Susceptibility across host species is independent of dietary protein to carbohydrate ratios

Twitter summary: No role of host diet in susceptibility to a novel viral pathogen across host species

Impact Statement: A successful host shift of a parasite from one susceptible species to a novel host can be influenced by many ecological factors. Changes in host diet can alter the immune response and outcomes of host–parasite interactions and could potentially alter the outcome of a virus host shift. To investigate, we infected 27 species of Drosophilidae with an RNA virus (DCV) across three food types with differing protein to carbohydrate ratios. We then looked at pathogen loads and survival of infected hosts compared to uninfected controls. Changes in the ratio of protein to carbohydrate did not alter susceptibility to DCV across host species.

Abstract

The likelihood of a successful host shift of a parasite to a novel host species can be influenced by environmental factors that can act on both the host and parasite. Changes in nutritional resource availability have been shown to alter pathogen susceptibility and the outcome of infection in a range of systems. Here we examined how dietary protein to carbohydrate altered susceptibility in a large cross infection experiment. We infected 27 species of Drosophilidae with an RNA virus on three food types of differing protein to carbohydrate ratios. We then measured how viral load and mortality across species was affected by

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

Introduction

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

Thomas, 1999; Harvell *et al.*, 2002; Ponton *et al.*, 2013; Hayman *et al.*, 2016; Cumnock *et al.*, 2018).

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

resources with life history traits (Kraaijeveld & Godfray, 1997; Lochmiller & Deerenberg, 2000; Cotter *et al.*, 2011; Knutie *et al.*, 2017).

From a parasite perspective infecting a host of suboptimal nutritional status may mean they encounter a weaker immune response and therefore infection and establishment is easier (Siva-Jothy & Thompson, 2002). However, once established the parasite may encounter its own resource limitations due to competition with an already depleted host, causing suboptimal growth and potentially affecting onward transmission. Therefore, predicting the outcome of the interaction between nutrition, host immunity and subsequent resistance is complex as the effects on the two parties may be divergent (Bedhomme *et al.*, 2004).

Multiple life history traits are moderated by resource availability, with condition dependency across reproductive traits, aging and lifespan (Lee *et al.*, 2008; Maklakov *et al.*, 2009; Camus *et al.*, 2017; Henry & Colinet, 2018; Henry *et al.*, 2020). Laboratory experiments on dietary restriction, where individuals experienced a reduction in nutrition without inducing malnutrition (differentiated from Calorie Restriction) have been found to extend life span in a range of organisms (Weindruch & Walford, 1982; Klass, 1983; Anderson *et al.*, 2003; Nakagawa *et al.*, 2012). The effects of dietary restriction appear to be explained by resource-mediated trade-offs between longevity and reproductive effort (but see review by (Moatt *et al.*, 2020)). Geometric frameworks – the use of artificial diets with known compositions of specific nutrients that develop an

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

To investigate the effect that host diet has on the likelihood of a pathogen host shift into different host species we infected 27 species of Drosophilidae with Drosophila C Virus (DCV) fed on three diets with varying ratios of protein to carbohydrates but comparable calorie content. We then measured the change in viral load and host mortality across these different diets. DCV is a positive sense

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

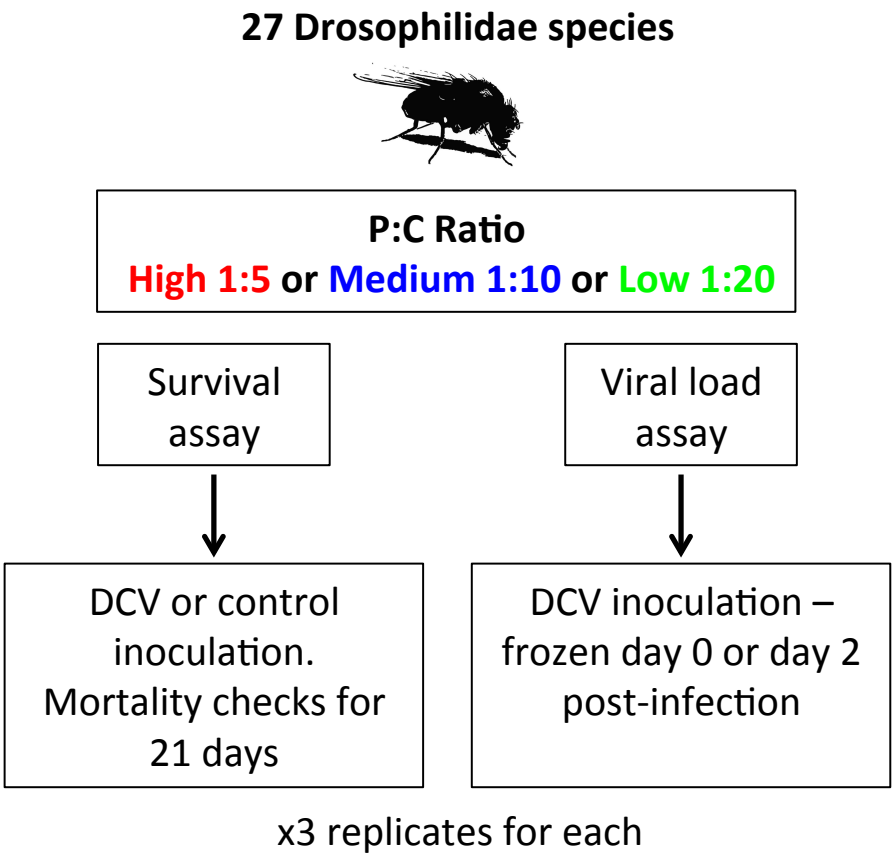
149 large proportion of the variation in DCV virulence (mortality) and viral load
150 (75% and 67% respectively) with high virulence being associated with high viral
151 loads (Longdon *et al.*, 2015). One of the fundamental steps needed for a
152 successful host shift is the ability of a pathogen to infect a novel host. Here we
153 ask if the nutritional environment alters the susceptibility to DCV following a
154 shift into a range of novel host species, and whether such patterns are consistent
155 across species.
156

157 **Methods**

158 **Diet preparation**

159 Three different cornmeal diets were used (*Supplementary of species used and*
160 *food type*). The standard cornmeal diet used in our lab comprised a 1:10 protein
161 to carbohydrate ratio and became our "Medium" - protein: carbohydrate ratio
162 diet treatment. We also developed two further diets that were approximately
163 isocaloric, a low protein: carbohydrate food (1: 20 protein to carbohydrate) and
164 a high protein: carbohydrate food (1: 5 protein carbohydrate); see
165 supplementary for full table of food recipes nutrient breakdown. These were
166 based around previous findings that suggested that in *D. melanogaster* lifespan
167 was maximized on a protein: carbohydrate ratio of around 1:16, and fitness -
168 measured as lifetime egg production at a ratio of 1:4 (Lee *et al.*, 2008). All diets
169 were manipulated by altering the dextrose and yeast amounts whilst
170 maintaining as close as possible the same calorie content at 142 Calories g/100
171 ml. Yeast was manipulated as it provides the majority of the protein as well as
172 other non-caloric nutritional requirements (Piper, 2017). Values were confirmed

173 using the Drosophila Dietary Composition Calculator (Lesperance & Broderick,
174 2020).



175

176 **Figure 1. Schematic of the experimental set up:** males from 27 species of
177 Drosophilidae were housed on three foods with different protein: carbohydrate
178 ratios before being inoculated with DCV or a control for a survival assay. Flies
179 were also inoculated with DCV and sampled immediately (day 0) or 2 days post
180 infection to measure the change in RNA viral load. For each treatment
181 (control/virus or day 0/day 2) there were three replicates, where each replicate
182 was a vial of flies (number of flies per vial described below)
183

184 **Viral Infections**

185 Twenty-seven different species of Drosophilidae were maintained in multi
186 generation populations, in Drosophila stock bottles (Fisherbrand) on 50 ml of
187 their respective food medium at 22˚C and 70% relative humidity with a 12-hour

188 light-dark cycle (See *Supplementary for species and food*). Each day, two vials of
189 0-1 day old male flies were randomly assigned to one of three potential food
190 types; low, medium or high, protein: carbohydrate ratio. The mating status of
191 flies was not controlled as some species may reach sexual maturity before
192 collection. We used male flies only for this study to remove any potential effect of
193 sex. Flies were kept on their respective food treatments for 5 days, and tipped
194 onto fresh vials of food every day (Broderick & Lemaitre, 2012; Blum *et al.*,
195 2013). After 5 days of acclimatisation on their food treatment flies were
196 experimentally infected with DCV. These collections and inoculations were
197 carried out over three replicate blocks, with each block being completed over
198 consecutive days (Figure 1). The order that the species were infected was
199 randomized each day, as was food treatment for each species.
200
201 We used Drosophila C virus (DCV) strain B6A (Longdon *et al.*, 2018), which is
202 derived from an isolate collected from *D. melanogaster* in Charolles, France
203 (Jousset *et al.*, 1972). The virus was prepared as described previously (Longdon
204 *et al.*, 2013). Briefly, DCV was grown in Schneider's Drosophila line 2 cells and
205 the Tissue Culture Infective Dose 50 (TCID50) per ml was calculated using the
206 Reed-Muench end-point method. Flies were anesthetized on CO_2 and inoculated
207 using a 0.0125 mm diameter stainless steel needle that was bent to a right angle
208 ~0.25mm from the end (Fine Science Tools, CA, USA). The bent tip of the needle
209 was dipped into the DCV solution (TCID50 = 6.32×10^9) and pricked into the
210 pleural suture on the thorax of the flies (Longdon *et al.*, 2015). We selected this
211 route of infection as oral inoculation has been shown to lead to stochastic
212 infection outcomes in *D. melanogaster*, with injection producing a more

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

238 to obtain a control and infected vial per species each day. Treatment (virus or
239 control) and the order in which fly species were inoculated were randomized
240 between blocks. Diet was randomized across days, so for a given food type a
241 control and viral infected vial was completed each day, and this was repeated
242 over consequent days until there was a control and infected for each species on
243 each food type (27 species × 2 treatments (control or challenged) × 3 diet
244 treatments × 3 experimental blocks). In total, we measured mortality in 9222
245 flies with a mean of 20 flies per replicate (range across species: 6–30 flies).
246

247 **Measuring the change in viral load**

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

263 carried out per sample with both the viral and endogenous control primers, with
264 replicates distributed across plates in a randomised block design. qRT-PCR was
265 performed on an Applied Biosystems StepOnePlus system using Sensifast Hi-Rox
266 Sybr kit (Bioline) with the following PCR cycle: 95˚C for 2 min followed by 40
267 cycles of: 95˚C for 5 sec followed by 60˚C for 30 sec. Each qRT-PCR plate
268 contained four standard samples. A linear model was used to correct the cycle
269 threshold (Ct) values for differences between qRT-PCR plates. Samples where
270 the technical replicates had Ct values more than 2 cycles apart after plate
271 correction were repeated. To estimate the change in viral load, we first
272 calculated ΔCt as the difference between the cycle thresholds of the DCV qRT-
273 PCR and the RpL32 endogenous control. For each species the viral load of day 2
274 flies relative to day 0 flies was calculated as $2^{-\Delta\Delta Ct}$; where ΔΔCt = ΔCt day0 – ΔCt
275 day2. The ΔCt day 0 and ΔCt day 2 is a pair of ΔCt values from a day 0 biological
276 replicate and a day 2 replicate.
277
278

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**

279 **Table 1: Ingredients for the experimental diet treatments**. Amounts given
280 are enough to produce ~100 vials of food, with calculated calories per 100ml.
281
282 **Effect of Body Size**

283 To account for any potential differences in body size between species, we
284 measured wing length as a proxy for body size (Huey *et al.*, 2006). During the
285 collections for the viral load assay males of each species were collected and
286 immediately stored in ethanol. Subsequently, wings were removed and
287 photographed under a dissecting microscope. Using ImageJ software (version
288 1.48) the length of the IV longitudinal vein from the tip of the proximal segment
289 to where the distal segment joins vein V was recorded, and the mean taken for
290 each species, overall there was a mean of 28 wings measured per species (range
291 20–35).
292
293 **Host phylogeny**

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

variation with 4 categories and estimated base frequencies. Additionally, the mitochondrial and nuclear data sets were partitioned into codon positions 1+2 and 3, with unlinked substitution rates and base frequencies across codon positions. The tree-shape prior was set to a birth-death process. The BEAST analysis was run twice to ensure convergence for 1000 million MCMC generations sampled every 10000 steps. On completion the MCMC process was examined using the program Tracer (version 1.7.1) (Rambaut *et al.*, 2014) to ensure convergence and adequate sampling, and the constructed tree was then visualised using FigTree (v1.4.4) (Rambaut, 2006).

Statistical analysis

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

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

Where y is the change in viral load of the ith biological replicate of host species h, for trait t. β are the fixed effects, with β_1 being the intercepts for each trait and β_2 being the effect of wing size. U_p are the random phylogenetic species effects and e

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

357 calculated correlations between traits from the variance covariance matrix from
358 the diet:species random effect ($u_{p:\mathrm{ht}}$). If the correlations between traits are close
359 to one and there is no change in the means or the variance, it would suggest that
360 there is no species-by-diet interaction. We confirmed our experimental design
361 and sample sizes had sufficient power to detect effects by down sampling a
362 similar dataset (Roberts *et al.*, 2018).
363

364 **Results**

365 In order to investigate the effect that host diet may have on the likelihood of
366 virus host shifts we quantified DCV viral load in 27 species of Drosophilidae that
367 had been housed on three different diets (Fig.2). Viral loads differed between
368 species, with a billion times more virus in the most susceptible compared to the
369 least susceptible species, consistent with previous studies (Longdon *et al.*, 2015;
370 Roberts *et al.*, 2018). Viral loads were highly repeatable, with the inter-specific
371 phylogenetic component (v_p), explaining a high proportion of the variation in
372 viral load across diets with little within species or measurement error (v_e)
373 (Repeatability = v_p /(v_p + v_e); Low = 0.92 (95% CI: 0.86, 0.96); Medium = 0.90
374 (95% CI: 0.84,0.96); High = 0.83 (95% CI: 0.75, 0.92).

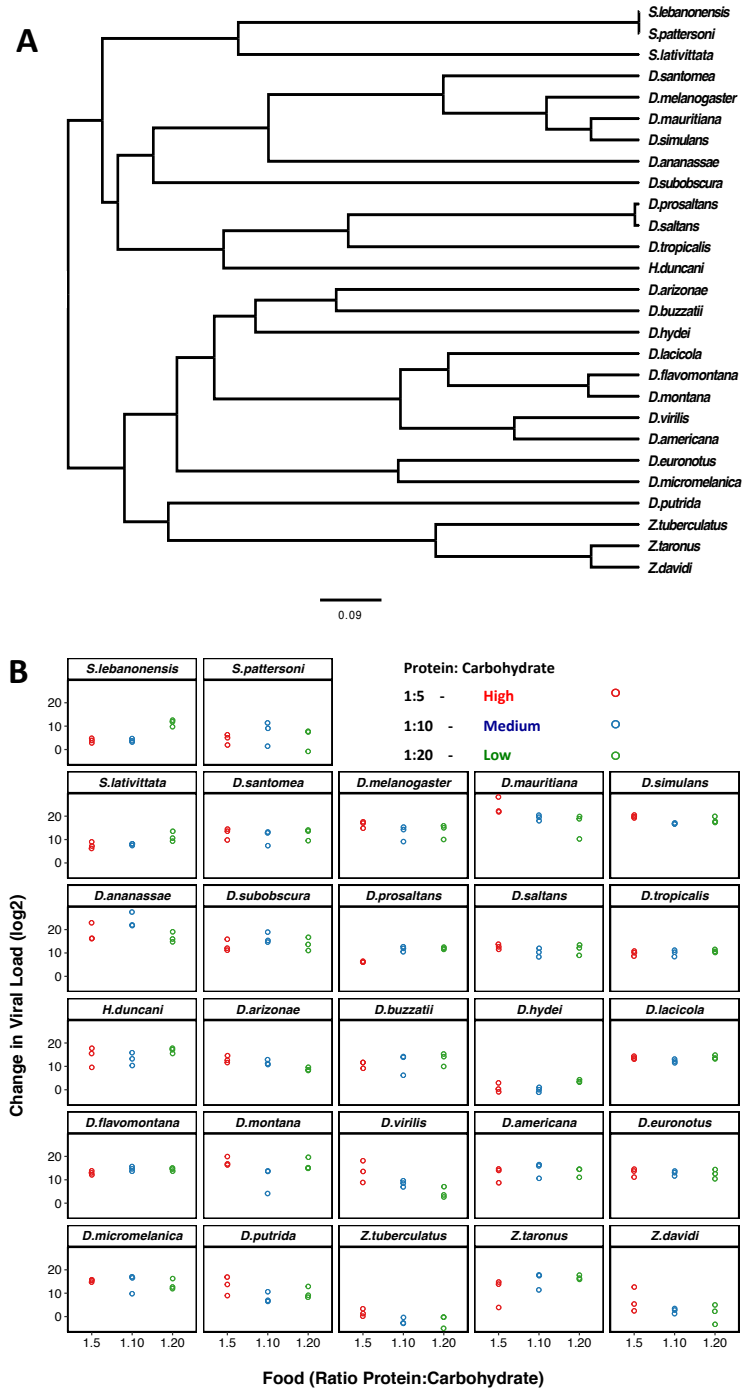


Figure 2. (A). Phylogeny of the 27 Drosophilidae host species. Scale bar is the number of substitutions per site. **(B) Change in RNA viral load (log_2) for the host species infected with DCV across the three different diets of differing protein: carbohydrate ratios.** Individual points are plotted with a small x-axis jitter and represent the change in viral load between day 0 and day 2-post infection. Panels are ordered as on the tips of the phylogeny in (A).

384 We also partitioned the inter-specific variance into that which can be explained
385 by a Brownian motion model of evolution on the host phylogeny (v_p), and a
386 species-specific component independent of the phylogeny (v_s). The proportion of
387 the between species variance that can be explained by the phylogeny can then be
388 calculated, using $v_p/(v_p + v_s)$ (Freckleton *et al.*, 2002), and can be equated to the
389 phylogenetic heritability or Pagel's lambda (Pagel, 1999; Housworth *et al.*, 2004).
390 We found that the host phylogeny explained a modest amount of the inter-
391 specific variation in viral loads across diets, however these estimates had broad
392 confidence intervals (Low = 0.20 (95% CI: 3.5 x10^{-6}, 0.63); Medium = 0.34 (95%
393 CI: 2.0 x 10^{-6}, 0.80); High = 0.51 (95% CI: 3.2 x 10^{-6}, 0.88), due to the model
394 struggling to separate out the phylogenetic and non-phylogenetic components.
395
396 In order to examine if the susceptibility of species responded in the same or
397 different ways to the changes in diet we examined viral load across the different
398 protein: carbohydrate ratios. We found strong positive inter-specific correlations
399 between viral loads across diet treatments suggesting the species are responding
400 in similar ways to the changes in ratios (Table 2). There was a decline in the
401 between species variance in the high diet compared to low and medium – but
402 this was not significantly different– (v_p: Low =77.13 (95% CI: 35.09, 125.50);
403 Medium =82.33 (95% CI: 37.55, 135.26); High =45.59 (95% CI: 19.61, 76.39) and
404 mean viral loads were similar across the diets (Low = 11.4 (95% CI: 5.3, 17.7);
405 Medium = 10.6 (95% CI: 3.6, 16.6); High = 10.6 (95% CI: 3.6, 16.7). Residual
406 variance did not differ significantly between treatments (Low = 6.45 (95% CI:
407 4.87, 8.04); Medium = 8.23 (95% CI: 6.45, 10.35); High = 8.32 (95% CI: 6.54,
408 10.6).

409

		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

410 **Table 2. Inter-specific correlations between viral load and mortality**
411 **measures across the different diet treatments.**
412

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

426 = 0.16 (95% CI: 2.58 x10^{-6}, 0.62); Medium = 0.18 (95% CI: 5.75 x 10^{-7}, 0.78); High
427 = 0.32 (95% CI: 7.75 x 10^{-8}, 0.87), which – like the viral load data – had broad
428 confidence intervals due to the model struggling to separate the phylogenetic
429 and non-phylogenetic components.
430
431 We found strong positive inter-specific correlations between the survival of
432 virus challenged flies across the diets, suggesting the species are responding in
433 similar ways to the dietary changes (Table 2). Among species variance in
434 mortality of virus infected flies was consistent across diets (Low = 0. 18 (95% CI:
435 0.07, 0.31); Medium = 0.16 (95% CI: 0.04, 0.30); High = 0.12 (95% CI: 0.04, 0.23)
436 as was the mean mortality (Low = 0.64 (95% CI: 0.47, 0.82); Medium = 0.58
437 (95% CI: 0.38, 0.75); High = 0.65 (95% CI: 0.47, 0.82). The residual variance was
438 also consistent across the diets (Low = 0.02 (95% CI: 0.01, 0.03); Medium = 0.02
439 (95% CI: 0.01, 0.04); High = 0.02 (95% CI: 0.01, 0.03).

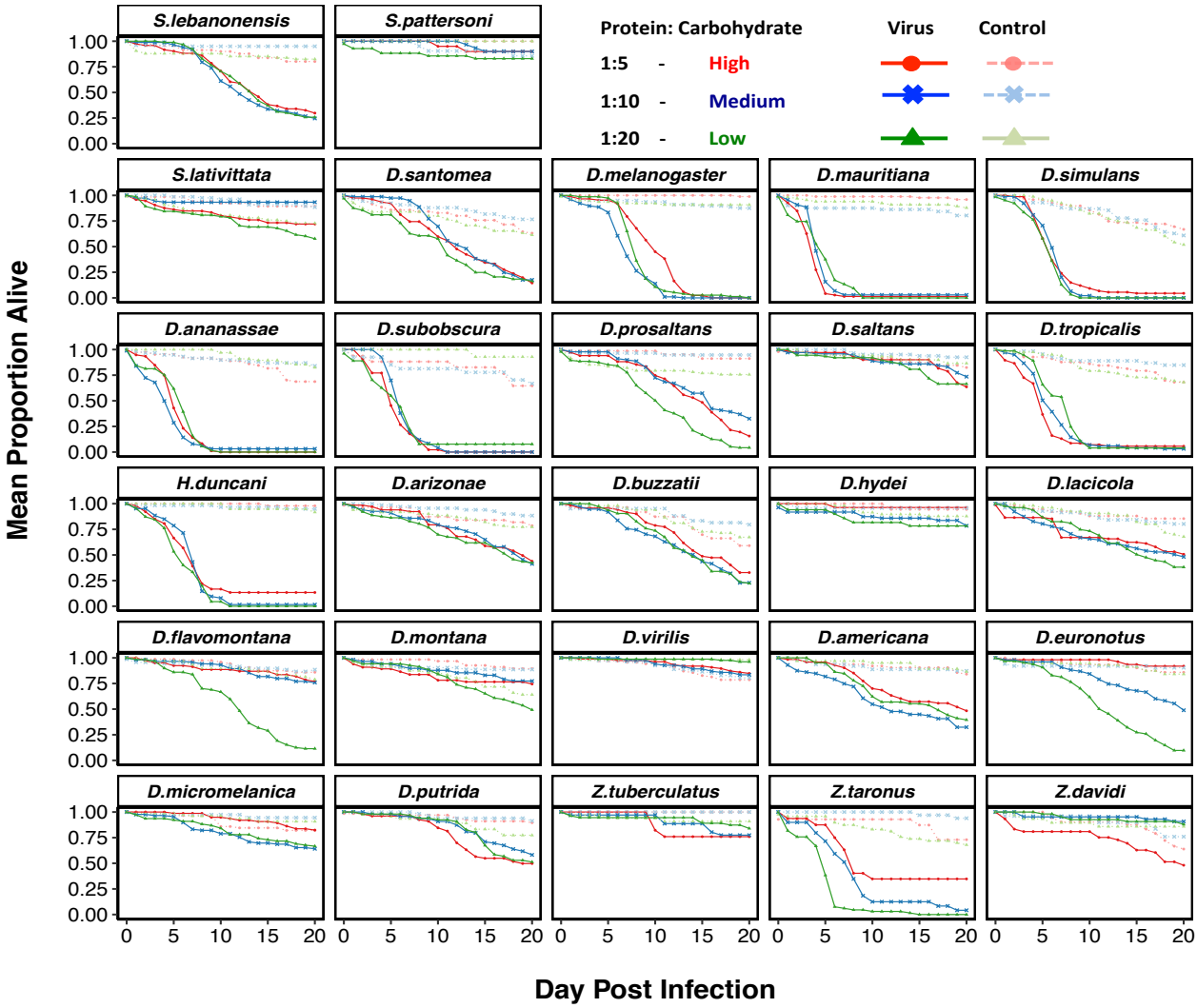


Figure 3. Mortality in 27 species of Drosophilidae housed on three different diets of varying protein: carbohydrate ratios. High- red circles, Medium - blue crosses and Low- green triangles and either control stabbed (dashed line) or virally challenged with DCV (solid lines). Panels are labelled in line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

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

452 survivorship of the different species, we also inoculated flies with a control
453 solution. There was far less mortality in the controls than the virus infected flies
454 (Fig. 3). There was inter-specific variation in control mortality (Low = 0.18 (95%
455 CI: 0.01, 0.59); Medium = 0.43 (95% CI: 0.01,0.76); High = 0.55 (95% CI: -0.72,
456 1.00) but this was not significantly correlated with survival of the virus infected
457 flies (survival of control versus virus infected on: Low = -0.11 (95% CI: -0.92,
458 0.75); Medium = 0.34 (95% CI: -0.48 0.97); High = 0.12 (95% CI: -0.82, 0.89). We
459 found no effect of wing length as a proxy for body size, (mean: -0.05, 95% CI: -
460 0.13, 0.05).
461

462 **Discussion**

463 We found dietary treatments of differing protein to carbohydrate ratios did not
464 alter the outcome of infection in 27 species of Drosophilidae infected with DCV.
465 We found strong positive inter-specific correlations across diets in both viral
466 load and mortality, suggesting that the species are in general responding in
467 similar ways to nutritional changes. Despite there being among species variation
468 in susceptibility, changes in diet did not affect viral loads, nor did they alter the
469 likelihood of surviving an infection. We found strong positive correlations
470 between mortality and viral load on each of the diets, suggesting the amount of
471 harm caused to a host is a result of virus accumulation within the infected host.
472

473 Both mounting and maintaining an immune response requires energy and
474 nutrients. During an acute immune challenge the provisioning of nutrients may
475 become more demanding for a host, with pathogen induced malabsorption
476 through damage to or obstruction of digestive tissues (Lochmiller & Deerenberg,

477 2000). DCV is known to cause severe pathology of the tissues of the digestive
478 tract with subsequent accumulation of food in the crop (food storage organ) and
479 obstruction in the intestine (Chtarbanova *et al.*, 2014). These physical symptoms
480 alter an infected hosts energy stores with infected flies showing significantly
481 reduced glycogen and triglyceride levels three to four days post infection
482 (Chtarbanova *et al.*, 2014). DCV infected flies also increase in body mass, with a
483 reduced food intake and reduced metabolism, suggesting that they experience
484 increased water retention (Thomas-Orillard, 1984; Arnold *et al.*, 2013;
485 Chtarbanova *et al.*, 2014). We therefore hypothesised that changing the ratio of
486 protein to carbohydrate in the diet may alter outcome of infection, and as species
487 may all have their own "optimal diet", that species may respond in different ways
488 to such changes. However, this does not appear to be the case.
489
490 Geometric frameworks for nutrition were developed in response to the fact that
491 what is "optimal" will depend on a balance of particular nutrients in the
492 organism and trait being investigated (Simpson & Raubenheimer, 1995; Archer
493 *et al.*, 2009; Cotter *et al.*, 2019). For example mice infected with *Salmonella* were
494 found to survive better on diets containing a higher ratio of protein to
495 carbohydrate (Peck *et al.*, 1992). As were army worm caterpillars infected with
496 bacteria, with survival increasing with dietary protein, suggesting high protein
497 requirements are associated with bacterial resistance (Povey *et al.*, 2009). A
498 recent study used 10 different protein: carbohydrate diets and challenged flies
499 with *Pseudomonas entomophila* bacteria (Savola *et al.*, 2020a). Survival on low
500 protein diets was found to be lower in infected flies and suggested protein was
501 important for survival during infection. This study also monitored lifespan and

502 reproduction in flies, and found that regardless of injury and infection, dietary
503 restriction extended lifespan and reduced reproductive output (Savola *et al.*,
504 2020a). One potential mechanism of the interaction of diet and infection has
505 been suggested in research using a model host-pathogen system *in vivo* and *in*
506 *vitro* (Wilson *et al.*, 2020). Caterpillars of *S. littoralis* challenged with the bacteria
507 *X. nematophila in vivo* and on high dietary protein had slower bacterial growth
508 with higher survival. When this was combined with *in vitro* experiments the
509 results suggested this was driven by the osmolality of the hosts' blood
510 (hemolymph) being altered by an increase in solutes in the high protein diets
511 slowing the bacterial growth (Wilson *et al.*, 2020).
512
513 Further research on the mechanistic basis of dietary effects on resistance is
514 needed for other pathogen taxa, including viruses. Immunity to DCV inoculation
515 in *D. melanogaster* has been reported to involve the JAK/STAT and Imd
516 pathways, and potentially phagocytosis (Van Rij *et al.*, 2006; Zhu *et al.*, 2013;
517 Lamiable *et al.*, 2016). Additionally, the RNAi pathway is a key antiviral defence
518 mechanism in *Drosophila* and DCV appears to have evolved to suppress this
519 response (Van Rij *et al.*, 2006). Although we find no interaction between dietary
520 protein:carbohydrate and susceptibility, the multifaceted immune response to
521 DCV may be energetically costly and other nutrients may interact with the ability
522 of a host to allocate resources between an immune response, damage repair and
523 the maintenance of homeostasis (Lochmiller & Deerenberg, 2000; Zuk & Stoehr,
524 2002; Schmid-Hempel, 2005; Sadd & Siva-Jothy, 2006). For example, lipid and
525 fats have been associated with *D. melanogaster* response to DCV viral infection;
526 peroxisomes were found to be required for host defense to infection, through

527 their primary function in lipid metabolism (Aubert *et al.*, 1995). The lipid level
528 across our diets was held constant, but this may be a potential area for further
529 study. There has been an increased use of a chemically defined (holidic) diet in
530 order to manipulate individual nutrients present in fly diets (Lee *et al.*, 2006).
531 Exome matched diets have been shown to alleviate trade-offs in fecundity and
532 longevity (Piper *et al.*, 2017). A possible extension of this would be to look at the
533 effect of matching diets to transcriptional changes during infection, and seeing if
534 this alleviates (or exacerbates) pathology.
535
536 Changes in diet have been shown to alter pathogen susceptibility in a number of
537 systems. We hypothesised that changes in diet could alter the potential outcomes
538 of virus host shifts. However, we found that overall changes in the ratio of
539 protein to carbohydrate did not alter susceptibility to DCV across host species in
540 this instance. This suggests dietary protein to carbohydrate ratios are not
541 universally important in determining susceptibility to pathogens. It is unclear if
542 the lack of studies showing no effect of diet reflect publication biases or whether
543 our model system is unusual. However, it highlights the need to examine the
544 importance of diet in explaining susceptibility to pathogens across a broad range
545 of host and pathogen taxa.
546

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