

## Article

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# Accepted Manuscript

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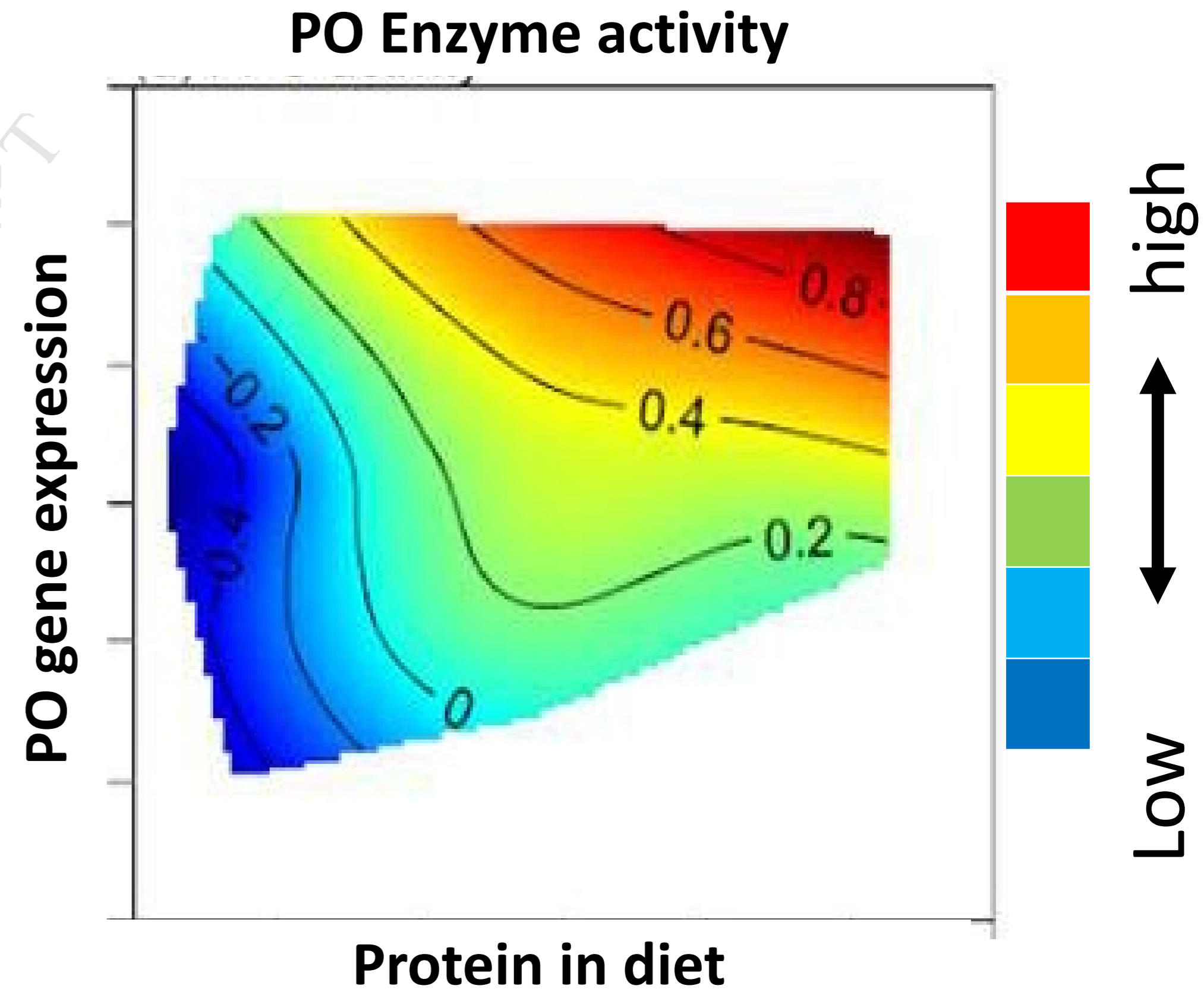
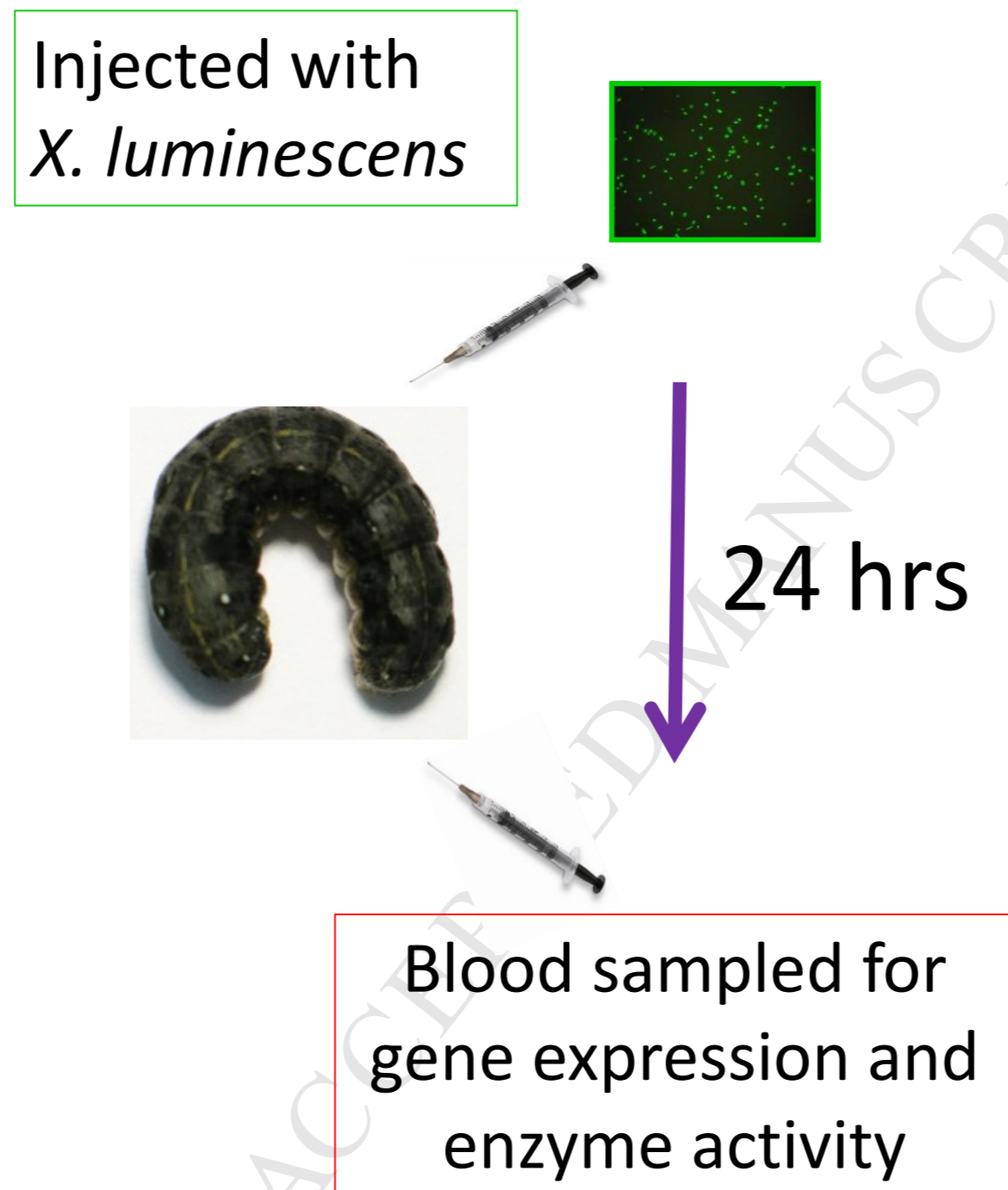
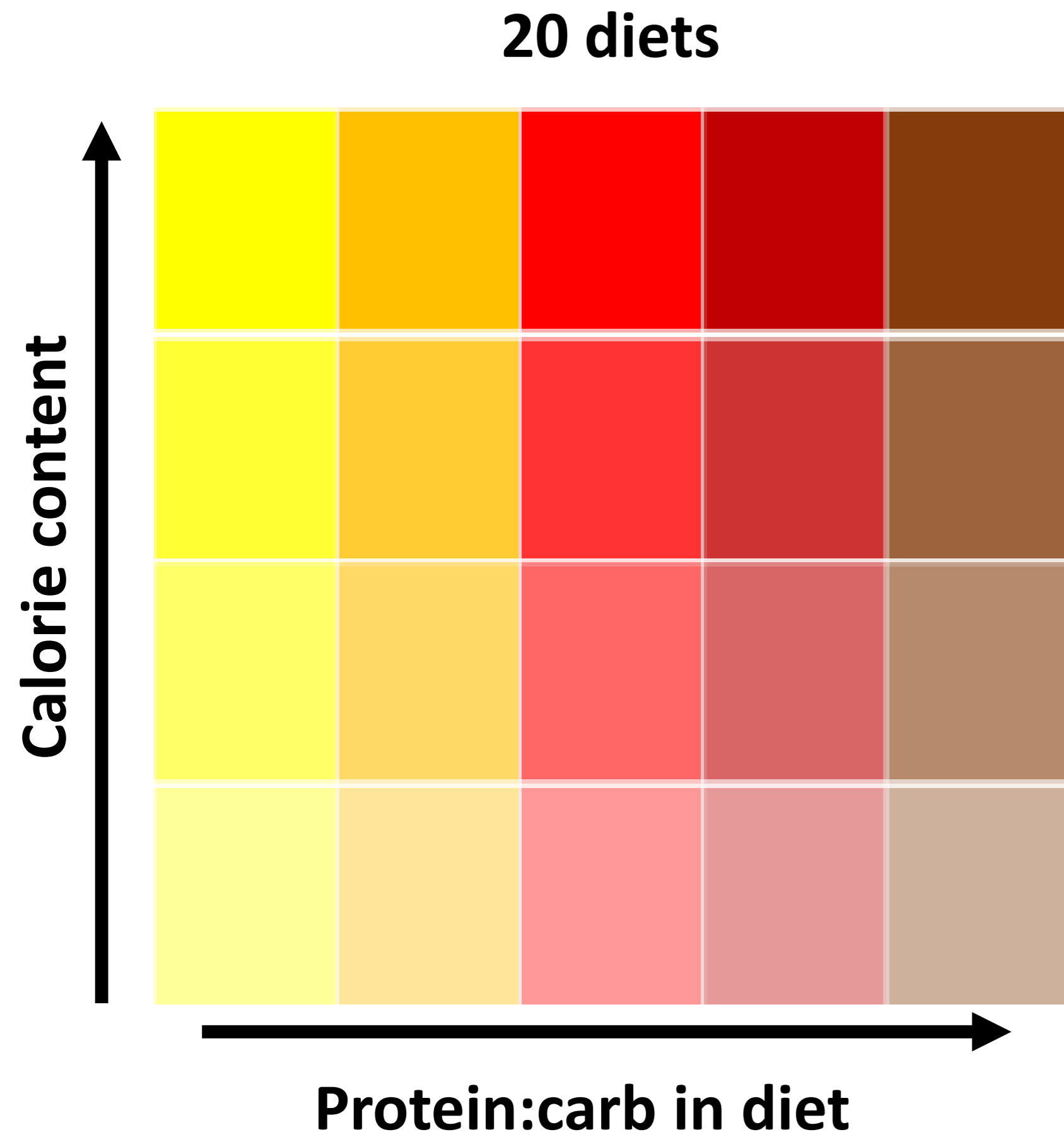
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1 **Diet modulates the relationship between immune gene expression and functional immune**  
2 **responses**

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24 **Abstract**

25 Nutrition is vital to health and the availability of resources has long been acknowledged as a key  
26 factor in the ability to fight off parasites, as investing in the immune system is costly. Resources have  
27 typically been considered as something of a “black box”, with the quantity of available food being  
28 used as a proxy for resource limitation. However, food is a complex mixture of macro- and  
29 micronutrients, the precise balance of which determines an animal’s fitness. Here we use a state-  
30 space modelling approach, the Geometric Framework for Nutrition (GFN), to assess for the first  
31 time, how the balance and amount of nutrients affects an animal’s ability to mount an immune  
32 response to a pathogenic infection.

33 *Spodoptera littoralis* caterpillars were assigned to one of 20 diets that varied in the ratio of  
34 macronutrients (protein and carbohydrate) and their calorie content to cover a large region of nutrient  
35 space. Caterpillars were then handled or injected with either live or dead *Xenorhabdus nematophila*  
36 bacterial cells. The expression of nine genes (5 immune, 4 non-immune) was measured 20 h post  
37 immune challenge. For two of the immune genes (PPO and Lysozyme) we also measured the  
38 relevant functional immune response in the haemolymph. Gene expression and functional immune  
39 responses were then mapped against nutritional intake.

40 The expression of all immune genes was up-regulated by injection with dead bacteria, but only those  
41 in the IMD pathway (Moricin and Relish) were substantially up-regulated by both dead and live  
42 bacterial challenge. Functional immune responses increased with the protein content of the diet but  
43 the expression of immune genes was much less predictable.

44 Our results indicate that diet does play an important role in the ability of an animal to mount an  
45 adequate immune response, with the availability of protein being the most important predictor of the  
46 functional (physiological) immune response. Importantly, however, immune gene expression  
47 responds quite differently to functional immunity and we would caution against using gene  
48 expression as a proxy for immune investment, as it is unlikely to be reliable indicator of the immune  
49 response, except under specific dietary conditions.

50 **Keywords: Nutritional ecology, host-pathogen interaction, immunity, *Spodoptera*, *Xenorhabdus*,**  
51 **diet, bacteria, resistance, tolerance, insect, Geometric Framework**

52

53

## 54 **Introduction**

55 It has long been recognised the role that “good nutrition” plays in human health, with both under-  
56 nutrition and obesity resulting in disease (Mokdad et al., 2001; Muller and Krawinkel, 2005; Samartin  
57 and Chandra, 2001). Poor nutrition can also impact the response to parasites, with evidence for both  
58 energy and protein deficits reducing the ability to fight infection (Kuvibidila et al., 1993) (Field et al.,  
59 2002) (Cunningham-Rundles et al., 2005). Studies have shown that starvation can compromise  
60 immune capability across a broad range of host taxa. For example, laboratory mice were found to  
61 have fewer T cells in the spleen and thymus during starvation, with numbers recovering once feeding  
62 was reinstated (Wing et al., 1988). Furthermore, injection with *Listeria monocytogenes* during  
63 starvation reduced the ability of the mice to develop antibodies against this bacterium (Wing et al.,  
64 1988). Food restriction, rather than starvation can have similar effects. Food-restricted Yellow-legged  
65 gulls, *Larus cachinnans*, were found to have reduced cell-mediated immunity (Alonso-Alvarez and  
66 Tella, 2001) and mice on a long-term calorie-restricted diet were found to die more rapidly from  
67 sepsis after gut puncture than those fed *ad libitum* (Alonso-Alvarez and Tella, 2001). Comparable  
68 responses have been shown in invertebrates; bumble bees died more rapidly during starvation if their  
69 immune systems were stimulated by artificial parasites, suggesting that mounting an immune response  
70 is energetically costly (Moret and Schmid-Hempel, 2000). Similarly, starved bumble bees were more  
71 likely to die from a gut parasite, *Crithida bombi*, than hosts with adequate nutrition (Brown et al.,  
72 2000).

73 However, nutrition is much more complex than simply a source of energy, being a vital mixture of  
74 macro- (carbohydrates, fats and proteins) and micro-nutrients (vitamins and minerals), the amount and  
75 balance of which determine an animal’s fitness (Simpson et al., 2004). Several studies have examined

76 how shifting the balance of macronutrients in the diet affects immune responses and the outcome of  
77 infection, without restricting the availability of calories (Graham et al., 2014; Lee et al., 2006; Ponton  
78 et al., 2011; Povey et al., 2009; Povey et al., 2014) . For example, caterpillars of the armyworms,  
79 *Spodoptera littoralis* and *Spodoptera exempta*, show improved immune responses and markedly  
80 higher survival after viral infection (Lee et al., 2006; Povey et al., 2014) and bacterial infection  
81 (Povey et al., 2009) when their diet is heavily protein-biased. Furthermore, when given the  
82 opportunity, infected caterpillars will “self-medicate” with protein, significantly improving their  
83 chances of survival (Lee et al., 2006; Povey et al., 2009; Povey et al., 2014) .

84 The studies above strongly suggest that it is the source of the energy in the diet that is key to the  
85 response to parasites, rather than the availability of energy *per se*. However, neither food restriction,  
86 nor the manipulation of macronutrient balance alone can determine the relative importance of either  
87 on host-parasite interactions. To address properly the role of nutrient availability on immunity, both  
88 the balance of nutrients in the diet and their quantity need to be simultaneously manipulated. The  
89 Geometric Framework for Nutrition (GFN) is a state-space model that allows the association of  
90 particular nutrient intakes with outcomes of interest (Simpson and Raubenheimer, 1995), for example,  
91 immunity (Ponton et al., 2011; Ponton et al., 2013). With the GFN, animals are restricted to diets in  
92 which both the balance and availability of nutrients are manipulated, forcing intakes over a wide  
93 region of nutrient space, encompassing both over- and under-nutrition, and thereby allowing the  
94 additive and interactive effects of specific nutrients on traits of interest to be modelled (Simpson and  
95 Raubenheimer, 1995).

96 The GFN approach has highlighted that the fundamental life-history trade-off between fecundity and  
97 longevity is mediated by nutrients across taxa, with longevity generally peaking at low-protein, high-  
98 carbohydrate ratios, whilst fecundity tends to peak at much higher relative protein intakes; as such, no  
99 diet can maximize both traits (*Drosophila*: (Lee et al., 2008); (Jensen et al., 2015); Field crickets:  
100 (Maklakov et al., 2008); Queensland Fruit fly; (Fanson et al., 2009); Mice: (Solon-Biet et al., 2015)).  
101 Similarly, using the GFN, it was found that different immune responses peak in different regions of  
102 nutrient space, thereby indicating a nutrient-mediated trade-off within the immune system, and, as for

103 fecundity and longevity, no single diet could maximize multiple immune responses (Cotter et al.,  
104 2011b). In a recent study, mice were restricted to one of 25 diets varying in their ratio of proteins, fats  
105 and carbohydrates and energy density, and their innate immune capacity was measured. It was shown  
106 that the balance of T cells indicative of healthy ageing was achieved on a low protein:NPE diet (non-  
107 protein energy i.e. carbohydrate plus fat), irrespective of calorie intake (Le Couteur et al., 2015).  
108 However, this powerful approach has not yet been taken to assess an animal's immune response to a  
109 pathogenic challenge.

110 Insects have a comparatively simple yet effective immune system that has numerous parallels to the  
111 innate immune response of vertebrates Vilmos, 1998 #1473; Leulier, 2008 #47751; Wiesner, 2010  
112 #77972} . It comprises cellular and humoral components that work together to fight invading  
113 pathogens. Hemocytes show phagocytic activity against microparasites, much like vertebrate  
114 macrophages, and can respond to macroparasites by forming a multi-layered envelope around the  
115 invader, in a process called encapsulation, which is subsequently melanised via the action of the  
116 phenoloxidase (PO) enzyme (Gupta, 1991). Phenoloxidase is stored in hemocytes in the form of an  
117 inactive precursor, Pro-phenoloxidase (PPO), which is activated upon detection of non-self  
118 (Gonzalez-Santoyo and Cordoba-Aguilar, 2012). This recognition occurs via the detection of  
119 pathogen-associated molecular patterns (PAMPs) such as the peptidoglycan or the lipopolysaccharide  
120 components of fungal and bacterial cell walls. Detection stimulates either the *Toll* (fungi and gram-  
121 positive bacteria) or *Imd* pathways (Gram-negative bacteria), via host *pattern recognition receptors*  
122 (PRRs) that result in the bespoke production of antimicrobial peptides and the upregulation of  
123 constitutive lysozymes, which form the humoral component of the response (Ligoxygakis, 2013;  
124 Wiesner and Vilcinskas, 2010).

125 The strength of the immune response can be measured using standard functional assays of  
126 antimicrobial activity or PPO activity in the haemolymph, and the strength of the encapsulation  
127 response or phagocytosis can be measured against synthetic parasites injected into the haemocoel (see  
128 (Wilson and Cotter, 2013) and references therein). These functional responses have been shown to be  
129 indicative of the ability of the animal to fight off parasites (e.g. (Lee et al., 2006; Paskewitz and



130 Riehle, 1994; Povey et al., 2009) and so are arguably meaningful measures of immune investment.  
131 However, gene expression is also often used as a proxy for investment in specific traits, e.g. immunity  
132 (Freitak et al., 2007; Jackson et al., 2011; Woestmann et al., 2017), but few of these studies consider  
133 how well the expression of the gene of interest predicts the functional response under the conditions  
134 in which they are tested.

135 There has been a great deal of research examining how well gene transcripts relate to protein  
136 abundance across individual genes, but with contradictory findings (Liu et al., 2016). This is not  
137 surprising as there are numerous steps between gene expression and the production of the protein, all  
138 of which can change the relationship between the two. In cell culture, under steady-state conditions,  
139 mRNA transcripts correlate well with protein abundance, typically explaining between 40 and 80% of  
140 the variation (Edfors et al., 2016; Jovanovic et al., 2015; Liu et al., 2016). However, multiple factors  
141 can affect this relationship. Upregulation of gene expression in response to a perturbation is expected  
142 to change the abundance of proteins concordantly, but there can be a delay in this process, such that  
143 there is a time lag between mRNA levels and protein abundance, the length of which may differ  
144 between genes (Gedeon and Bokes, 2012; Jovanovic et al., 2015). Some genes are constitutively  
145 transcribed and translation of the protein occurs only when the correct conditions are met, known as  
146 “translation on demand” (Hinnebusch and Natarajan, 2002), meaning that there is no correlation  
147 between mRNA and protein levels most of the time. The majority of ecological studies consider gene  
148 expression in whole animals, which are hugely more variable than cell cultures, and so we can expect  
149 the relationship between gene expression and protein abundance to be further weakened in natural  
150 systems. One aspect of variation in whole animals is the availability of resources. Protein production  
151 is costly, consuming ~50% of the ATP in growing yeast cells (Warner, 1999), so we can expect the  
152 availability of energy and amino acids to affect the speed and efficacy of translation (Liu et al., 2016).  
153 This means that the relationship between the expression of a gene and its protein is likely to change  
154 with the resource levels of the animal. To our knowledge, there are no studies comparing how the  
155 mRNA-protein relationship changes across nutrient space.

156 Here we address this gap using a model insect, *Spodoptera littoralis*, (Lepidoptera: Noctuidae), a  
157 generalist herbivore. We take a GFN approach, restricting caterpillars to diets that vary in their P:C  
158 ratio and energy content, thereby covering a large region of nutrient space. We then challenge the  
159 immune system by injecting caterpillars with live or dead bacteria, and measure the expression of 9  
160 genes (5 immune, 4 non-immune), and 3 functional immune responses, which are transcribed by two  
161 of the immune genes (PPO and lysozyme) in the hemolymph, thus allowing us to associate gene  
162 expression and functional immune responses to nutrient intake, and importantly, to assess how well  
163 gene expression predicts the immune response specifically associated with those genes under different  
164 dietary conditions.

165

## 166 **Material and methods**

### 167 **Host and pathogen cultures**

168 The *Spodoptera littoralis* culture was established from eggs collected near Alexandria in Egypt in  
169 2011. The colony was reared using single pair matings with around 150 pairs established each  
170 generation. Following mating of unrelated adult moths; eggs were laid within 2 days with larvae  
171 hatching after a further 3 days. *Spodoptera littoralis* spend approximately 2 weeks in the larval stage,  
172 about 7 days of which are spent in the 5th and 6th instars. Larvae were reared individually from the  
173 2nd instar on a semi-artificial wheat germ-based diet (Reeson et al., 1998) in 25 ml polypots until the  
174 final larval instar (6th), at which point they were used in the experiments as described below. Insects  
175 were maintained at 27°C under a 12:12 light: dark photo regime.

176 Bacteria were originally supplied by the laboratory of Givaudan and colleagues (Montpellier  
177 University, France; *Xenorhabdus nematophila* F1D3 GFP labelled, see (Sicard et al., 2004)). Pure *X.*  
178 *nematophila* F1D3 stocks were stored at -20°C in Eppendorf tubes (500 µl of *X. nematophila* F1D3 in  
179 nutrient broth with 500 µl of glycerol). Vortexing ensured that all *X. nematophila* F1D3 cells were  
180 coated in glycerol. To revive the stocks for use, 100 µl was added to 10 ml nutrient broth, and  
181 incubated at 28°C for up to 48 h (generally stocks had grown sufficiently after 24 hrs). On the day of

182 experimental bacterial challenge, a sub-culture of the stock was carried out, with 1 ml of the original  
183 stock added to 10 ml of nutrient broth and placed in a shaker-incubator for approximately 4 h. This  
184 ensured that the bacteria were in log phase prior to challenge. Following the sub-culture, a 1 ml  
185 sample was checked for purity under the microscope by looking for non-fluorescent cells, which  
186 would indicate contamination. The clean sample was then used to produce a serial dilution in nutrient  
187 broth from which the total cell count was determined with fluorescence microscopy, using a  
188 haemocytometer with improved Neubauer ruling. The remaining culture was diluted with nutrient  
189 broth to the appropriate concentration required for the bacterial challenge. The heat-killed treatment  
190 group was established by autoclaving the bacteria for 30 min at 121°C.

### 191 **Diet manipulation**

192 The aim of the experiments was to tease apart the importance of relative and absolute nutrient effects  
193 on immune gene expression and immune protein activity. Therefore, larvae were fed on one of 20  
194 chemically-defined diets that varied in both the protein to carbohydrate (P:C) ratio and calorie density  
195 based on (Simpson and Abisgold, 1985) (Table 1). This comprised five P:C ratios (5:1, 2:1, 1:1, 1:2,  
196 1:5) and four calorie densities (326, 612, 756 and 1112 kJ/100g diet; the remainder of the diet  
197 comprising indigestible cellulose (See Table S1 for information about the specific ingredients for each  
198 diet). Thus, the 20 diets could be described with respect to the absolute amount of proteins or  
199 carbohydrates, by their sum (calorie density), by their ratio (P:C) or by their interaction (P\*C). In  
200 addition, the absolute amounts of food eaten by the larvae on each diet were recorded so the absolute  
201 amount of protein or carbohydrate eaten as opposed to amounts offered could also be used. We were  
202 therefore able to define 30 alternative models for describing the relationship between the trait of  
203 interest (e.g. Toll expression), and host diet (Table 1). These were then compared using an  
204 information theoretic approach by comparing  $AIC_c$  values and other model metrics (Burnham and  
205 Anderson, 2003; Whittingham et al., 2006).

### 206 **Bacterial challenge**

207 *Xenorhabdus nematophila* is a highly pathogenic Gram-negative bacterium. In the wild, this species  
208 relies on the entomopathogenic nematode *Steinernema carpocapsae*, which vectors *X. nematophila*, to  
209 gain access to an insect host, where it rapidly multiplies, generally causing death within 24-48 hours  
210 (Georgis et al., 2006; Herbert and Goodrich-Blair, 2007). However, in the lab we can circumvent the  
211 requirement for the nematode by injecting *X. nematophila* directly into the insect haemocoel (Herbert  
212 and Goodrich-Blair, 2007).

213 **Experiment 1:** Within 24 h of moulting to the 6th instar, 400 larvae were divided into 20 groups (n =  
214 20 per group), placed individually into 90 mm diameter Petri dishes and provided with ~1.5 g of one  
215 of the 20 chemically-defined diets (Table 1). Within each diet, 10 larvae were allocated to the control  
216 group and 10 were assigned to the bacteria-challenged group. Following 24 h feeding on the assigned  
217 diets (at time, t = 0), 200 larvae were handled then replaced on their diet (control) whilst 200 larvae  
218 were injected with 5 µl of a heat killed LD50 dose of *X. nematophila* (averaging 1272 *X. nematophila*  
219 cells per ml nutrient broth) using a microinjector (Pump 11 Elite Nanomite) fitted with a Hamilton  
220 syringe (gauge = 0.5mm). The syringe was sterilised in ethanol prior to use and the challenge was  
221 applied to the left anterior proleg. Every 24 h up to 72 h (i.e. 48 h post infection), larvae were  
222 transferred individually to clean 90 mm Petri dishes containing 1.5 - 1.8 g of their assigned  
223 chemically-defined diet. 96 h after moulting into L6, the larvae had either pupated or were placed on  
224 semi-artificial diet until death or pupation. The amount of food eaten each day was determined by  
225 weighing the wet mass of the chemically-defined diet provided each day to the caterpillars, as well as  
226 weighing uneaten control diets each day (3 control diets per diet). The uneaten diet and control diet  
227 were then dried to a constant mass (for approx. 72 h), allowing the consumption per larva to be  
228 estimated.

229 **Experiment 2:** The set up for this experiment was identical to Experiment 1, except that each of the  
230 400 larvae was injected with 5 µl of either a heat-killed (control) or live LD50 dose of *X. nematophila*  
231 (averaging 1272 *X. nematophila* cells per ml nutrient broth).

232 **Hemolymph sampling**

233 Following challenge, hemolymph samples were obtained from all caterpillars at 20 h post infection.  
234 Hemolymph samples were obtained by piercing the cuticle next to the first proleg near the head with a  
235 sterile needle and allowing released hemolymph to bleed directly into an Eppendorf tube.  
236 Immediately following hemolymph sampling, 30  $\mu$ l of fresh hemolymph was added to a sterile ice-  
237 cooled Eppendorf containing 350  $\mu$ l of lysis buffer (RLT + Beta mercaptoethanol – 100:1) for later  
238 RNA extraction and qPCR analysis (Expts 1 and 2). The remainder of the hemolymph extracted was  
239 stored in a separate Eppendorf for further immune assays (Expt 2 only). All hemolymph samples were  
240 stored at  $-80^{\circ}\text{C}$  prior to processing.

#### 241 **Gene expression (Expts 1 and 2)**

242 RNA was extracted from hemolymph samples using Qiagen RNeasy mini kit following the  
243 manufacturers instructions with a final elution volume of 40  $\mu$ l. Extracts were quantified using the  
244 Nanodrop 2000 and diluted to 0.5  $\mu\text{g}/\mu\text{l}$  for cDNA synthesis. Prior to cDNA synthesis a genomic  
245 DNA elimination step was carried out by combining 12  $\mu$ l RNA (0.5  $\mu\text{g}$  total RNA) plus 2  $\mu$ l DNA  
246 wipeout solution and incubating at  $42^{\circ}\text{C}$  for 2 min, cDNA synthesis was carried out using Qiagen  
247 Quantitect Reverse Transcription kit in a final reaction volume of 20  $\mu$ l following the manufacturer's  
248 instructions, cDNA synthesis was carried out for 30 min at  $42^{\circ}\text{C}$  followed by 3 min incubation at  $95^{\circ}\text{C}$   
249  $^{\circ}\text{C}$  and stored at  $-20^{\circ}\text{C}$ . cDNA was diluted 1:5 for use as a qPCR template.

250 Primers and probes were synthesised by Primer Design and qPCR was performed in a reaction  
251 volume of 10  $\mu$ l with 1x Taqman FAST Universal PCR Master mix (Thermo Fisher), 0.25  $\mu\text{M}$  of each  
252 primer, 0.3  $\mu\text{M}$  probe and 2  $\mu$ l of a 1:5 dilution of cDNA. qPCR was carried on the ABI 7500 FAST,  
253 cycling parameters included an initial denaturation at  $95^{\circ}\text{C}$  for 20 sec followed by 40 cycles of  
254 denaturation at  $95^{\circ}\text{C}$ , 3 sec and annealing at  $60^{\circ}\text{C}$  for 30 sec. All PCRs were run in duplicate.

255 We selected five immune genes, three from the Toll immune pathway: Toll, Prophenoloxidae (PPO),  
256 which is the precursor of the phenoloxidase enzyme (PO), responsible for production of melanin  
257 during the encapsulation response, and lysozyme, which produces the antimicrobial lysozyme  
258 enzyme, active against Gram positive bacteria. We also selected two genes from the IMD immune

259 pathway, Moricin, which produces the AMP Moricin, active against Gram positive and negative  
260 bacteria, and Relish, which activates transcription of AMP genes (Ligoxygakis, 2013; Wiesner and  
261 Vilcinskas, 2010). We also selected three non-immune genes, Tubulin, a component of the  
262 cytoskeleton responsible for organelle and chromosomal movement. Armadillo (b-catenin), which  
263 facilitates protein-protein interactions and EF1, an elongation factor facilitates protein synthesis.  
264 These genes were selected, due to robust amplification, from a pool of potential endogenous controls  
265 that were tested in pilot studies. We also selected Arylphorin, which is primarily characterised as a  
266 storage protein (Telfer and Kunkel, 1991), however, it is up-regulated in response to bacterial  
267 infection and also in response to non-pathogenic bacteria in the diet of *Trichoplusia ni* caterpillars  
268 (Freitak et al., 2007) and so we did not have an a priori expectation as to its behaviour in this species.

269

#### 270 **Lysozyme assays (Expt 2 only)**

271 Bacterial agar plates were used to determine lytic activity. These were made by mixing 1.5% water  
272 agar and 1.5% freeze-dried *Micrococcus luteus* (Merck: M3770) potassium phosphate buffer in a 2:1  
273 ratio. 10 ml plates of the resulting solution were poured and 2 mm diameter holes punched in each  
274 plate. Each hole filled with 1 ml of ethanol saturated with phenylthiourea (PTU), in order to prevent  
275 melanisation of the samples. The ethanol evaporates, leaving the PTU in the hole. Following  
276 defrosting and vortexing of the stored hemolymph, each well was the filled with 1µl of hemolymph,  
277 with two technical replicates per sample. The plates were incubated at 30°C for 24 h, and the clear  
278 zones around the samples were measured using digital callipers. Lytic activity (mg/ml) was then  
279 calculated from a serial dilution of a hen egg white lysozyme (Merck: 62971; Standard series 0.01,  
280 0.05, 0.1, 0.5, 1 and 2 mg per ml in water).

#### 281 **Phenoloxidase assays (Expt 2 only)**

282 Following defrosting of the hemolymph samples, 10 µl of hemolymph was added to 450 µl of  
283 NaCac buffer (1.6g NaCac and 0.556g CaCl<sub>2</sub> l<sup>-1</sup> sterile distilled water). The solution was then split  
284 into two Eppendorfs (each containing 225 µl), in order to carry out assays for both proPO and PO.

285 To one Eppendorf, 25  $\mu$ l of NaCac buffer was added (PO assay), and to the other, 25  $\mu$ l of 20 mg  
286 per ml chymotrypsin in NaCac buffer was added (proPO activated). The samples were vortexed  
287 and incubated at 25 °C for 1 h. 90  $\mu$ l of each solution was placed in a well of a 96-well microplate  
288 with 90  $\mu$ l of 10 mM dopamine as a substrate. Two technical replicates were carried out per  
289 sample. Readings were taken every 15 secs for 10 mins at 490 nm and 25 °C using a Tecan infinite  
290 m200pro plate reader with Magellan software (V7.2). This range accounted for the linear stage of  
291 the reaction. The maximum rate of reaction was then used as an approximation of PO and proPO  
292 level. While many researchers still use L-dopa as a substrate for PO reactions, for insect POs,  
293 dopamine is the preferred substrate over L-dopa. It is the natural substrate for insects, it is more  
294 soluble than L-dopa and unlike L-dopa, is not subject to spontaneous darkening (Sugumaran,  
295 2002).

296

## 297 **Statistical analyses**

### 298 **Gene expression**

299 All statistical analyses were conducted using the *R* statistical package version 3.2.2 (R Core Team,  
300 2018). Gene expression data were normalised using NORMA-Gene (Heckmann et al., 2011), a data  
301 driven approach that normalises gene expression relative to other genes in the dataset rather than to  
302 specifically identified reference genes. It is particularly suited to data sets with limited numbers of  
303 assayed genes. Normalised gene expression data were then standardized using the mean ( $\mu$ ) and  
304 standard deviation ( $\sigma$ ) of each trait ( $Z = (X - \mu) / \sigma$ ) prior to analysis. The two experiments, run at  
305 different times, had only one treatment in common, (1 – handled vs heat-killed bacteria, 2- heat-killed  
306 vs live bacteria). For ease of interpretation, we wanted to analyse both experiments in a single model.  
307 To test the validity of this approach, we first compared the gene expression, physiological immune  
308 response data and the data for the total amount of food consumed across both experiments for the  
309 heat-killed treatment only. There was no significant difference between any of the measures across  
310 experiments, with the exception of the total amount of food eaten, and expression of the Moricin gene.

311 Therefore, all data were analysed in a single model, with the exception of those two response  
312 variables, where data from the two experiments were analysed separately.  
313 Data were analysed for each gene separately using linear mixed-effects models in the packages *lme4*  
314 (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2017). For each gene, the plate that the samples  
315 were run on was included as a random effect. A comparison was made of 90 candidate models for  
316 each gene, which comprised 30 models covering different combinations of dietary attributes (Table  
317 3), either alone, with bacterial treatments added or with bacterial treatment interacting with the dietary  
318 traits. AIC values were corrected for finite sample sizes ( $AIC_c$ ) to establish the most parsimonious  
319 models including likely nutritional attributes driving the observed data.  $AIC_c$  values and *Akaike*  
320 *weights* were estimated using the *MuMin* package (Bartoń, 2018). The relative weight of evidence in  
321 favour of one model over another (evidence ratio) is determined by dividing the *Akaike weight* of one  
322 model by another (Burnham and Anderson, 2003). In each case, the residuals from the best model  
323 were visually inspected for deviations from normality. Gene expression for Lysozyme, Arylophorin,  
324 PPO, EF1 and Tubulin were Tukey transformed prior to reanalysis using the package *rcompanion*  
325 (Mangiafico, 2017). For visualisation of the effects of the immune challenge treatment and diet on  
326 gene expression (Figures 2-5), residuals from the null model, containing just the random plate effect  
327 (Model 1, Table 3), were plotted as thin plate splines using the package *fields* (Nychka et al., 2017).  
328 Food consumption data were analysed in the same way as the gene expression data, with experiment  
329 included as a random effect.

330

### 331 **Physiological immune responses**

332 The same approach was taken for the physiological immune measurements, lysozyme, PPO and PO  
333 activity, except for these variables, standard linear fixed effects models were run as data were  
334 collected in a single experiment. The same set of 90 models as described above were fitted, with the  
335 addition of 180 extra models that included the additive and interactive effects of the expression of the  
336 relevant gene, after correction for the plate to plate variation (residuals from the null model containing  
337 the random effect of plate only) – the lysozyme gene for lysozyme activity and the PPO gene for PPO  
338 and PO activity.



## 339 **Survival**

340 Time to death was analysed for experiment 2, where larvae were injected with dead or live bacteria  
341 only. Data were analysed using Cox's proportional hazard models in the package (Therneau, 2015).  
342 The same sets of models as described above were fitted (Table 3), with the addition of 120 extra  
343 models that included the additive and interactive effects of Moricin gene expression, after correction  
344 for the plate to plate variation (residuals from the null model containing the random effect of plate  
345 only). For visualisation of the effects of the immune challenge treatment on survival (dead vs live  
346 bacteria), predicted curves for low and high levels of Moricin gene expression were generated using  
347 the package *Survminer* (Kassambara and Kosinski, 2018) using *ggplot2* (Wickham, 2016.). The  
348 effects of diet on time to death were plotted as thin plate splines using the package *fields* (Nychka et  
349 al., 2017).

350

## 351 **Results**

### 352 **How does consumption vary across diets and bacterial challenge treatments?**

353 The total amount of food consumed varied across the diets. For experiment 1, comparing handled  
354 caterpillars versus those injected with heat-killed bacteria, the best model predicting consumption was  
355 model 30 ( $Pe * Ce + Pe^2 + Ce^2$ ), but this was indistinguishable from the same model that included the  
356 additive effects of treatment ( $Treatment + Pe * Ce + Pe^2 + Ce^2$ ,  $\delta = 1.34$ ).

357 For experiment 2, comparing dead and live bacterial injections, the best model predicting  
358 consumption was model 20 ( $Co * R + Co^2 + R^2$ ), but as for the handled versus dead treatments in  
359 experiment 1, this model was indistinguishable from the same model that included the additive effects  
360 of treatment ( $Treatment + Co * R + Co^2 + R^2$ ,  $\delta = 0.51$ ).

361 For all treatment groups, it can be seen that consumption tended to increase as the calorie density of  
362 the diet decreased (Figure 1a,b,d,e), suggesting that food dilution constrained caterpillars from being  
363 able to take in sufficient nutrients, as expected, and that on the more calorie-dense diets caterpillars  
364 over-consumed nutrients. However, this increase in total consumption was more extreme on the high-

365 protein than on the low-protein diets, suggesting that caterpillars were willing to overeat protein to  
366 gain limiting carbohydrates.

367 Overall consumption tended to decrease with treatment - dead-bacteria treated caterpillars ate less  
368 than handled, and live-bacteria treated caterpillars ate less than dead-bacteria treated (Figure 1a vs b  
369 and d vs e). However, inspection of the intake arrays (Figures 1c,e), suggests that consumption was  
370 most reduced in both dead and live bacteria treatments on the highest protein diets.

371

### 372 **How does immune gene expression vary across diets and bacterial challenge treatments?**

373 For the immune genes (Toll, PPO, Lysozyme, Moricin and Relish), injection with dead bacteria  
374 resulted in up-regulation of gene expression relative to handled caterpillars (Figure 2). In contrast,  
375 injection with live bacteria either did not up-regulate gene expression relative to controls (Toll, PPO  
376 and Lysozyme), or did not up-regulate it as strongly (Moricin and Relish) (Figure 2). For the non-  
377 immune genes (Arylophorin, EF1, Armadillo and Tubulin), the variation in expression levels was  
378 lower; for Arylophorin, EF1 and Armadillo, live bacteria triggered the down-regulation of gene  
379 expression relative to handled caterpillars, whilst there was no effect for Tubulin (Figure 2). For  
380 Arylophorin, Armadillo and Tubulin, injection with dead bacteria up-regulated gene expression  
381 relative to handled caterpillars but there was no effect for EF1 (Figure 2). The best supported model  
382 for every gene tested was model 30, with the bacterial treatment interacting with the amount of  
383 protein and carbohydrate eaten ( $\text{Treatment} * (\text{Pe} * \text{Ce} + \text{Pe}^2 + \text{Ce}^2)$ ). However, although the fit of these  
384 models was generally good ( $r^2 > 0.26-0.86$ ), with the exception of Moricin, the amount of variation  
385 explained by the fixed part of the model was very low ( $r^2 < 0.12$ ; Table 4; Figures 3-5). This means  
386 that the majority of the variation in gene expression was caused by variation across plates. For  
387 Moricin, when comparing the handled and dead treatments, 74% of the variation explained by the  
388 model was explained by the fixed terms due to the massive up-regulation of Moricin in the dead-  
389 bacteria injected larvae (Figures 2, 3a,b). The difference between the dead and live treatment groups  
390 was much smaller and comparable to the other immune genes (Table 4, Figures 3c,d)

391 Variation in the expression of all of the genes was explained by main and interactive effects of the  
392 amount of protein and carbohydrate eaten, and in interaction with the bacterial treatment, suggesting  
393 that the response to diet for each gene differed across treatments. A visualisation of these response  
394 surfaces (Figures 3-5) shows that, for the immune genes, whilst there is general up-regulation between  
395 handled and dead bacterial challenges, the response surfaces are fairly flat, i.e. diet does not explain  
396 much variation in gene expression. However, for the live challenge, expression tends to peak at  
397 moderate protein but high carbohydrate intake, which corresponds to the highest intakes on the 33%  
398 protein diet for Toll, PPO, Lysozyme and Relish, and on the 17% protein diet for Moricin (Figures  
399 3,4). In contrast, the non-immune genes (Arylphorin, EF1, Armadillo and Tubulin), show a  
400 consistently weak response to the dietary manipulation, with much flatter surfaces on average than  
401 those shown by the immune genes (compare Figure 4 with Figure 5).

402

#### 403 **Does immune gene expression predict physiological immune responses?**

404 For the Lysozyme and PPO genes, we simultaneously measured functional lytic and PPO (and PO)  
405 activity in the hemolymph, allowing us to determine how well gene expression predicts the functional  
406 immune response. We had lytic and PO data only for Experiment 2, where larvae were challenged  
407 with live or dead bacteria.

408 For PPO activity, AICc could not discriminate between several of the diet models, with seven being  
409 equally well supported ( $\Delta < 2$ ; Table 5). Of these models, the top six contained protein and protein  
410 squared with additive or interactive effects of bacterial treatment or gene expression (Table 5). For the  
411 models that included treatment, the estimates show that PPO activity was increased with live bacterial  
412 infection. For PO activity, AICc could not discriminate between 11 different models ( $\Delta < 2$ ; Table  
413 6). However, the top three models were the same as for PPO, with protein plus protein squared with  
414 additive or interactive effects of PPO gene expression. Only two of the models contained treatment  
415 effects and both in interaction with diet components. For lytic activity in the hemolymph, three  
416 models were equally well supported, all of which contained Lysozyme gene expression interacting

417 with dietary components, which were either protein and protein squared, as for PO and PPO, or the  
418 P:C ratio (Table 7); none of the models contained treatment, suggesting that lysozyme activity is up-  
419 regulated in response to the presence of bacteria and not whether they are alive or dead. As for gene  
420 expression, the overall explanatory power of the models was quite low, ( $r^2 < 0.12$ ; Tables 5-7).

421 For ease of comparison, all 3 physiological immune traits were plotted against the protein content of  
422 the diet, as this model was common to all three traits, and the expression of the relevant gene, which  
423 featured in the majority of the selected models (Tables 5-7). The effect of treatment was excluded as it  
424 did not feature in the majority of the models. For each trait, activity in the hemolymph tended to  
425 increase with gene expression, as we might expect, but this was strongly moderated by the protein  
426 content of the diet (Figure 6). For PO and PPO activity, on low protein diets enzyme activity was low  
427 and there was little correspondence between gene expression and the physiological response, but as  
428 the protein content of the diet increased, this relationship became more linear (Figure 6a,b). For lytic  
429 activity the pattern was different in that enzyme activity increased strongly with the protein and less  
430 strongly with lysozyme gene expression up to about 45% protein, thereafter there was consistently  
431 high lytic activity across all levels of gene expression (Figure 6c).

432

### 433 **Does immune gene expression predict survival?**

434 Survival was reduced in the live bacterial treatment group relative to those injected with dead bacteria  
435 (Hazard ratios 1.25-1.31 for models without treatment interactions, Table 3), however, this effect was  
436 moderated by Moricin expression (Figure 7 a,b). In the dead-bacteria treatment group, Moricin did not  
437 explain time to death, but in the live-bacteria treatment group, larvae with high levels of Moricin  
438 expression had an increased risk of death relative to those with low expression (Figure 7 a,b; Hazard  
439 ratios 1.20-1.24 for models without GE interactions, Table 3). Of the top 5 models, 4 included the  
440 additive and interactive effects of protein and carbohydrate eaten as well as their squared terms (Table  
441 3). To visualise the effects of diet on survival we plotted thin-plate splines for time to death against  
442 the amount of protein and carbohydrate consumed. The patterns differ between dead and live bacterial

443 treatments. Time to death is overall shorter in the live treatment (note the shift of colour towards  
444 orange and blue). However, whilst time to death is affected by both protein and carbohydrate  
445 consumption in the dead treatment, with peak survival on high protein/low carbohydrate and vice  
446 versa, in the live treatment, time to death appears to be solely explained by protein availability (note  
447 the near-vertical contours). Low-protein diets resulted in the most rapid deaths and high-protein diets  
448 extended the time to death.

449

## 450 **Discussion**

451 Previous work has shown that immune responses can be strongly affected by the amount and/or  
452 balance of nutrients in the diet e.g. (Fernandes et al., 1976; Ingram et al., 1995; Kristan, 2008; Le  
453 Couteur et al., 2015; Lee et al., 2006; Nayak et al., 2009; Povey et al., 2009; Ritz and Gardner, 2006;  
454 Wallace et al., 1999; White et al., 2017). However, most of these studies covered only a relatively  
455 small region of nutrient space (Fernandes et al., 1976; Ingram et al., 1995; Lee et al., 2006; Nayak et  
456 al., 2009; Povey et al., 2009; Ritz and Gardner, 2006; White et al., 2017) and/or only tested innate  
457 responses (e.g. (Fernandes et al., 1976; Ingram et al., 1995; Le Couteur et al., 2015; Lee et al., 2006;  
458 Nayak et al., 2009; Povey et al., 2009; White et al., 2017) or the response to an artificial pathogen or  
459 immune stimulant (Cotter et al., 2011b). Here we addressed this gap by looking at both gene  
460 expression, functional immune responses and survival after both dead and live pathogen challenges  
461 over a broad region of nutrient space. Our major findings are that whilst functional immune responses  
462 (PPO, PO and lytic activity in the hemolymph) change as expected in response to the dietary  
463 manipulation, showing a clear elevation as the protein content of the diet increases, gene expression is  
464 much less predictable (Figures 3,4). Despite this, expression of the PPO and Lysozyme genes did  
465 predict PPO/PO and Lysozyme activity in the hemolymph, but this relationship was strongly  
466 dependent on the amount of protein in the diet (Figure 6), suggesting that using immune gene  
467 expression as an indicator of the efficacy of the immune response may be reliable only under specific  
468 dietary conditions. Furthermore, expression of the most responsive gene to infection (Moricin)  
469 strongly modulated survival, with high levels of expression resulting in reduced survival after

470 bacterial infection, suggesting that expression is a marker of bacterial load or ‘sickness’ as opposed to  
471 an indication of a robust immune response.

472 Our dietary manipulation was successful in inducing caterpillars to consume over a large region of  
473 nutrient space, allowing us to independently assess the effects of macronutrient composition and the  
474 calorie content of the diet on immunity. There was evidence for compensatory feeding; caterpillars  
475 did not consume the same amount of every diet. As expected, caterpillars ate more as the calorie  
476 density of the food decreased (Figure 1), but this varied across diets, such that consumption was  
477 highest on the high protein diets, suggesting that caterpillars were willing to over-eat protein to gain  
478 limiting carbohydrates. However, as has been found in previous studies (Adamo, 1998; Adamo et al.,  
479 2007; Exton, 1997; Lennie, 1999; Povey et al., 2014), we found some evidence for illness-induced  
480 anorexia. Caterpillars injected with live *X. nematophila* showed suppressed food consumption across  
481 all diets (Figure 1e – note the shift of colours towards oranges and blues). Interestingly, injection with  
482 dead *X. nematophila* did not induce this response, which suggests that it is not the triggering of an  
483 immune response that causes this change in consumption, but the presence of an actively replicating  
484 pathogen. This reduction in consumption was also consistent across diets, with infected caterpillars,  
485 on average, consuming just 77% of the food consumed by healthy caterpillars (Figure 1c).

486 In insects, two major pathways are triggered in response to microbial infection; typically, genes in the  
487 Toll pathway respond to infection by fungi and Gram-positive bacteria, whilst genes in the IMD  
488 pathway respond to Gram-negative bacteria (Broderick et al., 2009). Moricin and Lysozyme are  
489 triggered by Toll in Lepidoptera (e.g. (Zhong et al., 2016), but Moricin has also been shown to  
490 respond to Gram-negative bacteria and so may also be triggered by IMD (Hara and Yamakawa,  
491 1995). Of the 5 immune genes we tested, only the IMD genes, Moricin and Relish, were significantly  
492 up-regulated in response to infection with both dead and live bacteria. For the Toll genes (Toll, PPO  
493 and Lysozyme), gene expression was up-regulated by dead bacteria but not by live bacteria (Figure 2).  
494 However, even for Moricin and Relish, up-regulation was much stronger in response to dead than live  
495 bacteria. This may reflect a general down-regulation of gene expression during an active infection, as  
496 the non-immune genes typically show reduced gene expression in response to the live infection

497 compared to the controls. This may be driven by the illness-induced anorexia, with reduced  
498 consumption resulting in lower metabolic activity and consequently lower gene expression. However,  
499 there is evidence that *X. nematophila* can inhibit Cecropin, Attacin and Lysozyme gene expression (Ji  
500 and Kim, 2004; Park et al., 2007). It may be that, rather than specifically inhibiting AMP gene  
501 expression, *X. nematophila* inhibits the expression of all genes.

502 As Moricin was most strongly up-regulated in response to infection, we tested how its expression  
503 correlated with time to death in challenged caterpillars (dead vs live injection, Expt 2 only). Whilst  
504 Moricin expression had negligible effects on survival in the dead bacterial treatment, high levels of  
505 expression were indicative of an *increased* risk of death after live infection. Thus, high expression  
506 levels were not a good indicator of immune capacity, but rather signalled heavy bacterial loads or low  
507 tolerance. Distinguishing between these hypotheses would require data on bacterial loads at different  
508 time points after infection. Survival was also strongly affected by diet, with the longest survival times  
509 occurring on the highest protein diets after live-bacteria infection. High-protein diets have been  
510 implicated in increased survival after viral infection in this species (Lee et al., 2006) and after either  
511 bacterial or viral infection in the congener, *Spodoptera exempta* (Povey et al., 2009; Povey et al.,  
512 2014). However, none of these diets are associated with the highest gene expression for any immune  
513 gene, suggesting that high-protein diets may reduce the burden of infection via mechanisms other than  
514 improving the immune response.

515 *X. nematophila* is a Gram-negative bacterium, and is clearly triggering Moricin and Relish expression,  
516 but as Toll is only marginally up-regulated in response, it is probably the IMD pathway that is  
517 controlling this response. Another possible explanation for why live bacteria appear to trigger a down-  
518 regulation of gene expression is that our sampling protocol (20 h post-challenge) did not allow us to  
519 catch peak expression levels (note that bacterial loads tend to peak in *S. littoralis* at around 24h).

520 Expression of lysozyme and PPO in the Glanville fritillary butterfly was not up-regulated 24 h after  
521 injection with *M. luteus* cells (Woestmann et al., 2017) , whilst in the silkworm, up-regulation of  
522 lysozyme in response to fungal infection occurred in two peaks, from 9-18 h, and then between 30 and  
523 48 h (Hou et al., 2014) . This may be a fungal-specific response, or it might mean that we would have

524 seen higher gene expression had we assayed over an extended time period. It is also possible that the  
525 timing of gene expression peaks earlier after live, rather than dead bacterial injection, further studies  
526 would be required to elucidate the time course of gene expression for the different genes to be certain  
527 of this. However, as non-immune genes also appear to follow the same pattern, reduced expression in  
528 response to live vs dead bacteria, the hypothesis that infection results in down-regulation of gene  
529 expression in general is a reasonable assumption.

530 Arylphorin is primarily characterised as a storage protein (Telfer and Kunkel, 1991), however, it is  
531 up-regulated in response to bacterial infection and also in response to non-pathogenic bacteria in the  
532 diet of *Trichoplusia ni* caterpillars (Freitag et al., 2007). It has been shown to bind to fungal conidia in  
533 *Galleria mellonella* hemolymph, potentially working in coordination with antimicrobial peptides  
534 (Fallon et al., 2011). The lack of up-regulation here may be due to the use of a Gram-negative  
535 bacterial challenge; the up-regulation in *T. ni* was in response to a mixture of *E. coli* (G-ve) and  
536 *Micrococcus luteus* (G+ve), so it is not clear if both or just one of the bacteria caused the response.  
537 Another possibility is that Arylphorin levels are already expressed at maximal levels and cannot be  
538 further up-regulated. In *T. ni* caterpillars, Arylphorin is the most abundant protein in the hemolymph  
539 during the final instar (Kunkel et al. 1990). Its levels are known to increase throughout the final instar  
540 in *Spodoptera litura* (Yoshiga et al., 1997), and the point at which gene expression was measured here  
541 was 48-72 h into the final instar, which is shortly before pupation. The pattern of gene regulation for  
542 Arylphorin looks more like that shown by the non-immune genes, with little or no up-regulation in  
543 response to dead bacteria and down-regulation in response to live bacteria. Further studies would be  
544 required to assess the role of Arylphorin as a putative immune gene in this species.

545 For two of the immune genes, PPO and Lysozyme, we were able to simultaneously measure the  
546 activity of the relevant protein in the hemolymph as a measure of the functional immune response.  
547 Thus, we were able to assess how well gene expression predicts functional immune activity and  
548 whether this relationship changes with the diet. Here, we found that for each functional immune  
549 response, PPO activity, PO activity and lysozyme activity, expression of the relevant gene does  
550 predict the response, but only at certain intakes of protein (Figure 6). For example, PPO and PO



551 activity increase linearly with the expression of the PPO gene, but only above ~30% dietary protein  
552 (Figure 6). This suggests that the availability of dietary protein limits the translation of PPO mRNA  
553 into PPO protein, and the activation of PPO into PO. In contrast, the expression of the gene is not  
554 limited by protein availability, and so gene expression can be high when dietary protein is low, but it  
555 is ineffective as it does not result in a comparable functional immune response. The lytic response is  
556 also affected by dietary protein, however, in this case, the relationship between gene expression and  
557 lytic activity is consistently weak and above 45% protein maximal lytic activity is achieved at low  
558 gene expression, and increased expression does not improve the response. As for PPO, this suggests  
559 that protein limits the translation of lysozyme up to about 45% protein.

560 These results are not surprising when you consider the costs associated with the production of protein.  
561 It is estimated that only 10% of the energetic costs of protein production are spent on transcription;  
562 translation is much more energetically expensive and relies on the availability of amino acids to build  
563 the relevant protein (Warner, 1999). It is likely, therefore, that whilst transcription of immune genes  
564 might still be up-regulated in response to infection under low protein conditions, the translation of the  
565 protein might be reduced, impairing the correlation between mRNA and protein abundance. It is also  
566 possible that gene expression would be a better predictor of the functional response at different time  
567 points, if there is a lag between gene expression and protein translation. Again, this would require  
568 further investigation. However, given the much stronger relationship between the physiological  
569 immune responses and protein availability, it still seems likely that the relationship between the two  
570 will differ across diets. Our results suggest that caution should be used when interpreting gene  
571 expression as a measure of “investment” into a particular trait, or as a measure of the strength of a  
572 particular immune response. It is surprisingly common in ecological studies for gene expression to be  
573 used in this way without any attempt to correlate the expression of a gene with the production of the  
574 functional protein (Zylberberg, 2019). If dietary protein levels are limiting, then gene expression may  
575 be a poor indicator of the immune capacity of an animal. Here we have tested this just with immune  
576 genes for which we have good functional assays of the active protein, but it seems likely that this

577 would also be true of other classes of genes, for which gene expression is routinely used as an  
578 indicator of an organism's investment.

579 In summary, as expected, immune challenge with a live Gram-negative bacterium up-regulated  
580 immune genes in the IMD pathway, though all immune genes were up-regulated to a certain extent by  
581 the challenge with dead bacteria. While functional immune responses (PO, PPO and Lysozyme)  
582 typically improved with the protein content of the diet, gene expression varied non-linearly with diet  
583 composition. However, the expression of PPO and Lysozyme genes predicted PPO/PO and Lysozyme  
584 activity, but only when the availability of dietary protein was not limiting, suggesting that using gene  
585 expression as an indicator of investment in a trait is unlikely to be reliable, unless its relationship with  
586 diet is known.

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595

#### 596 **Author contributions**

597 KW, JAS, SCC, FP and SJS conceived the idea, RH, CER, JR, JAS & YT carried out the  
598 experiments, SCC analysed the data and wrote the first draft of the paper, all authors commented on  
599 and approved the final manuscript.

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601

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813 **Tables**814 **Table 1. Nutritional composition of the 20 chemically-defined diets**

<b>Diet</b>	<b>Protein</b>	<b>Carbs</b>	<b>Fats</b>	<b>Cellulose</b>	<b>Micro-nutrients</b>	<b>Energy</b>	<b>Ratio</b>	<b>P:C</b>
	<b>(g/100g diet)</b>	<b>(g/100g diet)</b>	<b>(g/100g diet)</b>	<b>(g/100g diet)</b>	<b>(g/100g diet)</b>	<b>(kJ/100g diet)</b>	<b>(%)</b>	
<b>1</b>	10.5	52.5	1.1	33.0	3.0	1112	0.17	1:5
<b>2</b>	7.0	35.0	1.1	54.0	3.0	756	0.17	1:5
<b>3</b>	5.6	28.0	1.1	62.4	3.0	612	0.17	1:5
<b>4</b>	2.8	14.0	1.1	79.2	3.0	326	0.17	1:5
<b>5</b>	21.0	42.0	1.1	33.0	3.0	1112	0.33	1:2
<b>6</b>	14.0	28.0	1.1	54.0	3.0	756	0.33	1:2
<b>7</b>	11.2	22.4	1.1	62.4	3.0	612	0.33	1:2
<b>8</b>	5.6	11.2	1.1	79.2	3.0	326	0.33	1:2
<b>9</b>	31.5	31.5	1.1	33.0	3.0	1112	0.50	1:1
<b>10</b>	21.0	21.0	1.1	54.0	3.0	756	0.50	1:1
<b>11</b>	16.8	16.8	1.1	62.4	3.0	612	0.50	1:1
<b>12</b>	8.4	8.4	1.1	79.2	3.0	326	0.50	1:1
<b>13</b>	42.0	21.0	1.1	33.0	3.0	1112	0.67	2:1
<b>14</b>	28.0	14.0	1.1	54.0	3.0	756	0.67	2:1
<b>15</b>	22.4	11.2	1.1	62.4	3.0	612	0.67	2:1
<b>16</b>	11.2	5.6	1.1	79.2	3.0	326	0.67	2:1
<b>17</b>	52.5	10.5	1.1	33.0	3.0	1112	0.83	5:1
<b>18</b>	35.0	7.0	1.1	54.0	3.0	756	0.83	5:1
<b>19</b>	28.0	5.6	1.1	62.4	3.0	612	0.83	5:1
<b>20</b>	14.0	2.8	1.1	79.2	3.0	326	0.83	5:1

815 See Table S1 for information about the specific ingredients for each diet

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818 **Table 2: Primer and probe sequences used for the qPCR analysis of immune gene expression**

Gene	Primers (5' - 3')	Probe (5' - 3')	Amplicon sizes (bp)	Efficiencies
<b>Toll</b>	FOR: AATGCTCGTGTTATCATGATC AAA REV: CGTGATCGTAGCCAGCGTTT	VIC- CTGGACCACCACTA ACGTCGTCGATTG- TAMRA	76	93.8%
<b>PPO</b>	FOR: GCTGTGTTGCCGAGAATG REV: AAATCCGTGGCGGTGTAGTC	VIC- CCGCGTATCCCGATC ATCATCCC-TAMRA	67	97.4%
<b>Lysozyme</b>	FOR: TGTGCACAAATGCTGTTGGA REV:CGAACTTGTGACGTTTGT AGATCTTC	VIC- ACATCACCTAGCTT CTCAGTGCGCC- TAMRA	76	96.6%
<b>Relish</b>	FOR: TCAACATAACAACACGGAGG AA REV: ATCAGGTACTAGGCAACTCAT ATC	6FAM - CCCACAAATTACTTG AAGATGAACAGGAC CC-TAMRA	82	95.3%
<b>Moricin</b>	FOR: GGCGCAGCGATTGGTAAA REV:GGTTTGAAGAAGGAATA GACATCATG	VIC- TCTCCGGGCGATTAA CATAGCCAGC- TAMRA	77	91.4%
<b>EF1</b>	FOR: TCAAGAACATGATCACTGGAA CCT REV: CCAGCGGCGACAATGAG	6FAM - CCAGGCCGATTGCG CCGT-TAMRA	94	94.0%
<b>Arylphorin</b>	FOR: CGTCAGATGCAGTCTTTAAGA TCTTC REV: TGCACGAACCAGTCCAGTTC	VIC- AATACCACGCCAAT GGCTATCCGGTT- TAMRA	112	96.7%
<b>Armadillo</b>	FOR: TGCACCAGCTGTCCAAGAAG REV: AAAGCGGCAACCATTTGC	6FAM- AAGCTTCTCGCCATG CTATTATGAACTCGC -TAMRA	70	92.8%
<b>Tubulin</b>	FOR: CGTGGAGCCCTACAACCTCTAT CC REV: GCCTCGTTGTGACCATGA	6FAM- ACCACCCACACCAC CCTTGAGCAC- TAMRA	81	100%

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823 **Table 3: Terms included in each of the basic models describing different attributes of the diet or**824 **the amount of protein or carbohydrate consumed.**

Terms included in model												
Model	P	C	P <sup>2</sup>	C <sup>2</sup>	Co	R	Co <sup>2</sup>	R <sup>2</sup>	Pe	Ce	Pe <sup>2</sup>	Ce <sup>2</sup>
1												
2	X											
3	X		X									
4		X										
5		X		X								
6	X	X										
7	X	X	X									
8	X	X		X								
9	X	X	X	X								
10	X	* X										
11	X	* X	X	X								
12					X							
13					X		X					
14						X						
15						X		X				
16					X	X						
17					X	X	X					
18					X	X		X				
19					X	* X						
20					X	* X	X	X				
21									X			
22									X		X	
23										X		
24										X		X
25									X		X	
26									X		X	X
27									X	X		X
28									X	X	X	X
29									X	* X		
30									X	* X	X	X

825

826 The table shows the terms included in each of the 30 basic models covering the different dietary

827 attributes. These models were also run including treatment as an additive or interactive effect, giving

828 90 models in total. **P** (protein) =grams of protein offered, **C** (carbohydrate) = grams of carbohydrate829 offered, **Co** (concentration) = percentage of the diet that comprises digestible nutrients (17%, 34%,830 42%, 63%), **R** (ratio) = percentage of protein in the digestible component of the diet (17%, 50% or831 83%); **Pe** (protein eaten) =grams of protein eaten, **Ce** (carbohydrate eaten) = grams of carbohydrate

832 eaten. For Pe and Ce this was calculated over the first 48 hours. Asterisks indicate interactions

833 between terms (e.g. Models 10 and 11 include the interaction between protein and carbohydrate

834 offered). Each of variables was also included as a squared term (e.g. **P<sup>2</sup>**). These 30 models were

835 modified by including additive or interactive effects of treatment (base 90 models for all analyses).

836 For the physiological traits and survival, the base 90 models were modified with the additive or

837 interactive effects of expression of the relevant genes (180 models).

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839

840 **Table 4: The best model selected by AICc to explain variation in gene expression across the diet**  
 841 **and bacterial treatments.**

Gene	Best Model	R <sup>2</sup> fixed	R <sup>2</sup> both
Toll	Treat*30	0.059	0.717
PPO	Treat*30	0.035	0.715
Lysozyme	Treat*30	0.104	0.736
Relish	Treat*30	0.120	0.378
Moricin (1)	Treat*30	0.741	0.741
Moricin (2)	Treat*30	0.097	0.264
Arylphorin	Treat*30	0.089	0.275
EF1	Treat*30	0.023	0.862
Armadillo	Treat*30	0.034	0.696
Tubulin	Treat*30	0.040	0.855

842

843 **Table 5: The best models selected by AICc to explain variation in PPO activity in the**  
 844 **haemolymph. GE represents gene expression for the PPO gene. Treat represents the immune**  
 845 **challenge treatment.**

Model	df	Log Likelihood	AICc	delta	weight	R <sup>2</sup>
3	4	-432.120	872.4	0.00	0.078	0.093
GE+3	5	-431.259	872.7	0.34	0.066	0.098
GE*3	7	-429.321	873.0	0.64	0.057	0.109
Treat+3	5	-431.737	873.7	1.30	0.041	0.095
Treat+GE*3	8	-428.617	873.7	1.34	0.040	0.113
Treat+GE+3	6	-430.716	873.7	1.34	0.040	0.101
7	5	-431.938	874.1	1.70	0.033	0.094

846

847 **Table 6: The best models selected by AICc to explain variation in PO activity in the**  
 848 **haemolymph. GE represents gene expression for the PPO gene. Treat represents the immune**  
 849 **challenge treatment.**

Model	df	Log Likelihood	AICc	delta	weight	R <sup>2</sup>
GE*3	7	-425.954	866.3	0.00	0.062	0.092
GE+3	5	-428.058	866.3	0.04	0.061	0.080
3	4	-429.363	866.9	0.58	0.047	0.072
GE+16	5	-428.350	866.9	0.62	0.046	0.078
GE+9	7	-426.378	867.1	0.85	0.041	0.090
16	4	-429.513	867.2	0.88	0.040	0.071
GE+Treat*17	10	-423.239	867.2	0.93	0.035	0.108
Treat*17	9	-424.471	867.5	1.26	0.033	0.100
GE+17	6	-427.775	867.8	1.55	0.029	0.081
GE+10	8	-425.777	868.0	1.75	0.026	0.093
GE+19	6	-427.887	868.0	1.77	0.026	0.081

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852 **Table 7: The best models selected by AICc to explain variation in lytic activity in the**  
 853 **haemolymph. GE represents gene expression for the lysozyme gene.**

Model	df	Log Likelihood	AICc	delta	weight	R <sup>2</sup>
GE*15	7	647.521	-1280.7	0.00	0.208	0.072
GE*18	9	649.465	-1280.3	0.34	0.176	0.080
GE*3	5	644.526	-1278.9	1.82	0.084	0.051

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858 **Table 8: The best models selected by AICc to explain variation in survival after bacterial (dead**  
859 **or live) injection. GE represents gene expression for the Moricin gene.**

Model	df	Log Likelihood	AICc	delta	weight	R <sup>2</sup>
Treat+GE*30	12	-1662.917	3350.8	0.00	0.139	0.127
Treat*GE*30	23	-1650.823	3351.1	0.30	0.120	0.186
Treat+GE+30	7	-1668.715	3351.8	0.99	0.085	0.098
Treat+GE+20	7	-1668.864	3352.1	1.29	0.073	0.097
GE*30	11	-1664.796	3352.4	1.61	0.062	0.118

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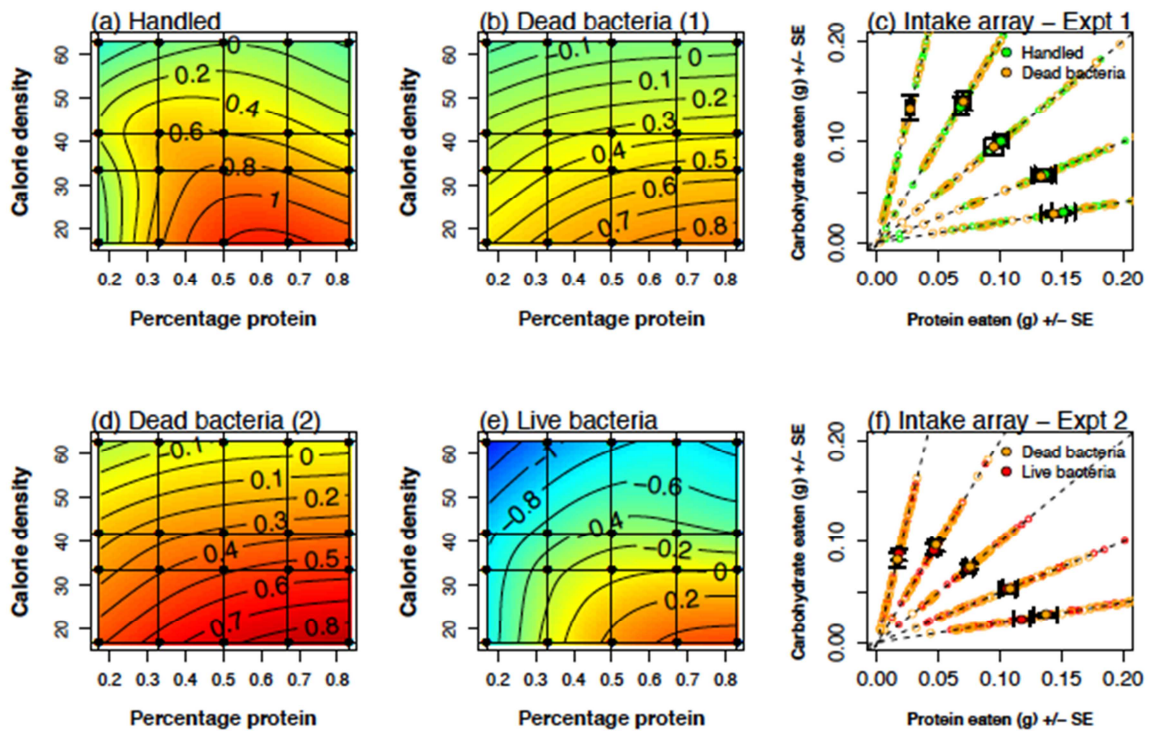
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864 **Figure legends**

865 **Figure 1 – The total amount of food eaten by caterpillars that were either (a) handled or (b)**  
 866 **injected with dead bacteria (Experiment 1) or (d) injected with dead bacteria versus (e) live**  
 867 **bacteria (Experiment 2). Blue colours indicate low consumption and red colours high**  
 868 **consumption. Colours are standardised within each experiment and are not comparable across**  
 869 **experiments. Numbers on the contour lines indicate z values for consumption. Intake arrays**  
 870 **indicating total consumption across the diets are shown separately for (c) experiment 1 and (d)**  
 871 **experiment 2.**

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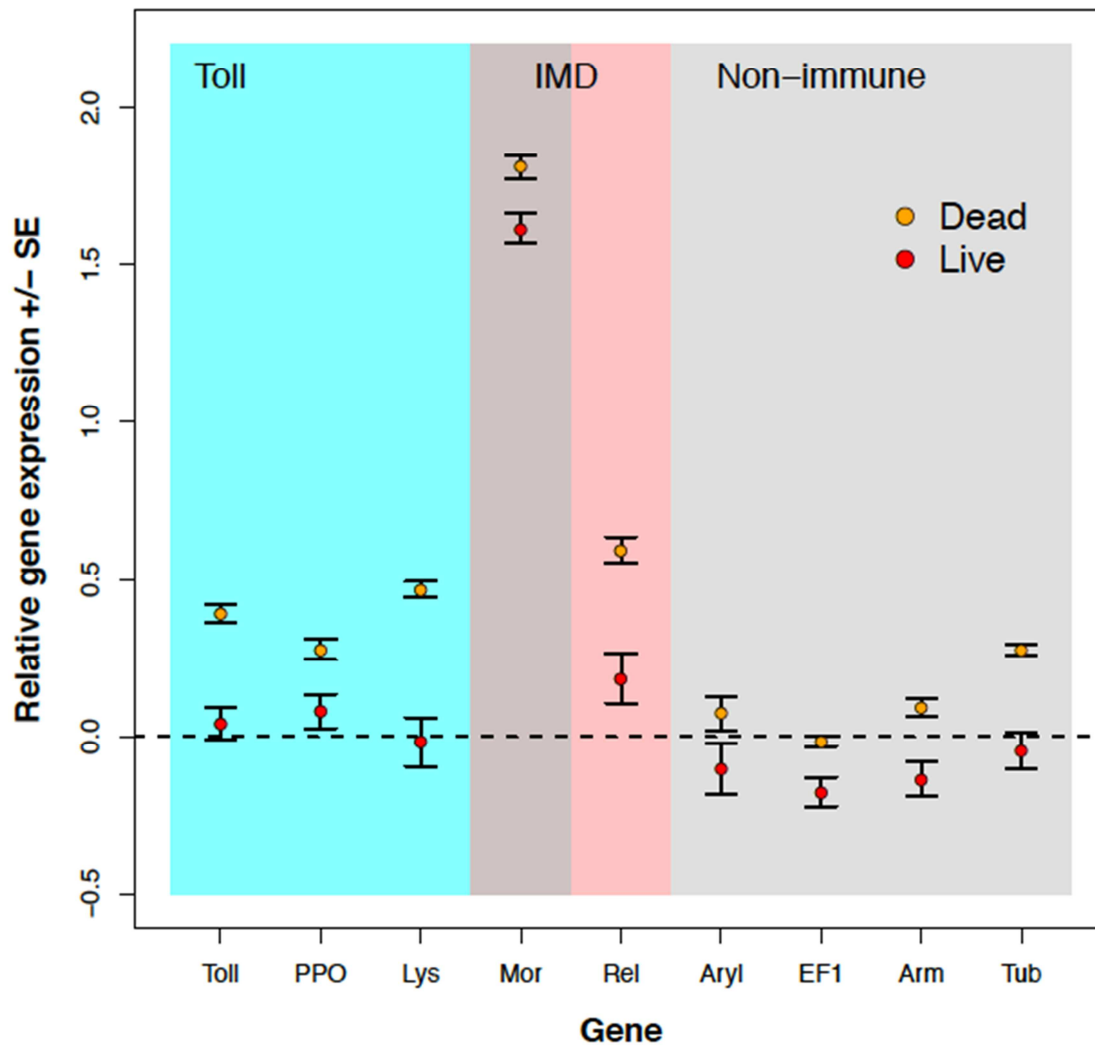
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876 **Figure 2 – Mean gene expression (+/- SE) for each of the immune genes and non-immune genes**  
 877 **in response to immune challenge treatment, relative to the ‘handled’ controls. Genes are**  
 878 **grouped by immune pathway Toll (blue zone: Toll, PPO, Lysozyme and Moricin), IMD (pink**  
 879 **zone: Moricin and Relish [11] [12] ) or classified as non-immune genes (grey zone; Arylophorin,**  
 880 **EF1, Armadillo and Tubulin). The black dashed line represents gene expression in the handled**  
 881 **group. Residuals from the model accounting for the random effects of ‘plate’ are plotted against**  
 882 **treatment. All models were re-run on untransformed data for ease of visualisation.**

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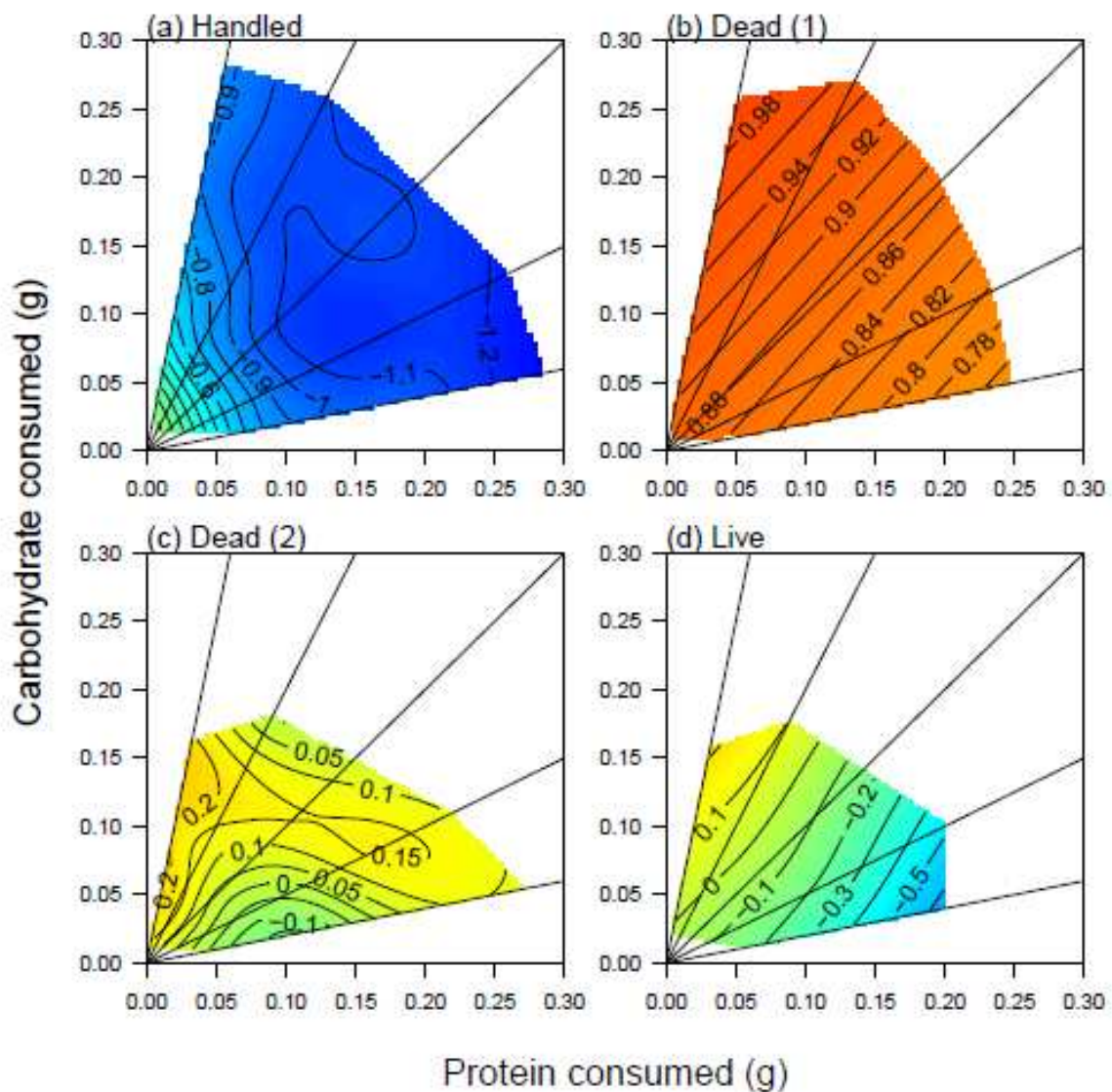


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885 **Figure 3 – Variation in Moricin expression across diets in haemolymph of caterpillars subject to**  
886 **different immune challenge treatments, (a) handled only, (b) injected with dead bacteria (Expt**  
887 **1), (c) injected with dead bacteria (Expt 2) and (d) injected with live bacteria. Blue colours**  
888 **indicate low gene expression and red colours high gene expression. Colours are standardised**  
889 **within each experiment and are not comparable across experiments.**

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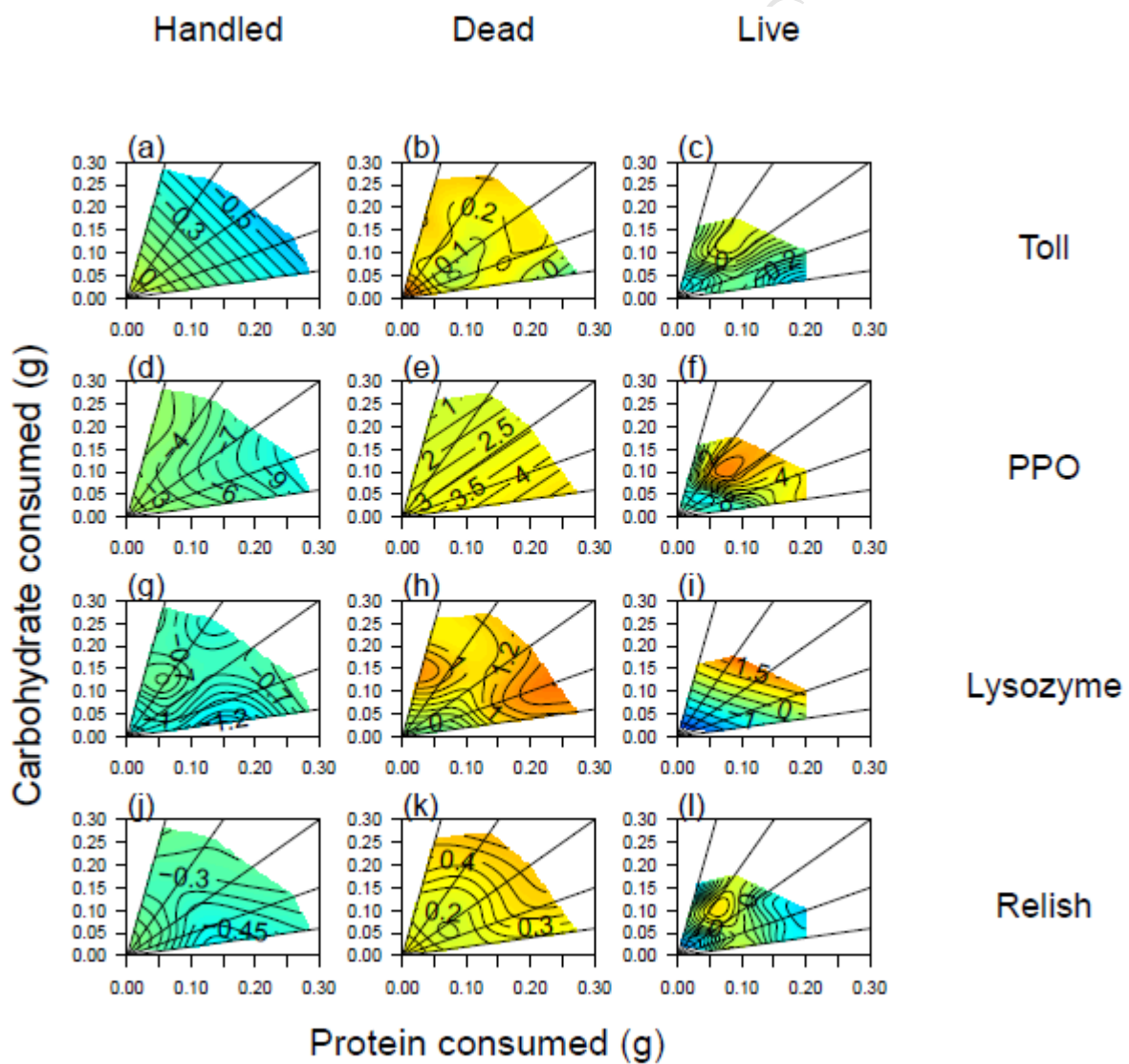
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894 **Figure 4 – Variation in immune gene expression across diets in haemolymph of caterpillars**  
 895 **subject to different immune challenge treatments, (a-c) Toll, (d-f) PPO, (g-i) Lysozyme and (j-l)**  
 896 **Relish. All figures in the first column are for the handled treatment, column 2 includes those**  
 897 **injected with dead bacteria and column 3, those injected with live bacteria. Blue colours**  
 898 **indicate low gene expression and red colours high gene expression.**

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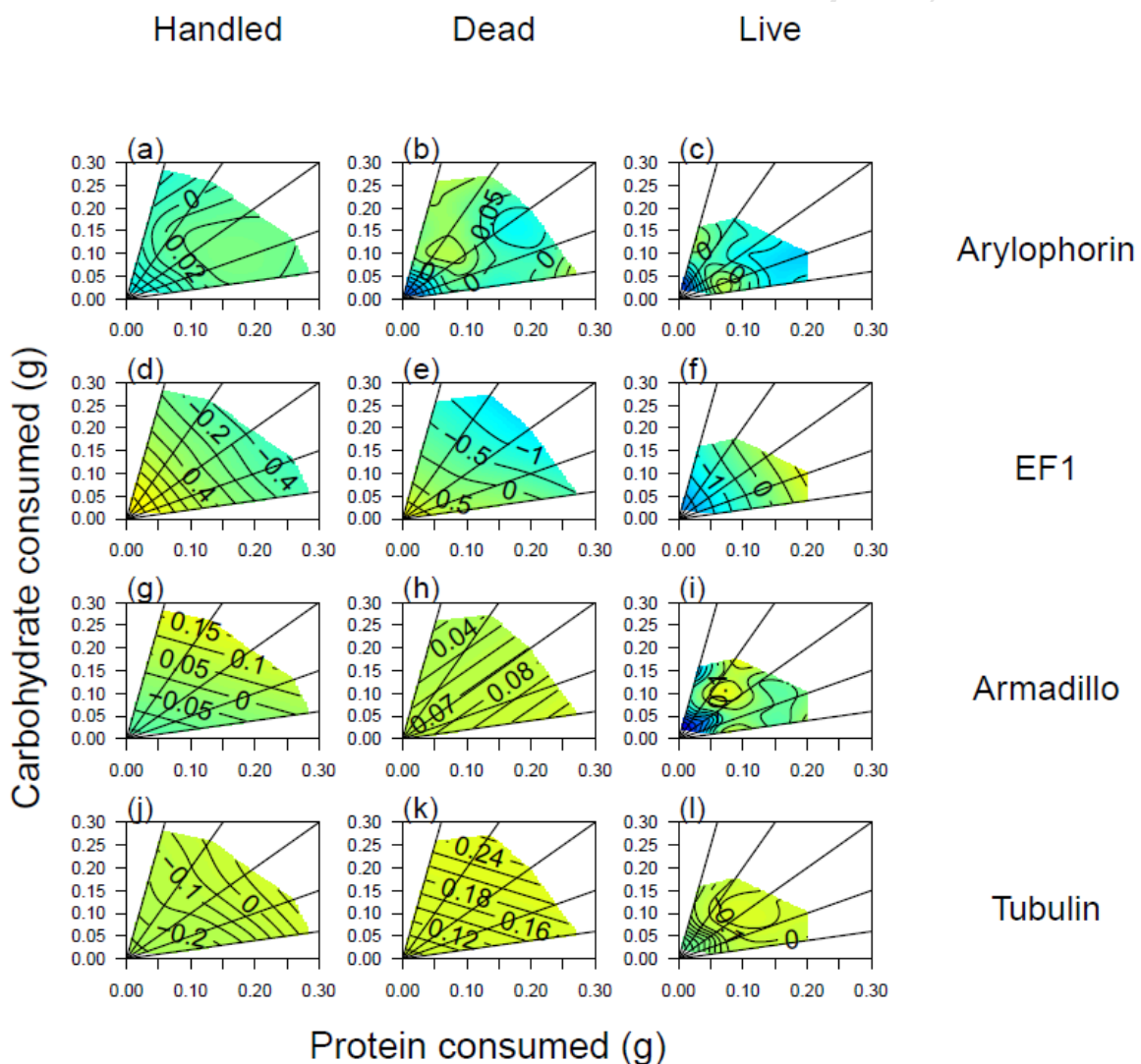
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904 **Figure 5 – Variation in non-immune gene expression across diets in haemolymph of caterpillars**  
 905 **subject to different immune challenge treatments, (a-c) Arylophorin, (d-f) EF1, (g-i) Armadillo**  
 906 **and (j-l) Tubulin. All figures in the first column are for the handled treatment, column 2**  
 907 **includes those injected with dead bacteria and column 3, those injected with live bacteria. Blue**  
 908 **colours indicate low gene expression and red colours high gene expression.**

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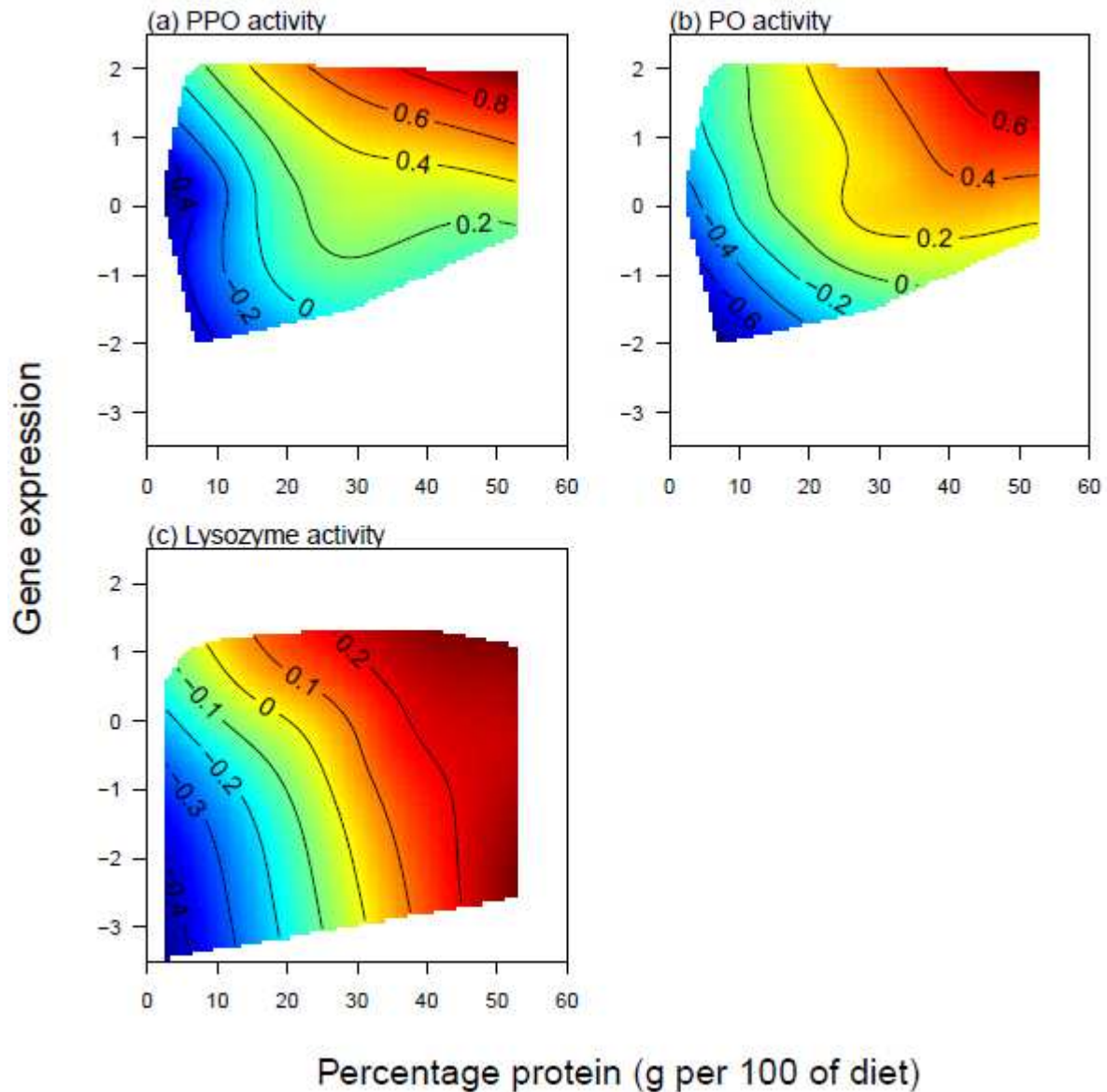


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912 **Figure 6 – Physiological immune responses vary with the protein content of the diet and the**  
913 **expression of the relevant gene. (a) PPO and (b) PO activity in the haemolymph in response to**  
914 **PPO gene expression and (c) lysozyme activity in the haemolymph in response to lysozyme gene**  
915 **expression. Blue colours indicate low activity and red colours high activity.**

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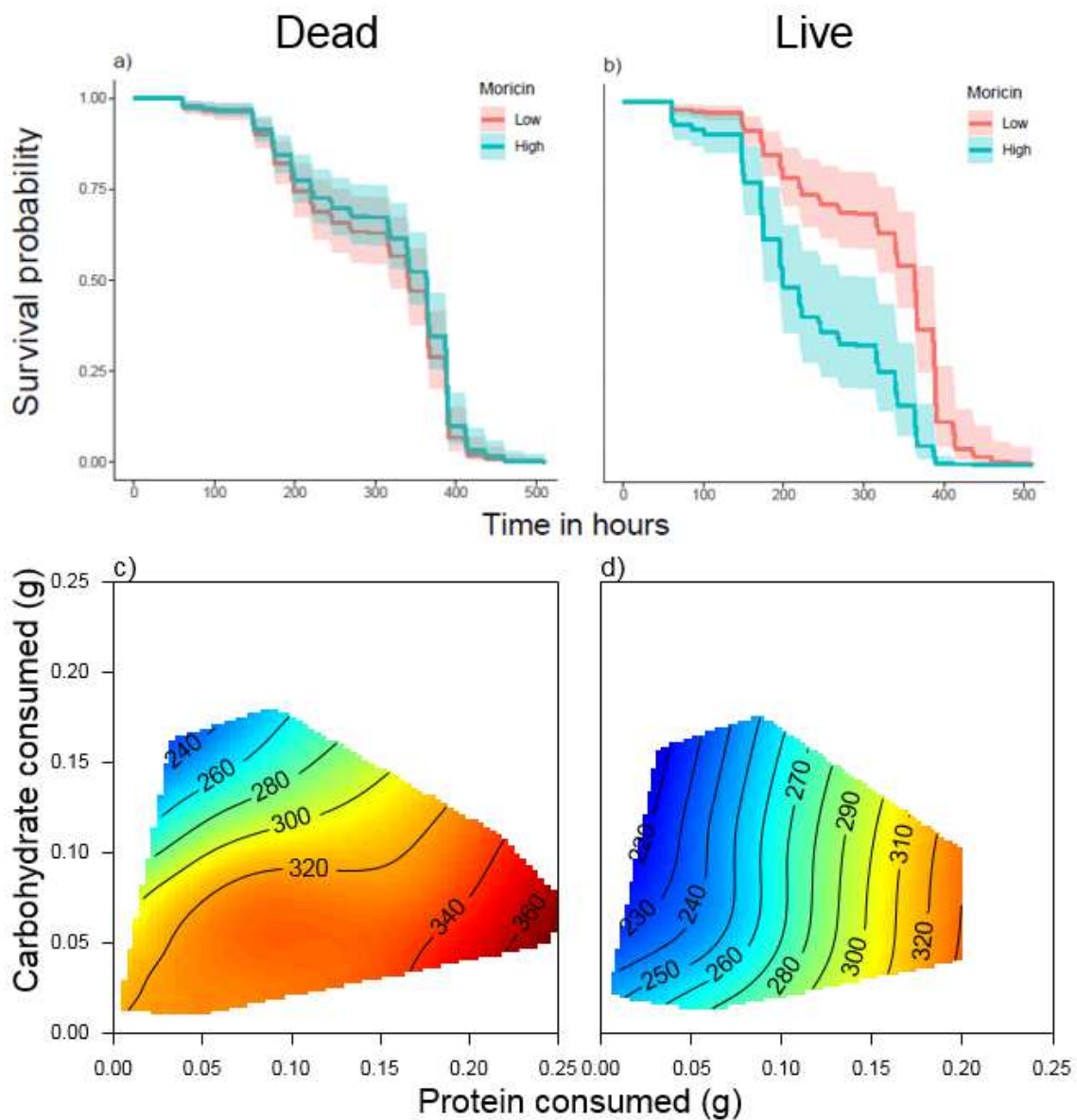


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920 **Figure 7 –Survival for larvae injected with either dead (a,c) or live (b,d) *X. nematophila***  
 921 **bacteria. Predicted survival curves (a,b) are plotted for model Treat\*GE\*30. Protein eaten and**  
 922 **carbohydrate eaten were set at mean values for each coefficient and Moricin gene expression**  
 923 **was set as either low (lower quartile) or high (upper quartile). To visualise the effects of diet on**  
 924 **survival, time to death (c,d) is plotted as thin plate splines against the amount of protein and**  
 925 **carbohydrate consumed. Blue colours indicate a short time to death and red colours a slow time**  
 926 **to death.**  
 927





ACCEPTED MANUSCRIPT

**Highlights**

- High protein diets improved survival after live bacterial infection
- Injection with dead bacteria increased expression of Toll and IMD immune genes.
- Injection with live bacteria inhibited immune gene expression (GE).
- The ratio and concentration of macronutrients in the diet affected GE.
- GE only predicted functional immune activity at high levels of dietary protein.