Dynamics of macronutrient self-medication and illness-induced anorexia in virally-infected insects

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Running headline: Dynamics of self-medication

Abstract

(1) Some animals change their feeding behaviour when infected with parasites, seeking out substances that enhance their ability to overcome infection. This "self-medication" is typically considered to involve the consumption of toxins, minerals or secondary compounds. However, recent studies have shown that macronutrients can influence the immune response, and that pathogen-challenged individuals can self-medicate by choosing a diet rich in protein and low in carbohydrates. Infected individuals might also reduce food intake when infected (i.e. illness-induced anorexia).

(2) Here, we examine macronutrient self-medication and illness-induced anorexia in caterpillars of the African armyworm (*Spodoptera exempta*) by asking how individuals change their feeding decisions over the time course of infection with a baculovirus. We measured self-medication behaviour across several full-sib families to evaluate the plasticity of diet choice and underlying genetic variation.

(3) Larvae restricted to diets high in protein (P) and low in carbohydrate (C) were more likely to survive a virus challenge than those restricted to diets with a low P:C ratio. When allowed free choice, virus-challenged individuals chose a higher protein diet than controls.

(4) Individuals challenged with either a lethal or sub-lethal dose of virus increased the P:C ratio of their chosen diets. This was mostly due to a sharp decline in carbohydrate intake, rather than an increased intake of protein, reducing overall food intake, consistent with an illness-induced anorexic response. Over time the P:C ratio of the diet decreased until it matched that of controls.

(5) Our study provides the clearest evidence yet for dietary self-medication using macronutrients, and shows that the temporal dynamics of feeding behaviour depends on

47 the severity and stage of the infection. The strikingly similar behaviour shown by
48 different families suggests that self-medication is phenotypically plastic and not a
49 consequence of genetically-based differences in diet choice between families.

50 **Keywords:** diet, geometric framework, immunity, Lepidoptera, NPV, parasite, pathogen,
51 resistance, *Spodoptera exempta*

52

53 **Introduction**

54 By definition, parasites reduce the fitness of their hosts by diverting hosts' nutritional
55 resources for their own growth and reproduction, and by causing other fatal or debilitating
56 effects (Schmid Hempel 2011). To counter this threat, and to minimise the costs of parasitic
57 infection, multicellular organisms have evolved an effective immune system to recognise and
58 attack invading parasites. But immune defences are costly; they can cause self-harm when
59 triggered (Sadd & Siva-Jothy 2006), and also demand nutritional resources that could
60 otherwise be channelled into growth and reproduction (e.g. Moret & Schmid-Hempel 2000;
61 Siva-Jothy & Thompson 2002; Cotter, Kruuk & Wilson 2004).

62 The nutritional state of the host can affect its ability to fight and resist an infection
63 (Chandra 1996; Lochmiller & Deerenberg 2000) such that increasing an organism's access to
64 resources can increase its resistance to parasites. For example, food-supplemented snowshoe
65 hares (*Lepus americanus*) experienced reduced nematode prevalence compared to controls
66 (Murray, Keith & Cary 1998), whilst experimental food restriction suppressed cell-mediated
67 immunity in yellow-legged gulls (*Larus cachinnans*, Alonso-Alvarez & Tella 2001).
68 Similarly, invertebrate studies have focused on the effect of nutrient deprivation or starvation
69 on immune function and/or parasite resistance, with the consensus being that reduced

resources compromise immunity (e.g. (Moret & Schmid-Hempel 2000; Siva-Jothy & Thompson 2002; Ayres & Schneider 2009) but see (Triggs & Knell 2012).

Often, energy is assumed to be the limiting resource that individuals must partition between traits and, indeed, mounting an immune response has been shown to increase the metabolic rate of both vertebrates (Demas *et al.* 1997) and invertebrates (Freitak *et al.* 2003). Despite the requirement for resources during an immune response, many animals display illness-induced anorexia, in which food intake is reduced immediately after an immune challenge (Kyriazakis, Tolkamp & Hutchings 1998; Adamo, Fidler & Forestell 2007). This may seem counter-intuitive but has been hypothesised to serve a number of possible functions, from reducing the risk of ingesting more parasites , to starving resident parasites of key macro- and micro-nutrients (see references in Kyriazakis, Tolkamp & Hutchings 1998 and Adamo, Fidler & Forestell 2007). However, beyond the intake of energy, feeding comprises the ingestion of nutrients in particular ratios, which are allocated to different functions within the body, and there is good evidence that over- as well as under-ingestion of certain nutrients can be costly (Simpson *et al.* 2004; Raubenheimer, Lee & Simpson 2005; Cotter *et al.* 2011). Animals that would benefit from reducing the intake of a particular nutrient that favours parasite growth might be forced to decrease food consumption overall.

In lepidopteran larvae, resistance to parasites has been shown to depend on the relative amounts of macronutrients (protein and carbohydrate) in the diet and the diet that optimises growth rates in uninfected individuals differs from the diet that optimises the immune response (Lee *et al.* 2006; Povey *et al.* 2009; Cotter *et al.* 2011), thus, we might expect organisms to modify their intake based on their current nutritional requirements. This behaviour is known as self-medication, which Singer, Mace & Bernays (2009) define as “a specific therapeutic and adaptive change in behaviour in response to disease or parasitism”. It is generally recognised that verification of therapeutic self-medication must satisfy three

95 criteria: (i) the behaviour should increase the fitness of infected individuals; (ii) it should
96 decrease or have no effect on the fitness of uninfected individuals; and (iii) the behaviour
97 should be specifically triggered by infection. There is evidence for therapeutic self-
98 medication from several studies of vertebrates, most famously from chimpanzees that use
99 plant-derived substances when infected with protozoan or helminth parasites (Huffman &
100 Seifu 1989; Fowler, Koutsioni & Sommer 2007), and some experimental studies of livestock
101 infected with gut nematodes using nitrogen-rich clover (Hutchings *et al.* 2003). There is also
102 evidence from insect species for medicinal use of plant secondary compounds, such nicotine,
103 pyrrolizidine alkaloids and iridoid glycosides (e.g. Krischik, Barbosa & Reichelderfer 1988;
104 Christe *et al.* 2003; Castella *et al.* 2008; Singer, Mace & Bernays 2009). More recent studies
105 have provided support for macronutrient self-medication in bacteria- or virus-challenged
106 caterpillars, (Lee *et al.* 2006; Povey *et al.* 2009). Although macronutrients are a ubiquitous
107 part of the diet and their use is not restricted to self-medication, nearly all documented cases
108 of self-medication involve increasing the amount of a nutrient or chemical that comprises
109 some fraction of the normal diet (see Raubenheimer & Simpson 2009).

110 Implicit in the notions of self-medication and illness-induced anorexia is that changes
111 in feeding behaviour should be dynamic, with the magnitude of the response depending on
112 the stage of infection and the host's capacity to resist or tolerate infection. To capture this
113 dynamic, studies must control for differences in feeding behaviour prior to and during
114 infection, i.e. dietary preferences should be compared longitudinally *within* groups pre- and
115 post-challenge. In addition, studies must consider the possibility that the capacity to self-
116 medicate could have a significant genetic component, such that the magnitude, direction or
117 timing of behavioural changes differs between families or genotypes (Lefevre *et al.* 2010).

118 Here, we assess the effects of dietary protein and carbohydrate balance on the
119 outcome of infection with nucleopolyhedrovirus (NPV) in larvae of the African armyworm,

120 *Spodoptera exempta*, and on the associated immune response. This is a natural host-pathogen
121 interaction in sub-Saharan Africa (Graham *et al.* 2012), and *S. exempta* larvae feed on a wide
122 range of graminaceous crops and pasture grasses that vary in their nutritional composition
123 (Yarro 1984; Rose, Dewhurst & Page 2000). Using artificial diets to control macronutrient
124 composition precisely, we measured the diet-choice of individuals from different full-sibling
125 families both before and after challenge with NPV, thus providing the strongest test yet for
126 dynamical self-medication using dietary macronutrients. In so doing, we also examined the
127 absolute amount of each macronutrient consumed to test whether sickness-induced anorexia,
128 and/or selective intake of specific nutrients, occurred in response to infection. Our study
129 tested the following specific predictions: (1) resistance to NPV will decline as the relative
130 protein-content of the diet is reduced, (2) diet-related resistance to NPV will be associated
131 with diet-related changes in immune function, providing a potential mechanism for changes
132 in resistance, (3) virus-challenged insects will prefer a diet rich in the macronutrient that
133 favours NPV resistance in the short term, and revert to diets similar to non-challenged
134 individuals when the infection is under control, (4) infection with NPV will trigger a short-
135 term anorexic response, limiting the potential for further exposure to the virus or starving it of
136 resources, and finally, (5) the degree of plasticity in the self-medication response will vary
137 among full-sibling families, consistent with genetic variation in the trait.
138

139 **Methods**

140 ***Insects and virus***

141 *S. exempta* is a major crop pest throughout sub-Saharan Africa and feeds mostly on
142 graminaceous plants, including the staple cereal crops maize, sorghum, millet, and rice, as
143 well as on a diverse range of pasture grasses (see Rose, Dewhurst & Page 2000) for a full

144 species list). As an outbreak pest species that frequently occurs at larval densities in excess of
145 100 per m^2 (Rose, Dewhurst & Page 2000; Graham *et al.* 2012), *S. exempta* larvae will
146 typically switch between plant species when feeding in mixed pastures, impacting on its
147 growth and fitness (Yarro 1984). A continuous culture of *S. exempta*, originally collected in
148 Tanzania, had been maintained at Lancaster University for four years (*ca.* 48 generations)
149 prior to the start of the experiments. More than 150 breeding pairs were established each
150 generation to ensure high genetic variability. From the third-instar onwards, larvae were
151 reared in isolation in 25ml plastic pots containing a wheatgerm-based semi-artificial diet
152 comprising *ca.* 33% protein and 29% carbohydrate. Larvae were kept at a constant
153 temperature of 25ºC under a 12h:12h light:dark regime. All experiments were performed
154 using newly-moulted final instar larvae.

155 The baculovirus, *Spodoptera exempta* nucleopolyhedrovirus (SpexNPV) occurs
156 naturally in *S. exempta* larvae and a recent study found that the prevalence of overt virus
157 disease at high-density larval outbreaks in Tanzania ranged between 0% and 17% (Graham *et*
158 *al.* 2012), though prevalences in excess of 90% have been reported in late-season outbreaks
159 elsewhere (Rose, Dewhurst & Page 2000). Larvae become infected when they ingest
160 vegetation contaminated by virus occlusion bodies released from cadavers, though vertical
161 transmission of virus is also common (Vilaplana *et al.* 2008; Vilaplana *et al.* 2010). To
162 generate sufficient virus for the experiments, virally-infected cadavers were homogenised
163 before being filtered through muslin and centrifuged at 1000 rpm for 5 minutes to remove
164 larval debris. The supernatant was then pelleted by spinning for 20 min at 3000g. The
165 resulting pellet was re-suspended in water and purified on a 50-60% discontinuous sucrose
166 gradient at 30000 g for 60 min. This purified virus was washed and pelleted three times in
167 distilled water and spun at 10000g for 30 min. The purified virus was stored at –20ºC until
168 needed. Dilutions needed for experiments were estimated using a Neubauer haemocytometer.

169

170 ***Viral inoculations***

171 Larvae were placed individually in Petri dishes (9 cm diameter), where they received
172 a diet plug, of approximately 100mg, inoculated with 1µl of either water (control), or a
173 solution of SpexNPV (Grzywacz *et al.* 2008). The amount of virus administered was either an
174 LD_{50} dose of 2000 occlusion bodies (OBs) per aliquot or an LD_{10} dose of 400 OBs per
175 aliquot (Povey 2008). The LD_{50} dose was used to quantify the effects of diet on virus-induced
176 mortality, while the LD_{10} dose was chosen to elicit a strong and specific defence response
177 while causing minimal mortality (Povey 2008). The diet plug used for the challenge
178 contained 14% protein and 28% carbohydrate, which has been found to be the optimal diet
179 for non-infected *S. exempta* larvae (Lee, Simpson & Raubenheimer 2004). The Petri dishes
180 were placed on trays in plastic bags to prevent the diet plugs from drying out and only larvae
181 that had consumed the entire plug were used in the experiments. After inoculation, larvae
182 were transferred to one of the experimental diets described below.

183

184 ***Artificial diets***

185 The experimental diets (based on Simpson & Abisgold 1985) varied in their soluble
186 protein and digestible carbohydrate content, and have been used previously in studies using *S.*
187 *exempta* (Lee, Simpson & Raubenheimer 2004). The protein portion of the diet consisted of a
188 3:1:1 ratio of casein, peptone and albumen, and the carbohydrate content consisted of a 1:1
189 ratio of sucrose and dextrin. Other constituents of the diets were Wesson's salts (2.4%),
190 cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and a vitamin mixture (0.2%).
191 The remaining portion of the diets was made up of cellulose, a non-nutritive bulking agent.
192 The dry ingredients were suspended at a 1 to 6 ratio w/v in 1% agar solution. Five diets were

used in total, in each case the protein and carbohydrate portion made up 42% of the final diet: 7% carbohydrate with 35% protein (7:35), 14:28, 21:21, 28:7, and 35:7; the remaining 58% of the dry ingredient was indigestible cellulose.

Experiment 1: The effects of P:C ratio on larval survival and diet-choice in insects challenged with a high dose (LD_{50}) of NPV

The aim of this experiment was to ask how dietary protein-to-carbohydrate (P:C) ratio affects larval survival and to determine whether, when given a choice, virally-challenged larvae actively select a diet that improves their survival.

No-choice treatment: 60 larvae per diet treatment were used in this experiment, 20 control larvae and 40 challenged with an LD_{50} dose of NPV (2000 OB per larva). All larvae were inoculated upon reaching the final instar and randomly placed on one of five diets varying in P:C ratio from extremely carbohydrate-biased to extremely protein-biased: 7:35, 14:28, 21:21, 28:14 or 35:7. Given a choice, healthy *S. exempta* choose a carbohydrate-biased diet (19:23) (Lee, Simpson & Raubenheimer 2004). Ten caterpillars (3 control and 7 virally-challenged) were discarded as they failed to consume the inoculated diet plug. Fresh diet blocks were provided each day post-infection until the larvae had ceased feeding at the pre-moult stage. All deaths were recorded to the nearest day, and checked for the presence of OBs, though viral loads were not quantified due to logistical constraints.

Self-selecting treatment: 60 final-instar larvae were weighed to the nearest 0.001g before being inoculated with either an LD_{50} dose of NPV (n = 32) or with distilled water (n = 28). After inoculation, larvae were placed in Petri dishes and given a choice between the two most

217 extreme diets (35:7 *vs.* 7:35), to maximise the chances of detecting an effect of viral
218 inoculation on diet choice. Diet blocks, each weighing between 0.7 - 1.3g, were replaced
219 daily until the larvae had ceased feeding at the pre-pupal stage. Uneaten food was dried to a
220 constant mass in a desiccating oven. Consumption was calculated as the difference between
221 the initial and final dry weight of each diet block. The initial dry weight of the blocks was
222 estimated using regression of control blocks for each diet type (Lee *et al.* 2006). From the dry
223 mass of food eaten, the amount of protein and carbohydrate consumed on each day was
224 estimated. Deaths were monitored daily until all larvae had died or pupated; viral infection
225 was confirmed by the presence of OBs.
226

227 ***Experiment 2: The effects of P:C ratio on immune function and diet-choice in insects***
228 ***challenged with a low dose (LD_{10}) of NPV***

229 This experiment tested whether immune responses were up-regulated in virally-
230 challenged larvae, and how diets with different P:C ratios affected those responses. We used
231 a low-dose viral challenge (LD_{10}) to stimulate a strong defence response whilst minimising
232 mortality. We also performed a second choice-test using this low viral dose to determine if
233 this was sufficient to change larval feeding behaviour. In addition larvae from 3 full sibling
234 families were split across the treatment groups to test for genetic effects on diet choice and
235 immune parameters.
236

237 *No-choice treatment:* On reaching the final instar, 160 larvae, 32 per diet treatment, were
238 inoculated with either an LD_{10} dose of virus (400 OB per larva) or distilled water, as
239 described above. Larvae were then provided with a diet block of one of the five chemically-
240 defined diets, as before. After being allowed to feed on the diets for 24h, haemolymph was

241 collected from the larvae. One larva died before haemolymph was collected and so was
242 discarded from the experiment. Phenoloxidase (PO) activity, antimicrobial activity and
243 haemocyte density were then measured for each sample (see below).
244
245 *Self-selecting treatment:* The methods for the self-selection treatment were as described in
246 Experiment 1, with the following modifications: larvae were placed on their assigned diets
247 for 24 h before viral inoculation. Larvae were given an LD_{10} viral dose and were provided
248 with the choice between a 14:28 diet and a 28:14 diet. These ratios were chosen as we wanted
249 to determine whether diet choice would be apparent even when the diets varied relatively
250 little in their nutritional composition.
251
252 *Antimicrobial activity:* Antimicrobial growth-inhibition assays were carried out as described
253 in Povey *et al.* (2009) using an agar-overlay technique (Rahalison *et al.* 1991) and the gram-
254 positive bacterium *Micrococcus luteus*. Briefly, 1µl samples of fresh haemolymph were
255 pipetted directly into labelled holes on the agar plates, which were incubated for 24 h at 37ºC.
256 Antimicrobial activity was measured as the radius of the clear zone of bacterial growth
257 inhibition around the holes in the plate. Measurements were made using *Image Pro Plus*
258 software 4.1 (Media Cybernetics, USA).
259
260 *Phenoloxidase activity and haemolymph protein levels:* Phenoloxidase is a key enzyme in the
261 prophenoloxidase cascade that generates highly cytotoxic quinones that can inactivate viral
262 pathogens. The end-point of this melanisation reaction is the production of melanin, which
263 can kill macroparasites and viral-infected cells. Following haemolymph collection, samples
264 for assaying phenoloxidase (PO) activity were frozen at -80ºC until needed. PO activity and
265 the amount of protein per sample were measured as described by (Povey *et al.* 2009). Briefly,

266 6µl of each haemolymph sample was mixed with 300µl of phosphate buffered saline (PBS),
267 100µl of the resulting solution was pipetted in duplicate into a microtitre plate with 4mM
268 dopamine and absorbance measured at 492nm over 10 minutes at 25 ºC on a VERSAmax
269 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Haemolymph protein levels
270 were determined using a standard curve created using a BSA standard (BioRad, Hercules,
271 CA, USA); 10µl of the haemolymph sample was added to wells in a microtitre plate
272 containing 200µl of the dye reagent and the resulting colour measured at 600nm.
273
274 *Haemocyte density:* Haemocytes are the immune cells of insects and are important effectors
275 against parasites and pathogens, including baculoviruses (Strand 2008). Immediately after
276 collection, 10µl of each haemolymph sample was added to 10µl of a 50:50
277 ethylenediaminetetraacetic acid (EDTA)/glycerol solution (Cotter, Kruuk & Wilson 2004)
278 and stored at -80ºC until needed. Haemocyte counts were performed by pipetting 8µl of the
279 haemolymph sample onto each side of an Improved Neubauer Haemocytometer (Hawksley,
280 Sussex, www.hawksley.co.uk). Haemocytes were counted in five non-adjacent squares on
281 each side of the haemocytometer; these were then summed to give an estimate of the
282 haemocyte density for each larva.
283
284 ***Statistical analyses***
285
286 *Experiment 1:* Survival analyses were performed using accelerated failure time (AFT) models
287 using the S-Plus 6.2 (Insightful Corp., Washington) statistical package. These describe the
288 relationship between the hazard function, or the risk of death, and a set of explanatory terms
289 (Cox 1972). The hazard function is the instantaneous probability of death for an individual

still alive. The interactive effects of *Treatment* (virally-inoculated or control) and *Diet* (the percentage protein content of the diet) on the instantaneous death rates were considered. The choice data were analysed using Restricted Estimate Maximum Likelihood (REML) mixed-effects models in *Genstat 14*, with caterpillar ID included as a random effect to account for multiple measures on each individual.

Experiment 2: Antimicrobial activity, PO activity and haemolymph protein levels were analysed using GLM in R (v2.13.1). PO activity, haemolymph protein levels and haemocyte density were log-transformed to obtain normally-distributed data to meet the assumptions of the GLM. *Family* and *Treatment* were included as factors and *Diet*, as both linear and quadratic terms, were included as independent variables in the model. As for Experiment 1, the self-selecting data were analysed using REML mixed-effects models in *Genstat 14* in which caterpillar ID was included as a random effect to account for multiple measures on each individual. The 3 individuals that died from viral infection were excluded from the consumption data.

Results

Experiment 1: The effects of P:C ratio on larval survival and diet-choice in insects challenged with a high dose (LD_{50}) of NPV

No-choice treatment: Larvae started to die from virus 4 days post-inoculation, and all larvae had either died or pupated by 10 days. Larval risk of death was affected by both viral inoculation (AFT model, *Treatment*: $\chi^2_1 = 82.30$, $p < 0.0001$) and the relative protein content of the diet (*Diet*, $\chi^2_1 = 33.35$, $p < 0.0001$). No other interactions were statistically significant. As expected, larvae inoculated with NPV had substantially lower survival than those in the

control group (mean survival: control = 98%, NPV-challenged = 54%; estimate ± se = -0.40 ± 0.09; Fig. 1). Whereas survival in the non-challenged insects was uniformly high (>95%) across diet treatments, in the virus-challenged larvae, survival increased with the ratio of protein to carbohydrates (estimate ± s.e. = 0.60 ± 0.01; Fig. 1), such that on the most protein-rich diet (35:7), 79% of the virally-challenged larvae survived, compared to just 33% on the most protein-poor diet (7:35).

Self-selecting treatment: Larvae that were inoculated with an LD_{50} dose of NPV chose a higher P:C ratio diet than larvae that given water only (REML: *Treatment*: $F_{1,55} = 6.93$, P = 0.011, Fig. 2a); there was no effect of time post-inoculation on diet choice and no significant interaction between these two factors (*Day*: $F_{3,166} = 1.44$, P = 0.232; *Day*Treatment*: $F_{3,163} = 0.54$, P = 0.657). There was also no effect of larval weight on the P:C ratio of the chosen diet (*Larval weight*: $F_{1,69} = 2.19$, P = 0.143). When we examined larvae that died from NPV separately from those that survived (giving three treatment groups – control, NPV-survived and NPV-died), there was a significant interaction between day and treatment (*Day*Treatment*: $F_{6,159} = 2.44$, P = 0.028). Larvae that survived viral challenge showed an early shift towards a high P:C ratio diet on day 1 compared to controls, whilst those that later died from viral infection did not increase their P:C preference until day 2 (Fig. 3a).

Analysis of total food consumption, a measure associated with illness-induced anorexia, showed that larger larvae consumed more food than smaller larvae (*Larval weight:* $F_{1,69} = 10.26$, P < 0.001). However, virally-challenged larvae also ate significantly less than the controls (*Treatment*: $F_{1,57} = 11.33$, P < 0.001; Fig 2b). As before, there was no effect of time post-inoculation on the daily amount of food consumed or a significant interaction between the two (*Day*: $F_{3,167} = 1.30$, P = 0.278; *Day*Treatment*: $F_{3,163} = 1.89$, P = 0.134). Considering larvae that died from NPV separately from those that survived, there was a

339 strong interaction between day and infection treatment (*Day*Treatment:* $F_{6,160}$ = 4.31, P <
340 0.001; Fig 3b). While control larvae and those that died from viral infection maintained a
341 similar level of food consumption over the 4 days, those that survived viral challenge
342 *decreased* their consumption as time went on (Fig. 3b).

343 These effects on the proportion and total amounts of the two foods eaten translated
344 into differences in amounts of protein and carbohydrate eaten. Consumption of both
345 macronutrients increased with larval weight (*Larval weight*: P - $F_{1,68}$ = 9.35, P = 0.003; C -
346 $F_{1,69}$ = 7.14, P = 0.009), but there were also significant interactions between infection
347 treatment and time (*Day*Treatment*: P - $F_{6,158}$ = 5.54, P < 0.001; C - $F_{6,160}$ = 2.92, P = 0.010;
348 Figs. 3c,d). Controls and those that died of infection maintained their protein intake during
349 the 4 days post inoculation. In contrast, survivors ate much higher levels of protein on day 1,
350 then decreased consumption steadily over the next 3 days (Fig. 3c). Carbohydrate
351 consumption, in contrast, was slightly higher in the controls on day 1, but whereas
352 consumption tended to increase over time for controls and those that died of infection, it fell
353 off significantly in those that survived infection (Fig. 3d).
354

355 ***Experiment 2: The effect of P:C ratio on immune function and diet choice in insects***
356 ***challenged with a low dose (LD_{10}) of NPV***

357

358 *No-choice treatment:* Mortality in this experiment was 8% and the analysis excludes
359 larvae that subsequently died of virus infection. Haemolymph protein levels increased with
360 the amount of protein in the diet, such that highest levels were at P:C = 35:7 (GLM: *Diet*:
361 $F_{1,157}$ = 25.13, P < 0.0001; $Diet^2$: $F_{1,155}$ = 0.15, P = 0.70; Fig 4a). However, protein levels did
362 not respond to NPV challenge or the interaction between viral treatment and dietary protein

363 intake (*Treatment*: $F_{1,156}$ = 1.17, P = 0.28; *Treatment*Diet*: $F_{1,151}$ = 1.51, P = 0.22;
364 *Treatment*Diet*^{2}: $F_{1,150}$ = 0.12, P = 0.72; Fig 4a). There was no significant variation between
365 families in haemolymph protein levels (*Family*: $F_{3,152}$ = 0.50, P = 0.68) and none of the
366 interactions with family were significant.

367 Phenoloxidase activity also increased with the protein content of the diet and peaked
368 at P:C = 35:7 (GLM: *Diet*: $F_{1,157}$ = 31.60, P < 0.0001; *Diet*^{2}: $F_{1,152}$ = 0.20, P = 0.65; Fig 4b),
369 with virus-treated insects exhibiting a small, but significant, reduction in PO activity
370 (*Treatment*: $F_{1,156}$ = 4.69, P = 0.032; Fig. 4b). The interaction terms were not significant
371 (*Treatment*Diet*: $F_{1,151}$ = 0.11, P = 0.74; *Treatment*Diet*^{2}: $F_{1,150}$ = 0.64, P = 0.42) and there
372 were no family effects (*Family*: $F_{3,153}$ = 1.02, P = 0.38) nor any significant interactions
373 between *Family* and other terms in the model.

374 Antimicrobial activity increased non-linearly with the protein content of the diet
375 (GLM: *Diet*: $F_{1,153}$ = 25.72, P < 0.0001; *Diet*^{2}: $F_{1,153}$ = 9.69, P = 0.002; Fig 4c), peaking on a
376 diet that was marginally protein-biased (P:C = 28:14). However, antibacterial activity did not
377 depend on NPV challenge (*Treatment*: $F_{1,149}$ = 0.32, P = 0.57; *Treatment*Diet*: $F_{1,148}$ = 0.57,
378 P = 0.45; *Treatment*Diet* ^{2}: $F_{1,147}$ = 0.007, P = 0.93), family-group (*Family*: $F_{3,150}$ = 1.86, P =
379 0.14), or interactions with *Family*.

380 Haemocyte density increased non-linearly with the protein content of the diet, but
381 peaked at P:C = 35:7 (GLM: *Diet:* $F_{1,153}$ = 111.06, P < 0.001; *Diet*^{2}: $F_{1,153}$ = 4.27, P < 0.001;
382 Fig 4d). However, in this case, being challenged with a low dose of NPV 24h previously
383 resulted in a stronger increase in the density of haemocytes in the haemolymph with
384 increasing protein content of the diet (*Treatment*Diet*: $F_{1,153}$ = 4.27, p = 0.04;
385 *Treatment*Diet*^{2}: $F_{1,152}$ = 0.36, p = 0.55). There were no significant differences between
386 families (*Family*: $F_{3,151}$ = 1.14, P = 0.33) and none of the interactions with *Family* were
387 statistically significant.

388

389 *Self-selecting treatment:* Before inoculation, both virus-challenged and control larvae
390 chose a P:C ratio that was significantly carbohydrate-biased (Fig. 5a). However, following
391 the challenge, the two treatment groups differed markedly in how their P:C diet-choice
392 changed over time (REML: *Day*Treatment*: $F_{4,210} = 22.35$, $p < 0.001$). The P:C ratio chosen
393 by control larvae on the day following inoculation was carbohydrate-biased (mean P:C ratio
394 = 1:1.5) and increased moderately over time, whereas virus-challenged larvae increased their
395 P:C ratio immediately after virus challenge to a strongly protein-biased diet (mean P:C ratio
396 = 1.5:1). This ratio then fell gradually over the next three days until the final ratio chosen was
397 not significantly different from that of control larvae. Diet was not affected by *larval weight*,
398 *Family*, or any of their interactions ($F < 0.55$, $P > 0.46$).

399 Total food consumption also varied significantly between control and virus-
400 challenged larvae. Before being inoculated, both virus-challenged and control groups
401 consumed a similar amount of food (Fig. 5b). Food consumption differed significantly among
402 families (REML: *Family*: $F_{2,73} = 4.35$, $p = 0.009$) and heavier larvae ate more food (*Larval*
403 *weight*: $F_{1,69} = 7.44$, $p = 0.008$). Following the virus challenge the two treatment groups
404 differed in total food consumption over time (*Day*Treatment*: $F_{4,191} = 4.35$, $p = 0.002$). While
405 control larvae ate a similar amount of food each day (Fig. 5b), the virus-challenged larvae
406 *decreased* their food consumption immediately following challenge and then *increased* it
407 steadily. By day 4, food consumption was the same for both groups (Fig. 5b). None of the
408 other interaction terms were statistically significant ($F < 1.73$, $P > 0.094$).

409 Consumption of the two macronutrients also exhibited temporal variation and a
410 significant effect of treatment, with the temporal change in nutrient consumption differing
411 between control and NPV-challenged caterpillars (P – *Day*Treatment*: $F_{4,194} = 3.23$, $p =$
412 0.014; C - *Day*Treatment*: $F_{4,189} = 6.15$, $p < 0.001$; Fig 5c,d). Whilst protein consumption

413 gradually *increased* in virus-challenged insects relative to controls on days 3 and 4 post-
414 inoculation, carbohydrate consumption *decreased* significantly on day 1 before returning to
415 pre-inoculation levels thereafter. Consumption increased with larval weight (REML: *Larval*
416 *weight*: P - $F_{1,69} = 5.90$, $p = 0.018$; C - $F_{1,68} = 7.69$, $p = 0.007$), and differed among families
417 (*Family*: P - $F_{2,73} = 4.25$, $p = 0.018$; C - $F_{2,72} = 4.86$, $p = 0.010$).
418

419 **Discussion**

420 Here, we provide the clearest evidence to date for therapeutic self-medication, *sensu*
421 Singer et al. (2009), using dietary macronutrients. Consistent with this phenomenon, *S.*
422 *exempta* larvae challenged with a high (LD_{50}) dose of nucleopolyhedrovirus chose a diet that
423 was rich in protein (containing ~50% more P than C) compared to that of uninfected control
424 larvae, which chose a diet that was carbohydrate-biased (~50% more C than P). By choosing
425 a relatively protein-rich diet, NPV-challenged insects improved their survival prospects from
426 less than 40% on foods containing the most carbohydrates (P:C = 7:35 and 14:28) to around
427 80% on the most protein-rich foods (P:C = 28:14 and 35:7) . In this and previous studies, the
428 survival of non-infected larvae was high and independent of P:C ratio, but larval growth rate
429 and overall performance (survival x larval growth rate) peaked on a diet that was slightly
430 carbohydrate-rich and dropped off dramatically on diets with an excess of protein (Lee,
431 Simpson & Raubenheimer 2004). Thus, the main criteria for self-medication are satisfied.
432 Comparison of overall feeding patterns of virus-challenged and control insects in both
433 experiments suggests that challenged individuals self-medicate on protein, but closer analysis
434 of the feeding dynamics supports a plastic response in which feeding behaviour changes as
435 the viral infection progresses. Among caterpillars that had been given a high (LD_{50}) dose of
436 virus, those which survived viral challenge behaved very differently from those that died. The

437 first day post inoculation was characterised by a sharp increase in P consumption and an
438 elevated P:C ratio in survivors relative to controls and casualties. P and C consumption then
439 declined in survivors over the course of experiment, resulting in a decrease in total food
440 consumption. Note that this dynamic is masked if survivors and casualties are lumped
441 together.

442 Experiment 2 showed that this change in behaviour was not simply caused by families
443 which naturally choose higher levels of protein being more likely to survive infection. We
444 also tested diet preference *before* infection so that we could be sure that any differences in
445 feeding behaviour were a response to the virus-challenge. Prior to inoculation, the digestible
446 component of the diet comprised around two-thirds carbohydrate and one-third protein. In the
447 non-challenged controls, the amount of protein in the diet remained low but gradually
448 increased as pupation approached. In contrast, sublethally-infected larvae radically changed
449 their feeding behaviour on a daily basis (Fig. 5) and this is likely to have coincided with
450 temporal changes in the viral infection process (Keddie, Aponte & Volkman 1989;
451 Washburn, Kirkpatrick & Volkman 1996; Cory & Myers 2003). On day 1, there was a
452 dramatic *reduction* in the amount of carbohydrate consumed by the virus-challenged larvae
453 and a decline in the overall feeding rate (Fig. 5b,d). This change in feeding behaviour
454 coincided with the period when virus released from the ingested occlusion bodies invades the
455 larval midgut epithelial cells and replicates in their nuclei. Importantly, the amount of protein
456 eaten by inoculated larvae was *maintained* at pre-infection levels, such that the percentage of
457 protein in the diet increased from less than 40% to approximately 60% in all of the families
458 we tested. By day 2, carbohydrate intake returned to pre-infection levels in the sub-lethally
459 infected insects, such that total food consumption increased and the overall P:C ratio declined
460 towards 1:1. This change in feeding behaviour coincided with a period when many infected
461 midgut cells are likely to have become melanised, encapsulated and/or sloughed into the gut

462 lumen to be replaced by healthy cells and, in some larvae, virus will have migrated into the
463 insect haemocoel to infect haemocytes and other tissues. By day 3, the total food-intake of
464 virus-challenged larvae continued to increase, perhaps to offset the reduced food
465 consumption earlier in the infection. Finally, by day 4, the dietary P:C ratio and total food
466 intake of virus-challenged caterpillars became comparable to that of non-infected control
467 larvae, presumably as the infection has been controlled and is no longer imposing a
468 nutritional demand on its host.

469 Although we detected genetic variation for nutrient consumption, this explained a
470 relatively small amount of the variation in feeding behaviour and was independent of
471 treatment or time post-infection. Rather, diet choice showed a high degree of phenotypic
472 plasticity and different families demonstrated the capacity to respond to infection by self-
473 medicating. Of particular note is that the immediate response following inoculation with a
474 sub-lethal dose of virus is that the larvae limit their consumption of carbohydrate, and food
475 intake overall, but maintain a constant level of protein ingested. This behaviour is consistent
476 with a form of illness-induced anorexia (Kyriazakis, Tolkamp & Hutchings 1998; Adamo,
477 Fidler & Forestell 2007). Specifically, the anorexic response could limit the ingestion of
478 further virus occlusion bodies with contaminated food, or it could be a mechanism by the host
479 to reduce calorie intake overall (or carbohydrate intake specifically) without sacrificing
480 protein consumption. Another explanation is that this is the most efficient mechanism by
481 which the host can alter the blend of ingested food to bias it towards proteins; this would be
482 an adaptive response if a protein-rich diet enhances resistance to the virus or limits the virus
483 replication rate.

484 To explore the impact of macronutrients on possible viral resistance mechanisms, we
485 assayed several aspects of immune function. In both virus-challenged and control larvae, the
486 haemolymph protein pool increased linearly with the amount of protein in the diet. Thus,

487 short-term changes in larval feeding behaviour are reflected in rapid changes in the
488 nutritional composition of their blood (see also Povey *et al.* 2009). The P:C composition of
489 the diet was also reflected in constitutive levels of phenoloxidase activity, antimicrobial
490 activity and haemocyte density, all three of which increased (linearly or non-linearly) with
491 increasing protein content of the diet, though unlike the other haemolymph properties, peak
492 antimicrobial activity was not achieved on the most protein-rich diet. This suggests that
493 larvae that switch from a carbohydrate-biased diet onto a diet that is relatively protein-rich
494 will generally have more haemocytes and higher levels of PO with which to melanise and
495 encapsulate virus-infected cells (Washburn, Kirkpatrick & Volkman 1996; Trudeau,
496 Washburn & Volkman 2001), as well as a greater capacity to combat concomitant microbial
497 infections. However, only PO activity and haemocyte density were significantly modulated
498 by viral infection, with virus-challenged larvae having marginally more haemocytes and
499 lower PO activity. Haemocytes are involved in the encapsulation of virus-infected tissues and
500 so their greater density in infected larvae may reflect their increased production following
501 infection. The reduction in PO activity in virus-infected larvae is counter-intuitive, but is
502 consistent with previous studies suggesting phenotypic and genetic trade-offs between
503 immune traits (Cotter *et al.* 2004; Cotter, Kruuk & Wilson 2004; Povey *et al.* 2009; Rao,
504 Ling & Yu 2010). Thus, whilst pre-ingestive behavioural plasticity allows infected
505 individuals to capture the resources required to mount an effective immune response, post-
506 ingestive internal trade-offs may constrain immune expression (Cotter *et al.* 2011). It is also
507 worth noting, however, that other important viral resistance mechanisms have not been
508 quantified in this study, such as the sloughing and replacement of infected midgut epithelial
509 cells, and the resource implications of these processes are not easily quantified.

510 Finally, this study builds on two previous investigations of the impact of
511 macronutrients on insect resistance to pathogens and the dietary choices insects make when

512 faced with a pathogen challenge (Lee et al. 2006; Povey et al. 2009). Each study used
513 different host-pathogen combinations, but broadly similar protocols in the same research
514 laboratory, providing the opportunity to explore the generality of their key findings. Lee et al.
515 (2006) found that *S. littoralis* larvae challenged with an LD_{50} dose of *S. littoralis* NPV had
516 highest survival on the diet with the highest relative protein content, as also observed here for
517 *S. exempta* and its specific NPV, so demonstrating the importance of protein for resisting
518 baculovirus across different host-virus combinations. Povey et al. (2009) conducted a similar
519 experiment using *S. exempta* challenged with the bacterium, *Bacillus subtilis*, suggesting that
520 protein is perhaps ubiquitously important for resisting entomopathogens. This comparison is
521 particularly revealing since the baculovirus infects orally, whereas the bacterium was injected
522 into the haemocoel, suggesting that dietary protein may benefit multiple defence mechanisms
523 in the gut, haemocoel and elsewhere. In diet-choice experiments, *S. littoralis* larvae that were
524 challenged with an LD_{30} dose of baculovirus ate significantly less food post-infection than
525 did the control larvae (Lee et al. 2006), so demonstrating a similar anorexic response to that
526 shown by the *S. exempta* larvae receiving an LD_{50} dose of virus in the present study
527 (Experiment 1). Moreover, in both these experiments, larvae that subsequently survived a
528 potentially lethal dose of virus chose a P:C ratio that was significantly more protein-rich than
529 those that succumbed. However, because of the high levels of virus-induced mortality in
530 prior experiments, and the fact that dietary preferences before viral-challenge were not
531 quantified, we could not exclude the possibility that these results depended on genetic or
532 other intrinsic differences in dietary preferences of larvae that predisposed them to dying of
533 NPV (Lee et al. 2006). Both of these deficiencies were remedied in Experiment 2 of the
534 present study by challenging *S. exempta* larvae with a low dose of virus and by quantifying
535 feeding preferences prior to virus challenge, so that we could monitor shifts in feeding
536 behaviour from pre- to post-infection. These clearly revealed that individuals from different

537 families all switched to a relatively protein-rich diet immediately following infection before
538 returning to a diet that resembled that of control larvae over the following days. It is also
539 worth noting that in none of these experiments did we quantify viral loads in dead or
540 surviving larvae and so we cannot rule out the possibility that protein-biased diets either alter
541 host tolerance or trigger the virus to switch to a vertically-transmitted mode. These
542 possibilities would make interesting avenues for further study.

543 In conclusion, as predicted, we showed that: (1) survival following virus challenge
544 declined as the relative protein-content of the diet was reduced;, (2) increasing dietary P:C
545 ratio resulted in higher levels of all immune traits, so providing a potential mechanism for
546 changes in resistance; (3) when given a choice between complementary diets, virus-
547 challenged insects temporarily increased the relative protein content of their diet, but in
548 insects challenged with a low viral dose this was achieved by reducing the intake of
549 carbohydrates whilst maintaining protein intake; (4) infection with a low-dose of NPV
550 triggered a short-term anorexic response, so limiting the potential for further exposure to the
551 virus or starving it of key resources. In contrast, we found little evidence for prediction (5),
552 that the degree of plasticity in the 'self-medication' response would vary between full-sibling
553 families. Whilst the total amounts of each macronutrient consumed varied between families,
554 the P:C ratio achieved did not, suggesting that this choice is not genetically-determined but is
555 a form of phenotypic plasticity common to all genotypes. Our results have clear implications
556 for the foraging behaviour of *S. exempta* larvae in the wild and may help explain the diverse
557 range of graminaceous plant species included in their diet (Yarro 1984; Rose, Dewhurst &
558 Page 2000).
559

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Figure legends

Figure 1. Survival curves for larvae restricted to one of five diets varying in their protein to carbohydrate ratios (P:C) and inoculated with either an LD_{50} dose of NPV or with water (controls). Data are taken from Experiment 1, no-choice treatment.

Figure 2. Effects of virus treatment on (a) the mean P:C ratio of the diet selected and (b) the total amount of food consumed. Larvae were inoculated with either an LD_{50} dose of NPV or with water (controls). Data are taken from Experiment 1, self-selecting treatment.

Figure 3. Effects of the outcome of infection (those that survived or died versus controls) on a) P:C ratio of the diet chosen, (b) the total amount of food consumed (c) the amount of protein consumed and (d) the amount of carbohydrate consumed. Data are taken from Experiment 1, self-selecting treatment.

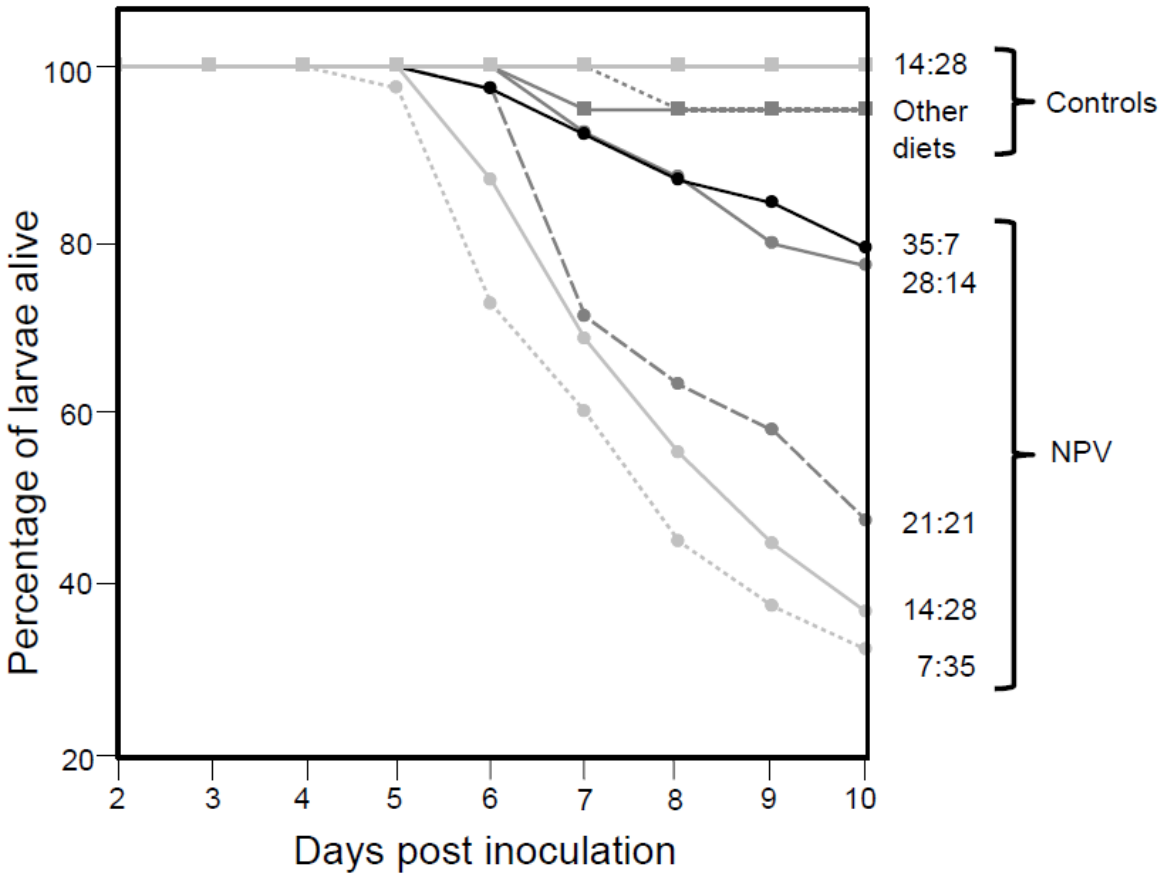
Figure 4. Effects of virus treatment and larval diet on (a) haemolymph protein levels, (b) haemolymph phenoloxidase activity, (c) haemolymph antimicrobial activity and (d) haemocyte density. Larvae were restricted to one of five diets varying in their P:C ratio following inoculation with either an LD_{10} dose of NPV or with water (controls). Data are taken from Experiment 2, no-choice treatment.

Figure 5. Effects of virus treatment on (a) the mean P:C ratio of the diet selected, (b) the total amount of food consumed, (c) the amount of protein consumed, and (d) the amount of carbohydrate consumed by virus-challenged and control larvae on each day of the experiment. For figure (a) separate lines are plotted for each family to illustrate the similarity in diet choice across genotypes. Data are taken from Experiment 2, self-selecting treatment.

722

723 **Figures**

724 ***Figure 1***



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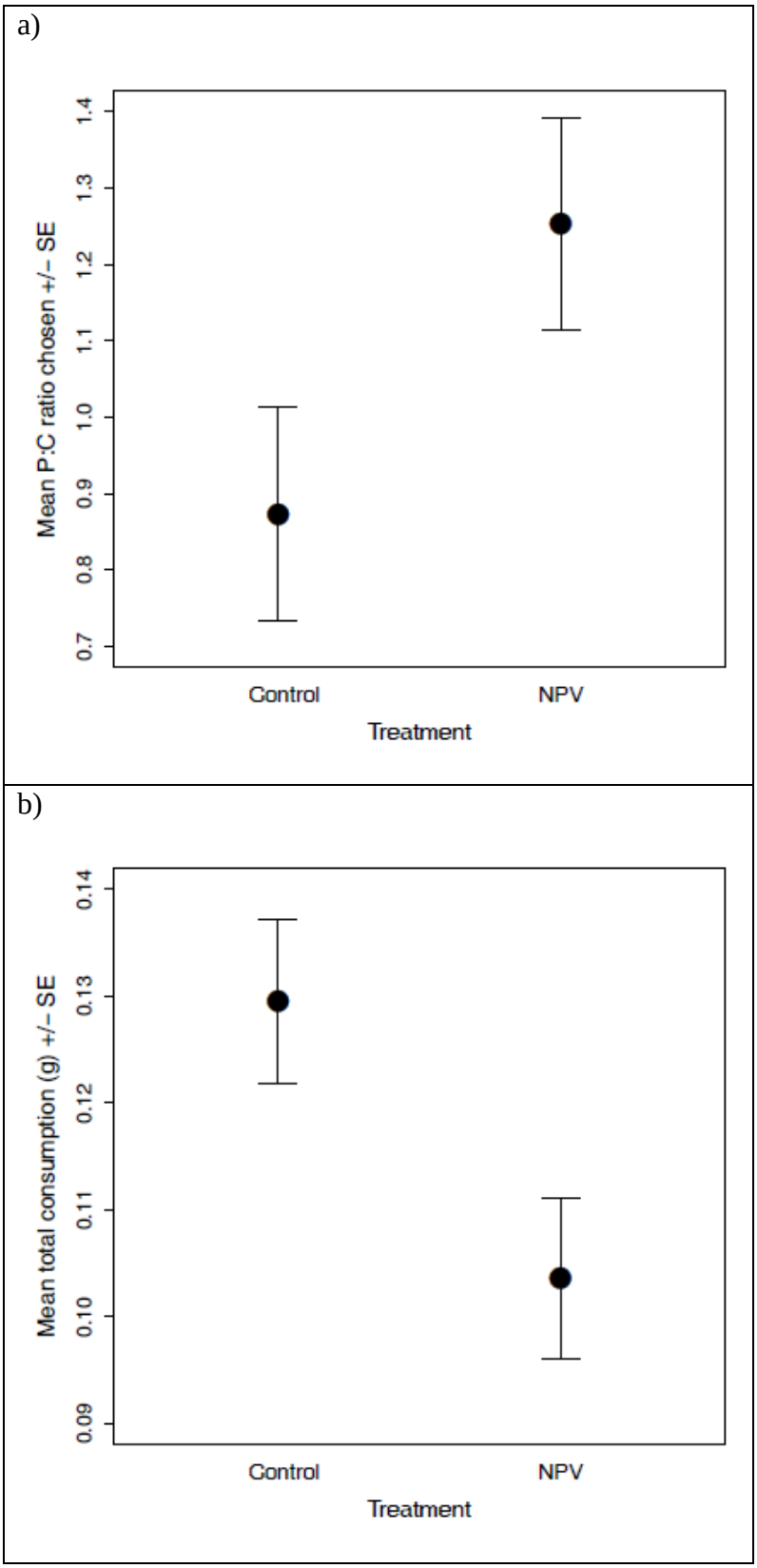
728 ***Figure 2***



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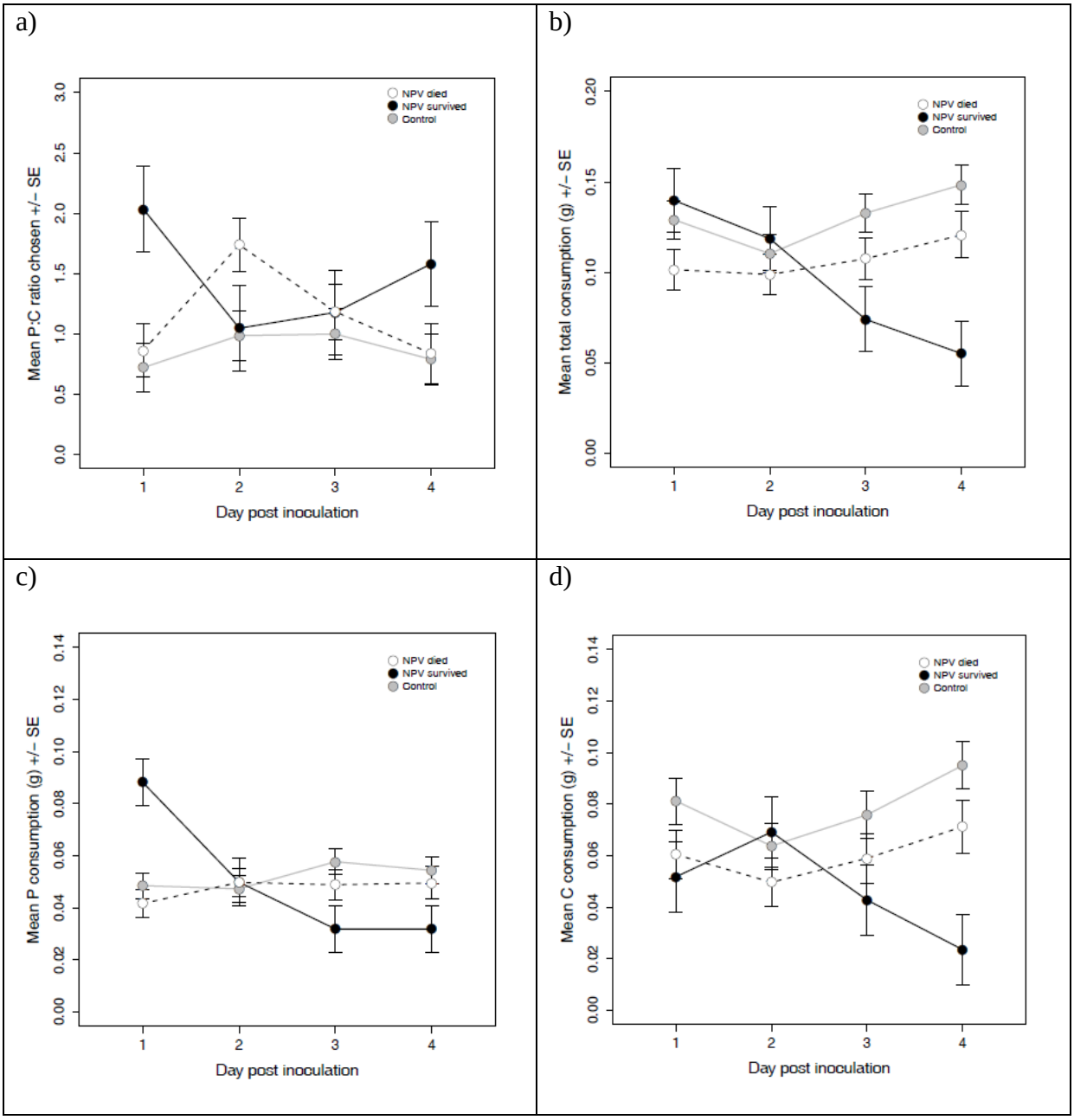
731 ***Figure 3***



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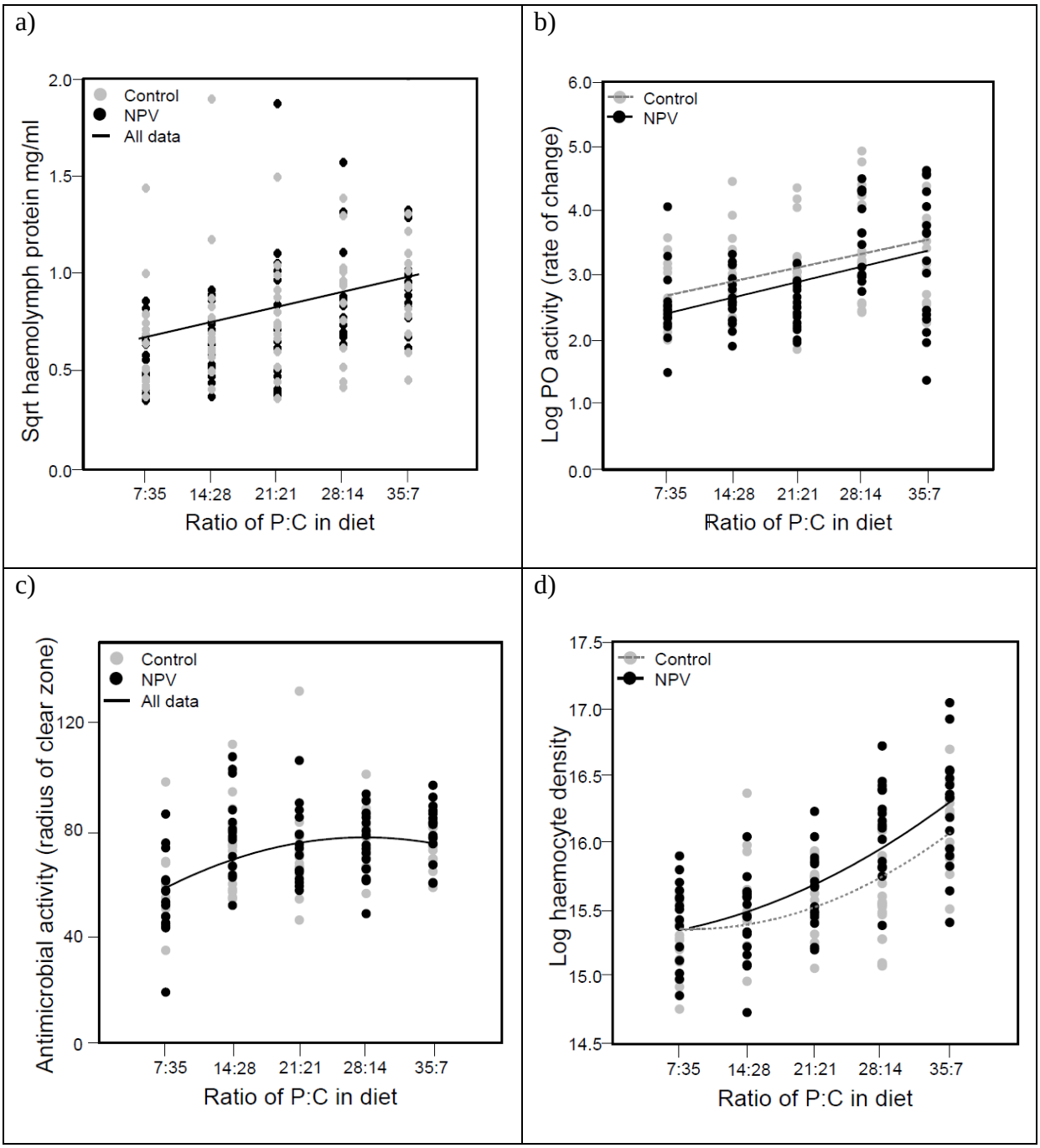
734 ***Figure 4***



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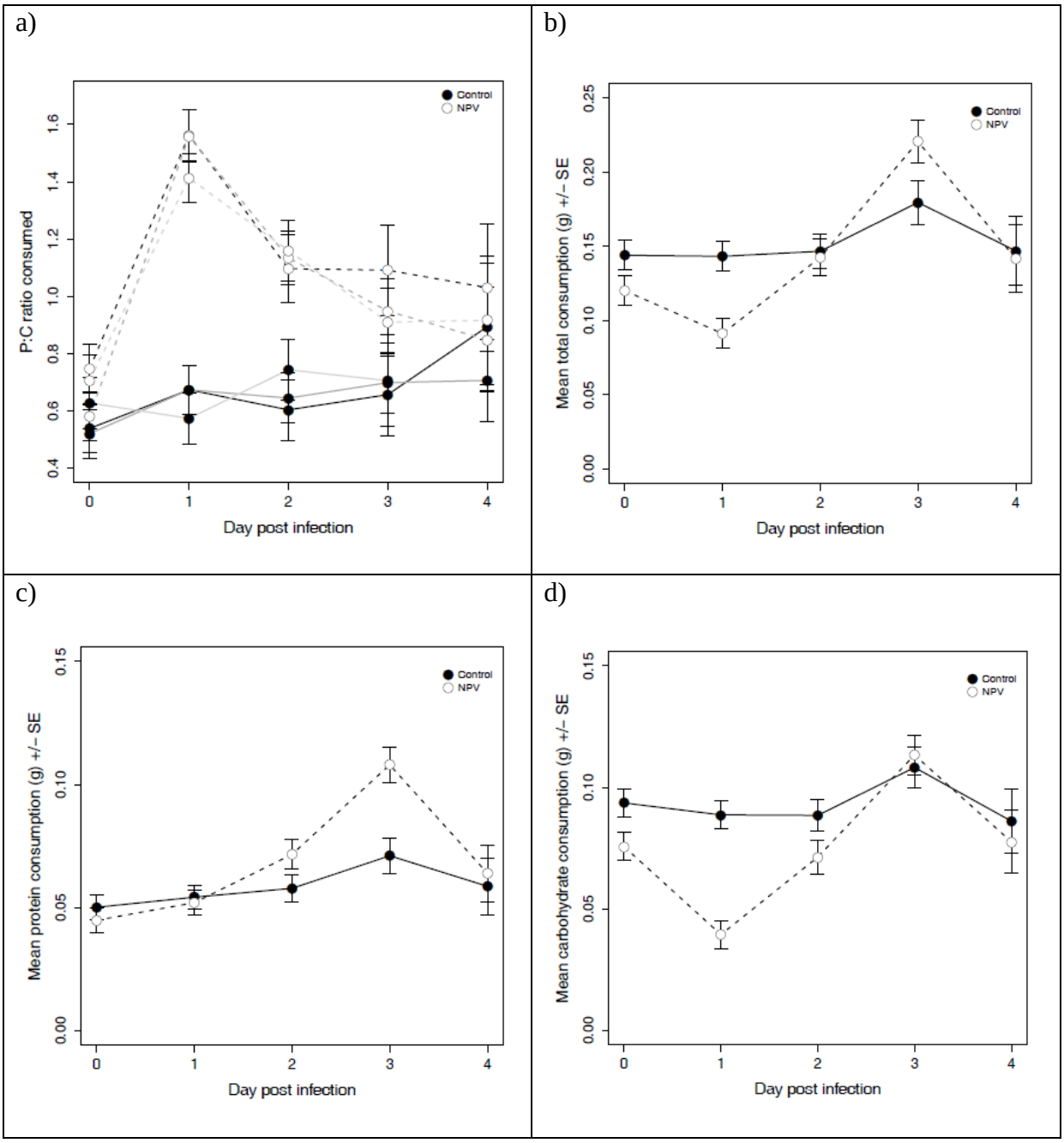
737 ***Figure 5***



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