Eating in a losing cause: anorexia and macronutrient manipulation by a cockroach fails to boost immunity

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Summary

1. Host-pathogen interactions can lead to dramatic changes in host feeding behaviour. One aspect of this includes self-medication, where infected individuals consume substances such as toxins, minerals or secondary compounds or alter their macronutrient consumption to enhance immune competence. Another aspect includes illness-induced anorexia, which is a general mechanism adopted by animals following infection. Anorexia is thought to assist host immunity directly or by limiting the nutritional resources available to pathogens.

2. Here, we recorded macronutrient preferences of the global pest cockroach, *Blatta orientalis* to investigate how shifts in host macronutrient dietary preference and quantity interact with immunity following sublethal bacterial infection.

3. We compared the survival of uninfected and infected individuals on diets enriched with carbohydrate (C) or protein (P) and conducted food-choice experiments after challenging hosts with the common entomopathogenic soil bacterium *Pseudomonas entomophila*. We then carried out a quantitative proteomic analysis and an antimicrobial activity assay of hemolymph from infected individuals that had been restricted to diets with defined macronutrient compositions.

4. We find that diets enriched for P reduce cockroach survival. However, following immune challenge by *P. entomophila*, cockroaches significantly reduced their overall nutrient intake, particularly of carbohydrates, and increased the ratio of protein (P:C) consumed. Surprisingly, these behavioural shifts did not significantly improve cockroach immunity or

survival, with negligible differences in immune protein abundance and antimicrobial activity between infected individuals placed on P- or C- rich diets.

5. We show that cockroach feeding behaviour can be dynamically modulated by a pathogen, resulting in an illness-induced anorexia-like feeding response and preference for P-enriched food. Interestingly however, our results also demonstrate that these responses hold no special advantage for the host's immune response. The lack of a benefit following the host's behavioural shift highlights a possible decoupling of dietary regulation from immunity in these invasive animals.

Introduction

Microbe symbioses form a fluctuating but universal backdrop to animal life. However, the evolutionary processes that drive animal hosts and their symbionts, including pathogens, operate at different scales and often in opposing directions (Dawkins & Krebs 1979), with the animal immune system acting as a key interface between host and symbiont ecology (Schmid-Hempel 2003). In addition to the core immune system, behavioural mechanisms have attracted increasing attention for their ability to co-ordinate host responses to infection (Simpson *et al.* 2015; Wong *et al.* 2015). While behaviour is the primary means by which animals interact with the biotic environment, its importance for a wide range of immune-related functions has only relatively recently come to the fore.

Hosts can respond behaviourally before infection has even taken place. This can include avoidance of pathogen transmission areas (e.g. defecation sites) and deterrence of disease vectors (Hart 2011; Moore 2013). Other prominent examples include activities falling within the category of "social immunity", which among insects can include pathogen detection

68 alarm behaviours (Rosengaus *et al.* 1999); grooming of conspecific group members
69 (Rosengaus *et al.* 1998; Reber *et al.* 2011); removal (Armitage *et al.* 2016) or even
70 destruction of infected individuals (Yanagawa *et al.* 2011). Such mechanisms are well
71 documented in many social insect lineages, where they contribute significantly to a number
72 of prophylactic mechanisms operating within societies (Schmid-Hempel 1998; Cremer *et al.*
73 2007). Other prophylactic social behaviours include the collection of secondary antimicrobial
74 compounds to prevent microbial growth in the nest environment (Castella *et al.* 2008;
75 Simone *et al.* 2009), in addition to the direct use – typically via feeding – of antimicrobials in
76 both individual and transgenerational prophylaxis (Lefevre *et al.* 2010; Lefevre *et al.* 2012;
77 Milan *et al.* 2012; de Roode *et al.* 2013; Kacsoh *et al.* 2013).

78 Once infection has occurred, the first and principal line of defence is the immune system.
79 Here, behavioural defensive adaptations can also play an important role in regulating or
80 augmenting the response to infection. As with prophylaxis, the role of feeding behaviour has
81 increasingly been viewed as a key mechanism by which animals can respond to infection
82 (Abbott 2014). Here, the selection of novel antimicrobial compounds, or the enrichment of
83 specific dietary elements can be employed as therapeutic treatment against pathogens (de
84 Roode *et al.* 2013). Fruit flies use ethanol therapeutically as well as prophylactically to
85 combat parasitoid wasp infection (Milan *et al.* 2012) whereas parasitoid fly-infected
86 *Grammia* caterpillars mix pyrrolizidine alkaloid-producing toxic plants into the normal diet to
87 assist parasitoid clearance, which comes at the expense of body growth (Singer *et al.* 2004;
88 Smilanich *et al.* 2011).

89 Infection-induced adaptive changes to feeding behaviour can also involve modifications to
90 the quantity and composition of macronutrients in the diet. Anorexia is a well-documented

91 response to infection in both vertebrates (Johnson *et al.* 1993; Konsman *et al.* 2002) and
92 invertebrates (Adamo *et al.* 2007; Ayres & Schneider 2009) and is thought to assist hosts in
93 limiting nutritional resources available to pathogens (Kluger & Rothenburg 1979). Anorexia
94 may also help by activating components of the immune system that are enhanced under
95 conditions of nutritional stress, such as autophagy (van Niekerk *et al.* 2016a; van Niekerk *et*
96 *al.* 2016b). In recent years, the balance of macronutrients itself has been examined as a way
97 for animals to regulate the response to infection. In particular, the proportion of protein has
98 been shown to be an important criterion in animal choice of diet following infection. In
99 *Spodoptera* moths, larvae select a diet enriched in protein following infection with a
100 generalist gram-positive bacterium and a host-specific DNA virus (Lee *et al.* 2006; Povey *et*
101 *al.* 2009; Povey *et al.* 2013), leading to enhanced antimicrobial activity in both cases. By
102 contrast, diets enriched in carbohydrate were selected when *Tenebrio* beetles and *Grammia*
103 caterpillars were infected with a rat tapeworm (Ponton *et al.* 2011) and a parasitoid fly
104 (Mason *et al.* 2014), respectively. In the latter study, this behaviour was also associated with
105 an enhanced melanization response.

106 The use of macronutrients by hosts to regulate immunity could in principle apply to any
107 animal that is not an obligate food specialist. However, virtually nothing is known about the
108 relationship between macronutrient diet choice and immunity outside of a handful of
109 holometabolous, mostly herbivorous, insect larvae. Holometabolous insects undergo
110 complete metamorphosis consisting of distinct larval, a pupal and an adult winged phase,
111 which are typically correlated with vastly different ecologies and corresponding
112 physiological, morphological and immunological conditions (McMahon & Hayward 2016). By
113 contrast, hemimetabolous insects undergo progressive molts where each larval instar closely

114 resembles the adult (Sehnal *et al.* 1996). Our understanding of the interface between diet,
115 behaviour and immunity would greatly benefit from studying animals that vary widely in
116 their taxonomy, ecology and development.

117 Our study addresses this by examining the interaction between macronutrient feeding
118 behaviour and immunity in the omnivorous cockroach, *Blatta orientalis*. We investigate the
119 macronutrient preferences of individuals in response to a range of sublethal immune
120 challenges, before examining the impact of macronutrients on host survival, immune
121 competence and finally, the expression of the host's proteome, which captures a critical
122 component of the host's response to a pathogen. Overall, our findings suggest that diet is
123 decoupled from immune regulation in cockroaches.

124

125 **Materials and Methods**

126 INSECTS AND BACTERIA

127 A breeding culture of sequential *Blatta orientalis* cohorts was established at the Federal
128 Institute for Materials Research and Testing (BAM) in June 2015, initially obtained from the
129 collection at the Federal Environment Agency, Berlin, which consists of a mixed population
130 of four independent genetic backgrounds maintained for 50 generations. Each generation
131 consists of a minimum of 150 breeding pairs of cockroaches to minimize the effects of
132 inbreeding. Each experimental cohort was maintained for approximately 190 days in the
133 dark at 26 °C and 50 % humidity. Prior to being placed on experimental (artificial) diets,
134 animals were reared on a mixture of 77.0 % dog biscuit powder, 19.2 % oat flakes and 3.8 %
135 brewer's yeast and supplied with water *ad libitum* and weekly with apple and carrot slices.

136 All experiments were conducted with males to minimise changes in physiology associated
137 with oogenesis. Each individual was used only once in each experiment. For the food choice
138 experiment and the survival on enforced diets, individuals from 3 different cohorts were
139 used. The generalist gram-negative bacterial pathogen *Pseudomonas entomophila* (strain
140 L48; DSM No. 28517) which is able to infect a variety of insect orders (Vallet-Gely *et al.* 2010)
141 was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell
142 Cultures. Bacteria were stored at -70 °C until use in experiments.

143

144 ARTIFICIAL DIETS

145 The artificial diets used in this study are based on isocaloric diets, as described elsewhere
146 (Lee *et al.* 2006; Povey *et al.* 2013), which was slightly modified to suit cockroach needs. We
147 employed two diets containing 35 % carbohydrate (C) and 7 % protein (P) or *vice versa*. The
148 carbohydrate portion consisted of sucrose while the protein portion consisted of casein,
149 peptone and albumin from eggs in a 3:1:1 ratio. Remaining ingredients are listed in
150 Supplementary Tab. 1. Diet blocks of approximately 0.125 cm^3 in size were dried at 50 °C for
151 2 days before being weighed and given to experimental cockroaches.

152

153 BACTERIAL INOCULATION

154 About 200 µl of an overnight culture of *P. entomophila* was mixed in 10 ml fresh liquid
155 medium (according to DSMZ instructions) and incubated at 28 °C and 140 rpm to an OD_{600nm}
156 of 0.55, representing 1.5 x 10^8 CFUs (determined by plating). The desired concentrations of
157 bacteria were subsequently obtained by diluting bacteria in insect Ringer's solution (0.024 g

158 calcium chloride, 0.021 g potassium chloride, 0.01 g sodium hydrogen carbonate, 0.45 g
159 sodium chloride, 200 ml dest. water). Cockroaches were anaesthetised with CO_2, abdomens
160 swabbed with 70 % ethanol, then injected with 2µl of bacterial solution directly into the
161 hemocoel using a glass capillary needle inserted between the 3rd and 4th abdominal
162 segment. Sublethal infections (high: 5.8×10^5, low: 5.8×10^3) and lethal (4.5×10^6) doses
163 were determined in pre-experiment injection assays.

164

165 DIET CHOICE FOLLOWING SUBLETHAL INFECTION

166 From each of 3 cohorts, 40 *B. orientalis* males (120 in total) were given free choice of
167 macronutrients by placing them together with 1 block of known weight of each artificial diet.
168 Individuals were kept for three days to accustom them to artificial diets, and to obtain a
169 baseline P:C ratio preference. Thereafter food blocks were collected, placed at 50°C until
170 completely dry, and then their weight loss was determined, equating to the amount eaten
171 by the cockroach. Experimental cockroaches were assigned randomly to one of the following
172 sublethal treatments (40 per treatment): 1) High infection (injected 5.8×10^5 *P. entomophila*
173 CFUs); 2) Low infection (injected 5.8×10^3 *P. entomophila* CFUs); 3) Wounding control
174 (injected Ringer's solution); 4) Unmanipulated control. Cockroaches were then placed on
175 new food blocks of both diets of known weight. The blocks were replaced daily for four days
176 and their loss of weight was again determined after drying at 50°C.

177

178 SURVIVAL ON ENFORCED DIET

179 From each of 3 cohorts, 20 *B. orientalis* males were placed on protein-rich diet (35 % P; 7 %
180 C) and another 10 were placed on carbohydrate-rich diet (7 % P; 35 % C). All individuals were
181 supplied with water *ad libitum*. Survival was checked twice weekly; food blocks and water
182 were changed once a week over 150 days.

183 SURVIVAL ON ENFORCED DIET FOLLOWING LETHAL INFECTION

184 One hundred and fifty *B. orientalis* males were equally assigned to one of the following
185 lethal treatments: 1) Infection (injected 4.5 x 10^6 *P. entomophila* CFUs); 2) Wounding control
186 (injected Ringer's solution); 3) Unmanipulated control. Half of the individuals from each
187 treatment were randomly assigned to either a protein- (35 % P; 7 % C) or carbohydrate-
188 enriched (7% P; 35% C) artificial diet and supplied with water *ad libitum*. Survival of each
189 individual was recorded for six days.

190

191 IMMUNE ASSAYS

192 **Immune challenge and hemolymph collection**

193 One hundred and twenty *B. orientalis* males were immune-challenged by injecting 2 µl
194 Ringer's solution containing 5.8 x 10^5 *P. entomophila* CFUs. Half were assigned to the
195 protein- (35 % P; 7 % C) and the other half to the carbohydrate-enriched (7 % P; 35 % C)
196 artificial diet and supplied with water *ad libitum*. After 24 hrs, the hemolymph from each
197 individual was collected by cutting the first 2 leg pairs and placing the cockroach head-first
198 into a spin-column (Sigma-Aldrich) in a 1.5 ml tube containing propylthiouracil (to inhibit
199 phenol-oxidase activity). They were then centrifuged at 500 *g* for up to 5 min or until 10 µl of

200 hemolymph was collected. The hemolymph of 10 individuals of the same treatment was
201 pooled resulting in 6 pools per treatment and stored at -70 °C until needed.

202 **Bacteria growth inhibition assay**

203 The bacterial growth inhibition of the cockroach hemolymph was measured using a plate
204 reader assay. First, 10 µl Mueller-Hinton broth were added to each well of a 384-well
205 polypropylene plate. Then 10 µl hemolymph was loaded in the second column of the plate.
206 One well contained the hemolymph of one pool of animals (in total 12 wells loaded with
207 hemolymph). Wells in the first column which did not contain hemolymph served as the
208 negative control. A seven-step serial dilution of the hemolymph was performed (with the
209 last 10 µl being discarded) and 10 µl *P. entomophila* in Mueller-Hinton broth with an OD_{600} of
210 0.005 was added to each well containing hemolymph, as well as to a further column not
211 containing hemolymph, which served as a positive control for unsuppressed bacterial
212 growth. OD_{600} was measured in a plate reader (BioTek) every 10 min for 16 hrs.

213

214 PROTEOMIC ANALYSIS BY MASS SPECTOMETRY

215 A detailed description of protein sample preparation and liquid chromatography-mass
216 spectrometry and data processing is described in Supplementary file 1. Initially, we carried
217 out *de novo* transcriptome sequencing to generate a peptide database for *B. orientalis*.
218 Briefly, RNA was extracted from *B. orientalis* by homogenizing individuals in pre-cooled Trizol
219 (Thermo Fisher Scientific) and recovering RNA using chloroform extraction and isopropanol
220 precipitation, and treatment with TurboDNase (Ambion) according to manufacturer's
221 instructions. mRNA libraries were enriched and prepared using a NEXTflexTM Rapid

222 Directional mRNA-seq Kit protocol (Bioo Scientific) before being sequenced on an Illumina
223 NextSeq500/550 platform at the Berlin Center for Genomics in Biodiversity Research
224 (BeGenDiv). Raw data were processed and annotated as described elsewhere (He *et al.*
225 2018) (Supplementary file 1). For proteomic analysis, protein identification and label-free
226 quantification was performed using MaxQuant (v1.6.0.1) with Andromeda search engine
227 (Cox & Mann 2008; Cox *et al.* 2011; Tyanova *et al.* 2015). Raw data were matched against an
228 in-house protein database of *B. orientalis* created by *de novo* transcriptome sequencing (see
229 above). Trypsin was selected as enzyme allowing a maximum of two missed cleavages. The
230 minimum peptide length was set to 7 amino acids and the false discovery rate (FDR) for
231 peptide and protein identification was set to 0.01.

232

233 STATISTICAL ANALYSIS

234 P:C ratios, the amounts of P and C eaten as well as total consumption differences between
235 treatments were analysed on the first day following infection using Bonferroni-corrected
236 Wilcox rank sum tests.

237 The food-choice data were analyzed using a Generalized linear mixed model (GLMM) with an
238 underlying beta family distribution. Analyses were run in the glmmADMB package v0.8.3.3
239 (Fournier *et al.* 2012; Skaug *et al.* 2014) in R 3.4.3 (R Core Team 2018) in conjunction with
240 the R2admb package v0.7.16 (Bolker *et al.* 2017). GLMMs examined whether a response
241 variable consisting of proportion of P consumed (amount of P eaten divided by the amount
242 of diet available) or proportion of C (amount of C eaten divided by the amount of diet
243 available) was influenced by treatment (high infection; low infection; wounded; and

244 unmanipulated) and day post infection, as well as an interaction between treatment and
245 day. Minimal adequate models were derived by stepwise-model simplification and
246 comparison via ANOVA. Individual and cohort were treated as random effects to account for
247 multiple measurements and origin. Comparisons among treatment levels were carried out
248 with post-hoc Tukey tests using a Bonferroni correction, using package multcomp v1.4-8
249 (Hothorn *et al.* 2008). Individuals 16, 48 82, 84 and 91 were removed prior to analysis due to
250 the presence of fungal growth on the artificial diet blocks.

251 The effect of treatment (infection and control) and diet (P- and C-enriched) on survival were
252 analysed using Cox proportional hazard models in R 3.3.1 with the survival package v2.39-5
253 (Therneau 2015). Bacterial growth inhibition data were analyzed using R package nparLD
254 v2.1 (Noguchi *et al.* 2012) using default parameters.

255

256 **Results**

257 DIET CHOICE FOLLOWING SUBLETHAL INFECTION

258 Before wounding or infection, cockroaches of all treatments preferred an average P:C ratio
259 of approximately 0.28 (Fig. 1A). The unmanipulated animals preferred this ratio over the
260 course of the experiment. By contrast, highly infected individuals selected a P:C ratio of
261 approximately 1.3 whereas low infected and wounded cockroaches preferred an
262 intermediate ratio of approximately 1.0 P:C on the first day post-infection (p.i.) (Wilcox rank
263 sum test: high vs. low: W= 595.0, $p = 0.098$; low vs. wounded: W = 412.5, $p > 0.1$; wounded
264 vs. unmanipulated: W = 713.5, $p < 0.001$). All manipulated groups returned to baseline P:C
265 ratios by day 4 p.i..

266 Final minimal GLMMs consisted of the fixed terms treatment and day without an interaction
267 since the model with a treatment*day interaction did not significantly improve the model
268 (ANOVA for model comparison, $p > 0.1$). Cockroaches that were wounded differed
269 significantly from unmanipulated cockroaches in their preferred P proportion on the first day
270 following treatment (P proportion chosen: 0.14 vs 0.06, wounded vs. unmanipulated,
271 respectively: $z = -3.348$ $p = 0.005$), as did cockroaches infected with a high (P proportion
272 chosen: 0.17 vs 0.06, high vs. unmanipulated respectively: $z = -7.416$, $p < 0.001$) or low
273 bacterial dose (P proportion chosen: 0.14 vs 0.06, low vs. unmanipulated respectively: $z = -$
274 2.809, $p = 0.029$). Cockroaches infected with a high bacterial dose also selected a higher
275 proportion of P compared to individuals exposed to both a low bacterial dose (P proportion
276 chosen: 0.17 vs 0.14, high vs. low respectively: $z = -3.718$, $p = 0.001$) or to wounding (P
277 proportion chosen: 0.17 vs 0.14, high vs. wounded respectively: $z = -2.808$, $p = 0.029$).
278 However, individuals that were wounded or were infected with a low bacterial dose did not
279 select a significantly different proportion of P (P proportion chosen: 0.14 vs 0.14, low vs.
280 wounded respectively: $z = 0.625$, $p = 1.000$). Concerning the proportion of C, the pattern is
281 slightly different. Only highly infected cockroaches on the first day p. i. differed significantly
282 from unmanipulated (C proportion chosen: 0.16 vs 0.26, high vs. unmanipulated
283 respectively: $z = 5.270$, $p < 0.001$). All the other treatments were not significantly different
284 from each other (Supplementary Tab. 2).

285 P, C as well as total food consumption varied significantly over the course of the experiment
286 (Fig. 1B-D). Individual cockroaches ate on average 0.6 mg P and 2.2 mg C under
287 unmanipulated conditions. Conversely, in all manipulated groups the amount of P eaten
288 declined immediately after infection (Wilcox rank sum test: wounded vs. unmanipulated: W

289 = 77.0, $p < 0.001$; low vs. unmanipulated: W = 66.5, $p < 0.001$; high vs. unmanipulated: W =
290 76.0, $p < 0.001$). The same accounts for the consumption of C (Wilcox rank sum test:
291 wounded vs. unmanipulated: W = 11.0, $p < 0.001$; low vs. unmanipulated: W = 7.0, $p < 0.001$;
292 high vs. unmanipulated: W = 6.0, $p < 0.001$). As a result, the total amount eaten was reduced
293 in the same way immediately following treatment (Wilcox rank sum test: wounded vs.
294 unmanipulated: W = 0.0, $p < 0.001$; low vs. unmanipulated: W = 0.0, $p < 0.001$; high vs.
295 unmanipulated: W = 0.0, $p < 0.001$). By the 2nd day, consumption across all groups began to
296 recover, reaching pre-treatment levels by the 4th day p.i..

297

298 SURVIVAL ON ENFORCED DIET WITH AND WITHOUT LETHAL INFECTION

299 The median (50 %) survival time for *B. orientalis* males placed on a P-rich diet was 82 days,
300 whereas the mortality of males placed on a C-rich diet did not exceed 30 % over the course
301 of the experiment (150 days) (Fig. 2A). By the end of the experiment males restricted to P-
302 rich diet showed a significantly higher mortality (86.44 %) compared to those on C-rich diet
303 (27.59 %; Cox proportional hazard regression P vs. C: $z = 5.974$, $p < 0.001$). When injected
304 with a lethal dose of *P. entomophila*, cockroaches started to die on day 1 p.i. (Fig. 2B), and
305 diet did not have a significant effect on survival following infection (Cox proportional hazard
306 regression P vs. C: $p > 0.10$). In both cases around 70 % of the individuals had died by day 6
307 p.i..

308

309 BACTERIA GROWTH INHIBITION ASSAY

310 The inhibitory effect of the hemolymph (N = 6 per dilution per treatment) of immune-
311 challenged *B. orientalis* males on bacterial growth was not diet-dependent (ANOVA: C 1:4 vs.
312 P 1:4: $F_1 = 0.13$, $p > 0.10$; C 1:8 vs. P 1:8: $F_1 = 0.02$, $p > 0.10$; Fig. 3) resulting in similar
313 suppression of bacterial growth regardless of whether cockroaches ate protein- or
314 carbohydrate-rich diet, with only dilution level influencing bacterial growth: hemolymph
315 diluted 1:4 and 1:8 suppressed *P. entomophila* growth for the first 600 min and 420 min,
316 respectively, after which bacteria started to slowly replicate. By the end of quantification,
317 bacteria exposed to the 1:8 hemolymph dilutions (from both P and C-enforced diets) had
318 reached a significantly higher OD than in controls (ANOVA: C 1:8 vs. Control: $F_1 = 6.51$, p =
319 0.011; P 1:8 vs. Control: $F_1 = 4.59$ p = 0.032) whereas both 1:4 dilutions did not.

320

321 PROTEOMIC ANALYSIS BY MASS SPECTOMETRY

322 A total number of 3514 peptide hits were identified and assembled into 750 proteins by
323 MaxQuant. After filtering, 387 different proteins were identified and quantified in the
324 hemolymph of infected *B. orientalis* males fed on a P-rich vs. a C-rich diet (N = 6 per
325 treatment) (Supplementary Tab. 3), of which 65 are putative immune-related genes. Overall,
326 Apolipophorin was the most abundant protein making up approximately 70 % of the whole
327 hemolymph protein content. Other highly abundant proteins were Transferrin, Gelsolin,
328 Heterochromatin-associated protein MENT and an Insulin-like growth factor-binding protein
329 complex. We identified 17 proteins that showed significant changes in abundance following
330 diet treatment (Fig. 4 and Supplementary Tab. 3). Infected individuals on a C-rich diet were
331 significantly enriched for Hexokinase type II, which is involved in carbohydrate metabolism
332 (glycolysis) (Yanagawa 1978), in addition to Carbonyl reductase I-like, which is involved in

333 NADPH-dependent reduction of active substrates including endogenous and xenobiotic
334 carbonyl compounds (Hoffmann & Maser 2007). Additionally, Tropomyosin which is a
335 calcium-dependent regulator of muscle contraction (Pomés *et al.* 2007), and Acyl-CoA-
336 binding protein, which carries out lipid-binding transport and suppresses glucose-induced
337 insulin secretion (Færgeman *et al.* 2007) were more abundant. Furthermore, a L-galactose
338 dehydrogenase-like protein was enriched but its function is not known in insects. Conversely
339 infected individuals on a P-rich diet were significantly enriched for Alpha-amylase, which is
340 involved in carbohydrate metabolism (Terra & Ferreira 1994) and Proteasome subunit alpha
341 type-3, which is involved in protein degradation (Rivett 1993). Additionally, Hemolymph
342 lipopolysaccharide-binding protein-like (2 isoforms), which binds carbohydrates (foreign
343 particles) (Jomori & Natori 1991) and Extracellular superoxide dismutase, which carries out
344 superoxide metabolic processing (Felton & Summers 1995) were detected. Glutamine
345 synthetase is involved in glutamate and glutamine catabolism and biosynthesis (Smartt *et al.*
346 1998) while Adenylate kinase isoenzyme 1 and Hexamerin are associated with ATP
347 metabolism (Fujisawa *et al.* 2009) and amino-acid and energy storage, respectively
348 (Burmester 1999). There was also an enrichment of Ankyrin-1, although its function in
349 insects remains unclear.
350

351 **Discussion**

352 Under normal conditions, extensive protein consumption shortens the lifespan of many
353 insects including ants, honeybees and flies (Lee *et al.* 2008; Dussutour & Simpson 2009;
354 Fanson *et al.* 2009; Grandison *et al.* 2009; Cook *et al.* 2010; Pirk *et al.* 2010), a finding that is
355 corroborated in our and another study of cockroaches (Hamilton *et al.* 1990). Here, we find

356 that male *B. orientalis* cockroaches showed 45 % higher mortality (Fig. 2A) when restricted
357 to a P- vs a C-rich diet. One explanation for this consistent observation across study
358 organisms is that elevated levels of proteins increase TOR signaling. TOR serves as a nutrient
359 sensor linked to macronutrient intake and metabolism, causing a broad anabolic response
360 that is life-shortening over the long term (reviewed in (Simpson & Raubenheimer 2009).
361 Other explanations could relate to the toxic effects of breaking down nitrogenous products,
362 and the enhanced production of mitochondrial radical oxygen species, DNA and protein
363 oxidative modifications, membrane fatty acid composition and mitochondrial metabolism
364 (reviewed in (Simpson & Raubenheimer 2009). The higher abundance of extracellular
365 superoxide dismutase in cockroach males fed on a P-rich diet (Fig. 4; Supplementary Tab. 3)
366 supports this explanation.

367 Unsurprisingly, male cockroaches consumed low amounts of protein under normal
368 conditions. But this preference shifted dramatically following infection. As with caterpillars
369 (Povey *et al.* 2013), highly infected male cockroaches switched to a P-rich diet. Furthermore,
370 cockroaches adapted their feeding behaviour to the severity of the infection. Lowly infected
371 and wounded (Ringer-injected) individuals chose an intermediate (balanced) P:C ratio,
372 whereas uninfected and highly infected individuals chose a significantly enriched diet for C
373 and P, respectively. This demonstrates that cockroaches quantitatively regulate their
374 behavioural response to infection. Additionally, our data suggest host-driven adaptation as
375 opposed to pathogen manipulation because wounded individuals also reduced their
376 carbohydrate intake. Wounding elicits a localized immune response in insects (Haine *et al.*
377 2007), suggesting a form of prophylactic behavior since it is likely that microbes can enter
378 the hemolymph via damaged cuticle (Siva-Jothy *et al.* 2005).

379 In contrast to caterpillars and other organisms which can modulate their immune response
380 with diet, changes in cockroach dietary preference did not influence any of the immune
381 parameters measured. In caterpillars, a shift from a carbohydrate- to a protein-biased diet
382 following *Bacillus subtilis* (gram-positive) or baculovirus infection led to an increase of
383 antibacterial and phenoloxidase activity and hemocyte density, and resulted in higher
384 survival (Povey *et al.* 2009; Povey *et al.* 2013). By contrast, a switch to a protein-biased diet
385 did not influence cockroach hemolymph antimicrobial activity (Fig. 3) or survival (Fig. 2B) nor
386 have a major impact on the production of immune related proteins (Fig. 4; Supplementary
387 Tab. 3). Only two hemolymph lipopolysaccharide-binding protein isoforms, which may play a
388 role in pathogen recognition by binding foreign particles (Jomori & Natori 1991) were more
389 abundant in the hemolymph of P-rich fed infected cockroaches, but overall our findings
390 indicate that the behavioural changes adopted by cockroaches are limited in their ability to
391 alter infection outcome.

392 The overrepresentation of proteins participating in C- and P metabolism in C- vs P-rich diets,
393 respectively (Fig. 4), demonstrate that the diets altered cockroach physiology in the
394 expected direction. For example, the higher abundance of alpha-amylase in the hemolymph
395 of *B. orientalis* males feeding on P-rich diet shows these individuals were metabolizing lower
396 quantities of C. Alpha-amylase is thought to be involved in the breakdown of glycogen,
397 which is the major glucose storage compound in animals. It is employed if not enough
398 carbohydrates are present in the diet (Mohamed 2004).

399 Overall, our results suggest that cockroaches may not be able to self-medicate using
400 macronutrients, but that they do engage in a typical anorexia response, as has been shown
401 in macronutrient self-medication in caterpillars (Adamo *et al.* 2007; Povey *et al.* 2009; Povey

402 *et al.* 2013). Illness-induced anorexia offsets physiological trade-offs between launching
403 immune responses and food digestion. A previous study demonstrated that crickets reduce
404 their food intake, especially for lipids, following infection with the bacterium *Serratia*
405 *marcescens* (Adamo *et al.* 2010). High hemolymph lipid levels are associated with decreased
406 concentrations of monomeric apolipophorin III, a lipid transporter, and higher susceptibility
407 to *S. marcescens* infection (Adamo *et al.* 2008). In other insects, anorexia can have a direct
408 impact on immunity. For example, in *Drosophila*, starvation can modify antimicrobial peptide
409 production and lead to reduced melanisation (Ayres & Schneider 2009).

410 The apparent lack of a link between macronutrient dietary selection and male cockroach
411 immunity is unexpected. One possible explanation is that future food availability and quality
412 may be far less predictable in omnivorous pest organisms like cockroaches (Raubenheimer &
413 Jones 2006). A recent genomic study reports major expansions of cockroach gene families
414 linked to chemoreception, detoxification and innate immunity (Li *et al.* 2018), indicating that
415 adaptations in these pathways may have been essential for enabling cockroaches to thrive in
416 unpredictable, antigen-rich environments. Indeed, while cockroach survival was reduced on
417 an enforced P-rich diet, a negative effect could only be observed well over 40 days after
418 exposure. In such organisms, there could be a clear advantage in reducing regulatory
419 interactions between host diet and immunity. An additional point to consider is that in
420 contrast to several previous studies, we performed experiments on adult individuals and not
421 larvae, which have different resource allocation strategies and consumption rates in general
422 (Boggs 2009). In holometabolous insects, most of the resources in larvae are allocated to
423 growth, maintenance and storage whereas in adults, they are allocated to reproduction and
424 maintenance. Consequently, there has been great emphasis on a trade-off between growth

425 and immunity in the larval stage of herbivorous insects (reviewed in (Singer *et al.* 2014)).
426 Here, the need for fast growth competes with the requirement to provide protection from
427 parasite and pathogen-induced mortality. Given that proteins and amino acids are a crucial
428 limiting factor in herbivorous diets, we argue that a trade-off between two essential life-
429 history parameters that depend strongly on P – growth and survival – could be particularly
430 pronounced in herbivorous insects (Schoonhoven *et al.* 2005; Simpson & Raubenheimer
431 2012).

432 In conclusion, we find that *B. orientalis* males modulate their macronutrient feeding
433 behaviour following infection by dramatically reducing food intake and favouring a diet
434 containing a higher P-ratio. We also show that a P-rich diet eventually leads to significantly
435 reduced host lifespan, and that male cockroaches avoid such a diet under normal conditions.
436 To our surprise, the observed behavioural response to immune challenge did not positively
437 influence the antimicrobial activity or proteomic profile of host immunity. Overall, our
438 findings support the concept of a generalized host-directed response to microbial challenge
439 in cockroaches, based on anorexia and the limitation of carbohydrate intake. Such a
440 response is likely to be beneficial to the host, but primarily as a means of avoiding
441 contaminated food and reducing pathogen access to resources, rather than facilitating cross-
442 talk with the immune system. Our findings indicate that dietary composition and immunity
443 may be decoupled in cockroaches resulting from adaptations to detoxification and innate
444 immunity linked to survival in antigen-rich and nutritionally diverse environments. Our study
445 highlights the importance of understanding variation in natural diet, development, and
446 ecology when exploring the link between nutrition and animal immunity.

447

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623

624 **Authors' contribution**

625 T.S. and D.P.M. conceived and coordinated the study; T.S. designed and conducted the
626 experiments; T.S. and D.P.M. wrote the manuscript; S. H. and P. R. J. conducted the
627 proteomic database preparation by de novo transcriptome sequencing; A.R.-R. guided the
628 sample preparation for the proteomic analysis; B. K. and C. W. conducted LC-MS/MS and
629 corresponding data analysis. All authors contributed to drafting and revising the manuscript.

630

631 **Data accessibility**

632 All data associated with the study will be made available in Dryad.

633

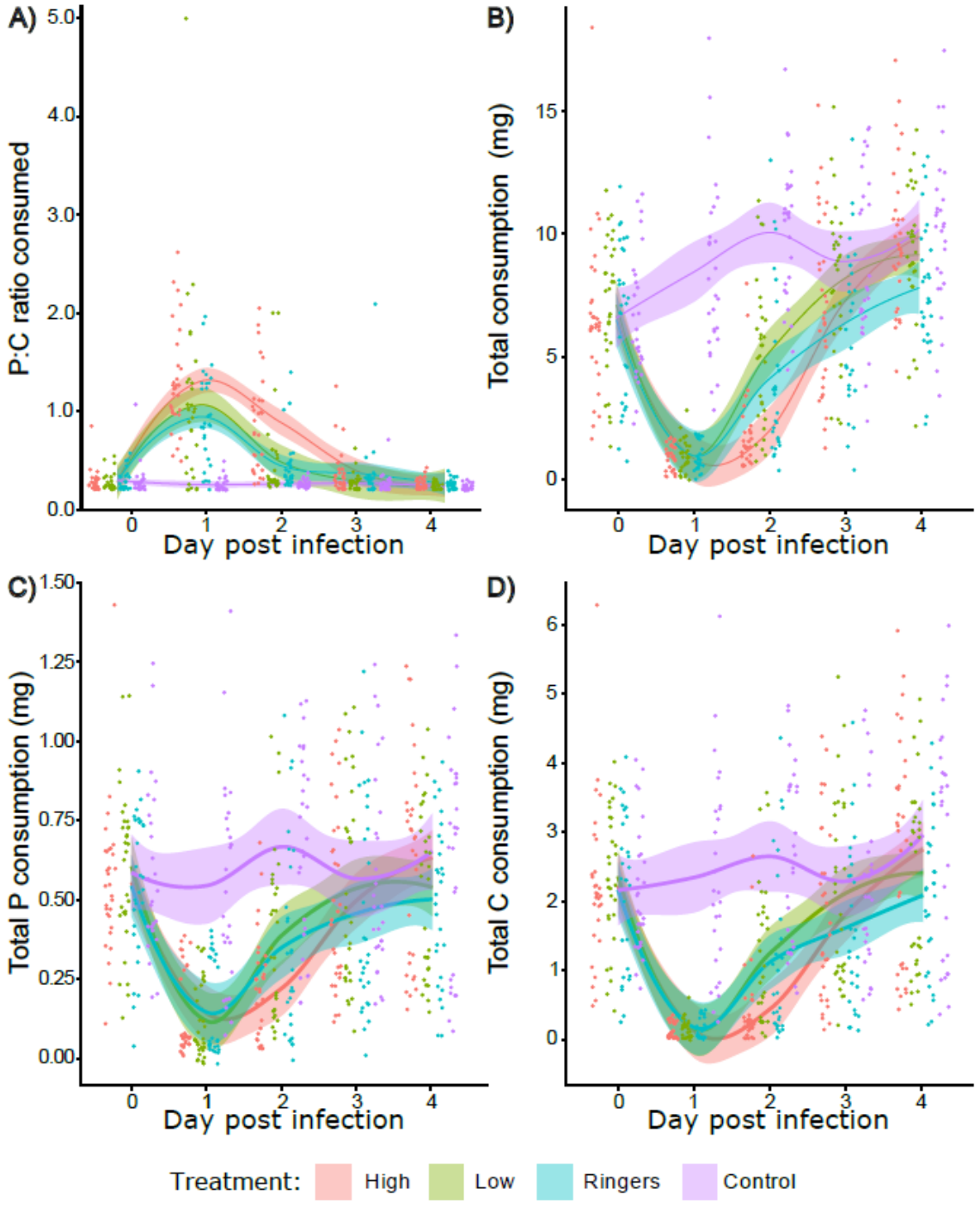
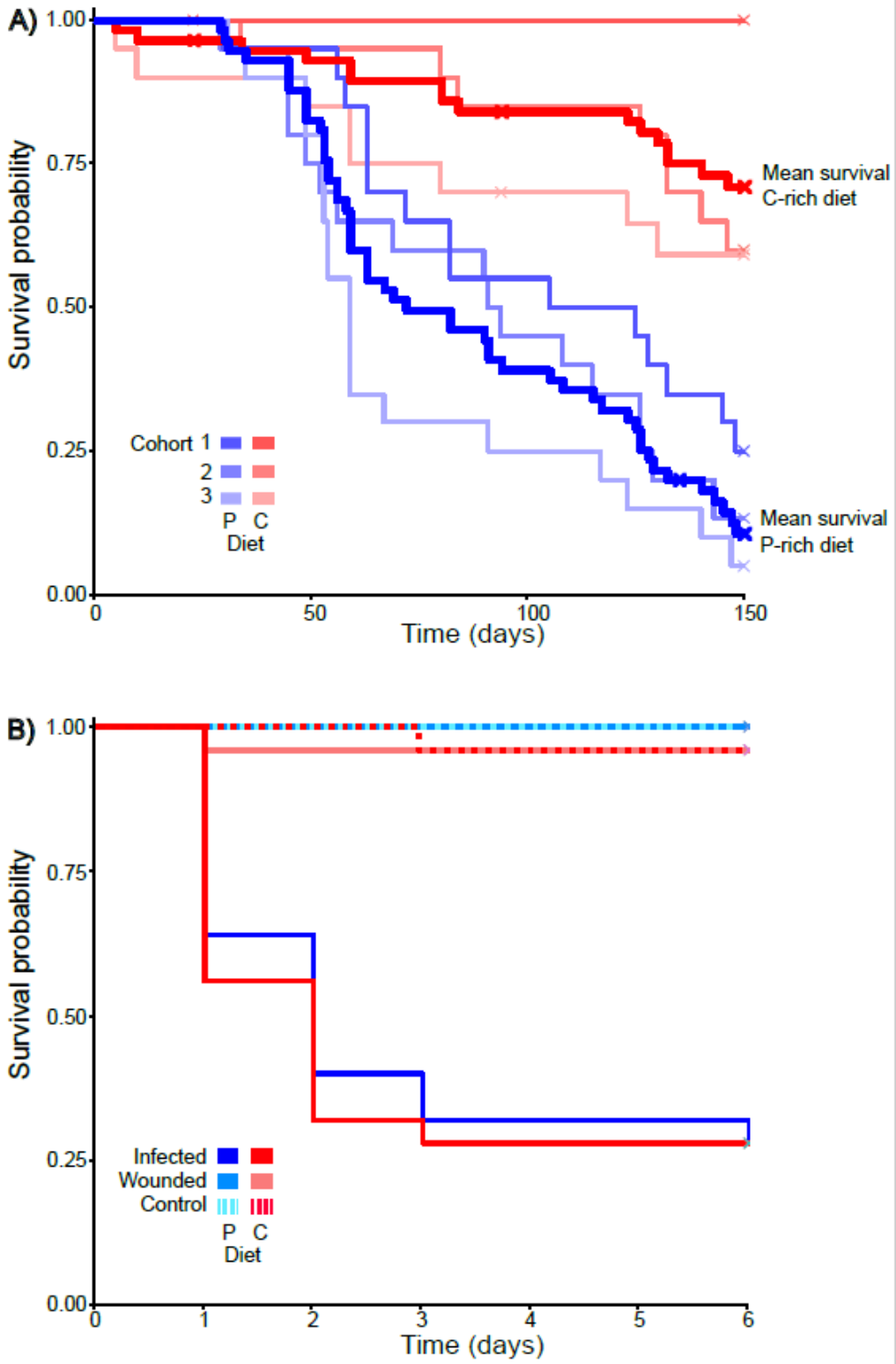


Fig. 1: Effect of bacterial infection with *P. entomophila* (high load, low load), Ringer's solution or no manipulation (control) of *B. orientalis* males on: **A)** P:C ratio chosen, **B)** total consumption, **C)** P consumption, **D)** C consumption. Note different scales used for total P- and C- consumption. Area surrounding mean curves indicate 95% confidence intervals.

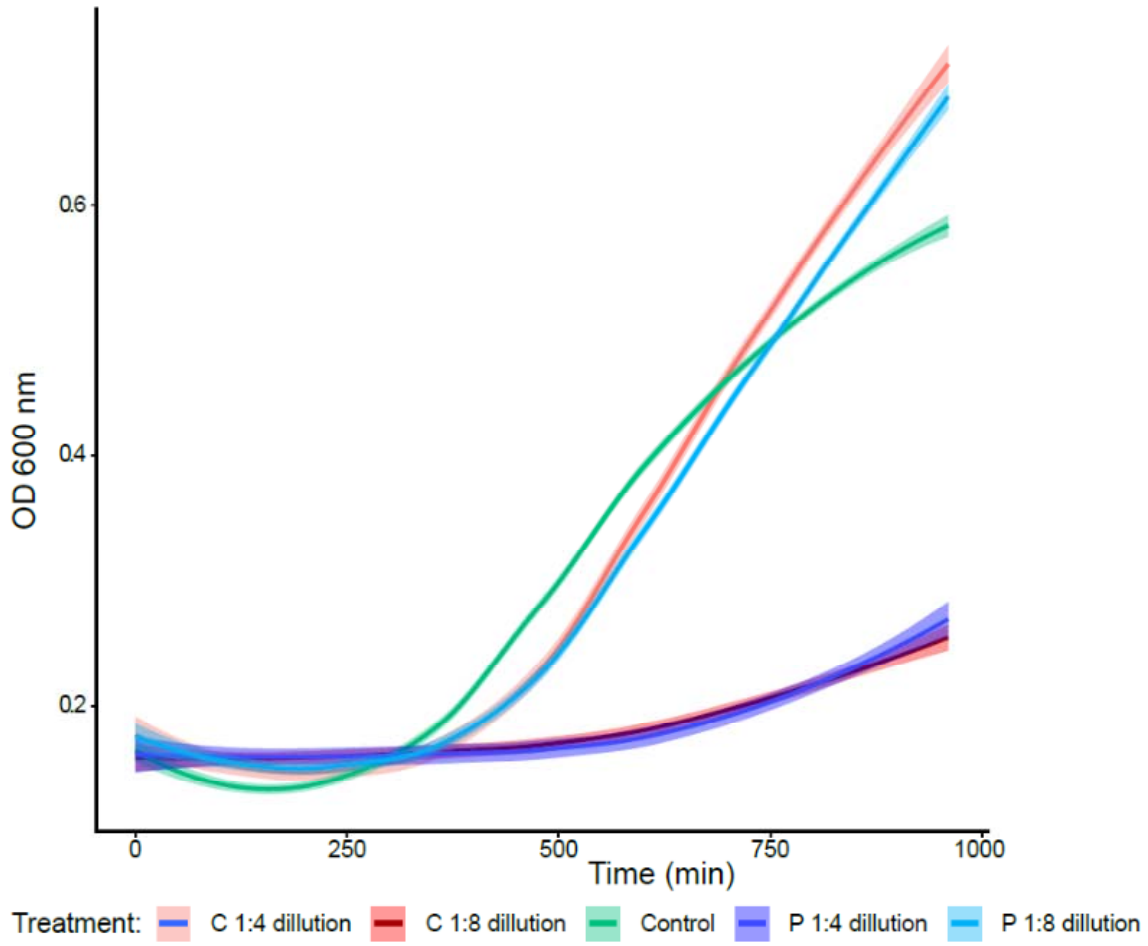


639

640 Fig. 2: Kaplan-Meier survival curves of: **A)** Unmanipulated *B. orientalis* males restricted to P-
641 rich (35 % protein and 7 % carbohydrate) or C-rich (7 % protein and 35 % carbohydrate)

642 diets. Survival data for three independent cohorts (1,2,3) for P- and C-rich diets are given in
643 blue and red respectively, with mean population survival on each diet indicated by a thick
644 bold line. Note the long period at the beginning of the experiment where no clear survival
645 differences between diets are observable. **B)** *B. orientalis* males restricted to P-rich (35 %
646 protein and 7 % carbohydrate) or C-rich (7 % protein and 35 % carbohydrate) diet and
647 infected with an LD_{50} of *P. entomophila* (infected), Ringer's solution (wounded) or
648 unmanipulated (control).

649



650

651 Fig. 3 Impact of diet on *in vitro* growth inhibition of *P. entomophila* in *B. orientalis*
652 hemolymph (1:4 and 1:8 dilution). A bacterial solution without hemolymph served as the
653 control.

654

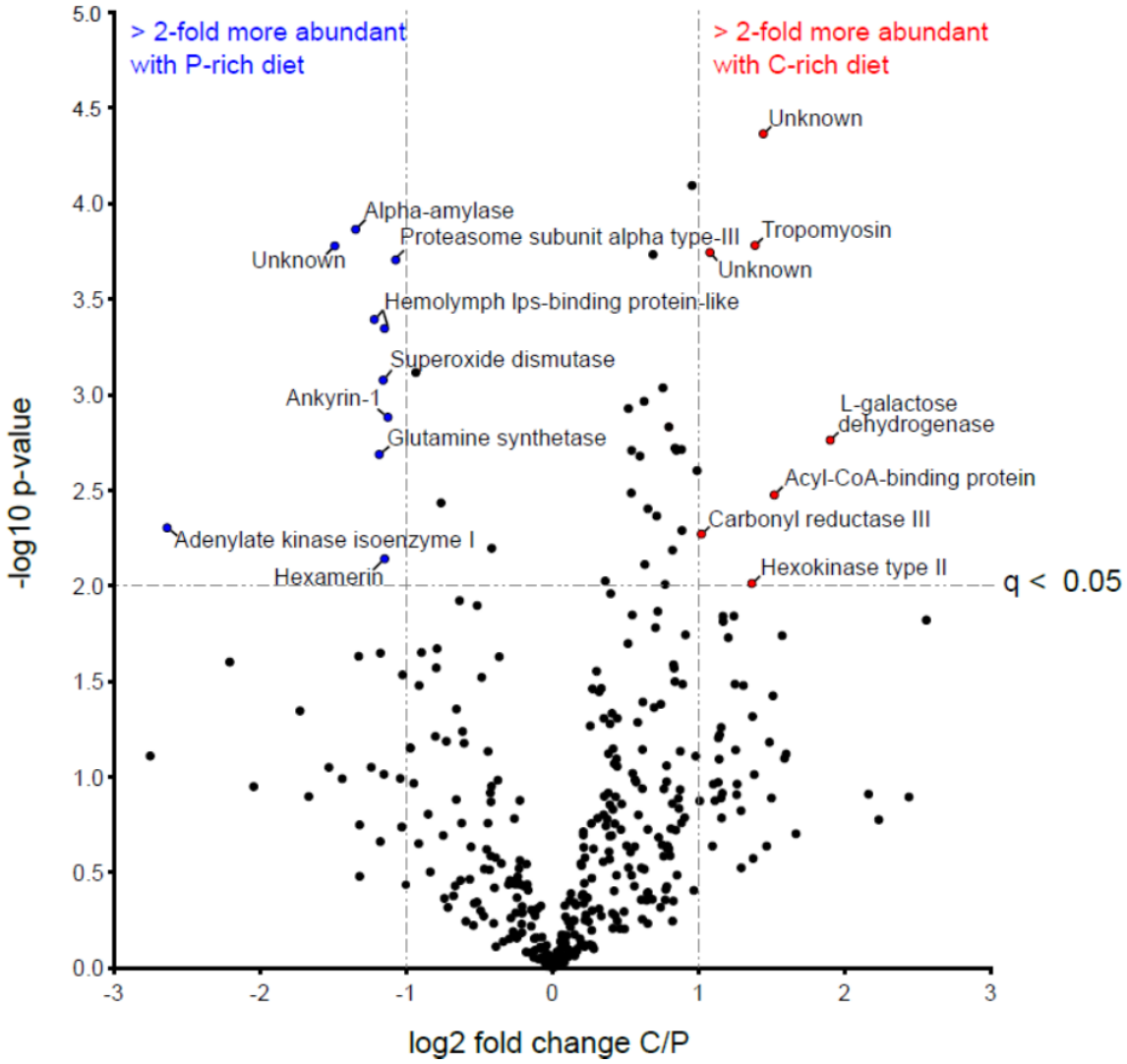


Fig. 4 Effect of diet on abundance of male *B. orientalis* hemolymph proteins following immune challenge (high dose). Points in blue and red reflect proteins that are significantly (>2) more abundant in P- and C-rich diets respectively.