Diet modulates the relationship between immune gene expression and physiological immune responses

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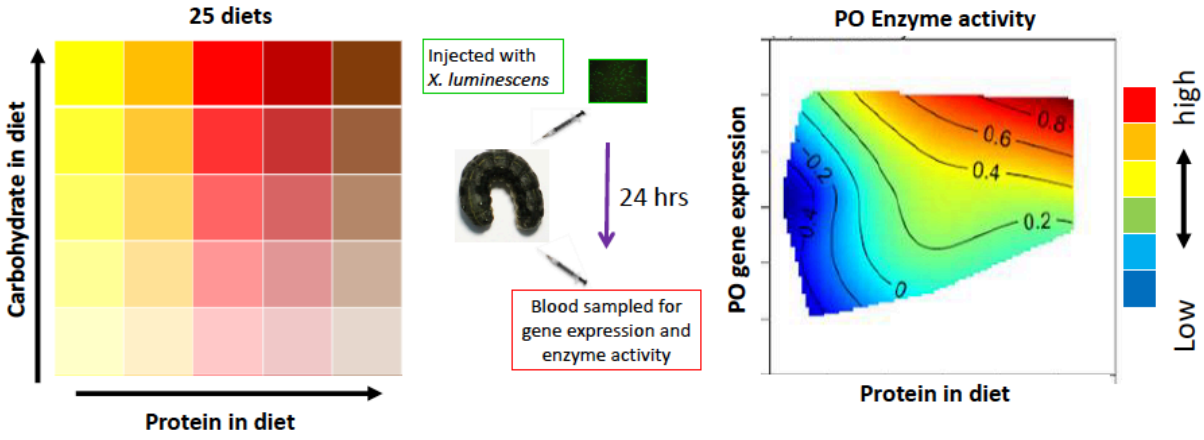
Graphical abstract



Abstract

Nutrition is vital to health and the availability of resources has long been acknowledged as a key factor in the ability to fight off parasites, as investing in the immune system is costly. Resources have typically been considered as something of a "black box", with the quantity of available food being used as a proxy for resource limitation. However, food is a complex mixture of macro- and micronutrients, the precise balance of which determines an animal's fitness. To unpick the effects of the balance and amount of nutrients on an animal's ability to mount an immune response, we used the Geometric Framework for Nutrition (GFN).

Spodoptera littoralis caterpillars were assigned to one of 25 diets that varied in the ratio of macronutrients (protein and carbohydrate) and their energy density to cover a large region of nutrient space. Caterpillars were then handled or injected with either live or dead *Xenorhabdus nematophila* bacterial cells. The expression of 9 genes (6 immune, 3 non-immune) was measured 24 h post immune challenge. For two of the immune genes (PPO and Lysozyme) we also measured the relevant functional immune response in the blood. Gene expression and functional immune responses were then mapped against nutritional intake.

The expression of all immune genes was upregulated by injection with dead bacteria, but only those in the IMD pathway (Moricin and Relish) were substantially upregulated by both dead and live bacterial challenge. Functional immune responses increased with the protein content of the diet but the expression of immune genes was much less predictable. Nonetheless, immune gene expression did predict the relevant functional immune response, but the relationship between the two varied over nutrient space, such that you could infer functional activity from gene expression only when protein availability was not limiting.

Our results indicate that diet does play an important role in the ability of an animal to mount an adequate immune response, with the availability of protein being the most important predictor of the functional immune response. However, gene expression alone responds quite differently to functional

immunity and we would caution against using gene expression as a proxy for immune investment, as it is unlikely to be reliable indicator of the immune response, except under specific dietary condition.

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

1. Introduction

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

However, nutrition is much more complex than simply a source of energy, being a vital mixture of macro- (carbohydrates, fats and proteins) and micro-nutrients (vitamins and minerals), the amount and

71 balance of which determine an animal's fitness (Simpson et al. 2004). Several studies have examined
72 how shifting the balance of macronutrients in the diet affects immune responses and the outcome of
73 infection, without restricting the availability of calories (Lee et al. 2006, Povey et al. 2009, Ponton et
74 al. 2011a, Graham et al. 2014, Povey et al. 2014). For example, caterpillars of the armyworms,
75 *Spodoptera littoralis* and *Spodoptera exempta*, show improved immune responses and markedly
76 higher survival after viral infection (Lee et al. 2006, Povey et al. 2014) and bacterial infection (Povey
77 et al. 2009) when their diet is heavily protein-biased. Furthermore, when given the opportunity,
78 infected caterpillars will "self-medicate" with protein, significantly improving their chances of
79 survival (Lee et al. 2006, Povey et al. 2009, Povey et al. 2014).

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

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

98 thereby indicating a nutrient-mediated trade-off within the immune system, and, as for fecundity and
99 longevity, no single diet could maximize multiple immune responses (Cotter et al. 2011). In a recent
100 study, mice were restricted to one of 25 diets varying in their ratio of proteins, fats and carbohydrates
101 and energy density, and their innate immune capacity was measured. It was shown that the balance of
102 T cells indicative of healthy ageing was achieved on a low protein, high non-protein energy diet,
103 irrespective of calorie intake (Le Couteur et al. 2015). However, this powerful approach has not yet
104 been taken to assess an animal's immune response to a pathogenic challenge.

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

120 The strength of the immune response can be measured using standard functional assays of
121 antimicrobial activity or PPO activity in the blood, and the strength of the encapsulation response or
122 phagocytosis can be measured against synthetic parasites injected into the haemocoel (see (Wilson
123 and Cotter 2013) and references therein). These functional responses have been shown to be
124 indicative of the ability of the animal to fight off parasites (e.g. (Paskewitz and Riehle 1994, Lee et al.

2006, Povey et al. 2009) and so are arguably meaningful measures of immune investment. However, gene expression is also often used as a proxy for investment in specific traits, e.g. immunity (Freitak et al. 2007, Jackson et al. 2011, Woestmann et al. 2017), but few of these studies consider how well the expression of the gene of interest predicts the functional response under the conditions in which they are tested.

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

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

2. Material and methods

2.1 Host and pathogen cultures

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

Bacteria were originally supplied by the laboratory of Givaudan and colleagues (Montpellier University, France; *Xenorhabdus nematophila* F1D3 GFP labelled, see (Sicard et al. 2004)). Pure *X. nematophila* F1D3 stocks were stored at -20°C in Eppendorf tubes (500 µl of *X. nematophila* F1D3 in nutrient broth with 500 µl of glycerol). Vortexing ensured that all *X. nematophila* F1D3 cells were coated in glycerol. To revive the stocks for use, 100 µl was added to 10 ml nutrient broth, and incubated at 28°C for up to 48 h (generally stocks had grown sufficiently after 24 hrs). On the day of experimental bacterial challenge, a sub-culture of the stock was carried out, with 1 ml of the original

177 stock added to 10 ml of nutrient broth and placed in a shaker-incubator for approximately 4 h. This
178 ensured that the bacteria were in log phase prior to challenge. Following the sub-culture, a 1 ml
179 sample was checked for purity and then used to produce a serial dilution in nutrient broth from which
180 the total cell count was determined with fluorescence microscopy, using a haemocytometer with
181 improved Neubauer ruling. The remaining culture was diluted with nutrient broth to the appropriate
182 concentration required for the bacterial challenge. The heat-killed treatment group was established by
183 autoclaving the bacteria for 30 min at 121ºC.

184 **2.2 Diet manipulation**

185 The aim of the experiments was to tease apart the importance of relative and absolute nutrient effects
186 on immune gene expression and immune protein activity. Therefore, larvae were fed on one of 20
187 chemically-defined diets (Table 1; based on (Simpson and Abisgold 1985)) that varied in both the
188 protein to carbohydrate (P:C) ratio and calorie density. This comprised five P:C ratios (5:1, 2:1, 1:1,
189 1:2, 1:5) and four calorie densities (326, 612, 756 and 1112 kJ/100g diet; the remainder of the diet
190 comprising indigestible cellulose). Thus, the 20 diets could be described with respect to the absolute
191 amount of protein or carbohydrates, by their sum (calorie density), by their ratio (P:C) or by their
192 interaction (P*C). In addition, the absolute amounts of food eaten by the larvae on each diet were
193 recorded so the absolute amount of protein or carbohydrate eaten as opposed to amounts offered could
194 also be used. We were therefore able to define 30 alternative models for describing the relationship
195 between the trait of interest (e.g. Toll expression), and host diet (Table 1). These were then compared
196 using an information theoretic approach by comparing AIC_c values and other model metrics (Burnham
197 and Anderson 2003, Whittingham et al. 2006).

198 **2.3 Bacterial challenge**

199 *Xenorhabdus nematophila* is a highly pathogenic gram-negative bacterium. In the wild, this species
200 relies on the entomopathogenic nematode *Steinernema carpocapsae*, which vectors *X. nematophila,* to
201 gain access to an insect host, where it rapidly multiplies, generally causing death within 24-48 hours
202 (Georgis et al. 2006, Herbert and Goodrich-Blair 2007). However, in the lab we can circumvent the

requirement for the nematode by injecting *X. nematophila* directly into the insect haemocoel (Herbert and Goodrich-Blair 2007).

Experiment 1: Within 24 h of moulting to the 6th instar, 400 larvae were divided into 20 groups (n = 20 per group), placed individually into 90 mm diameter Petri dishes and provided with ~1.5 g of one of the 20 chemically-defined diets (Table 1). Within each diet, 10 larvae were allocated to the control group and 10 were assigned to the bacteria-challenged group. Following 24 h feeding on the assigned diets (at time, t = 0), 200 larvae were handled then replaced on their diet (control) whilst 200 larvae were injected with 5 µl of a heat killed LD50 dose of *X. nematophila* (averaging 1272 *X. nematophila* cells per ml nutrient broth) using a microinjector (Pump 11 Elite Nanomite) fitted with a Hamilton syringe (gauge = 0.5mm). The syringe was sterilised in ethanol prior to use and the challenge was applied to the left anterior proleg. Every 24 h up to 72 h (i.e. 48 h post infection), larvae were transferred individually to clean 90 mm Petri dishes containing 1.5 - 1.8 g of their assigned chemically-defined diet. 96 h after moulting into L6, the larvae had either pupated or were placed on semi-artificial diet until death or pupation.

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

2.4 Blood sampling

Following challenge, haemolymph samples were obtained from all caterpillars at 20 h post infection. Haemolymph samples were obtained by piercing the cuticle next to the first proleg near the head with a sterile needle and allowing released haemolymph to bleed directly into an Eppendorf tube. Immediately following haemolymph sampling, 30 µl of fresh haemolymph was added to a sterile ice-cooled Eppendorf containing 350 µl of lysis buffer (RLT + Beta mercaptoethanol – 100:1) for later RNA extraction and qPCR analysis. The remainder of the haemolymph extracted was stored in a separate Eppendorf for further immune assays. All haemolymph samples were stored at -80°C prior to processing.

The amount of food eaten each day was determined by weighing the wet mass of the chemically-defined diet provided each day to the caterpillars, as well as weighing uneaten control diets each day (3 control diets per diet). The unused diet and control diet were then dried to a constant mass (for approx. 72 h), allowing the consumption per larva to be estimated.

2.5 Lysozyme assays

Bacterial agar plates, consisting of 10 ml of 1.5% agar:potassium phosphate buffer (2:1) and 50 mg of freeze-dried *Micrococcus luteus*, were used to determine lytic activity. 2 mm diameter holes were punched in each plate and each hole filled with 1 ml of ethanol saturated with phenylthiourea (PTU), in order to prevent melanisation of the samples. The ethanol evaporates, leaving the PTU in the hole. Following defrosting and vortexing of the stored haemolymph, each well was the filled with 1µl of haemolymph, with two technical replicates per sample. The plates were incubated at 30°C for 24 h, and the clear zones around the samples were measured using digital callipers. Lytic activity (mg/ml) was then calculated from a serial dilution of a hen egg white lysozyme standard (0.01, 0.05, 0.1, 0.5, 1 and 2 mg per ml in water).

2.6 Phenoloxidase assays

Following defrosting of the haemolymph samples, 10 µl of haemolymph was added to 450 µl of NaCac buffer. The solution was then split into two Eppendorfs (each containing 225 µl), in order to carry out assays for both proPO and PO. To one Eppendorf, 25 µl of NaCac buffer was added (PO assay), and to the other, 25 µl of 20 mg per ml chymotrypsin in NaCac buffer was added (proPO activated). The samples were vortexed and incubated at 25 °C for 1 h. 90 µl of each solution was placed in a well of a 96-well microplate with 90 µl of 10 mM dopamine as a substrate. Two technical replicates were carried out per sample. Readings were taken every 15 secs for 10 mins at 490 nm and 25 °C using a Tecan infinite m200pro plate reader with Magellan software (V7.2). This range accounted for the linear stage of the reaction. The maximum rate of reaction was then used as an approximation of PO and proPO level.

255

256 **2.7 Gene expression**

257 RNA was extracted from haemolymph samples using Qiagen RNeasy mini kit following the
258 manufacturers instructions with a final elution volume of 40 µl. Extracts were quantified using the
259 Nanodrop 2000 and diluted to 0.5 µg/µl for cDNA synthesis. Prior to cDNA synthesis a genomic
260 DNA elimination step was carried out by combining 12 µl RNA (0.5 µg total RNA) plus 2 µl DNA
261 wipeout solution and incubating at 42 °C for 2 min, cDNA synthesis was carried out using Qiagen
262 Quantitect Reverse Transcription kit in a final reaction volume of 20 µl following the manufacturer's
263 instructions, cDNA synthesis was carried out for 30 min at 42 °C followed by 3 min incubation at 95
264 °C and stored at -20 °C. cDNA was diluted 1:5 for use as a qPCR template.

265 Primers and probes were synthesised by Primer Design and qPCR was performed in a reaction
266 volume of 10 µl with 1x Taqman FAST Universal PCR Master mix (Thermo Fisher), 0.25 µM of each
267 primer, 0.3 µM probe and 2 µl of a 1:5 dilution of cDNA. qPCR was carried on the ABI 7500 FAST,
268 cycling parameters included an initial denaturation at 95 °C for 20 sec followed by 40 cycles of
269 denaturation at 95 °C, 3 sec and annealing at 60 °C for 30 sec.

270

271 **2.8 Statistical analyses**

272 All statistical analyses were conducted using the *R* statistical package version 3.2.2 (R Core Team,
273 2013). Gene expression data were normalised using NORMA-Gene (Heckmann et al. 2011), a data
274 driven approach that normalises gene expression relative to other genes in the dataset rather than to
275 specifically identified reference genes. It is particularly suited to data sets with limited numbers of
276 assayed genes. Normalised gene expression data were then standardized using the mean (µ) and
277 standard deviation (σ) of each trait ($Z = (X- \mu)/\sigma$) prior to analysis. Data were analysed for each gene
278 separately using linear mixed-effects models in the packages *lme4* and *lmerTest*. For each gene, the
279 plate that the samples were run on was included as a random effect and this was nested within
280 experiment. A comparison was made of 90 candidate models for each gene, which comprised 30

281 models covering different combinations of dietary attributes (Table 3), either alone, with bacterial
282 treatments added or with bacterial treatment interacting with the dietary traits. AIC values were
283 corrected for finite sample sizes (AIC_c) to establish the most parsimonious models including likely
284 nutritional attributes driving the observed data. AIC_c values and *Akaike weights* were estimated using
285 the *MuMin* package in *R*. The relative weight of evidence in favour of one model over another
286 (evidence ratio) is determined by dividing the *Akaike weight* of one model by another (Burnham and
287 Anderson 2003). For visualisation of the effects of the immune challenge treatment and diet on gene
288 expression (Figures 2-10), residuals from the null model, containing just the random plate effect
289 (Model 1, Table 3), were plotted. Food consumption data were analysed in the same way as the gene
290 expression data, with experiment included as a random effect.
291 The same approach was taken for the physiological immune measurements, lysozyme, PPO and PO
292 activity, except for these variables, standard linear models were run as data were collected in a single
293 experiment. The same sets of models as described above were fitted, with the addition of 60 extra
294 models that included the additive and interactive effects of the expression of the relevant gene, after
295 correction for the plate to plate variation (residuals from the null model containing the random effect
296 of plate only) – the lysozyme gene for lysozyme activity and the PPO gene for PPO and PO activity.

297

298 **3. Results**

299 **3.1 How does consumption vary across diets and bacterial challenge treatments?**

300 The total amount of food consumed varied across the diets. The best model predicting consumption
301 was model 20 including the additive effects of treatment (Treatment+Co*R+Co^2+R^2). Inspection of
302 the coefficients suggested that only the live bacteria treatment group was different and so the two
303 controls (handled and dead bacteria) were collapsed into a single "control" group for further analysis.
304 Again, the best model was model 20, the next best model was model 18 (Treatment+Co+R+R^2; delta
305 = 8.77). For both treatment groups, it can be seen that consumption tended to increase as the diet
306 concentration decreased (Figure 1a,b), suggesting that food dilution constrained caterpillars from
307 being able to take in sufficient nutrients, as we expected, and that on the more concentrated diets

caterpillars over-consumed nutrients due the extreme nutrient density. However, this increase in total consumption was more extreme on the high-protein than on the low-protein diets, suggesting that caterpillars were willing to overeat protein to gain limiting carbohydrates. The data were also visualised as an intake array (Figure 1c), and the same pattern can be seen, with tighter control of carbohydrate than protein intake (the curve of points is tilted towards the horizontal rather than the vertical axis).

Overall, consumption was significantly reduced in the caterpillars injected with live bacteria compared to controls (Treatment: $F_{1,1189} = 28.23$, $P<0.001$; Figure 1). It is clear from the intake array (Figure 1c), that whilst live challenged caterpillars ate less, they did so consistently across the diets, suggesting that infection did not change their "rules of compromise" i.e. their willingness to over- or under-eat specific nutrients when confined to a suboptimal diet.

3.2 How does immune gene expression vary across diets and bacterial challenge treatments?

For the immune genes (Toll, PPO, Lysozyme, Moricin and Relish), injection with dead bacteria resulted in up-regulation of gene expression relative to handled caterpillars (Figure 2). In contrast, live bacteria either did not up-regulate gene expression relative to controls (Toll, PPO and Lysozyme), or did not up-regulate it as strongly (Moricin and Relish) (Figure 2). For the non-immune genes (Arylophorin, EF1, Armadillo and Tubulin), the variation in expression levels was lower; for EF1 and Armadillo, live bacteria triggered the down-regulation of gene expression relative to handled caterpillars, whilst there was no effect for Tubulin (Figure 2). For Armadillo and Tubulin, dead bacteria upregulated gene expression relative to handled caterpillars but there was no effect for EF1 (Figure 2). For Arylphorin, the best supported model was the null model, where the only variation explained ($r^2 = 0.15$) was due to the random, plate to plate variation; diet and treatment explained no variation in Arylphorin expression levels (Table 4, Figures 2 & 8). The best supported model for every other gene tested was model 30, with the bacterial treatment interacting with the amount of protein and carbohydrate eaten (Treatment*(Pe*Ce+Pe^2+Ce^2)). However, although the fit of these

models was generally good ($r^2 > 0.68$), with the exception of Moricin, the amount of variation explained by the fixed part of the model was very low ($r^2 < 0.12$; Table 4; Figures 3-5, 7-10). This means that the majority of the variation in gene expression was caused by random variation across plates. For Moricin, 64% of the variation explained by the model was explained by the fixed terms; in contrast only 4.7% was of the variation was due to between-plate variation.

Variation in the expression of all of the genes was explained by main and interactive effects of the amount of protein and carbohydrate eaten, and in interaction with the bacterial treatment, suggesting that the response to diet for each gene differed across treatments. A visualisation of these response surfaces (Figures 3-11) shows that, for the Toll genes (Toll, PPO and Lysozyme), whilst there is general up-regulation between handled and dead bacterial challenges, the response surfaces are fairly flat, i.e. diet does not explain much variation in gene expression. However, for the live challenge, expression peaks at moderate protein but high carbohydrate intake (Figures 3-5). For the IMD genes (Moricin and Relish), the response to dead bacteria is remarkably similar, with expression peaking at the highest intakes on the 33% protein diet (Figures 6, 7). The response to live bacteria is slightly different: whilst Relish still peaks at 33% protein, albeit at much lower levels of consumption, Moricin peaks at 17% protein (Figures 6, 7). In the handled larvae, Moricin expression was typically low but peaked at the lowest food consumption levels (Figure 6a).

The non-immune genes (Arylphorin, EF1, Armadillo and Tubulin), show a consistently weak response to the dietary manipulation, with much flatter surfaces on average than those shown by the immune genes (compare Figures 3-7 with Figures 7-11). Arylphorin shows a weak but similar response to live and dead bacterial challenge, with the lowest expression levels occurring at the lowest consumption (Figure 8). For EF1, Armadillo and Tubulin, expression increases with consumption after live challenge only, peaking on the balanced diet (50% protein) for EF1 and Tubulin (Figures 9,11) and between 33 and 50% protein for Armadillo (Figure 10).

3.3 Does immune gene expression predict physiological immune responses?

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

For PPO activity, AICc could not discriminate between several of the diet models, with six being equally well supported (delta < 2; Table 5). Of these models, the top five contained protein and protein squared with additive or interactive effects of bacterial treatment or gene expression (Table 5). For PO activity, AICc could not discriminate between 10 different models (delta < 2; Table 6). However, the top three models were the same as for PPO, with protein plus protein squared with additive or interactive effects of PPO gene expression. For lytic activity in the haemolymph, three models were equally well supported, all of which contained Lysozyme gene expression interacting with dietary components, which were either protein and protein squared, as for PO and PPO, or the P:C ratio (Table 7). As for gene expression, the overall explanatory power of the models was quite low, (r^2< 0.11; Tables 5-7).

For ease of comparison, all 3 physiological immune traits were plotted against the protein content of the diet, as this model was common to all three traits, and the expression of the relevant gene, which featured in the majority of the selected models (Tables 5-7). For each trait, activity in the haemolymph tended to increase with gene expression, as we might expect, but this was moderated by the diet (Figure 11). For PO and PPO activity, on low protein diets enzyme activity was low and there was little correspondence between gene expression and the physiological response, but as the protein content of the diet increased, this relationship became fairly linear (Figure 11a,b). For lytic activity the pattern was slightly different in that the relationship with Lysozyme gene expression was fairly linear on low protein diets, but as the protein content increased, this relationship disappeared with consistently high activity across all levels of gene expression.

4. Discussion

Previous work has shown that immune responses can be strongly affected by the amount and/or balance of nutrients in the diet (Fernandes et al. 1976, Ingram et al. 1995, Wallace et al. 1999, Lee et al. 2006, Ritz and Gardner 2006, Kristan 2008, Nayak et al. 2009, Povey et al. 2009, Le Couteur et al. 2015, White et al. 2017). However, most of these studies only covered a relatively small region of nutrient space (Fernandes et al. 1976, Ingram et al. 1995, Lee et al. 2006, Ritz and Gardner 2006, Nayak et al. 2009, Povey et al. 2009, White et al. 2017) and/or only tested innate responses (Fernandes et al. 1976, Ingram et al. 1995, Lee et al. 2006, Nayak et al. 2009, Povey et al. 2009, Le Couteur et al. 2015, White et al. 2017) or they relied on the response to an artificial pathogen or immune stimulant (Cotter et al. 2011). Here we addressed this gap by looking at both gene expression and functional immune responses to both dead and live pathogen challenges over a broad region of nutrient space. Our major findings are that whilst functional immune responses (PPO, PO and lytic activity in the haemolymph) change as expected in response to the dietary manipulation, showing a clear improvement as the protein content of the diet increases, gene expression is much less predictable (Figures 3-11). Despite this, expression of the PPO and Lysozyme genes did predict PPO/PO and Lysozyme activity in the haemolymph, but this relationship was strongly dependent on the amount of protein in the diet (Figure 12), suggesting that using immune gene expression as an indicator of the efficacy of the immune response may be reliable only under specific dietary conditions.

Our dietary manipulation was successful in inducing caterpillars to consume over a large region of nutrient space, allowing us to independently assess the effects of macronutrient composition and the calorie content of the diet on immunity. There was evidence for compensatory feeding, caterpillars did not consume the same amount of every diet. As expected, caterpillars ate more as the nutrient density of the food decreased (Figure 1), but this varied across diets, such that consumption was highest on the high protein diets, suggesting that caterpillars were willing to over-eat protein to gain limiting carbohydrates. However, as has been found in previous studies (Exton 1997, Adamo 1998, Lennie 1999, Adamo 2005, Povey et al. 2014), we found evidence for illness-induced anorexia. Caterpillars injected with live *X. nematophila* showed suppressed food consumption across all diets

(Figure 1). Interestingly, injection with dead *X. nematophila* did not induce this response, which suggests that it is not the triggering of an immune response that causes this change in consumption, but the presence of an actively replicating pathogen. This reduction in consumption was also consistent across diets, with infected caterpillars, on average, consuming just 77% of the food consumed by healthy caterpillars (Figure 1c).

In insects, two major pathways are triggered in response to microbial infection; typically, Toll responds to infection by fungi and gram-positive bacteria, whilst IMD responds to gram-negative bacteria (Broderick et al. 2009). Moricin and Lysozyme are triggered by Toll in Lepidoptera (e.g. (Zhong et al. 2016), but Moricin has also been shown to respond to gram-negative bacteria and so may also be triggered by IMD (Hara and Yamakawa 1995). Of the 5 immune genes we tested, only the IMD genes, Moricin and Relish, were significantly up-regulated in response to infection with both dead and live bacteria. For the Toll genes, (Toll, PPO and Lysozyme), mean gene expression was up-regulated by dead bacteria but not by live bacteria (Figure 2). However, even for Moricin and Relish, up-regulation was much stronger in response to dead than live bacteria. This may reflect a general down-regulation of gene expression during an active infection, as the non-immune genes typically show reduced gene expression in response to the live infection compared to the controls. This may be driven by the illness-induced anorexia, with reduced consumption resulting in lower metabolic activity and consequently lower gene expression. However, there is evidence that *X. nematophila* can inhibit Cecropin, Attacin and Lysozyme gene expression (Ji and Kim 2004, Park et al. 2007). It may be that, rather than specifically inhibiting AMP gene expression, *X. nematophila* inhibits the expression of all genes.

X. nematophila is a gram-negative bacterium, and is clearly triggering Moricin and Relish expression, but as Toll is only marginally up-regulated in response, it is probably the IMD pathway that is controlling this response. Another possible explanation for why live bacteria appear to trigger a down-regulation of gene expression is that our sampling protocol (24 h post-challenge) did not allow us to catch peak expression levels. Expression of lysozyme and PPO in the Glanville fritillary butterfly was not up-regulated 24 h after injection with *M. luteus* cells (Woestmann et al. 2017), whilst in the

440 silkworm, up-regulation of lysozyme in response to fungal infection occurred in two peaks, from 9-18
441 h, and then between 30 and 48 h (Hou et al. 2014). This may be a fungal-specific response, or it might
442 mean that we would have seen higher gene expression had we assayed over an extended time period.

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

458 For two of the immune genes, PPO and Lysozyme, we were able to simultaneously measure the
459 activity of the relevant protein in the blood as a measure of the functional immune response. Thus, we
460 were able to assess how well gene expression predicts functional immune activity and whether this
461 relationship changes with the diet. Here, we found that for each functional immune response, PPO
462 activity, PO activity and lysozyme activity, expression of the relevant gene does predict the response,
463 but only at certain intakes of protein (Figure 12). For example, PPO and PO activity increase linearly
464 with the expression of the PPO gene, but only above ~30% dietary protein for PPO and 40% dietary
465 protein for PO (Figure 12). This suggests that the availability of dietary protein limits the translation
466 of PPO mRNA into PPO protein, and the activation of PPO into PO. In contrast, the expression of the

gene is not limited by protein availability, and so gene expression can be high when dietary protein is low, but it is ineffective as it does not result in a comparable functional immune response. The lytic response is also affected by dietary protein, however, in this case, the relationship between gene expression and lytic activity is linear between about 5 and 35% dietary protein, but above that maximal lytic activity is achieved at low gene expression and increased expression does not improve the response. As for PPO, this suggests that protein limits the translation of lysozyme up to about 35% protein.

These results are not surprising when you consider the costs associated with the production of protein. It is estimated that only 10% of the energetic costs of protein production are spent on transcription; translation is much more energetically expensive and relies on the availability of amino acids to build the relevant protein (Warner 1999). It is likely, therefore, that whilst transcription of immune genes might still be up-regulated in response to infection under low protein conditions, the translation of the protein might be reduced, impairing the correlation between mRNA and protein abundance. Our results suggest that caution should be used when interpreting gene expression as a measure of "investment" into a particular trait, or as a measure of the strength of a particular response. It is surprisingly common in ecological studies for gene expression to be used in this way without any attempt to correlate the expression of a gene with the production of the functional protein. [4] If dietary protein levels are limiting, then gene expression may be a poor indicator of the immune capacity of an animal. Here we have tested this just with immune genes for which we have good functional assays of the active protein, but it seems likely that this would also be true of other classes of genes, for which gene expression is routinely used as an indicator of an organism's investment.

In summary, as expected, immune challenge with a live gram-negative bacterium up-regulated immune genes in the IMD pathway, though all immune genes were up-regulated to a certain extent by the challenge with dead bacteria. While functional immune responses (PO, PPO and Lysozyme) typically improved with the protein content of the diet, gene expression varied non-linearly with diet composition. However, the expression of PPO and Lysozyme genes predicted PPO/PO and Lysozyme activity, but only when the availability of dietary protein was not limiting, suggesting that using gene

494 expression as an indicator of investment in a trait is unlikely to be reliable, unless its relationship with
495 diet is known.

496 5. Acknowledgements

497 This study was funded by a standard research grant awarded to KW, JAS and SJS by the
498 UK's *Biotechnology and Biological Sciences Research Council* (BB/I02249X/1). SCC was supported
499 by a *Natural Environment Research Council* Fellowship (NE/H014225/2) We are grateful to Alain
500 Givaudan and colleagues (Montpellier University, France) for gifting the *Xenorhabdus*
501 *nematophila* F1D3 bacteria, and to Esmat Hegazi for his support with *Spodoptera littoralis*. We thank
502 Phill Nott for technical assistance.
503

504 6. Author contributions

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

508

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706 **Tables**

707 **Table 1. Nutritional composition of the 20 chemically-defined diets**

Diet	Protein (g/100g diet)	Carbs (g/100g diet)	Fats (g/100g diet)	Cellulose (g/100g diet)	Micro (g/100g diet)	Cal (kJ/100g diet)	Ratio (%)	P:C
1	10.5	52.5	1.1	33.0	4.0	1112	0.17	1:5
2	7.0	35.0	1.1	54.0	4.0	756	0.17	1:5
3	5.6	28.0	1.1	62.4	4.0	612	0.17	1:5
4	2.8	14.0	1.1	79.2	4.0	326	0.17	1:5
5	21.0	42.0	1.1	33.0	4.0	1112	0.33	1:2
6	14.0	28.0	1.1	54.0	4.0	756	0.33	1:2
7	11.2	22.4	1.1	62.4	4.0	612	0.33	1:2
8	5.6	11.2	1.1	79.2	4.0	326	0.33	1:2
9	31.5	31.5	1.1	33.0	4.0	1112	0.50	1:1
10	21.0	21.0	1.1	54.0	4.0	756	0.50	1:1
11	16.8	16.8	1.1	62.4	4.0	612	0.50	1:1
12	8.4	8.4	1.1	79.2	4.0	326	0.50	1:1
13	42.0	21.0	1.1	33.0	4.0	1112	0.67	2:1
14	28.0	14.0	1.1	54.0	4.0	756	0.67	2:1
15	22.4	11.2	1.1	62.4	4.0	612	0.67	2:1
16	11.2	5.6	1.1	79.2	4.0	326	0.67	2:1
17	52.5	10.5	1.1	33.0	4.0	1112	0.83	5:1
18	35.0	7.0	1.1	54.0	4.0	756	0.83	5:1
19	28.0	5.6	1.1	62.4	4.0	612	0.83	5:1
20	14.0	2.8	1.1	79.2	4.0	326	0.83	5:1

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

Gene	Primers (5′ - 3′)	Probe (5′ - 3′)
Toll	FOR: AATGCTCGTGTTATCATGATCAAA REV: CGTGATCGTAGCCAGCGTTT	VIC-CTGGACCACCACTAACGTCGTCGATTG-TAMRA
PPO	FOR: GCTGTGTTGCCGCAGAATG REV: AAATCCGTGGCGGTGTAGTC	VIC-CCGCGTATCCCGATCATCATCCC-TAMRA
Lysozyme	FOR: TGTGCACAAATGCTGTTGGA REV:CGAACTTGTGACGTTTGTAGATCTTC	VIC-ACATCACCCTAGCTTCTCAGTGCGCC-TAMRA
Moricin	FOR: GGCGCAGCGATTGGTAAA REV:GGTTTGAAGAAGGAATAGACATCATG	VIC-TCTCCGGGCGATTAACATAGCCAGC- TAMRA
Relish	FOR: TCAACATAACAACACGGAGGAA REV: ATCAGGTACTAGGCAACTCATATC	6FAM -CCCACAAATTACTTGAAGATGAACAGGACCC-TAMRA
EF1	FOR: TCAAGAACATGATCACTGGAACCT REV: CCAGCGGCGACAATGAG	6FAM -CCAGGCCGATTGCGCCGT-TAMRA
Arylphorin	FOR: CGTCAGATGCAGTCTTTAAGATCTTC REV: TGCACGAACCAGTCCAGTTC	VIC-AATACCACGCCAATGGCTATCCGGTT-TAMRA
Armadillo	FOR: TGCACCAGCTGTCCAAGAAG REV: AAAGCGGCAACCATTTGC	6FAM-AAGCTTCTCGCCATGCTATTATGAACTCGC-TAMRA
Tubulin	FOR: CGTGGAGCCCTACAACTCTATCC REV: GCCTCGTTGTCGACCATGA	6FAM-ACCACCCACACCACCCTTGAGCAC-TAMRA

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Table 3: Terms included in each of the basic models describing different attributes of the diet.

	Terms included in model													
Model	**P**		**C**	**P^2**	**C^2**	**Co**		**R**	**Co^2**	**R^2**	**Pe**		**Ce**	**Pe^2**	**Ce^2**
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

The table shows the terms included in each of the 30 basic models covering the different dietary attributes. These models were also run including treatment as an additive or interactive effect, giving 90 models in total. **P** (protein) =grams of protein offered, **C** (carbohydrate) = grams of carbohydrate offered, **Co** (concentration) = percentage of the diet that comprises digestible nutrients (17%, 34%, 42%, 63%), **R** (ratio) = percentage of protein in the digestible component of the diet (17%, 50% or 83%); **Pe** (protein eaten) =grams of protein eaten, **Ce** (carbohydrate eaten) = grams of carbohydrate eaten. Asterisks indicate interactions between terms (e.g. Models 10 and 11 include the interaction between protein and carbohydrate offered). Each of variables was also included as a squared term (e.g. **P^2**).

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

Gene	Best Model	R^2 fixed	R^2 both
Toll	30	0.057	0.726
PPO	30	0.034	0.750
Lysozyme	30	0.117	0.689
Moricin	30	0.638	0.686
Relish	30	0.056	0.363
Arylphorin	30	0.066	0.301
EF1	30	0.023	0.862
Armadillo	30	0.034	0.696
Tubulin	30	0.050	0.825

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

Model	df	Log Likelihood	AICc	delta	weight	R^2
3	4	-432.120	872.4	0.00	0.088	0.093
GE+3	5	-431.259	872.7	0.34	0.074	0.098
GE*3	7	-429.321	873.0	0.64	0.064	0.109
Treat + 3	5	-431.737	873.7	1.30	0.046	0.095
Treat+GE*3	6	-430.716	873.7	1.34	0.045	0.101
7	5	-431.938	874.1	1.70	0.038	0.094

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

Model	df	Log Likelihood	AICc	delta	weight	R^2
GE*3	7	-425.954	-1699.5	0.00	0.062	0.092
GE+3	5	-428.058	-1699.5	0.04	0.061	0.080
3	4	-429.363	-1698.9	0.58	0.047	0.072
GE+16	5	-428.350	-1698.9	0.62	0.046	0.078
GE+9	7	-426.378	-1698.7	0.85	0.041	0.090
16	4	-429.513	-1698.6	0.88	0.040	0.071
Treat*17	9	-424.471	-1698.2	1.26	0.033	0.100
GE+17	6	-427.775	-1698.0	1.55	0.029	0.081
GE+10	8	-425.777	-1697.8	1.75	0.026	0.093
GE+19	6	-427.887	-1697.7	1.77	0.026	0.081

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

Model	df	Log Likelihood	AICc	delta	weight	R^2
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

Figure legends

Figure 1 – The total amount of food eaten by caterpillars that were (a) either handled or injected with dead bacteria (control) or (b) injected with live bacteria. Blue colours indicate low consumption and red colours high consumption. Numbers on the contour lines indicate consumption in grams. (c) Intakes across both groups were also visualised with an intake array. Individual consumption data points are plotted in open circles and the average intakes (+/-SE) per treatment group for each protein to carbohydrate ratio are plotted in blue (control) and red (live bacterial challenge).

Figure 2 – Mean gene expression (+/- SE) for each of the immune genes and non-immune genes in response to immune challenge treatment, relative to the controls. Genes are grouped by immune pathway Toll (blue zone: Toll, PPO, Lysozyme and Moricin), IMD (pink zone: Moricin and Relish**[11] [12]**) or classified as non-immune genes (grey zone; Arylophorin, EF1, Armadillo and Tubulin). The black dashed line represents gene expression in the handled group.

Figure 3 – Variation in Toll expression across diets in haemolymph of caterpillars subject to different immune challenge treatments, (a) handled only, (b) injected with dead bacteria and (c) injected with live bacteria. Blue colours indicate low gene expression and red colours high gene expression.

Figure 4 – Variation in PPO expression across diets in haemolymph of caterpillars subject to different immune challenge treatments, (a) handled only, (b) injected with dead bacteria and (c) injected with live bacteria. Blue colours indicate low gene expression and red colours high gene expression.

Figure 5 – Variation in Lysozyme expression across diets in haemolymph of caterpillars subject to different immune challenge treatments, (a) handled only, (b) injected with dead bacteria and (c) injected with live bacteria. Blue colours indicate low gene expression and red colours high gene expression.

Figure 6 – Variation in Moricin expression across diets in haemolymph of caterpillars subject to different immune challenge treatments, (a) handled only, (b) injected with dead bacteria and (c) injected with live bacteria. Blue colours indicate low gene expression and red colours high gene expression.

Figure 7 – Variation in Relish expression across diets in haemolymph of caterpillars subject to different immune challenge treatments, (a) handled only, (b) injected with dead bacteria and (c) injected with live bacteria. Blue colours indicate low gene expression and red colours high gene expression.

Figure 8 – Variation in Arylphorin expression across diets in haemolymph of caterpillars subject to different immune challenge treatments, (a) handled only, (b) injected with dead bacteria and (c) injected with live bacteria. Blue colours indicate low gene expression and red colours high gene expression.

Figure 9 – Variation in EF1 expression across diets in haemolymph of caterpillars subject to different immune challenge treatments, (a) handled only, (b) injected with dead bacteria and (c) injected with live bacteria. Blue colours indicate low gene expression and red colours high gene expression.

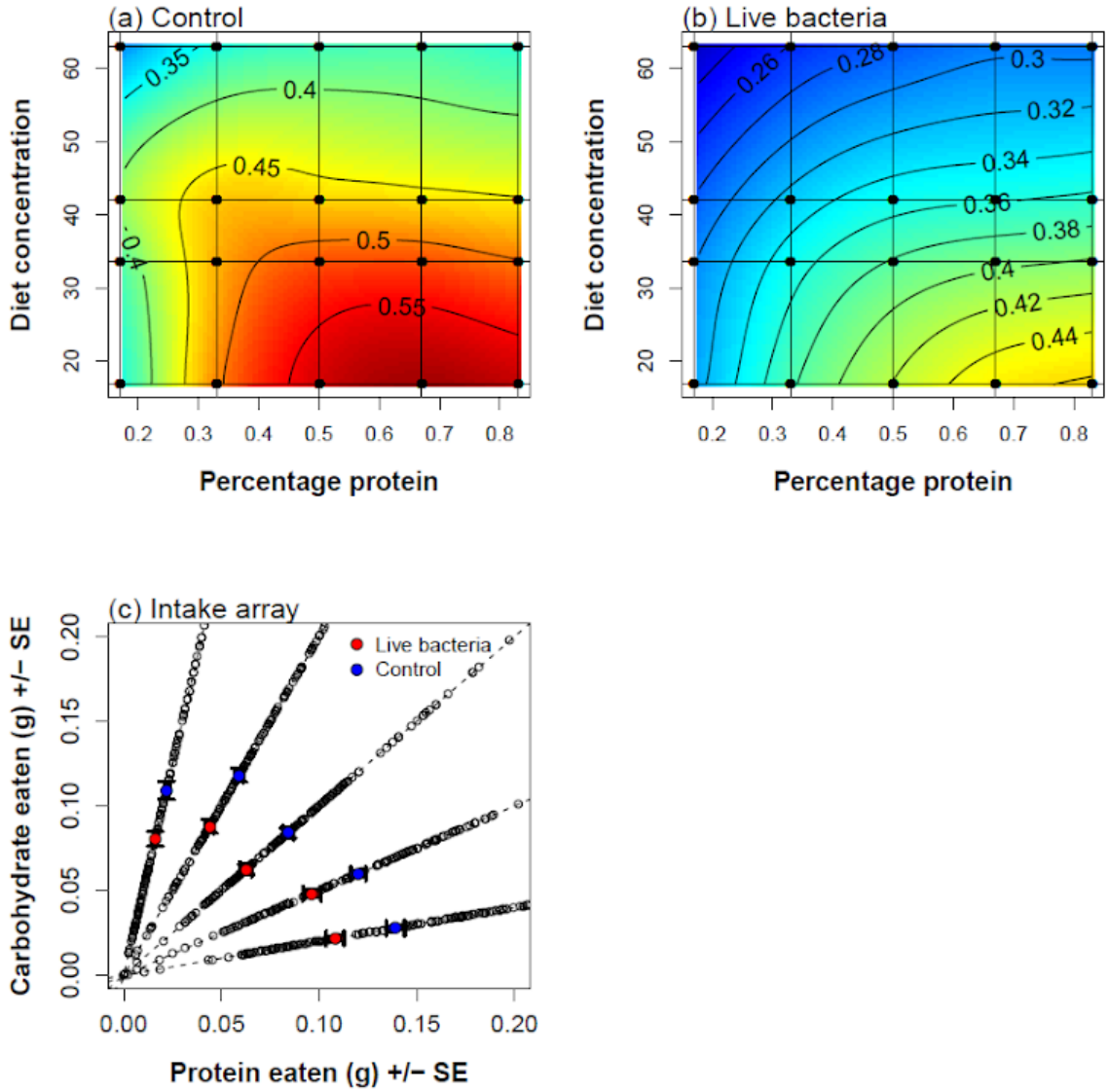
Figure 10 – Variation in Armadillo expression across diets in haemolymph of caterpillars subject to different immune challenge treatments, (a) handled only, (b) injected with dead bacteria and (c) injected with live bacteria. Blue colours indicate low gene expression and red colours high gene expression.

Figure 11 – Variation in Tubulin expression across diets in haemolymph of caterpillars subject to different immune challenge treatments, (a) handled only, (b) injected with dead bacteria and (c) injected with live bacteria. Blue colours indicate low gene expression and red colours high gene expression.

Figure 12 – Physiological immune responses vary with the protein content of the diet and the expression of the relevant gene. (a) PPO and (b) PO activity in the haemolymph in response to PPO gene expression and (c) lysozyme activity in the haemolymph in response to lysozyme gene expression. Blue colours indicate low enzyme activity and red colours high enzyme activity.

811 **Figures**

812 **Figure 1 - Total food eaten**



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819 **Figure 2 - Mean gene expression across treatments**

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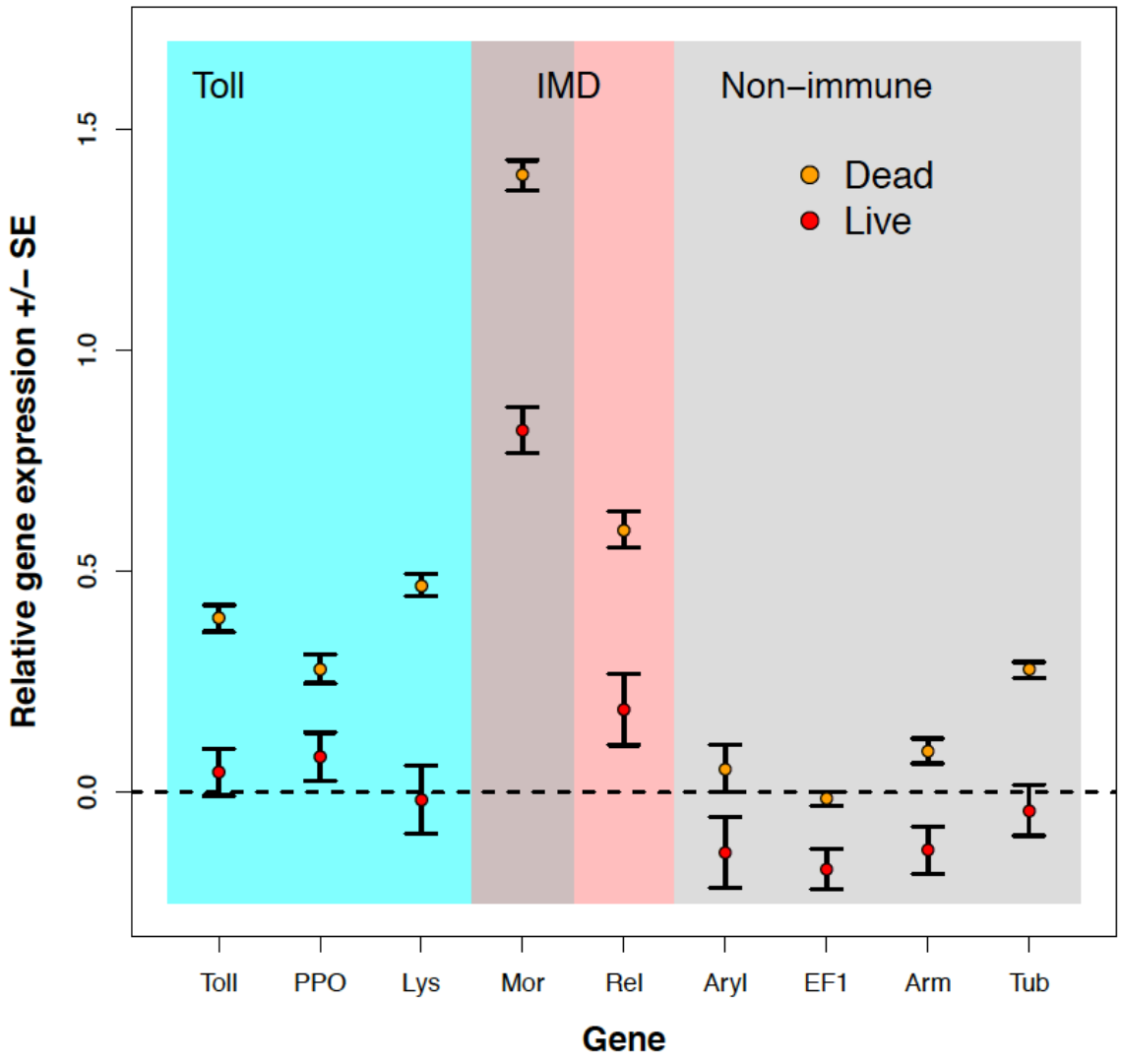

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822 **Figure 3 - Toll**

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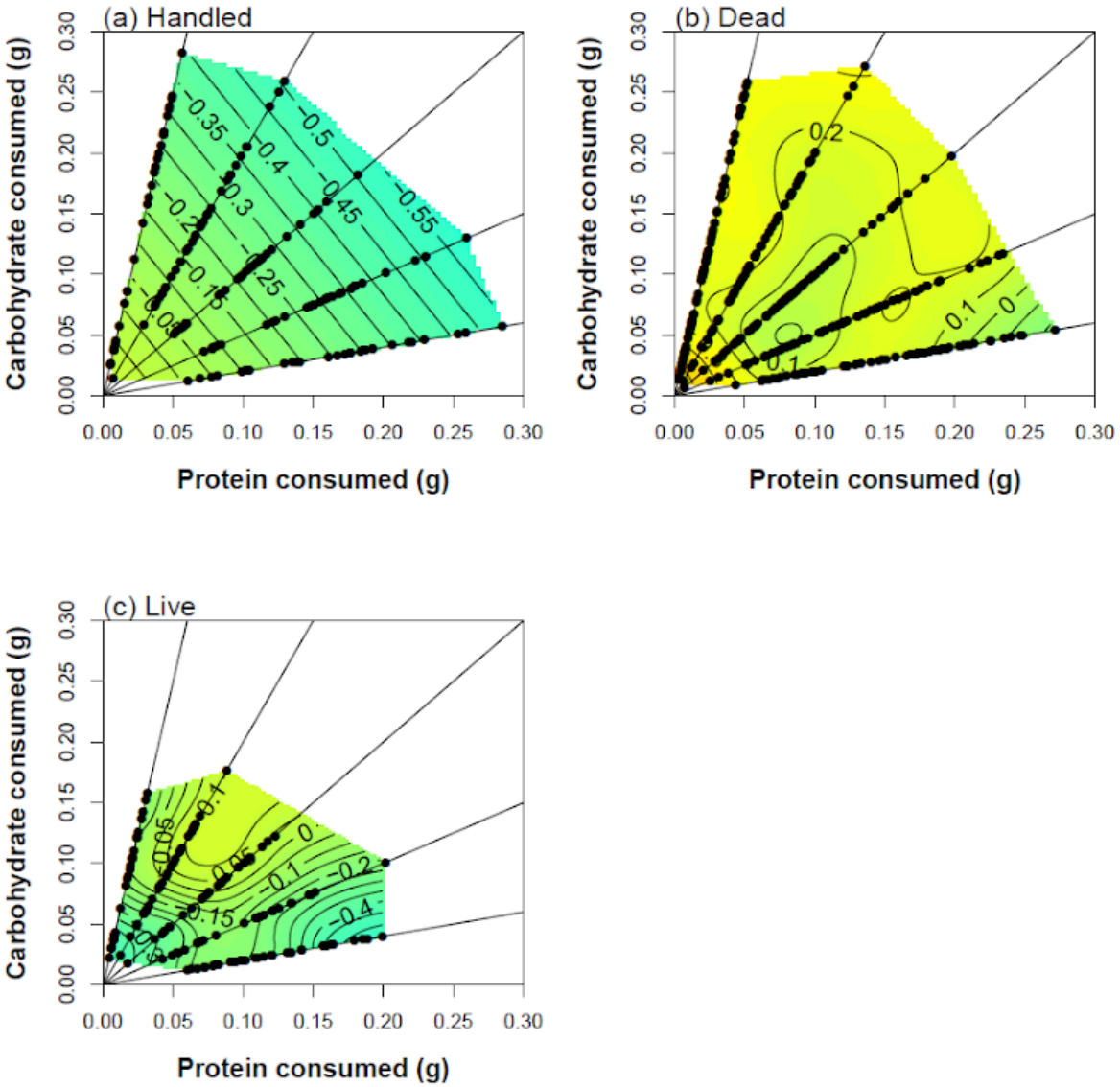

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826 **Figure 4 – PPO**

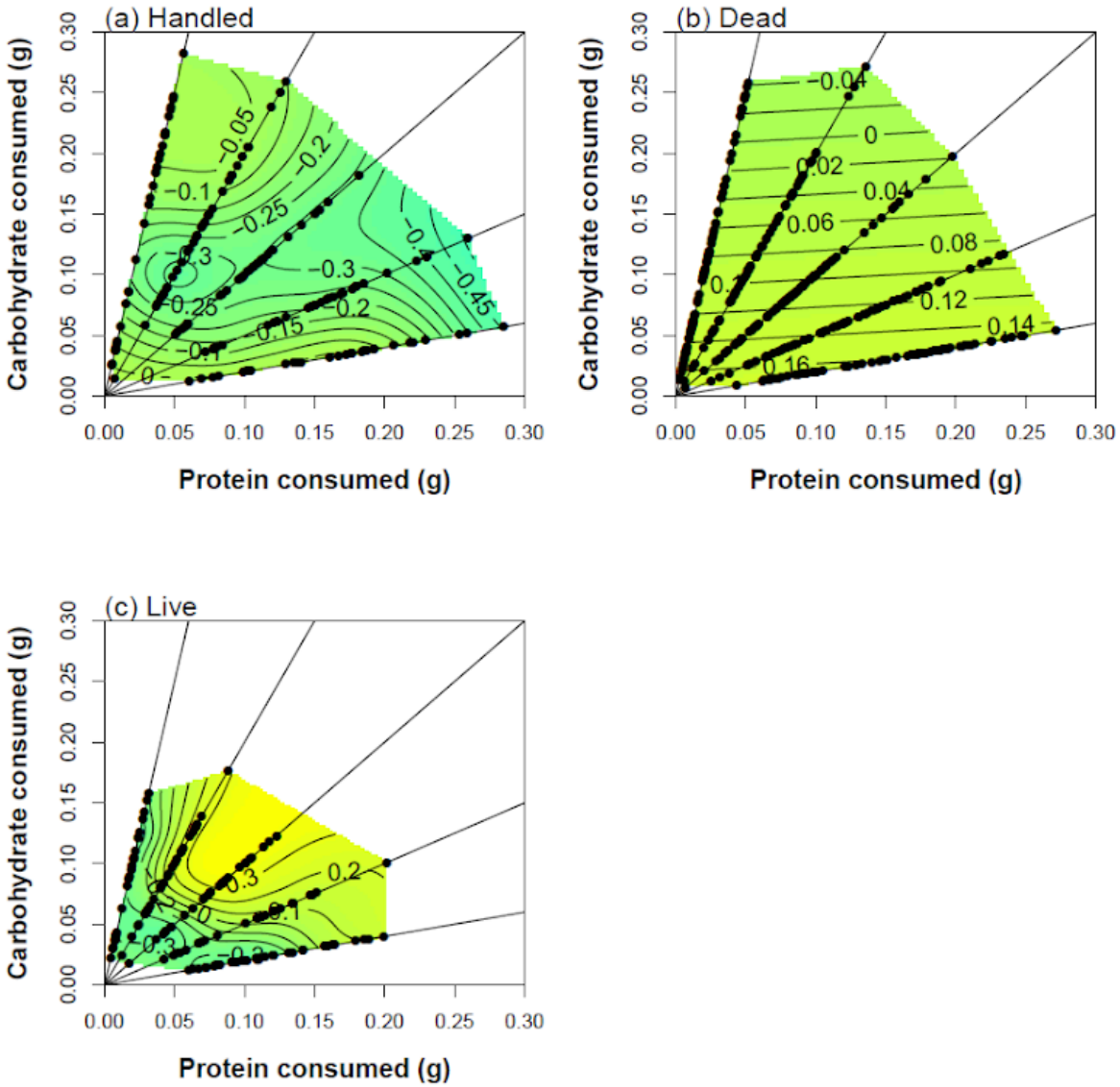

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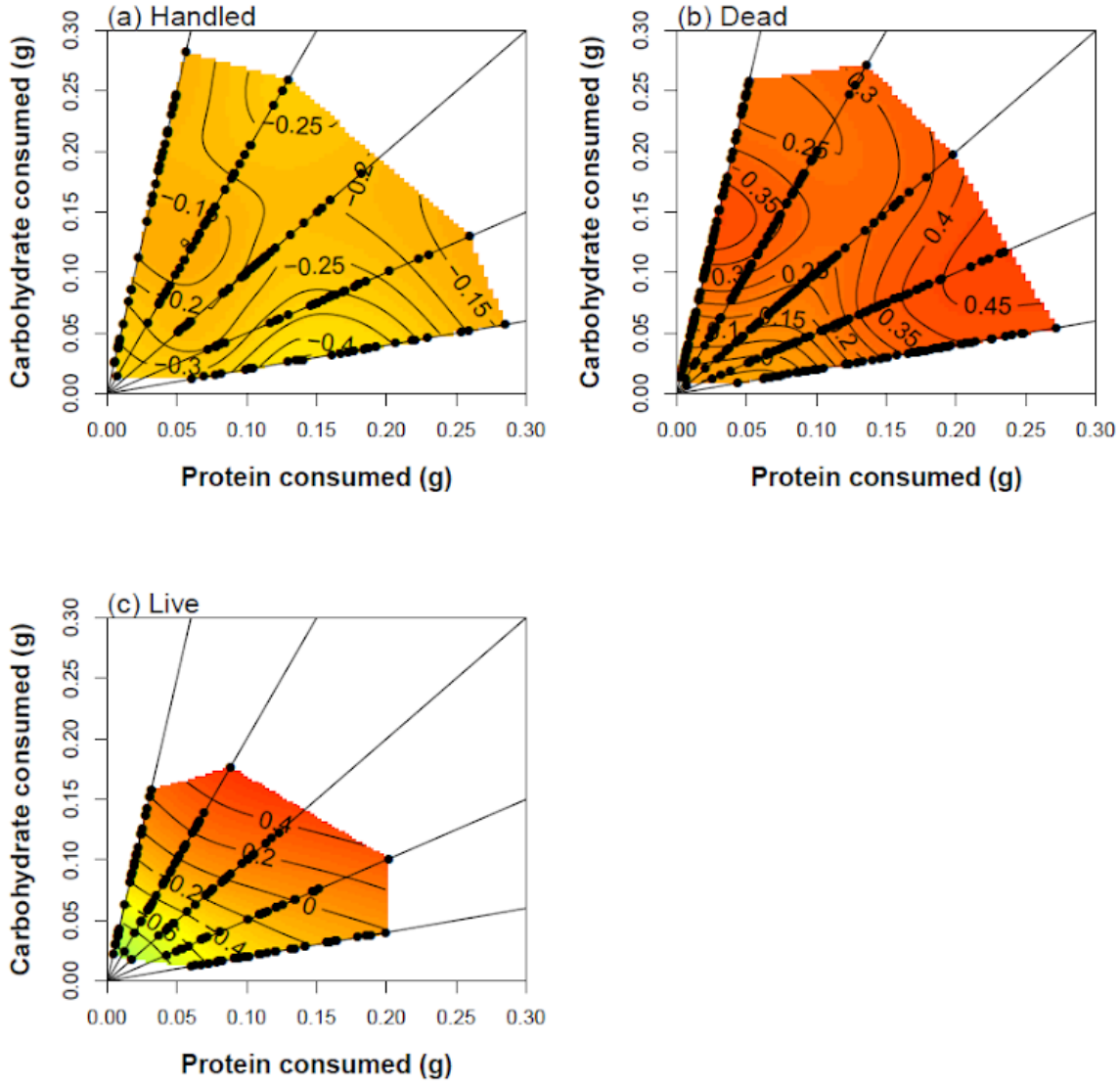
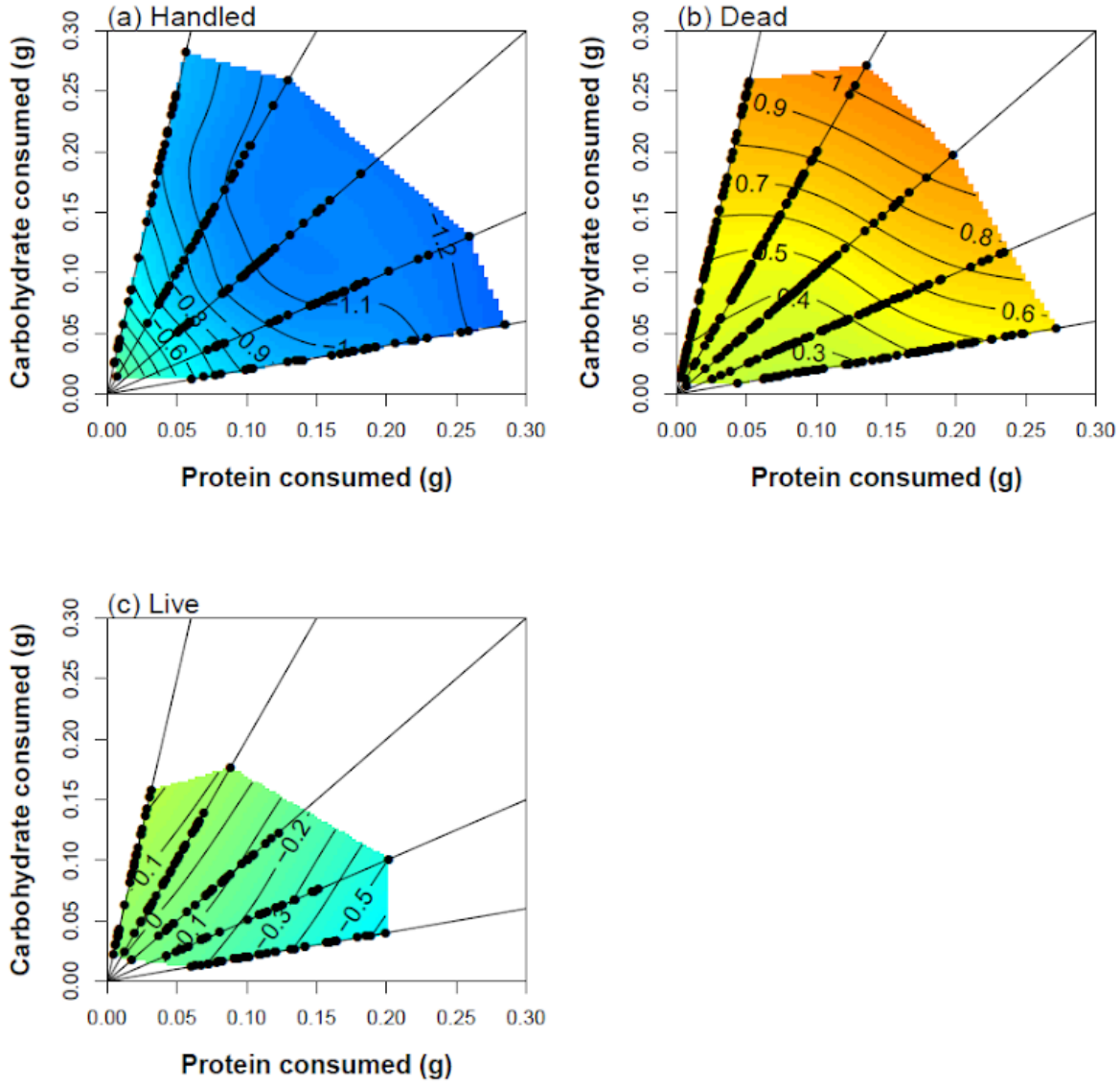
Figure 5 – Lysozyme



834 **Figure 6 - Moricin**



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838 **Figure 7 – Relish**

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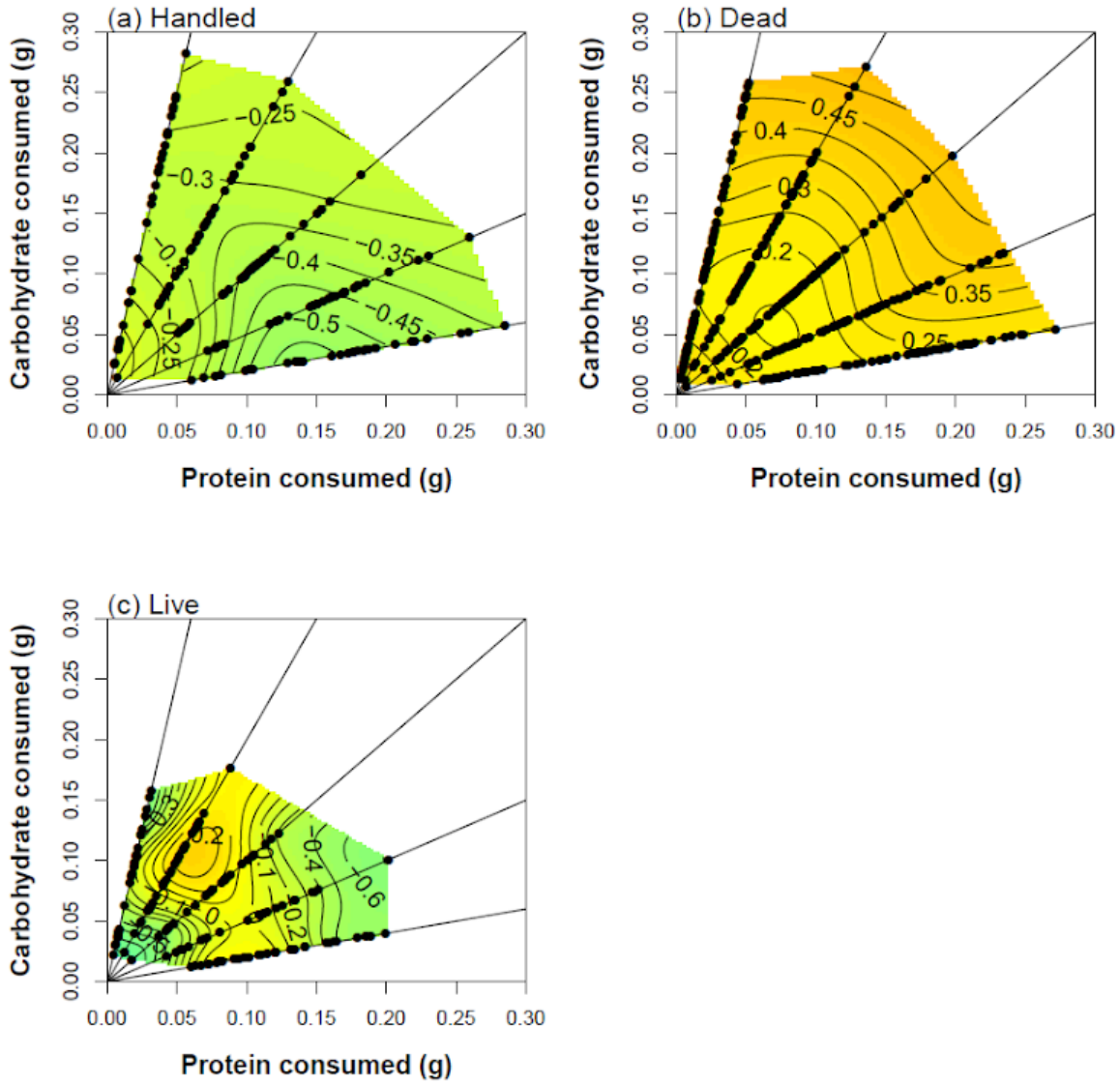

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842 **Figure 8 - Arylphorin**

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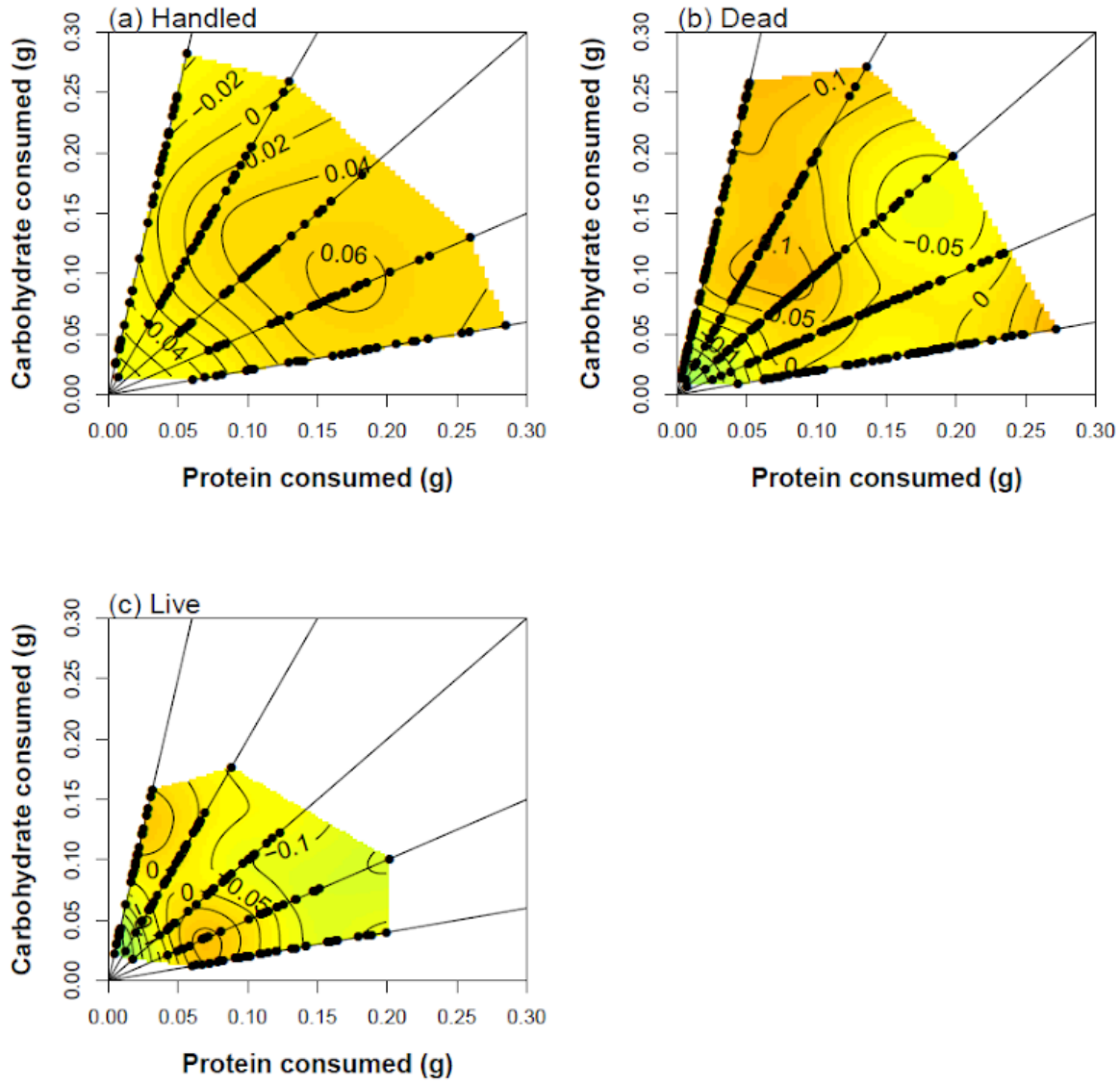

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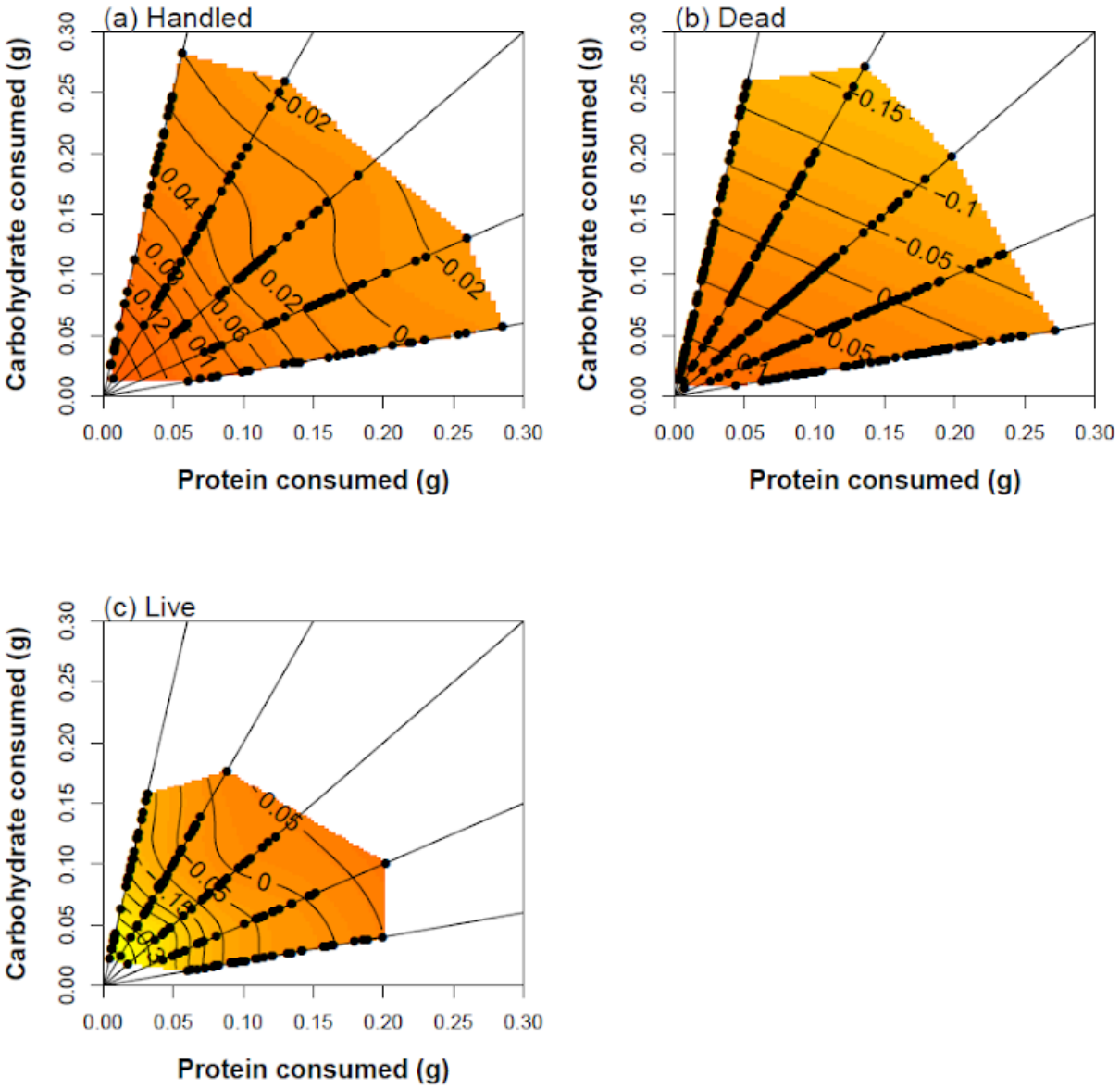
845 **Figure 9 – EF1**



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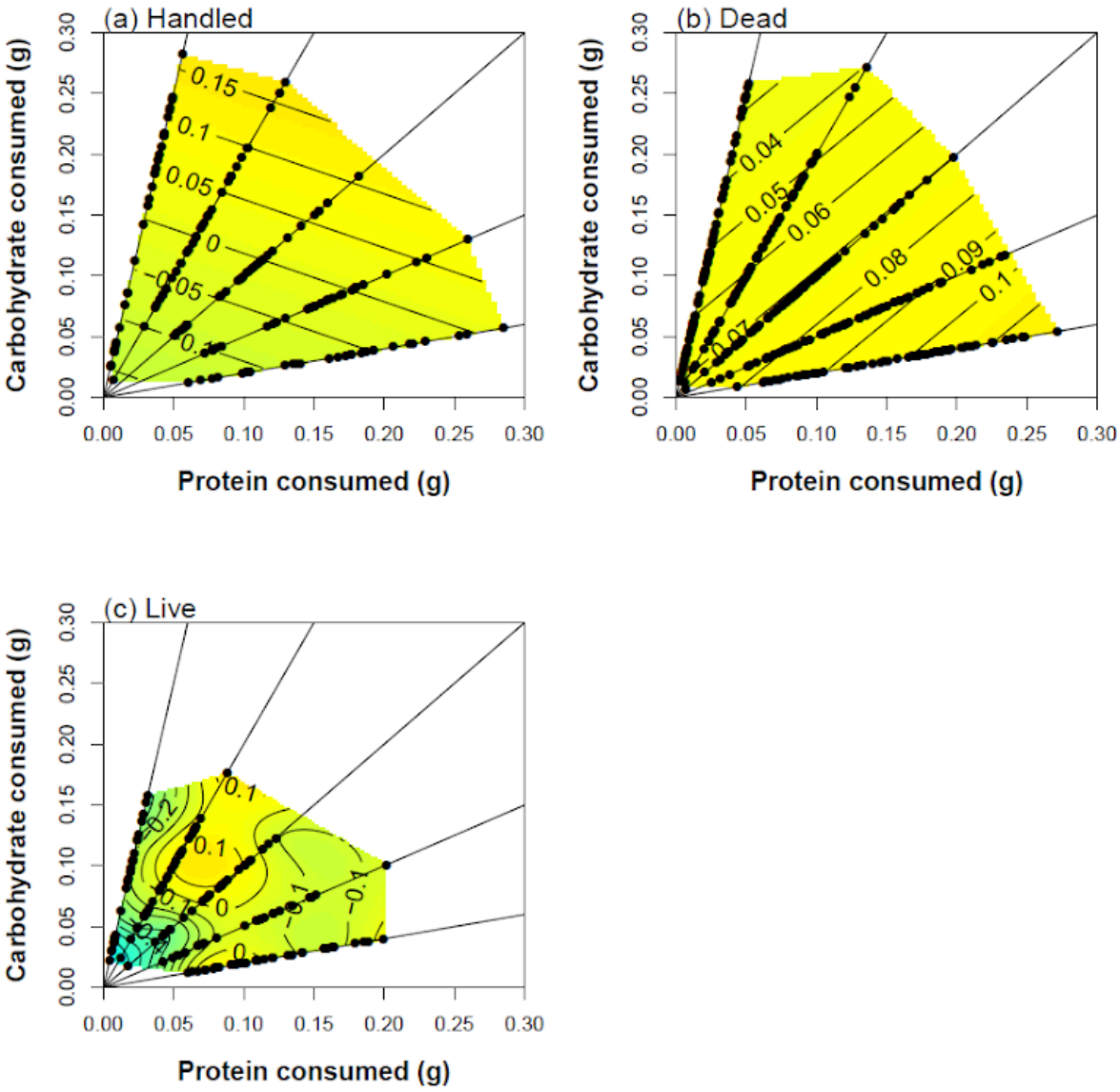
849 **Figure 10 – Armadillo**



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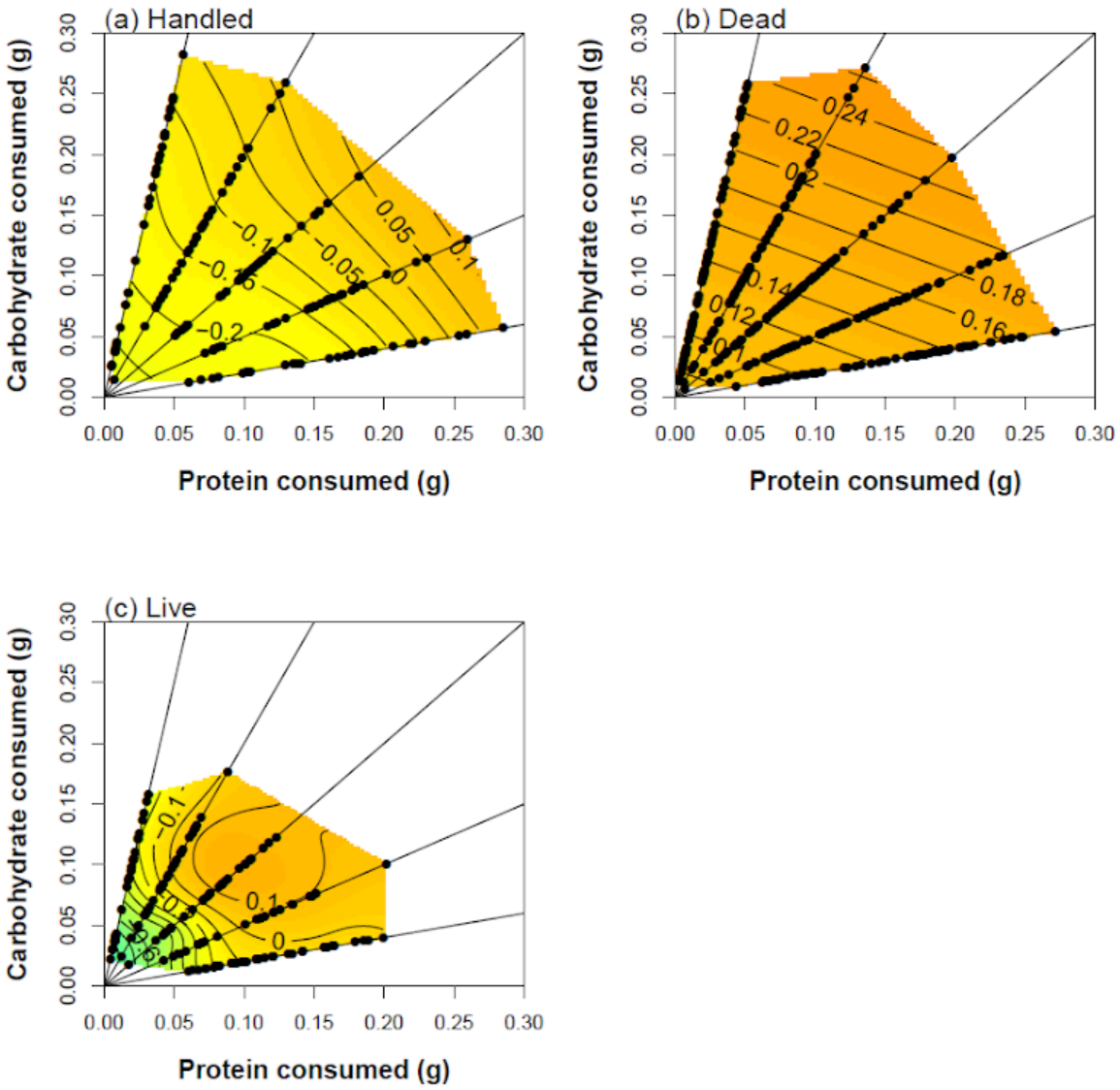
853 **Figure 11 – Tubulin**



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858 **Figure 12 – Lysozyme, PPO and PO activity**

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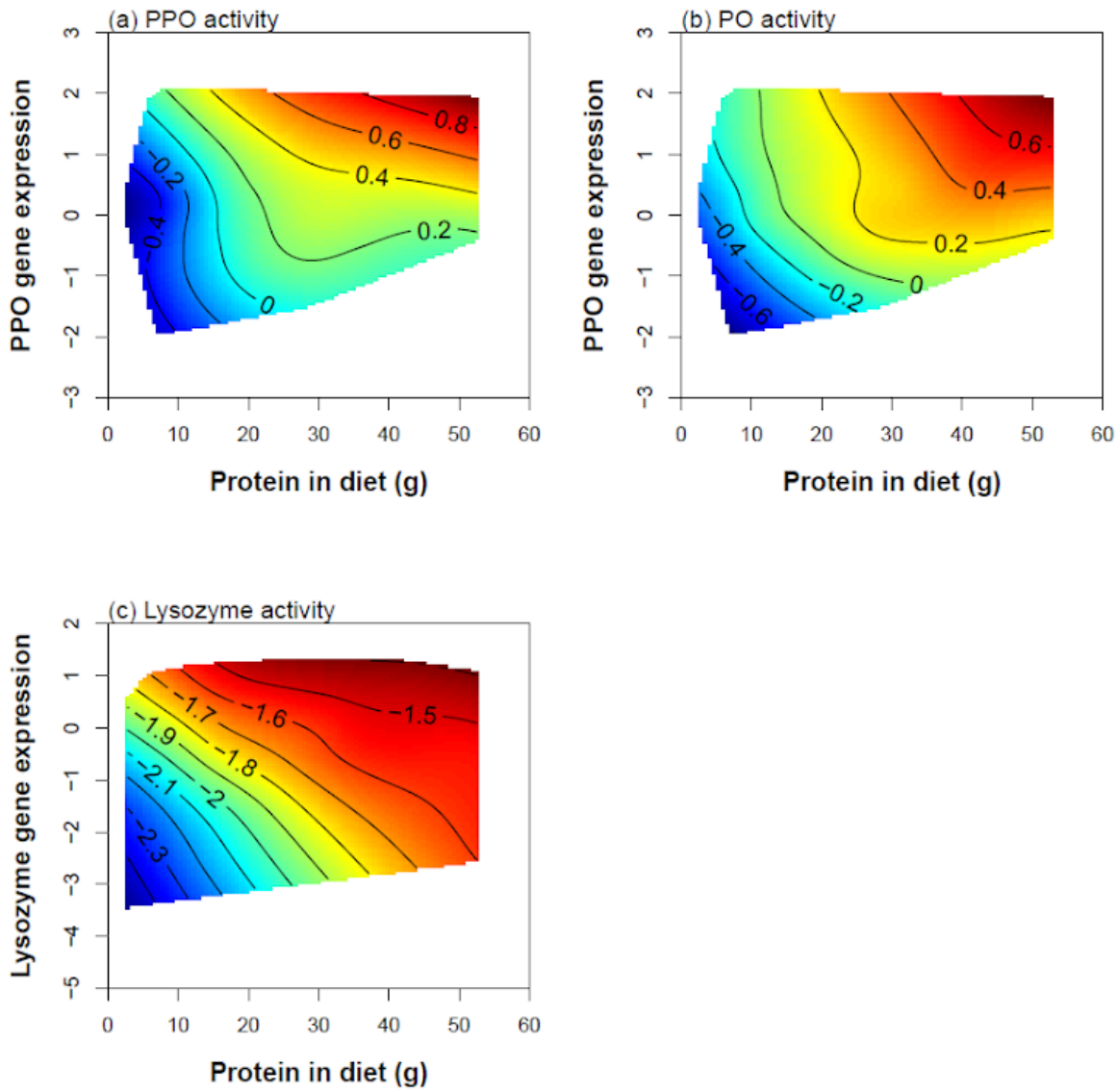

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