vial containing modified Lewis medium (Lewis,
193 1960, see P:C 1:6 diet in Table S1). The stress treatments were applied following Savola *et al.*,
194 (2020, modified from Dieppois *et al.*, 2015; Troha and Buchon, 2019), with the overnight *P.*
195 *entomophila* bacterial solution re-suspended in 30 ml Luria-Bertani (LB) medium in the
196 morning and left to grow for three hours prior to dilution from a known OD value to correspond
197 to an OD value of 0.001. This level was chosen from a pilot study (Figure S2) and is slightly
198 lower than a previous experiment in adults (Savola et al. 2020), as the diluted OD of 0.005 had
199 much lower survival compared to the previous experiment. Each block's bacterial culture was
200 established from a set of isogenic bacterial cultures grown overnight in LB medium, aliquoted
201 in 20-25% glycerol 200 µl quantities and stored at -80°C. A subset of flies from each block of
202 infections were plated on *Pseudomonas* isolating agar to confirm the infection treatments were
203 successful (following Gupta *et al.*, 2017, see supplementary information).

204 *ADULT TRAIT MEASUREMENTS:*

205 The number of eggs a female laid was counted from day 2 onwards for each block. For
206 the first 14 days, eggs were counted daily and females were tipped into new vials.
207 Subsequently, egg counts were performed every second day and stopped on day 98 for
208 logistical reasons. This is an accurate proxy for lifetime egg production, as females in our
209 previous experiment with adult P:C diet manipulation laid on average 99.37% (± 2.34%) of
210 their lifetime eggs by day 98 (Savola et al. 2020). If a fly died on a day when eggs were not
211 counted, an extra egg count was performed on the day of death. Survival was checked daily.

212 *STATISTICAL METHODS:*

213 The data were analysed using R software, version 3.5.2 (R Core Team 2014). All graphs
214 were drawn using ggplot2 (Wickham 2016). All traits were analysed using generalised linear

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215 mixed models (GLMM). Models using a Poisson distribution were checked for zero inflation
216 and overdispersion using the *DHARMa* package (Hartig 2019). In all models, even though we
217 altered the P:C ratio of diets, diet was analysed as a continuous covariate as the percentage of
218 protein in the diet (Table S1). To allow for non-linear effects, the quadratic term of protein
219 percentage was also included. To avoid scaling errors, all continuous covariates were
220 standardised to a mean of zero and a standard deviation of one. This was done separately for
221 each test due to different sample sizes for different measures. Stress treatment was analysed as
222 a categorical fixed effect. For all models with two-, or three-way interactions, for displaying
223 summaries of LRT results of main effects or two-way interactions, parameter estimates and
224 associated standard deviations are from separate models not including the associated two-, or
225 three-way interactions. All model prediction plots were made with all random effects set to 1
226 and diets are shown as the percentage of protein in the diet.

227 We analysed survival to a number of developmental stages: egg-to-pupa, pupa-to-adult
228 and egg-to-adult using linear models assuming a Gaussian distribution. For egg-to-pupa and
229 egg-to-adult survival, we included the number of eggs in each vial as a covariate to control for
230 differences in initial egg number on how many pupae or adults developed in each vial. This is
231 essentially the same as modelling viability as percentages or as a proportion, which is often
232 done in other larval diet studies (e.g. Andersen *et al.*, 2010; Sentinella, Crean and
233 Bonduriansky, 2013; Kutz, Sgrò and Mirth, 2019), however our method does not bound the
234 data at 100% or 1. Similarly, for egg-to-pupa survival, we included the number of pupae in
235 each vial as a covariate. All models were visually analysed for normality. Development time
236 was analysed through GLMM with a Poisson error distribution using the lme4 R package
237 (Bates et al. 2015) with the "bobyqa" optimiser with 100'000 iterations. We included vial as a
238 random effect to account for any within vial effects, for example different larval densities and
239 repeated measurements. For clarity of presentation, model predictions were made at either the
240 average number of eggs or pupae per vial.

241 For adult survival, Kaplan-Meier survival curves were made using the survminer R
242 package (Kassambara and Kosinski 2018) with diet as a factor. As our data did not conform to
243 the assumptions of proportional hazards (global term of cox.zph function Chisq = 54.98, p =
244 <0.001), we used an event history model following Moatt *et al.* (2019; Savola et al. 2020),
245 implemented through a binomial GLMM in the lme4 R package (Bates et al. 2015) with the
246 "bobyqa" optimiser with 100'000 iterations. This model is similar to the Cox proportional
247 hazards model, however the results in this case estimate a per day mortality risk, which we will

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248 refer to as mortality. Individuals in the dataset were scored daily as 0 for alive and once as 1
249 for dead. The model included day as a random effect to account for differences in survival
250 between days, and individual ID to account for multiple measures of an individual. To confirm
251 the results of the survival model, we further analysed the data as lifespan using a GLMM in the
252 package glmmTMB (Brooks et al. 2017) with a negative binomial distribution and block as a
253 random effect to account for differences between infection days. Even though the data do not
254 conform to the Cox proportional hazards assumptions, we checked for consistency with the
255 result of the above analysis using a Cox proportional hazards model in the R Survival package
256 (Therneau 2015).

257 For reproduction, various measures were analysed. As adult diet might have an
258 increasing influence on the number of eggs produced as flies get older, for example due to
259 compensatory feeding, we analysed measures of early-life egg production. We ran separate
260 analyses on the number of eggs produced prior to stress treatments, seven days in total, and
261 eggs produced in the seven days post stress treatments to test for early-life differences in
262 reproduction and if these were affected by stress treatment. Only flies left alive on the last day
263 of egg counts were included in these analyses. These data were analysed using a GLMM with
264 a negative binomial distribution and including a zero-inflation term with the glmmTMB R
265 package (Brooks et al. 2017). Lifetime egg production (to day 98) was analysed with an
266 identical model to the other reproduction models with all flies included, with an additional
267 model including lifespan as a predictor. Mean centered lifespan was included to account for
268 selective disappearance and block was included as a random effect. To analyse daily egg
269 production, all egg counts corresponding for a span of two days were divided by two and
270 rounded down to the nearest integer to match earlier daily egg counts. Daily egg production
271 was analysed using a GLMM with negative binomial distribution and including a zero-inflation
272 term. As well as the fixed effects described above, age was included as a linear and non-linear
273 term as well as the interactions between these and all other fixed effects. Individual ID and
274 block were included as random effects.

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275 **RESULTS:**

276 *EFFECTS OF LARVAL NUTRITION ON LARVAL TRAITS:*

277 P:C of the larval diet had a significant effect on how many individuals developed from
278 eggs to adults, where larvae reared on higher P:C were more likely to develop to adults (Figure
279 1A & Figure S3A; Table S3A; Protein = 2.20 (± 0.55), F = 15.90, p = <0.001). With higher
280 numbers of eggs in a vial, more adults developed (Table S3A; Average number of eggs = 0.72
281 (± 0.03), F = 762.19, p = <0.001). Separating this result into effects on larval and pupal
282 viability, there was a significant effect of P:C on the numbers of eggs developing into pupae
283 (Figure 1B & Figure S3B; Table S3A; Protein = 1.79 (± 0.52), F = 9.13, p = 0.003; Average
284 number of eggs = 0.86 (± 0.03), F = 1165.5, p = <0.001). There was a marginally non-
285 significant effect of P:C on the number of adults developing from pupae (Figure 1C & Figure
286 S3C; Table S3C; Protein = 0.70 (± 0.45), F = 3.81, p = 0.052). As expected, with more pupae
287 in a vial, more adults developed (Figure 1C & Figure S3C; Table S3C; Pupae = 0.82 (± 0.02),
288 F = 1228.5, p = <0.001).

289 P:C in the larval diet also had an effect on the development time to adulthood, with
290 higher larval P:C resulting in shorter development time (Figure 1D & Figure S4, Table S3;
291 Protein = -0.22 (± 0.01), Chi-squared = 191.75, p = <0.001). This relationship is quadratic,
292 suggesting that intermediate P:C diets had a quicker development time in comparison to the
293 high or low P:C diets, or that the rate of reduction in development time plateaued at the highest
294 P:C diets (Figure 1D & Figure S4, Table S4; $Protein^2$ = 0.16 (± 0.01), Chi-squared = 183.61, p
295 = <0.001). Vials with higher average number of eggs had a slightly longer development time
296 (Table S4; Average number of eggs = 0.03 (± 0.01), Chi-squared = 26.80, p = <0.001).

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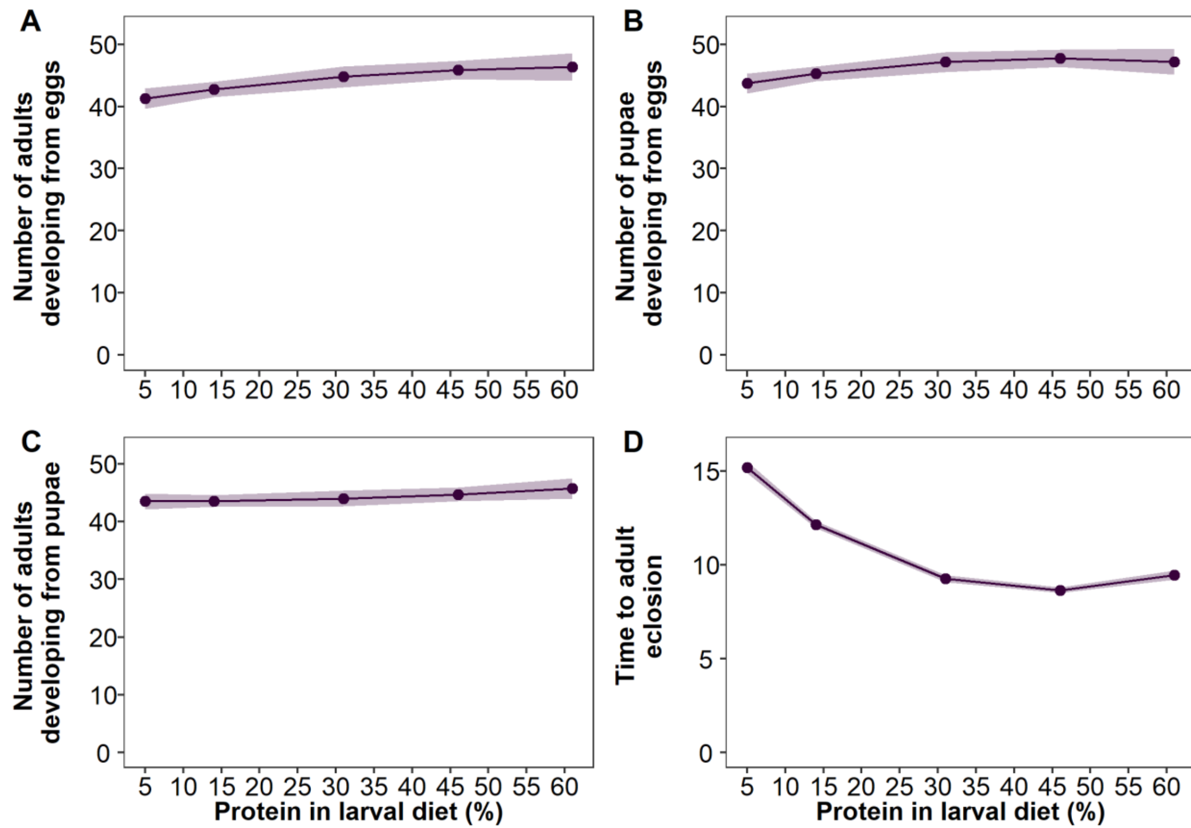

297

298 **Figure 1:** Model predictions of the effects of larval P:C (shown as the corresponding 299 percentage of protein) on various larval traits: (A) the number of adults developing having 300 controlled for the number of eggs laid (50 (± 19) eggs on average); (B) the number of pupae 301 eclosing having controlled for the number of eggs laid (50 (± 19) eggs on average); (C) the 302 number of adults developing having controlled for the number of pupae formed (46 (± 18) 303 pupae on average); and (D) the average time taken for adult eclosion. All predictions are based 304 on either vials starting with the overall mean number of eggs (50 (± 19) eggs) (A, B, D), or the 305 overall mean number of pupae (46 (± 18) pupae) (C). Shaded areas are 95% confidence 306 intervals. Protein and protein^2 are mean centered to standard deviation of 1. See S3 for viability 307 data as percentages.

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EFFECTS OF LARVAL NUTRITION ON ADULT TRAITS AND SURVIVAL AFTER STRESS:

P:C of larval diet had no effect on adult mortality regardless of stress treatment (Figure 2B & Figure S5; Table S5 & Table S6). Stress treatment had a significant effect on mortality, where infected flies had a higher risk of death (Figure 2B & Figure S5; Table S5; Treatment Chi-squared = 76.67, p = <0.001; Infection = 1.18 (□ 0.13); Injury = 0.24 (± 0.17)). Analysing the survival data as lifespan, the same patterns were observed, where stress treatment had a significant effect on lifespan, with infected flies having shorter lifespans (Figure S6 & Figure S7; Table S7 & Table S8; Treatment Chi-squared = 99.28, p = <0.001; Infection = -1.00 (± 0.10); Injury = -0.10 (± 0.10)). The results of a Cox proportional hazards model show the same patterns (Figure S8; Table S9).

Larval P:C had a significant effect on early-life egg production prior to stress treatments, where increasing larval P:C increased early-life egg production which then levelled off at very high P:C diets (Figure 2B & Figure S11; Table S14; Protein = -0.29 (± 0.06), chi-squared = 3.71, p = 0.054; Protein^2 = -0.20 (± 0.04), p = <0.001). There were no significant effects of larval diet or stress treatments on egg production in the seven days following stress treatments (Figure 2C & Figure S12; Table S15 & Table S16).

The effect of larval P:C on lifetime egg production was similar to the effect on early-life reproduction, where increasing P:C in the larval diet increased lifetime egg production (Figure 2D & Figure S9; Table S10; Protein = 0.11 (± 0.05), Chi-squared = 5.73, p = 0.02). The effect of P:C was non-linear (Figure 2D; Table S10; Protein^2 = -0.11 (± 0.04), Chi-squared = 8.01, p = 0.005), with egg production reaching a peak at intermediate P:C and not increasing further at higher P:C. Stress treatment had a significant effect on lifetime egg production, where infected flies produced fewer eggs (Figure 2D; Figure S9; Table S10; Treatment chi-squared = 8.81, p = 0.01; Infection = -0.18, (± 0.08); Injury = 0.04 (± 0.07)). Flies produced more eggs with longer lifespan (Table S11, Lifespan = 0.69 (± 0.04), Chi-squared = 275.77, p = <0.001). There was no significant interaction between larval P:C and stress treatment (Table S11). A model not accounting for lifespan showed the same pattern with stress treatments having a significant effect on egg production, where infected individuals produced fewer eggs (Figure S10; Table S12 & Table S13; Stress treatment chi-squared = 80.68, p = <0.001; Infection = -0.82 (± 0.09); Injury = -0.04 (± 0.09)). Increasing larval P:C resulted in higher lifetime egg production, however this pattern was not quadratic (Figure S10; Table S12 & Table S13; Protein = 0.12 (± 0.04), Chi-squared = 5.91, p = 0.02; Protein^2 = 0.07 (± 0.05), Chi squared =

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340 2.10, $p = 0.15$). Even though the models differ slightly, the P:C patterns are broadly similar for
341 both models, where egg production plateaus at the highest P:C level, as also seen in the raw
342 data (Figure 2D, S9 & S10).

343 In general, flies across all larval diets and stress treatments showed similar patterns in
344 egg laying over their lifespan (Figure 2E & Figure S13). Egg production was highest early in
345 life and then declined (Figure 2E & Figure S13). At mean centered P:C and lifespan, there was
346 a slowing in the rate of decline of egg laying with age, such that the rate of decline is high when
347 young and slows as flies get older (Table S17 & Table S19; Age = -0.60 (± 0.03), chi-squared
348 = 2175.5, p = <0.001; Age^2 = -0.03 (± 0.02), chi-squared = 26.14, p = <0.001). As with lifetime
349 egg production, there was a significant non-linear effect of P:C, with intermediate P:C diets
350 resulting in higher daily egg production in the control stress treatment at mean age and lifespan
351 (Figure S13; Table S17 & Table S19; $Protein^2$ = -0.05 (± 0.05), chi-squared = 12.26, p =
352 0.0005). Lifespan had no effect on daily egg production (Table S19, Lifespan = 0.02 (± 0.02),
353 $p = 0.16$).

354 The pattern of ageing in reproduction was broadly similar across larval diets and adult
355 stress treatments (Figure 2E & Figure S13). However, there were some significant two-, and
356 three-way interactions, which indicate small differences in the pattern of reproductive ageing
357 across diets and stress treatments. There was a significant two-way interaction between P:C
358 and the linear and quadratic effect of age, suggesting that with higher P:C, ageing in egg
359 production was quicker and the linear effect of age was highest at intermediate P:C diets
360 (Figure S13; Table S18 & Table S19; Protein:Age = -0.10 (± 0.02), chi-squared = 30.48, p =
361 <0.001; Protein:Age^2 = 0.10 (± 0.02), chi-squared = 23.94, p = <0.001). There was also a
362 significant two-way interaction between age and the quadratic effect of P:C, suggesting that
363 the rate of ageing was highest at intermediate P:C (Figure S13; Table S18 & Table S19;
364 $Protein^2$:Age = 0.12 (± 0.02), chi-squared = 22.62, p = <0.001). Stress treatments had
365 significant effects on these two-way interactions (Figure 2E & Figure S13; Table S19,
366 Treatment:Protein:Age^2 chi-squared = 14.41, p = 0.001, Treatment:$Protein^2$:Age chi-squared =
367 11.21, p = 0.004), where in injured flies these terms were smaller compared to the control
368 individuals (Table S19; Injury:Protein:Age^2 = -0.08 (± 0.03); Injury:$Protein^2$:Age = -0.10 (±
369 0.03)). This suggests that there was less of an effect of P:C on aging in the injured flies. Stress
370 treatment also had a significant effect on ageing, where infected flies had a more negative
371 decline in egg laying with age than control individuals (Figure 2E & Figure S13; Table S19;
372 Treatment:Age chi-squared = 18.05, p = <0.001; Infection:Age = -0.25 (± 0.06)).

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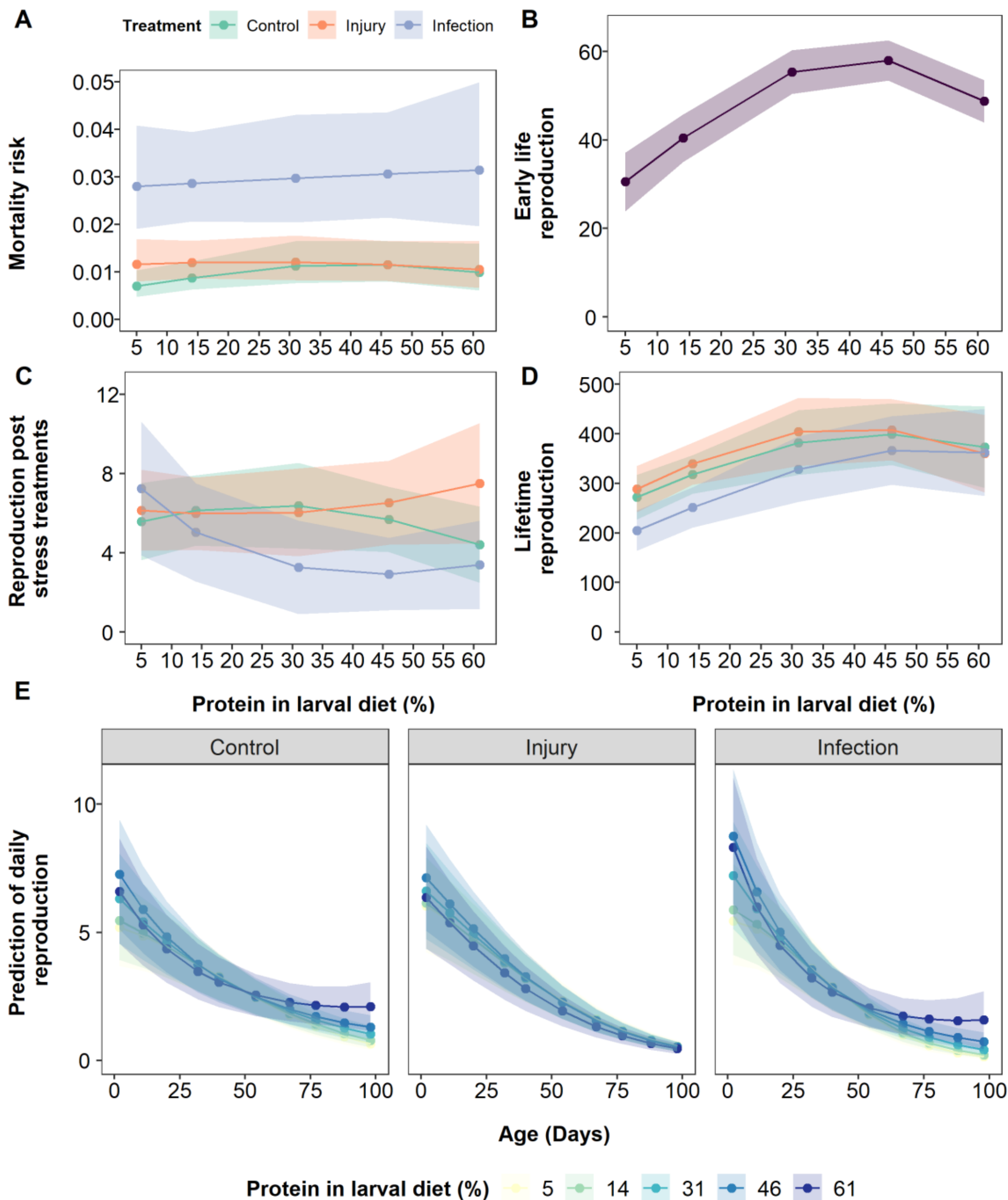

373

374 **Figure 2:** Model predictions of the effects of larval diet P:C (shown as the corresponding
375 percentage of protein) and adult stress treatment on various adult life-history traits: (A) per day
376 mortality risk; (B) egg production in the first 7 days of adulthood (prior to stress treatments);
377 (C) egg production across the 7 days after stress treatments; (D) lifetime egg production (up to
378 day 98); (E) reproductive ageing in terms of daily egg production. Adult flies were infected
379 with a bacterial pathogen (blue data points and lines in A, C, D), injured by a pinprick (orange
380 data points and lines in A, C, D) or with no treatment (green data points and lines in A, C, D).
381 Lifespan is accounted in the model to account for selective disappearance in (D). Shaded areas
382 are 95% confidence intervals. Protein and protein^2 (A-E), and age and age^2 (E) are mean
383 centered to standard deviation of 1.

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384 **DISCUSSION:**

385 The main objective of our study was to test whether larval diets ranging in P:C content
386 affected adult survival post-infection. We predicted that adults that developed on lower P:C
387 diets would have worse survival post-infection, due to the demonstrated importance of dietary
388 protein for the response to infection (e.g. Lee *et al.*, 2006; Povey *et al.*, 2009; Cotter *et al.*,
389 2019; Savola *et al.*, 2020). However, our results provide no evidence for an effect of larval P:C
390 diet on adult lifespan, regardless of stress treatment. Similarly, although intermediate P:C in
391 the larval diet increased lifetime and early-life reproduction, there were no interactions between
392 larval diet and stress treatment on reproduction, except for a smaller effect of P:C on the
393 senescent decline in egg production for injured flies. Infection did however have effects on
394 many life-history traits, specifically reducing lifespan and lifetime egg production, and
395 increasing the rate of senescence in egg laying. These results suggest that, in this study,
396 although P:C in larval diet affected larval and adult life-history traits, and exposure to infection
397 in adulthood affected adult life-history traits, these effects did not interact very strongly.

398 Previous studies have suggested links between larval diet and the ability of adults to
399 cope with environmental stress such as infection. For example, lower P:C nymphal diet with
400 matching adult diet decreased adult survival post-infection measured for five days in female
401 *Gryllus texensis* crickets (Kelly and Tawes 2013). Similarly, adult *Anopheles gambiae*
402 mosquitoes raised as larvae on increasing CR had lower melanising ability (Suwanchaichinda
403 and Paskewitz 1998). Studies measuring components of the immune system without a direct
404 stressor have also shown that adults raised on non-optimal diets had lower adult immune
405 function, with short term starvation (De Block and Stoks 2008) or caloric restriction (Rolff et
406 al. 2004). Similarly, adult *D. melanogaster* raised on higher P:C diets had higher levels of
407 *Diptericin A* and *Metchnikowin* AMP transcription (Fellous and Lazzaro 2010). This is
408 particularly relevant to our study, as AMPs are important in bacterial defence (reviewed in
409 Zhang and Gallo, 2016) and in *D. melanogaster* AMPs including *Metchnikowin* and *Diptericin*
410 are upregulated with *P. entomophila* infection (Liehl et al. 2006; Chakrabarti et al. 2012).
411 However, in this previous study, only the amount of yeast in diet was altered without reducing
412 carbohydrates thus altering both calorie and P:C content. Consequently, increased calories may
413 be driving these effects and not the increase in P:C. As previous studies have all manipulated
414 calories, perhaps the caloric value of juvenile diet affects adult immunity and not the
415 macronutrient content. By only manipulating the P:C content of larval diets, our results suggest
416 that macronutrient ratio does not affect adult survival post-infection.

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417 Outside of differences in the type of diet manipulation, another factor that could explain
418 our contrasting findings to previous research is the type of stress experienced. For example, *D.*
419 *melanogaster* flies raised on higher P:C had a longer chill coma recovery time (Andersen et al.
420 2010) and worse starvation resistance (Davies et al. 2018), however better heat coma and
421 desiccation resistance (Andersen et al. 2010). It has been suggested that these larval diet effects
422 are a result of effects on general body condition or specific tissues (Fellous and Lazzaro 2010),
423 the production of heat shock proteins (Andersen et al. 2010), and more directly, lipid storage
424 through eating a diet richer in carbohydrates as larvae (e.g. Roeder and Behmer, 2014; Kim,
425 Jang and Lee, 2020). Our results suggest this does not seem true for immune responses, even
426 though the fat body is an organ linked to both larval feeding and immune responses (reviewed
427 in Arrese and Soulages, 2010). Larval feeding may therefore differentially affect adult stress
428 response where certain diets are better for specific environmental stressors.

429 Another consideration is the timing of the stressor, as previous studies have applied
430 stressors closer to eclosion. Our seven day lag post-eclosion could allow enough time for
431 compensatory mechanisms (reviewed in Nestel *et al.*, 2016), such as compensatory feeding
432 (Raubenheimer and Simpson 1993) to mask any effects of larval diet. For example, adults that
433 developed on low P:C diets could have eaten enough protein to survive injury and infection to
434 a similar level to adults that developed on higher P:C. Two studies that altered both larval and
435 adult diets found that adult environment was the main determinant of life-history traits (Davies
436 et al. 2018; Duxbury and Chapman 2020), however, in one there were small and complex
437 differences in female lifetime reproduction between larval and adult diet combinations
438 (Duxbury and Chapman 2020). It would therefore be interesting to repeat our experiment and
439 expose adults to injury and infection stress immediately upon eclosion.

440 There was no effect of larval diet on survival or lifespan, as seen in other studies (Tu
441 and Tatar 2003; Houslay et al. 2015; Davies et al. 2018). In adults, altering P:C affects lifespan,
442 with lifespan maximised on either intermediate (e.g. Lee, 2015; Kim, Jang and Lee, 2020;
443 Savola *et al.*, 2020) or low P:C diets (e.g. Lee *et al.*, 2008; Maklakov *et al.*, 2008; Jensen *et al.*,
444 2015). A small number of studies have suggested an effect of larval diet P:C on adult lifespan,
445 but results have been inconsistent (lifespan maximised on high (Duxbury and Chapman 2020),
446 intermediate (Runagall-McNaull et al. 2015; Kim et al. 2019), and low (Economos 1984;
447 Stefana et al. 2017) P:C diets). A more consistent role has been suggested for calories, with
448 adult lifespan decreasing with larval calorie restriction (May et al. 2015; Adler et al. 2016;
449 Hooper et al. 2017; Krittika et al. 2019). Given a meta-analysis found no overall effect of early-

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450 life diet on lifespan (English and Uller 2016), such contrasting findings suggest no clear effect
451 of larval diet on adult lifespan and instead suggest that lifespan is more determined by adult
452 diet.

453 Lifetime and early-life reproduction increased with increasing larval P:C and then
454 declined slightly at the highest P:C. Similarly, ovariole number has been show to peak at
455 intermediate larval P:C in *Zaprionus indianus* (Matavelli et al. 2015) and in *Drosophila*
456 *melanogaster* (Rodrigues et al. 2015). Many larval studies lack this decline at the highest P:C
457 diets (e.g. Tu and Tatar, 2003; Andersen *et al.*, 2010; Silva-Soares *et al.*, 2017; Duxbury and
458 Chapman, 2019; Kim *et al.*, 2019). Protein is often the limiting nutrient in egg production
459 (reviewed in Wheeler, 1996; Boggs, 2009), but can have a toxic effect when consumed at very
460 high levels (reviewed in Simpson and Raubenheimer, 2009), which may explain the plateauing
461 at very high P:C due to larvae of worse condition developing to adults. Due to the highest P:C
462 ratio also including the lowest carbohydrate content, this effect may also be due to a limiting
463 effect of carbohydrates on development. As nutrients for egg production can be acquired from
464 adult feeding and nutrient requirements can differ between species (reviewed in Wheeler,
465 1996), this limit may not always appear. These P:C effects could also arise through the general
466 increase in body condition with higher P:C in larval diet (Runagall-McNaull et al. 2015).
467 Overall, there appears to be an increase in adult reproduction with increasing P:C in the larval
468 diet, but this effect may plateau at very high P:C levels.

469 Infection reduced lifetime egg production, a typical response in insects (reviewed in
470 Schwenke et al. 2016), however there was no interaction between larval P:C and reproduction
471 post-infection. When accounting for the overall shorter lifespan of infected flies, their
472 reproduction was comparable to the injured or unstressed flies. In the week after stress
473 treatments, all treatment groups produced the same number of eggs. This suggests that with
474 infection, individuals were able to produce more eggs earlier in life but then egg numbers
475 declined. This was reflected in the reproductive ageing results, as infection increased the rate
476 of senescence in egg laying. This could be evidence of terminal investment (Clutton-Brock
477 1984), specifically fecundity compensation, as flies shifted their egg production earlier as a
478 response to infection, as is a common outcome after infection (reviewed in Kutzer and
479 Armitage, 2016).

480 In general, the patterns of reproductive ageing were quite similar across treatments, but
481 there were some interactions between P:C in the larval diet and stress treatment. Overall, at
482 intermediate P:C, ageing in egg production was quickest. Similar results in reproductive ageing

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483 have been found in studies altering adult P:C (e.g. Jensen *et al.*, 2015; Savola *et al.*, 2020).
484 These results are also similar to previous studies focusing on ageing in egg laying where adults
485 raised on higher P:C and/or calories as larvae appear to have quicker ageing in egg laying (Tu
486 and Tatar, 2003; Hooper *et al.*, 2017, but see May, Doroszuk and Zwaan, 2015). As these
487 studies manipulated calories, here we show there are minor changes in ageing patterns also
488 with P:C manipulation of the larval diet.

489 For the larval traits, we predicted that larvae would have more successful and quicker
490 development on higher P:C diets (Britton and Edgar 1998; Colombani et al. 2003; Chang 2004;
491 Mirth and Riddiford 2007). Development time was quickest on intermediate P:C, and egg-to-
492 pupae and egg-to-adult viability were higher on higher P:C, as seen in previous studies (e.g.
493 Andersen et al. 2010; Silva-Soares et al. 2017; Kutz et al. 2019, but see Houslay *et al.*, 2015;
494 Davies *et al.*, 2018; Gray, Simpson and Polak, 2018). However, despite being statistically
495 different, the effect of diet on egg-to-pupae and egg-to-adult viability was small. There was no
496 effect of P:C on pupae-to-adult viability, suggesting that all of the diets used in our study
497 allowed larvae to pupate successfully. Studies often do not report pupae-to-adult viability,
498 however more extreme diets could affect this trait as different sources of carbohydrates have
499 been shown to affect pupae-to-adult viability (Nash and Chapman 2014). On the highest P:C
500 diet, development time was slightly slower, which is also a common finding (e.g. Lee et al.
501 2012; Rodrigues et al. 2015; Kutz et al. 2019), again potentially due to toxic effects of high
502 P:C diets (reviewed in Simpson and Raubenheimer, 2009) or due to a limitation of
503 carbohydrates for development. Vials with more eggs took longer to develop, most likely due
504 to larval density effects (e.g. Ludewig *et al.*, 2017; Klepsatel, Procházka and Gáliková, 2018;
505 Henry, Tarapacki and Colinet, 2020; Than, Ponton and Morimoto, 2020). Our work adds to
506 growing evidence of the importance of macronutrients in larval diet for larval development and
507 suggest that, with some exceptions, intermediate P:C diets are better for key larval traits.

508 **CONCLUSIONS AND FUTURE WORK:**

509 The results of this study suggest that larval dietary P:C has no effect on adult survival
510 with or without stress treatment, and thus larval P:C does not alter the long-term consequences
511 of injury or infection on survival. Intermediate P:C larval diets were optimal for many traits
512 pre-, and post-metamorphosis. Individuals were the quickest to develop into adults on
513 intermediate larval P:C, and subsequent adults produced the most early-life and lifetime eggs.
514 Larvae were more likely to develop into adults on higher P:C. Therefore, our results add to the
515 growing evidence that larval diet affects adult life-history traits, but the long-term

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516 consequences of infection and injury are not altered. To understand the effects of larval diet on
517 the ability of adults to respond to infection further, we suggest experiments exposing adults to
518 infection immediately after eclosion to avoid potential for compensatory feeding. Furthermore,
519 using a fully factorial experiment combining variation in larval and adult diet could help to
520 disentangle the differences between larval and adult feeding on stress responses such as
521 infection and injury.

522

523 **ACKNOWLEDGEMENTS:**

524 This work was supported by the Biotechnology and Biological Sciences Research Council
525 (BBSRC) grant number BB/M010996/1. We thank Joshua Moatt for helpful advice and
526 comments on the data analysis and write-up, Katy Monteith for laboratory training and
527 practical advice as well as help with data collection at the end of the experiment along with
528 Paul McNeil and Paulina Mika, and Anu Halonen for data on confirmation of infection from
529 one day of missing data.

530

531 **CONFLICTS OF INTEREST:**

532 The authors declare no conflicts of interest.

533

534 **AUTHORS'S CONTRIBUTIONS:**

535 ES, PV and CW designed the experiment. ES did the study and analysed the data with help
536 from CW. ES wrote the paper with help from CW and PV.

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