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1 **Viral susceptibility across host species is largely independent of dietary**
2 **protein to carbohydrate ratios**
3
4 **Twitter summary**: No role of host diet in susceptibility to a novel viral pathogen
5 across host species
6
7 **Impact Statement:** A successful host shift of a parasite from one susceptible
8 species to a novel host can be influenced by many ecological factors. Changes in
9 host diet can alter the immune response and outcomes of host–parasite
10 interactions and could potentially alter the outcome of a virus host shift. To
11 investigate, we infected 27 species of Drosophilidae with an RNA virus (DCV)
12 across three food types with differing protein to carbohydrate ratios. We then
13 looked at pathogen loads and survival of infected hosts compared to uninfected
14 controls. Changes in the ratio of protein to carbohydrate did not alter
15 susceptibility to DCV across host species.
16
17 **Abstract**
18 The likelihood of a successful host shift of a parasite to a novel host
19 species can be influenced by environmental factors that can act on both the host
20 and parasite. Changes in nutritional resource availability have been shown to
21 alter pathogen susceptibility and the outcome of infection in a range of systems.
22 Here we examined how dietary protein to carbohydrate altered susceptibility in
23 a large cross infection experiment. We infected 27 species of Drosophilidae with
24 an RNA virus on three food types of differing protein to carbohydrate ratios. We
25 then measured how viral load and mortality across species was affected by

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26 changes in diet. We found that changes in the protein:carbohydrate in the diet
27 did not alter the outcomes of infection, with strong positive inter-species
28 correlations in both viral load and mortality across diets, suggesting no species
29 by diet interaction. Mortality and viral load were strongly positively correlated,
30 and this association was consistent across diets. This suggests changes in diet
31 may give consistent outcomes across host species, and may not be universally
32 important in determining host susceptibility to pathogens.
33

34 **Introduction**

35 A key driver of pathogen host shifts – where a pathogen jumps from one host
36 species to another – is environmental change (Hoberg & Brooks, 2015; Carlson *et*
37 *al.*, 2020). For a host shift to successfully occur a novel host must first be exposed
38 to a parasite, which must then be able to replicate and successfully infect the
39 host, before sufficient onward transmission (Woolhouse *et al.*, 2005). Ecological
40 factors can therefore influence the likelihood of host shifts by altering species
41 distributions and abundances making encounters more likely, or by acting as
42 stressors that alter physiological factors including immunity or virulence. The
43 main ecological factor studied has been temperature, which can have
44 asymmetrical impacts on hosts and parasites and potentially alter the likelihood
45 of host shifts (Brooks & Hoberg, 2007; Hoberg & Brooks, 2015; Kirk *et al.*, 2018;
46 Roberts *et al.*, 2018). The role of other ecological traits such as resource
47 availability, humidity, population density and geographical range, or within host
48 ecological traits such as metabolic rate, have been less well studied in explaining
49 the outcomes of host shifts, despite an increasing understanding of the role such
50 factors play in effecting the outcomes of host parasite interactions (Blanford &

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51 Thomas, 1999; Harvell *et al.*, 2002; Ponton *et al.*, 2013; Hayman *et al.*, 2016;
52 Cumnock *et al.*, 2018).
53
54 Nutrition can shape the outcome of host-parasite interactions through its ability
55 to moderate both parasite virulence and host resistance (Ponton *et al.*, 2011,
56 2013; Pike *et al.*, 2019). The nutritional resources of a host can impact its ability
57 to resist infection as immune responses are thought to be costly to both maintain
58 and activate (Kraaijeveld & Godfray, 1997; Lochmiller & Deerenberg, 2000;
59 McKean *et al.*, 2008; Cotter *et al.*, 2011; Kutzer & Armitage, 2016; Knutie *et al.*,
60 2017). Nutrition is known to have long term consequences, with developmental
61 nutritional status being shown to have latent or even trans-generational effects
62 on immune responses (in *Drosophila*: Fellous & Lazzaro, 2010; Savola *et al.*,
63 2020b and reviewed in Grueber *et al.*, 2018). Hosts can also show behavioural
64 modifications in feeding upon infection; parasite-induced anorexia is thought to
65 be an adaptive host response (Ayres & Schneider, 2009; Rao *et al.*, 2017). In
66 some cases hosts actively increase the consumption of certain nutrients in their
67 diet for example, the African armyworm – *S. exempta* upon infection with a
68 baculovirus displays macronutrient self-medication (Povey *et al.*, 2009).
69 Nutrition may constrain the amount of investment that a host can allocate to an
70 energetically demanding immune response (Kraaijeveld & Godfray, 1997;
71 Lochmiller & Deerenberg, 2000; Cotter *et al.*, 2011; Knutie *et al.*, 2017), and
72 coping with costs associated with a parasite burden if infection does become
73 established (Sheldon & Verhulst, 1996). A suboptimal nutritional status may lead
74 a host to be unable to suppress or tolerate a parasite challenge they may
75 otherwise have been able to resist; or have reduced fitness from a trade off in

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76 resources with life history traits (Kraaijeveld & Godfray, 1997; Lochmiller &
77 Deerenberg, 2000; Cotter *et al.*, 2011; Knutie *et al.*, 2017).
78
79 From a parasite perspective infecting a host of suboptimal nutritional status may
80 mean they encounter a weaker immune response and therefore infection and
81 establishment is easier (Siva-Jothy & Thompson, 2002). However, once
82 established the parasite may encounter its own resource limitations due to
83 competition with an already depleted host, causing suboptimal growth and
84 potentially affecting onward transmission. Therefore, predicting the outcome of
85 the interaction between nutrition, host immunity and subsequent resistance is
86 complex as the effects on the two parties may be divergent (Bedhomme *et al.*,
87 2004).
88
89 Multiple life history traits are moderated by resource availability, with condition
90 dependency across reproductive traits, aging and lifespan (Lee *et al.*, 2008;
91 Maklakov *et al.*, 2009; Camus *et al.*, 2017; Henry & Colinet, 2018; Henry *et al.*,
92 2020). Laboratory experiments on dietary restriction, where individuals
93 experienced a reduction in nutrition without inducing malnutrition
94 (differentiated from Calorie Restriction) have been found to extend life span in a
95 range of organisms (Weindruch & Walford, 1982; Klass, 1983; Anderson *et al.*,
96 2003; Nakagawa *et al.*, 2012). The effects of dietary restriction appear to be
97 explained by resource-mediated trade-offs between longevity and reproductive
98 effort (but see review by (Moatt *et al.*, 2020)). Geometric frameworks – the use of
99 artificial diets with known compositions of specific nutrients that develop an

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100 understanding of dimensional nutrient space – have been used to examine the
101 consequences of different ratios of macronutrients across a range of
102 organisms(Simpson & Raubenheimer, 1995, 2011; Raubenheimer & Simpson,
103 1999). In *Drosophila* different life-history traits were optimized at different
104 protein-carbohydrate intakes (Lee *et al.*, 2008; Skorupa *et al.*, 2008; Fanson *et al.*,
105 2009; Jensen *et al.*, 2015). Across multiple species, low protein to carbohydrate
106 ratios reduce reproductive rates but maximise lifespan (Nakagawa *et al.*, 2012;
107 Le Couteur *et al.*, 2016). However, individuals with diets higher in protein and
108 lower in carbohydrates have greater reproductive rates but shorter life spans.
109 When given a choice of diet, individuals have been shown to optimise
110 reproduction over lifespan (Bunning *et al.*, 2016). Host dietary frameworks have
111 been used to examine effects on bacterial pathogens (Povey *et al.*, 2009; Cotter *et*
112 *al.*, 2019; Savola *et al.*, 2020b; Wilson *et al.*, 2020), viral pathogens (Lee *et al.*,
113 2006; Povey *et al.*, 2014), and individual aspects of immunity and gene
114 expression (Cotter *et al.*, 2011, 2019; Keaton Wilson *et al.*, 2019). In particular,
115 studies of viral infection in insects have found that high dietary protein leads to
116 increased resistance (Lee *et al.*, 2006) indicating there may be higher protein
117 costs of resistance.

118

119 To investigate the effect that host diet has on the susceptibility of different host
120 species we infected 27 species of Drosophilidae, with Drosophila C Virus (DCV)
121 fed on three diets with varying ratios of protein to carbohydrates but
122 comparable calorie content. We then measured the change in viral load and host
123 mortality across these different diets. DCV is a positive sense RNA virus in the

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124 family *Dicistroviridae*. DCV was isolated from *D. melanogaster* although has also
125 been detected in the closely related *D. simulans* (Christian, 1987), and in the wild
126 it is thought to be transmitted faecal-orally (Jousset *et al.*, 1972). Infection of DCV
127 by inoculation is highly pathogenic in adult flies causing increased mortality
128 rates, metabolic and behavioural changes and nutritional stress in the midgut,
129 causing similar pathologies to those seen in starvation (Christian, 1987; Arnold
130 *et al.*, 2013; Chtarbanova *et al.*, 2014; Vale & Jardine, 2017). DCV shows specific
131 tissue tropism in *D. melanogaster*, with infection of the heart tissue, fat body,
132 visceral muscle cells around the midgut and food storage organ (crop) causing
133 reduced defecation, food blockage and dehydration/starvation (Ferreira *et al.*,
134 2014). Infection progresses in a similar manner following both oral or septic
135 inoculation, with the same tissues ultimately becoming infected (Cherry &
136 Perrimon, 2004; Arnold *et al.*, 2013; Chtarbanova *et al.*, 2014; Ferreira *et al.*,
137 2014). If hosts are in a nutritional environment that allows for investment in
138 immune function or damage repair, they may be more able to resist, or tolerate a
139 novel infection (Ponton *et al.*, 2011, 2013; Pike *et al.*, 2019). This could then lead
140 to different outcomes following a host shift, either the host could manage to
141 suppress the parasite or avoid infection entirely, or could become infected and
142 minimise parasite damage (Lazzaro & Little, 2009; Howick & Lazzaro, 2014).
143 Alternatively hosts may be fully susceptible to infection, and enriched resources
144 may act to enhance pathogen virulence by enabling within host pathogen growth
145 (Hall *et al.*, 2009; Pike *et al.*, 2019). Previous work has demonstrated that
146 following inoculation into a novel host species, the host phylogeny is an
147 important determinant of susceptibility to DCV (Longdon *et al.*, 2011, 2015). The
148 host phylogeny explains a large proportion of the variation in DCV virulence

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149 (mortality) and viral load (75% and 67% respectively) with high virulence being
150 associated with high viral loads (Longdon *et al.*, 2015). One of the fundamental
151 steps needed for a successful host shift is the ability of a pathogen to infect a
152 novel host. Here we ask if the nutritional environment alters the susceptibility to
153 DCV following a shift into a range of novel host species, and whether such
154 patterns are consistent across species.
155
156 **Methods**
157 **Diet preparation**
158 Three different cornmeal diets were used (*Supplementary of species used and*
159 *food type*). The standard cornmeal diet used in our lab comprised a 1:10 protein
160 to carbohydrate ratio and became our "Medium" - protein: carbohydrate ratio
161 diet treatment. We also developed two further diets that were approximately
162 isocaloric, a low protein: carbohydrate food (1: 20 protein to carbohydrate) and
163 a high protein: carbohydrate food (1: 5 protein carbohydrate); see
164 supplementary for full table of food recipes nutrient breakdown. These were
165 based around previous findings that suggested that in *D. melanogaster* lifespan
166 was maximized on a protein: carbohydrate ratio of around 1:16, and fitness -
167 measured as lifetime egg production at a ratio of 1:4 (Lee *et al.*, 2008). All diets
168 were manipulated by altering the dextrose and yeast amounts whilst
169 maintaining as close as possible the same calorie content at 142 Calories g/100
170 ml. Yeast was manipulated as it provides the majority of the protein as well as
171 other non-caloric nutritional requirements (Piper, 2017). Values were confirmed
172 using the Drosophila Dietary Composition Calculator (Lesperance & Broderick,
173 2020).

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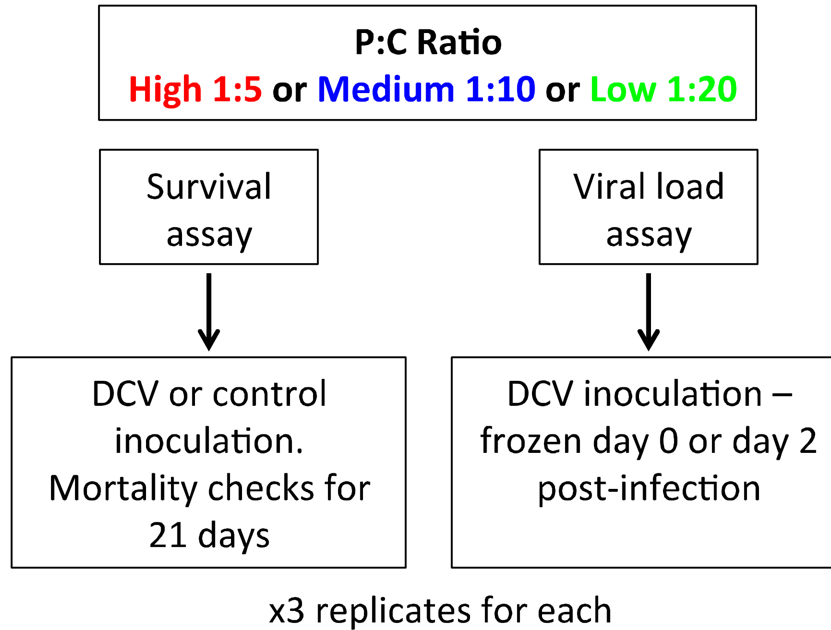

183 **Viral Infections**

184 Twenty-seven different species of Drosophilidae were maintained in multi
185 generation populations, in Drosophila stock bottles (Fisherbrand) on 50 ml of
186 their respective food medium at 22˚C and 70% relative humidity with a 12-hour
187 light-dark cycle (See *Supplementary for species and food*). Each day, two vials of
188 0-1 day old male flies were randomly assigned to one of three potential food

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189 types; low, medium or high, protein: carbohydrate ratio. The mating status of
190 flies was not controlled as some species may reach sexual maturity before
191 collection. We used male flies only for this study to remove any potential effect of
192 sex. Flies were kept on their respective food treatments for 5 days, and tipped
193 onto fresh vials of food every day (Broderick & Lemaitre, 2012; Blum *et al.*,
194 2013). After 5 days of acclimatisation on their food treatment flies were
195 experimentally infected with DCV. These collections and inoculations were
196 carried out over three replicate blocks, with each block being completed over
197 consecutive days (Figure 1). The order that the species were infected was
198 randomized each day, as was food treatment for each species.
199
200 We used Drosophila C virus (DCV) strain B6A (Longdon *et al.*, 2018), which is
201 derived from an isolate collected from *D. melanogaster* in Charolles, France
202 (Jousset *et al.*, 1972). The virus was prepared as described previously (Longdon
203 *et al.*, 2013). Briefly, DCV was grown in Schneider's Drosophila line 2 cells and
204 the Tissue Culture Infective Dose 50 (TCID50) per ml was calculated using the
205 Reed-Muench end-point method. Flies were anesthetized on CO_2 and inoculated
206 using a 0.0125 mm diameter stainless steel needle that was bent to a right angle
207 ~0.25mm from the end (Fine Science Tools, CA, USA). The bent tip of the needle
208 was dipped into the DCV solution (TCID50 = 6.32×10^9) and pricked into the
209 pleural suture on the thorax of the flies (Longdon *et al.*, 2015). We selected this
210 route of infection as oral inoculation has been shown to lead to stochastic
211 infection outcomes in *D. melanogaster*, with injection producing a more
212 reproducible infection, that has been found to follow a similar course to an oral
213 infection, with the same tissues ultimately becoming infected by both methods

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214 (Cherry & Perrimon, 2004; Chtarbanova *et al.*, 2014; Ferreira *et al.*, 2014;
215 Merkling & van Rij, 2015). One vial of inoculated flies was immediately snap
216 frozen in liquid nitrogen to provide a time point zero samples to be used as a
217 reference sample to control for relative viral dose. The second vial of flies were
218 infected and then placed back into a new vial of their respective food treatment.
219 After 2 days (+/- 1 hour) flies were snap frozen in liquid nitrogen. This time
220 point was chosen based on previous studies that show a clear increase in viral
221 growth but little mortality at 2 days post infection (Longdon *et al.*, 2015; Roberts
222 *et al.*, 2018). Each experimental block contained a day 0 and day 2 replicate for
223 each species, at each diet (27 species × 3 diet treatments × 3 experimental
224 blocks). In total, we quantified viral load in 7580 flies in 474 biological replicates
225 (biological replicate = change in viral load from day 0 to day 2 post-infection),
226 with a mean of 16 flies per replicate (range across species = 8-28).
227
228 **Survival**
229 In order to measure the effect of diet on virulence we also carried out a survival
230 assay where mortality was recorded following infection. The same infection
231 protocol was carried out as above; one vial of flies was infected with DCV whilst
232 the other was injected using a clean virus free needle dipped in *Drosophila*
233 Ringer's solution (Sullivan *et al.*, 2000) (Figure 1). Flies were maintained in vials
234 as described above and tipped onto their respective fresh food every 2 days. The
235 number of dead flies was counted every day for 21 days. The survival assay was
236 carried out across three blocks with infections carried out over consecutive days,
237 to obtain a control and infected vial per species each day. Treatment (virus or
238 control) and the order in which fly species were inoculated were randomized

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239 between blocks. Diet was randomized across days, so for a given food type a
240 control and viral infected vial was completed each day, and this was repeated
241 over consequent days until there was a control and infected for each species on
242 each food type (27 species × 2 treatments (control or challenged) × 3 diet
243 treatments × 3 experimental blocks). In total, we measured mortality in 9222
244 flies with a mean of 20 flies per replicate (range across species: 6–30 flies).
245

246 **Measuring the change in viral load**

247 The change in RNA viral load from day 0 to day 2-post infection was measured
248 using quantitative Reverse Transcription PCR (qRT-PCR). Frozen flies were
249 homogenised in Trizol reagent (Invitrogen) using a bead homogeniser for 30
250 seconds (Bead Ruptor 24; Omni international) and stored at -80˚C for later
251 extraction. Total RNA was extracted from the Trizol homogenised flies in a
252 chloroform isopropanol extraction, reverse-transcribed with Promega GoScript
253 reverse transcriptase and random hexamer primers. Viral RNA load was
254 expressed relative to the endogenous control housekeeping gene *RpL32*. Primers
255 were designed to match the homologous sequence in each species and crossed
256 an intron-exon boundary so will only amplify mRNA. The primers in *D.*
257 *melanogaster* were *RpL32* qRT-PCR F (5'-TGCTAAGCTGTCGCACAAATGG -3') and
258 *RpL32* qRT-PCR R (5'- TGCGCTTGTTCGATCCGTAAC -3') (see supplementary
259 table and Longdon *et al.*, 2011). DCV primers were 599F (5'-GACACTGCCTTT
260 GATTAG-3') and 733R (5'CCCTCTGGGAACTAAATG-3') as previously described
261 (Longdon *et al.*, 2015). Two qRT-PCR reactions (technical replicates) were
262 carried out per sample with both the viral and endogenous control primers, with
263 replicates distributed across plates in a randomised block design. qRT-PCR was

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264 performed on an Applied Biosystems StepOnePlus system using Sensifast Hi-Rox
265 Sybr kit (Bioline) with the following PCR cycle: 95˚C for 2 min followed by 40
266 cycles of: 95˚C for 5 sec followed by 60˚C for 30 sec. Each qRT-PCR plate
267 contained four standard samples. A linear model was used to correct the cycle
268 threshold (Ct) values for differences between qRT-PCR plates. Samples where
269 the technical replicates had Ct values more than 2 cycles apart after plate
270 correction were repeated. To estimate the change in viral load, we first
271 calculated ΔCt as the difference between the cycle thresholds of the DCV qRT-
272 PCR and the RpL32 endogenous control. For each species the viral load of day 2
273 flies relative to day 0 flies was calculated as $2^{-\Delta\Delta Ct}$; where ΔΔCt = ΔCt day0 – ΔCt
274 day2. The ΔCt day 0 and ΔCt day 2 is a pair of ΔCt values from a day 0 biological
275 replicate and a day 2 replicate.
276
277

Diet	Ratio Protein: Carb	Cornmeal (g)	Dextrose (g)	Yeast (g)	Agar (g)	Nipagin (ml)	dH20 (L)	Calories per 100ml
High	**1:5**	**176**	**131.2**	**84**	**22**	**29**	**1**	**142.60**
Medium	**1:10**	**176**	**176**	**38**	**22**	**29**	**1**	**142.30**
Low	**1:20**	**176**	**203**	**10**	**22**	**29**	**1**	**142.02**

278 **Table 1: Ingredients for the experimental diet treatments**. Amounts given
279 are enough to produce ~100 vials of food, with calculated calories per 100ml.
280
281 **Effect of Body Size**
282 To account for any potential differences in body size between species, we
283 measured wing length as a proxy for body size (Huey *et al.*, 2006). During the

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284 collections for the viral load assay males of each species were collected and
285 immediately stored in ethanol. Subsequently, wings were removed and
286 photographed under a dissecting microscope. Using ImageJ software (version
287 1.48) the length of the IV longitudinal vein from the tip of the proximal segment
288 to where the distal segment joins vein V was recorded, and the mean taken for
289 each species, overall there was a mean of 28 wings measured per species (range
290 20–35).
291

292 **Host phylogeny**

293 The host phylogeny was inferred as described previously (Longdon *et al.*, 2015)
294 using seven genes (mitochondrial; *COI, COII*, ribosomal; *28S* and nuclear; A*dh,*
295 *SOD, Amyrel, RpL32*). Publicly available sequences were downloaded from
296 Genbank or were Sanger sequenced. In total we had *RpL32* sequences for all 27
297 species, *28S* from 24 species, *Adh* from 24 species, *Amyrel* from 15 species, *COI*
298 from 27 species, *COII* from 27 species and *SOD* from 12 species. For each gene
299 the sequences were aligned in Geneious (version 9.1.8) (Kearse *et al.*, 2012)
300 using the global alignment setting, with free end gaps and a cost matrix of 70%
301 similarity. The phylogeny was inferred using BEAST (v1.10.4) (Drummond *et al.*,
302 2012), with genes partitioned into three groups; mitochondria, ribosomal and
303 nuclear, with their own molecular clock models. A random starting tree was
304 used, with a relaxed uncorrelated lognormal molecular clock. Each of the
305 partitions used a HKY substitution model with a gamma distribution of rate
306 variation with 4 categories and estimated base frequencies. Additionally, the
307 mitochondrial and nuclear data sets were partitioned into codon positions 1+2

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308 and 3, with unlinked substitution rates and base frequencies across codon
309 positions. The tree-shape prior was set to a birth-death process. The BEAST
310 analysis was run twice to ensure convergence for 1000 million MCMC
311 generations sampled every 10000 steps. On completion the MCMC process was
312 examined using the program Tracer (version 1.7.1) (Rambaut *et al.*, 2014) to
313 ensure convergence and adequate sampling, and the constructed tree was then
314 visualised using FigTree (v1.4.4) (Rambaut, 2006).
315

316 **Statistical analysis**

317 We used phylogenetic mixed models to look at the effects of host relatedness on
318 mortality and viral load across the three diet treatments. We fitted all models
319 using a Bayesian approach in the R package MCMCglmm version 2.29 (Hadfield,
320 2010) in RStudio (R version 3.5.1) (R Development Core Team, 2005). We used a
321 multivariate model with mortality of the controls, mortality of the virus infected
322 flies and viral load at each of the diets as the response variables. Mortality was
323 calculated as the mean portion of flies alive each day for each vial. The model
324 took the following form:
325

326 $$y_{hit} = \beta_{1:t} + wingsize\beta_{2:t} + u_{p:ht} + e_{hit}$$

327

328 Where y is the change in viral load of the ith biological replicate of host species *h*,
329 for trait *t*. β are the fixed effects, with β_1 being the intercepts for each trait and β_2
330 being the effect of wing size. U_p are the random phylogenetic species effects and e
331 the model residuals. The models were also run with a species-specific
332 component independent of phylogeny ($u_{s:ht}$) that allow us to estimate the

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333 proportion of variation that is not explained by the host phylogeny (v_s) (Longdon
334 *et al.*, 2011). The main model was run without this term as it struggled to
335 separate the phylogenetic and non-phylogenetic terms. Our main model
336 therefore assumes a Brownian motion model of evolution (Felsenstein, 1973).
337 The random effects and the residuals are assumed to be multivariate normal
338 with a zero mean and a covariance structure $\mathbf{V_p} \otimes \mathbf{A}$ for the phylogenetic affects
339 and $\mathbf{V_e} \otimes \mathbf{I}$ for the residuals ($\otimes$ here is the Kronecker product). **A** is the
340 phylogenetic relatedness matrix, **I** is an identity matrix and the **V** are 9×9
341 (co)variance matrices describing the (co)variances between viral load, mortality
342 in virus infected, and mortality in controls each at the 3 different diet levels. The
343 phylogenetic covariance matrix, $\mathbf{V_p}$ – describes the inter-specific variances in
344 each trait and the inter-specific covariances between them. The residual
345 covariance matrix, $\mathbf{V_e}$ describes the within-species variance that includes both
346 any actual within-species effects and also any measurement or experimental
347 error. The off-diagonal elements in $\mathbf{V_e}$ (the covariances) are unable to be
348 estimated because no vial has multiple measurements – so were set to zero. The
349 MCMC chain was run for 1300 million iterations with a burn-in of 30 million
350 iterations and a thinning interval of 1 million. Results were tested for sensitivity
351 to the use of different priors by being run with different prior structures (see
352 supplementary R code), which gave qualitatively similar results. We also ran
353 models with the data subset into viral load and mortality that gave similar
354 results. All confidence intervals (CI's) reported are 95% highest posterior
355 density intervals. In order to test for the interaction between diet and species we
356 calculated correlations between traits from the variance covariance matrix from
357 the diet:species random effect ($u_{p:\mathrm{h}t}$). If the correlations between traits are close

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358 to one and there is no change in the means or the variance, it would suggest that
359 there is no species-by-diet interaction. We confirmed our experimental design
360 and sample sizes had sufficient power to detect effects by down sampling a
361 similar dataset (Roberts *et al.*, 2018).
362

363 **Results**

364 In order to investigate the effect that host diet may have on the likelihood of
365 virus host shifts we quantified DCV viral load in 27 species of Drosophilidae that
366 had been housed on three different diets (Fig.2). Viral loads differed between
367 species, with a billion times more virus in the most susceptible compared to the
368 least susceptible species, consistent with previous studies (Longdon *et al.*, 2015;
369 Roberts *et al.*, 2018). Viral loads were highly repeatable, with the inter-specific
370 phylogenetic component (v_p), explaining a high proportion of the variation in
371 viral load across diets with little within species or measurement error (v_e)
372 (Repeatability = v_p /(v_p + v_e); Low = 0.92 (95% CI: 0.86, 0.96); Medium = 0.90
373 (95% CI: 0.84,0.96); High = 0.83 (95% CI: 0.75, 0.92).

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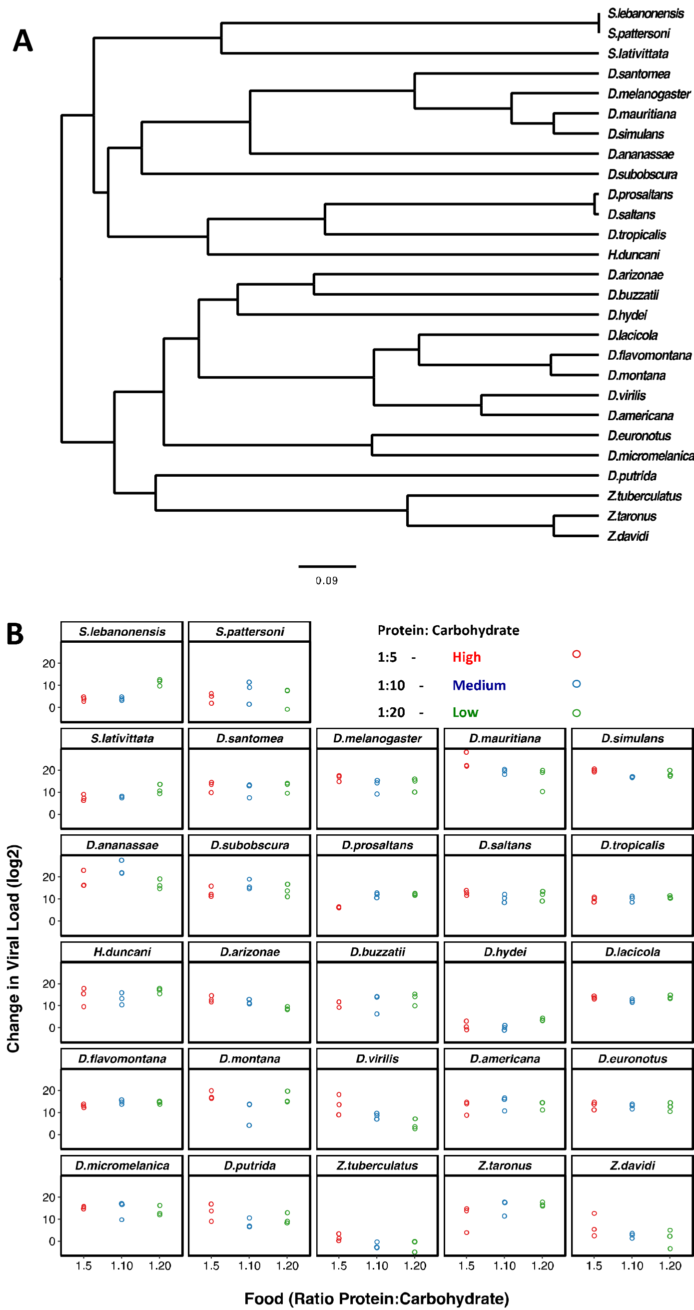

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383 We also partitioned the inter-specific variance into that which can be explained
384 by a Brownian motion model of evolution on the host phylogeny (v_p), and a
385 species-specific component independent of the phylogeny (v_s). The proportion of
386 the between species variance that can be explained by the phylogeny can then be
387 calculated, using $v_p/(v_p + v_s)$ (Freckleton *et al.*, 2002), and can be equated to the
388 phylogenetic heritability or Pagel's lambda (Pagel, 1999; Housworth *et al.*, 2004).
389 We found that the host phylogeny explained a modest amount of the inter-
390 specific variation in viral loads across diets, however these estimates had broad
391 confidence intervals (Low = 0.20 (95% CI: 3.5 x10^{-6}, 0.63); Medium = 0.34 (95%
392 CI: 2.0 x 10^{-6}, 0.80); High = 0.51 (95% CI: 3.2 x 10^{-6}, 0.88), due to the model
393 struggling to separate out the phylogenetic and non-phylogenetic components.
394

395 In order to examine if the susceptibility of species responded in the same or
396 different ways to the changes in diet we examined viral load across the different
397 protein: carbohydrate ratios. We found strong positive inter-specific correlations
398 between viral loads across diet treatments suggesting the species are responding
399 in similar ways to the changes in ratios (Table 2). There was a decline in the
400 between species variance in the high diet compared to low and medium – but
401 this was not significantly different– (v_p: Low =77.13 (95% CI: 35.09, 125.50);
402 Medium =82.33 (95% CI: 37.55, 135.26); High =45.59 (95% CI: 19.61, 76.39) and
403 mean viral loads were similar across the diets (Low = 11.4 (95% CI: 5.3, 17.7);
404 Medium = 10.6 (95% CI: 3.6, 16.6); High = 10.6 (95% CI: 3.6, 16.7). Residual
405 variance did not differ significantly between treatments (Low = 6.45 (95% CI:
406 4.87, 8.04); Medium = 8.23 (95% CI: 6.45, 10.35); High = 8.32 (95% CI: 6.54,
407 10.6).

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408

		Interspecific Correlation	95% CIs
Viral load	Low - Medium	0.93	0.85, 0.98
	Medium - High	0.83	0.66, 0.96
	Low - High	0.80	0.63, 0.96
Survival in virus challenged	Low - Medium	0.93	0.81, 0.99
	Medium - High	0.88	0.71, 0.99
	Low - High	0.90	0.73, 0.99

409 **Table 2. Inter-specific correlations between viral load and mortality**
410 **measures across the different diet treatments.**
411

412 As similar pathogen loads can cause different levels of harm to their hosts (Roy &
413 Kirchner, 2000; Boots, 2008; Råberg *et al.*, 2009) we examined if virus induced
414 mortality differed across diets over a 20 day period after viral challenge (Fig. 3).
415 We found differences in the virulence (mortality) caused by DCV between host
416 species, with some species seeing no apparent change in mortality over the
417 experimental period compared to sham infected controls, (e.g. *S. pattersoni* and
418 *D. saltans*), whilst other species show higher susceptibility with up to 50% of
419 flies dead by day 10 post infection (e.g. *D. simulans* and *D. melanogaster*). As with
420 the viral load data we calculated the repeatability of survival in these virus
421 infected flies which was high in all cases (Repeatability; Low = 0.90 (95% CI:
422 0.78, 0.98); Medium = 0.87 (95% CI: 0.71,0.97); High = 0.98 (95% CI: 0.85, 1.00).
423 We also calculated the proportion of between species variance that can be
424 explained by the phylogeny for the virus infected flies (Phylogenetic effect: Low

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425 = 0.16 (95% CI: 2.58 x10^{-6}, 0.62); Medium = 0.18 (95% CI: 5.75 x 10^{-7}, 0.78); High
426 = 0.32 (95% CI: 7.75 x 10^{-8}, 0.87), which – like the viral load data – had broad
427 confidence intervals due to the model struggling to separate the phylogenetic
428 and non-phylogenetic components.
429
430 We found strong positive inter-specific correlations between the survival of
431 virus challenged flies across the diets, suggesting the species are responding in
432 similar ways to the dietary changes (Table 2). Among species variance in
433 mortality of virus infected flies was consistent across diets (Low = 0. 18 (95% CI:
434 0.07, 0.31); Medium = 0.16 (95% CI: 0.04, 0.30); High = 0.12 (95% CI: 0.04, 0.23)
435 as was the mean mortality (Low = 0.64 (95% CI: 0.47, 0.82); Medium = 0.58
436 (95% CI: 0.38, 0.75); High = 0.65 (95% CI: 0.47, 0.82). The residual variance was
437 also consistent across the diets (Low = 0.02 (95% CI: 0.01, 0.03); Medium = 0.02
438 (95% CI: 0.01, 0.04); High = 0.02 (95% CI: 0.01, 0.03).

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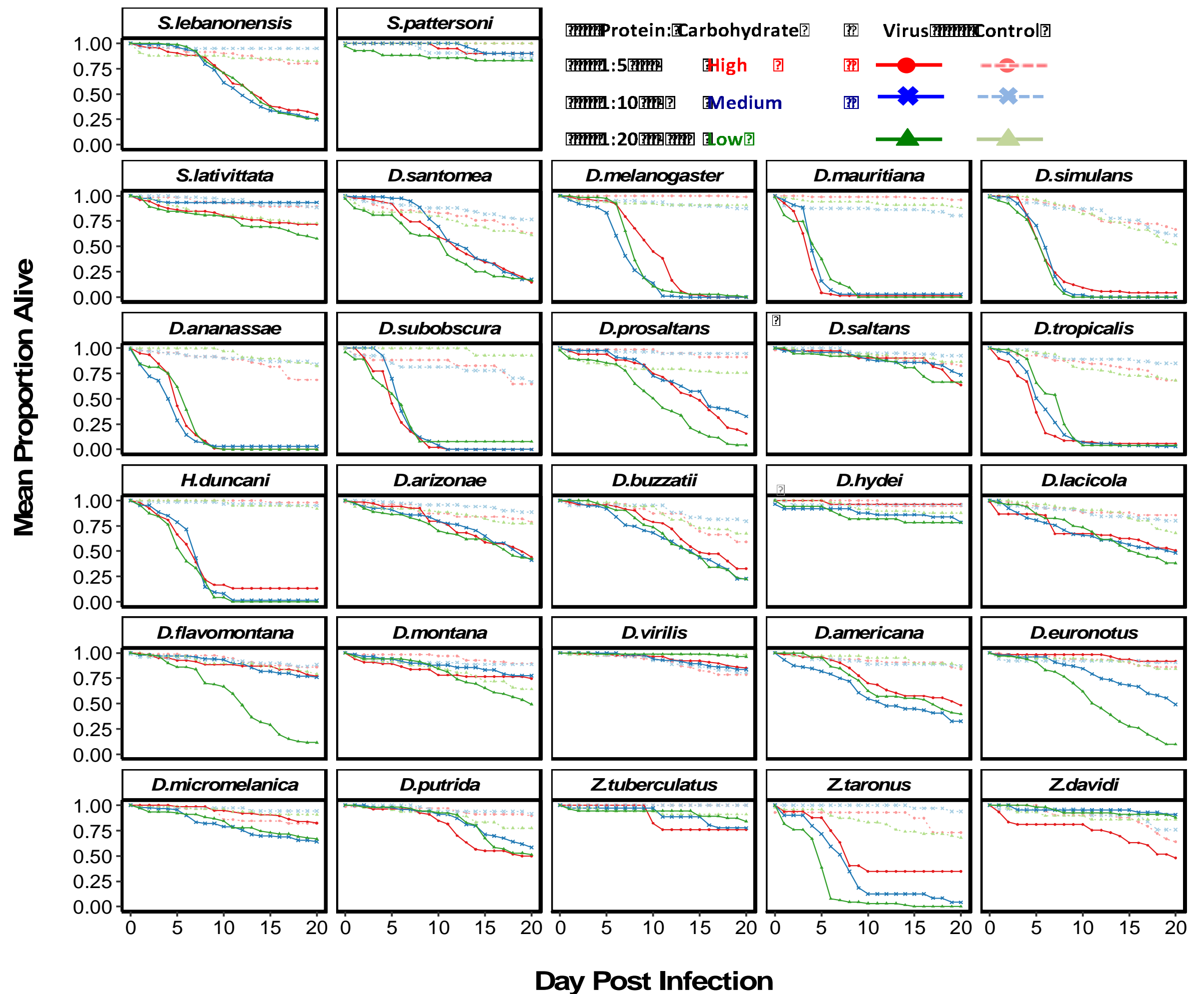

439

440 **Figure 3. Mortality in 27 species of Drosophilidae housed on three different**
441 **diets of varying protein: carbohydrate ratios.** High- red circles, Medium - blue
442 crosses and Low- green triangles and either control stabbed (dashed line) or
443 virally challenged with DCV (solid lines). Panels are labelled in line with the tips
444 in Figure 2A.

445

446 We found that there were strong positive correlations between mortality and
447 RNA viral load (interspecific correlations between viral load and survival of virus
448 infected flies: Low = 0.89 (95% CI: 0.78, 0.98); Medium = 0.85 (95% CI: 0.67,
449 0.97); High = 0.67 (95% CI: 0.35, 0.90). To confirm that these differences are due
450 to mortality caused by the virus rather than intrinsic differences in the

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451 survivorship of the different species, we also inoculated flies with a control
452 solution. There was far less mortality in the controls than the virus infected flies
453 (Fig. 3). There was inter-specific variation in control mortality (Low = 0.18 (95%
454 CI: 0.01, 0.59); Medium = 0.43 (95% CI: 0.01,0.76); High = 0.55 (95% CI: -0.72,
455 1.00) but this was not significantly correlated with survival of the virus infected
456 flies (survival of control versus virus infected on: Low = -0.11 (95% CI: -0.92,
457 0.75); Medium = 0.34 (95% CI: -0.48 0.97); High = 0.12 (95% CI: -0.82, 0.89). We
458 found no effect of wing length as a proxy for body size, (mean: -0.05, 95% CI: -
459 0.13, 0.05).
460
461 **Discussion**
462 We found dietary treatments of differing protein to carbohydrate ratios did not
463 alter the outcome of infection in 27 species of Drosophilidae infected with DCV.
464 We found strong positive inter-specific correlations across diets in both viral
465 load and mortality (Table 2), suggesting that the species are in general
466 responding in similar ways to nutritional changes. Despite there being among
467 species variation in susceptibility, generally changes in diet did not affect viral
468 loads, nor did they alter the likelihood of surviving an infection. We found strong
469 positive correlations between mortality and viral load on each of the diets,
470 suggesting the amount of harm caused to a host is a result of virus accumulation
471 within the infected host.
472
473 Although the point estimates of the inter-specific correlations are close to one
474 (Table 2) – suggesting overall there is limited evidence for interactions between
475 species and diet, some species do appear to show differences in mortality on

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476 different diets (e.g. *D. euronotus* and *D. flavomontana*, Figure 3). These patterns
477 however, are not present when looking at the viral load data for these species,
478 and our power analysis suggests we have enough power to detect interaction
479 effects with our present experimental design. Therefore, further experiments
480 designed to look specifically at the differences within species are required to
481 determine if these patterns of mortality would hold true.
482
483 Both mounting and maintaining an immune response requires energy and
484 nutrients. During an acute immune challenge the provisioning of nutrients may
485 become more demanding for a host, with pathogen induced malabsorption
486 through damage to or obstruction of digestive tissues (Lochmiller & Deerenberg,
487 2000). DCV is known to cause severe pathology of the tissues of the digestive
488 tract with subsequent accumulation of food in the crop (food storage organ) and
489 obstruction in the intestine (Chtarbanova *et al.*, 2014). These physical symptoms
490 alter an infected hosts energy stores with infected flies showing significantly
491 reduced glycogen and triglyceride levels three to four days post infection
492 (Chtarbanova *et al.*, 2014). DCV infected flies also increase in body mass, with a
493 reduced food intake and reduced metabolism, suggesting that they experience
494 increased water retention (Thomas-Orillard, 1984; Arnold *et al.*, 2013;
495 Chtarbanova *et al.*, 2014). We therefore hypothesised that changing the ratio of
496 protein to carbohydrate in the diet may alter outcome of infection, and as species
497 may all have their own “optimal diet”, that species may respond in different ways
498 to such changes. However, this does not appear to be the case.
499

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500 Geometric frameworks for nutrition were developed in response to the fact that
501 what is "optimal" will depend on a balance of particular nutrients in the
502 organism and trait being investigated (Simpson & Raubenheimer, 1995; Archer
503 *et al.*, 2009; Cotter *et al.*, 2019). For example mice infected with *Salmonella* were
504 found to survive better on diets containing a higher ratio of protein to
505 carbohydrate (Peck *et al.*, 1992). As were army worm caterpillars infected with
506 bacteria, with survival increasing with dietary protein, suggesting high protein
507 requirements are associated with bacterial resistance (Povey *et al.*, 2009). A
508 recent study used 10 different protein: carbohydrate diets and challenged flies
509 with *Pseudomonas entomophila* bacteria (Savola *et al.*, 2020a). Survival on low
510 protein diets was found to be lower in infected flies and suggested protein was
511 important for survival during infection. This study also monitored lifespan and
512 reproduction in flies, and found that regardless of injury and infection, dietary
513 restriction extended lifespan and reduced reproductive output (Savola *et al.*,
514 2020a). One potential mechanism of the interaction of diet and infection has
515 been suggested in research using a model host-pathogen system *in vivo* and *in*
516 *vitro* (Wilson *et al.*, 2020). Caterpillars of *S. littoralis* challenged with the bacteria
517 *X. nematophila in vivo* and on high dietary protein had slower bacterial growth
518 with higher survival. When this was combined with *in vitro* experiments the
519 results suggested this was driven by the osmolality of the hosts' blood
520 (hemolymph) being altered by an increase in solutes in the high protein diets
521 slowing the bacterial growth (Wilson *et al.*, 2020).
522
523 Further research on the mechanistic basis of dietary effects on resistance is
524 needed for other pathogen taxa, including viruses. Immunity to DCV inoculation

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525 in *D. melanogaster* has been reported to involve the JAK/STAT and Imd
526 pathways, and potentially phagocytosis (Van Rij *et al.*, 2006; Zhu *et al.*, 2013;
527 Lamiable *et al.*, 2016). Additionally, the RNAi pathway is a key antiviral defence
528 mechanism in *Drosophila* and DCV appears to have evolved to suppress this
529 response (Van Rij *et al.*, 2006). Although we find no interaction between dietary
530 protein:carbohydrate and susceptibility, the multifaceted immune response to
531 DCV may be energetically costly and other nutrients may interact with the ability
532 of a host to allocate resources between an immune response, damage repair and
533 the maintenance of homeostasis (Lochmiller & Deerenberg, 2000; Zuk & Stoehr,
534 2002; Schmid-Hempel, 2005; Sadd & Siva-Jothy, 2006). For example, lipid and
535 fats have been associated with *D. melanogaster* response to DCV viral infection;
536 peroxisomes were found to be required for host defense to infection, through
537 their primary function in lipid metabolism (Aubert *et al.*, 1995). The lipid level
538 across our diets was held constant, but this may be a potential area for further
539 study. There has been an increased use of a chemically defined (holidic) diet in
540 order to manipulate individual nutrients present in fly diets (Lee *et al.*, 2006).
541 Exome matched diets have been shown to alleviate trade-offs in fecundity and
542 longevity (Piper *et al.*, 2017). A possible extension of this would be to look at the
543 effect of matching diets to transcriptional changes during infection, and seeing if
544 this alleviates (or exacerbates) pathology.
545
546 Changes in diet have been shown to alter pathogen susceptibility in a number of
547 systems. We hypothesised that changes in diet could alter the potential outcomes
548 of virus host shifts. However, we found that overall changes in the ratio of
549 protein to carbohydrate did not alter susceptibility to DCV across host species in

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550 this instance. This suggests dietary protein to carbohydrate ratios are not
551 universally important in determining susceptibility to pathogens. It is unclear if
552 the lack of studies showing no effect of diet reflect publication biases or whether
553 our model system is unusual. However, it highlights the need to examine the
554 importance of diet in explaining susceptibility to pathogens across a broad range
555 of host and pathogen taxa.
556

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