1 **Food quality affects the expression of antimicrobial peptide genes upon**
2 **simulated parasite attack in the larvae of greater wax moth**
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34

35 ## Abstract

36

37 Predator-prey interactions are an important evolutionary force affecting the immunity of the
38 prey. Parasitoids and mites pierce the cuticle of their prey, which respond by activating the
39 immune system against predatory attacks. Immunity is a costly function for the organism that
40 often competes with other life history traits for limited nutrients. We tested whether the
41 expression of antimicrobial peptides (AMP) of the larvae of the greater wax moth *Galleria*
42 *mellonella* changes as a consequence of an insertion of a nylon monofilament, which acts like a
43 synthetic parasite. The treatment was done for larvae grown on a high-quality diet and a low-
44 quality diet. The expression of *Gloverin* and *6-tox* were upregulated in response to the insertion
45 of the nylon monofilament. The expression of *6-tox*, *Cecropin-D* and *Gallerimycin* were
46 significantly higher in the 'low-quality diet' group than in the 'high-quality diet' group. Since
47 food quality seems to affect AMP gene expression in *G. mellonella* larvae, it should always be
48 controlled for in studies on bacterial and fungal infections in *G. mellonella*.

49

50

51 ## Introduction

52

53 Immunity is the ability of the organism to protect itself against invasions of foreign bodies such
54 as bacteria, viruses, parasitoids, parasites and toxic substances. The immune system keeps the
55 body healthy and free from infections, but each organism also has to allocate resources to a
56 variety of other life history functions—such as reproduction and development—in order to
57 improve its lifetime fitness (Stearns, 1992; Dillon et al., 2013; Minkov et al., 2015; Ellison
58 2017). The resources available to satisfy competing functions of an individual are limited

59 (Stearns, 1992). Susceptibility to disease is therefore higher under circumstances in which
60 investment in immunity is compromised. On the other hand, an over-active immune system may
61 cause considerable self-harm to an organism (Kraaijeveld & Godfray, 1997; Jensen et al., 2006;
62 Sadd & Siva-Jothy, 2006; Spottiswoode, 2008; Schmid-Hempel, 2011). Since immune function
63 is energetically expensive (e.g., Lochmiller & Deerenberg, 2000; Muehlenbein & Bribiescas,
64 2005; Ardia et al., 2012; Krams et al., 2012), nutritional quantity and quality are of particular
65 importance in life history trade-offs (Moret & Schmid-Hempel, 2000; Morehouse et al., 2010;
66 Ponton et al., 2013; Povey et al., 2013).

67 The innate immune response has physical and chemical barriers that exist as the first line
68 of defense against infectious pathogens (Schmid-Hempel, 2011). When ectoparasites attempt to
69 pierce insect exoskeleton (Smith, 1988; Robb & Forbes, 2005), the immune system reacts to the
70 challenge by attempting to encapsulate the feeding tubes of mites in a coating of cellular
71 materials and chemical deposits (e.g., Rantala et al., 2000; Krams, et al., 2011; Robb & Forbes,
72 2005). Therefore, the strength of immunity is often accessed via an encapsulation response to
73 the implantation of a nylon monofilament which acts as if the insert were a real parasite piercing
74 the host's exoskeleton. The encapsulation response is one of the frontline defenses during
75 pathogen invasion (de Melo et al., 2013). This response is correlated not only with
76 encapsulation of parasites (Paskewitz & Riehle, 1994; Gorman et al., 1998) but also with other
77 measures of immunity, such as the phenoloxidase cascade (Rantala et al., 2000, 2002, 2003) and
78 resistance to an entomopathogenic fungal disease (Rantala & Roff, 2007). During the
79 encapsulation reaction, the organism acts via its cellular and humoral responses. Haemocytes
80 phagocytose small foreign particles, or attach themselves to large foreign objects (Gupta, 1986;
81 Kanost, 2009; Krams et al., 2013). The foreign object may become completely encapsulated and
82 isolated from other host tissues as haemocytes attach to its surface and ultimately enclose it
83 (Grimstone et al., 1967; Lavine & Strand, 2002). This cellular response is aided also by a

84 humoral response, which consists of proteins such as antimicrobial peptides (AMP) that are able
85 to interfere with a parasitic intruder and regulate coagulation and melanization of haemolymph
86 (Hancock et al., 2006; Lavine & Strand, 2002; Schmid-Hempel, 2011).

87 The encapsulation is linked to all immune signaling pathways of insects (Lemaitre &
88 Hoffmann, 2007). Moreover, its strength largely depends on the availability and the nutritional
89 value of food (Krams et al., 2014) which makes the strength of encapsulation response difficult
90 to predict. In this study we investigated the expression of various immunity-related AMP genes
91 during the insertion of a nylon monofilament in haemocoel of the larvae of the greater wax moth
92 (*Galleria mellonella*) grown either on high-quality / diverse food or low quality food. AMPs
93 belong to an early component of innate immune response towards bacterial and fungal
94 infections. AMPs act as antibiotics that impose a lethal effect against invading organisms
95 (Zasloff, 2002; Brogden, 2005; Brown et al., 2009; Mylonakis et al., 2016) and modulate
96 pathogen load in the host's body (Kaneko et al., 2007). Higher expressions of certain AMP
97 genes were found to be associated with a 'dark morph' melanic strain of *G. mellonella* larvae,
98 making melanic insects able to mount an immediate immune response against invading fungi
99 (Dubovskiy et al., 2013a). We therefore expected that AMP genes would be more expressed in
100 the larvae with the activated immunity that are grown on high-quality macronutrient-rich food
101 than when grown on simple food of low nutritional value.
102
103

104 ## Materials and methods

105

106 **Insects, treatment groups and food quality**

107

108 We studied a captive population of *G. mellonella* consisting of individuals collected from

109 natural populations in Estonia in summer 2014. The moths were reared in 2.4 liter plastic boxes
110 at 28 ± 1°C in the dark in Sanyo MIR-253 incubators. To study the effects of diet diversity on
111 the expression of AMP genes of *G. mellonella* larvae, we assigned them to groups differing in
112 the macronutritional diversity/energetic value of the food. Each larva was kept individually in a
113 plastic container (50 ml) with a lid and wire-mesh to allow ventilation and to prevent
114 individuals from escaping.

115 In this study we had groups of *G. mellonella* larvae that differed in food quality and
116 activation of the immune system via nylon monofilament. All larvae received diverse food *ad*
117 *libitum* from hatching till day 14 posthatch (Krams *et al*., 2013) (Figure 1). The larvae were
118 subsequently assigned into the following four groups: (1) the 'high-quality diet / immune
119 treatment' group, (2) the 'high-quality diet / control' group, (3) the 'low-quality diet / immune
120 treatment' group and (4) the 'low-quality diet / control' group (Fig. 1). In the 'high-quality diet /
121 immune treatment' group the larvae were grown on diverse high-quality food provided *ad*
122 *libitum* until day 30 when each larva was subjected to a challenge to their immune system so
123 that a sterile nylon monofilament implant (2 mm length, 0.18 mm diameter, knotted at one end)
124 was inserted through their cuticle between the 3rd and 4th sternite (Krams et al., 2014) for 10 h
125 at 28 ± 0.5 °C. Upon this treatment the implants were removed and the larvae were used for
126 gene expression analysis. The larvae in the 'high-quality diet / control' group received high-
127 quality food until day 30 posthatch when their bodies were used for gene expression analysis
128 (Fig. 1). The immune system of these larvae was not activated by the nylon implants. The 'low-
129 quality diet / immune treatment' group was grown on *ad libitum* food of low quality from day
130 14 till day 30 posthatch and they were implanted with the nylon monofilament for 10 hours. The
131 'low-quality diet / control' group received *ad libitum* food of low quality between days 14 and
132 30 posthatch and the immune system of these individuals was not affected by nylon implants
133 before their bodies were studied for the expressions of AMP genes.

134 The high-quality diet consisted of a homogenized mix of equal proportions of honey,
135 glycerol, bee-wax, dried milk, wheat flour, dry yeast, distilled water and two servings of corn
136 meal. The food was not autoclaved. The amount of energy contained in this food was estimated
137 as ca. 16.90 kJ/g. The low-quality diet consisted of natural bee-wax with a 5% admixture of
138 corn meal. Bee-wax is a natural polymer produced by bees; it is considered to have a low
139 nutritional value (3.03 kJ/g) and it is hard to process in the gut. However, we have observed the
140 ability of some wild progenitors of our study population to reproduce solely on bee-wax (Krams
141 et al., 2014). What is more, larvae of *G. mellonella* have been recently found to consume
142 polymer polyethylene producing ethylene glycol (Bombelli et al., 2017). Thus, the larvae of the
143 low-quality food group received slightly better food than pure wax. The environment containing
144 high-quality food such as used in this study matches the situation that the *G. mellonella* larvae
145 enjoy during their initial stages of invasion into the beehive when the honeycomb contains bee
146 larvae, honey and pollen (Barjac & Thomson, 1970). The low-quality food environment of this
147 study matches those situations in which previous generations of greater wax moths have left
148 their larvae with honeycomb cells that contain no bee larvae, honey or pollen.
149
150 **RNA extraction and quantitative real-time PCR**
151
152 The highest concentrations of AMPs are found in tissues exposed to microorganisms or cell
153 types that are involved in host defense such as epithelia and glandular structures (Ouellette &
154 Selsted, 1996; Ganz, 2003). Since experimental treatments done in this study involved
155 manipulations with food and cuticular defense, these may affect AMP secretion both in the
156 midgut and cuticle. Therefore, we studied the expression of AMP genes from the whole body of
157 the larvae *G. mellonella*.

158 The larvae were chilled on ice for 15 min, surface-sterilized with 70% ethanol and their
159 whole bodies were disrupted in liquid nitrogen. We pooled six individual larvae for each
160 treatment group. RNA was obtained from three replicates of each of the four groups (96 larvae
161 in total). The larval bodies were further homogenized in 1 ml of Trizol reagent (Sigma-Aldrich),
162 and RNA was extracted according to the manufacturer's recommendations. RNA integrity was
163 confirmed by ethidium bromide gel staining, and quantities were determined
164 spectrophotometrically.

165 Levels of steady-state transcripts were determined from cDNA samples by real-time
166 quantitative PCR (RT-PCR) using DDC_t protocol with the 7500 Real-Time PCR System
167 (Applied Biosystems) and SYBR Green PCR mix (Qiagen), relative to two reference genes, *18S*
168 *rRNA* (AF286298; forward primer: CACATCCAAGGAAGGCAG, reverse primer:
169 AGTGTACTCATTCCGATTACGA) and *translation elongation factor 1-alpha* (EF1;
170 AF423811; forward primer: AACCTCCTTACAGTGAATCC, reverse primer:
171 ATGTTATCTCCGTGCCAG) (Vogel et al., 2011). Six target genes were investigated, coding
172 for AMPs: *Gloverin* (strong activity against gram-positive bacteria and weak activity against
173 gram-negative bacteria) (forward primer: AGATGCACGGTCCTACAG, reverse primer:
174 GATCGTAGGTGCCTTGTG), *Gallerimycin* (strong effect against filamentous fungi) (forward
175 primer: GAAGTCTACAGAATCACACGA, reverse primer: ATCGAAGACATTGACATCCA)
176 (Schuhmann et al., 2003), *6-tox* (an atypical defensin-derived immune-related peptide expressed
177 in midgut against invading bacteria) (forward primer: GACGAACTGCGAAGAATTATC,
178 reverse primer: TGTCTGTCTTGAGTTGCATATTG) (Lee et al., 2010), *Galiomicin* (strong
179 antifungal effect and limited effect against bacteria) (forward primer:
180 GTGCGACGAATTACACCTC, reverse primer: TACTCGCACCAACAATTGAC) and
181 *Cecropin D* (strong activity against Gram-negative bacteria and fungi, weak activity against
182 Gram-positive bacteria) (forward primer: CTGCGCCATGTTCTTCA, reverse primer:

183 TCGCATCTCTGATCCTCTG), the *Toll-like receptor 18-Wheeler* (necessary for activation of
184 all AMP genes in *Drosophila* larvae (Ligoxygakis et al., 2002), not affected by bacterial or
185 fungal infections in *G. mellonella* larvae (Dubovskiy et al., 2013b) (forward primer:
186 CGCTCTAGAATCGCATCGGCAACATCACC, reverse primer:
187 CGCGAATTCCGGAGAGATTCAGCCACAGCA). The primers were obtained from Metabion
188 International AG (Planegg, Germany).
189
190 **Statistical analysis**
191
192 The data from three independently repeated experimental trials were pooled after confirming
193 that "trial" as a factor had no significant effect on data variation (two-way ANOVA)
194 (Dubovskiy et al., 2013b). Individual gene comparisons were made with Kruskall-Wallis,
195 followed by Tukey's HSD post hoc tests if the non-parametric one-way ANOVA indicated a
196 significant variation.
197
198
199 **Results**
200
201 The results show a significant variation between the four diet/immunity treatment groups of the
202 larvae in the expression of *Gallerimycin* (Kruskal-Wallis chi-squared = 9.974, df = 3, *P*-value =
203 0.019), *Gloverin* (Kruskal-Wallis chi-squared = 9.462, df = 3, *P*-value = 0.024), *Cecropin-D*
204 (Kruskal-Wallis chi-squared = 9.974, df = 3, *P*-value = 0.019) and *6-tox* (Kruskal-Wallis chi-
205 squared = 10.385, df = 3, *P*-value = 0.016), while the expression of *Galiomicin* (Kruskal-Wallis
206 chi-squared = 6.846, df = 3, *P*-value = 0.077) and the *Toll-like receptor 18-Wheeler* (Kruskal-

207 Wallis chi-squared = 3.00, df = 3, P-value = 0.392) did not differ significantly between the
208 groups.

209 Post hoc tests revealed that the *Gallerimycin* AMP gene was expressed at a significantly
210 higher level in the ‘high-quality diet / control’ group than in the ‘low-quality diet / control’
211 group (Tukey HSD test: $P = 0.002$). *Gallerimycin* expressions were higher in the ‘high-quality
212 diet / control’ group than in the ‘high-quality diet / immune challenge’ group (Tukey HSD test:
213 $P = 0.005$) and in the ‘low-quality diet / immune challenge’ group (Tukey HSD test: $P = 0.003$).
214 However, we did not find any significant differences between the ‘low-quality diet / control’
215 and the ‘high-quality diet / immune challenge’ groups, nor the ‘low-quality diet / immune
216 challenge’ and the ‘high-quality diet / immune challenge’ groups, nor the ‘low-quality diet /
217 control’ and the ‘low-quality diet / immune challenge’ groups (all $P > 0.05$) (Figure 2).

218 The expression of *Gloverin* AMP gene was higher in the ‘low-quality diet / immune
219 challenge’ group than in the ‘low-quality diet / control’ group (Tukey HSD test: $P < 0.001$). The
220 expression of *Gloverin* in the ‘low-quality diet / immune challenge’ group was higher than in
221 the ‘high-quality diet / control’ group (Tukey HSD test: $P < 0.001$) and in the ‘high-quality diet
222 / immune challenge’ group (Tukey HSD $P < 0.001$). The *Gloverin* gene expression did not
223 differ between the ‘high-quality diet / immune challenge’ group of *G. mellonella* and the ‘low-
224 quality diet / control group’ and the ‘high-quality diet / control’ group (all $P > 0.05$) (Figure 3).

225 The *Cecropin-D* AMP gene was upregulated in the ‘high-quality diet / control’ group
226 compared to the ‘low-quality diet / control’ group (Tukey HSD test: $P = 0.001$). The expression
227 of *Cecropin-D* was higher in the ‘high-quality diet / control’ group than in the ‘high-quality diet
228 / immune challenge’ (Tukey HSD test: $P = 0.019$) and the ‘low-quality diet / immune challenge’
229 groups (Tukey HSD test: $P = 0.008$). The expression in the ‘low-quality diet / control’ group
230 was not statically different from the *Cecropin-D* gene expression in the ‘high-quality diet /
231 immune challenge’ group (Figure 4).

232 The expression of *6-tox* AMP gene was the highest in the 'high-quality diet / immune
233 challenge' group and it was significantly higher than the *6-tox* AMP gene expressions in the
234 'high-quality diet / control' group (Tukey HSD test: $P = 0.032$) and in the 'low-quality diet /
235 control' group (Tukey HSD test: $P < 0.001$). However, the expression of *6-tox* gene in the
236 'high-quality diet / immune challenge' group did not differ statistically from its expression in
237 the 'low-quality diet / immune challenge' group (Figure 5).
238
239
240 **Discussion**
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242 Evidence shows that *G. mellonella* larvae increase expression of *Gallerimycin*, *Gloverin*,
243 *Galiomicin* and *Cecropin-D* genes when infected by filamentous fungi (Wojda et al., 2009; Mak
244 et al., 2010; Xu et al., 2012; Dubovskiy et al., 2013a). Some studies suggest that the potency of
245 AMP depends on the fungal species and the strain (Zhang et al., 2009; Fullaondo et al., 2011).
246 Thus, the broad-spectrum AMP genes may be activated by a wide range of factors (Schuhmann
247 et al., 2003; Mak et al., 2010). For example, Dubovskiy et al. (2013a,b) observed an enormous
248 variation in expression of immunity-related AMP genes against fungal infections and explained
249 this as a simultaneous action of different kinds of stressors that work in concert with factors
250 linked to melanism, stress adaptation, detoxification, and inflammation. Our results support the
251 previous findings, showing that AMP gene expressions are highly variable also during
252 implantation of the nylon monofilament mimicking the parasite/parasitoid attack. The immune
253 responses against nylon monofilament insertion and fungal infections may be similar because
254 both the insert and fungi penetrate the insect cuticle in a similar way. However, our results show
255 that there are considerable differences between the effects caused by fungal infections and
256 insertion of the nylon monofilament. We also suggest that the quality / diversity of larval diet

257 may cause a considerable source of variation in the expression of AMP genes (see also Adamo
258 et al., 2016) and that food-borne effects interfere with the effects caused by insertion of the
259 nylon monofilament.

260 *Gloverin* and *6-tox* were the AMPs that became increasingly expressed because of the
261 nylon monofilament. However, the upregulation of *Gloverin* and *6-tox* was seen only in the
262 'low-quality food / immune treatment' group. *Cecropin-D* and *Gallerimycin* expressions were
263 downregulated upon implantation of the nylon monofilament. This was seen only in the 'high-
264 quality diet / immune treatment' group, suggesting that the more diverse high-quality diet may
265 fuel some other parts of the immune defense rather than activate the production of *Cecropin-D*
266 and *Gallerimycin*. This might be supported by a non-significant upregulation of *Cecropin-D*
267 gene expression in the 'low-quality diet / immune treatment' group. We did not find any
268 significant changes in expressions of *18-Wheeler*, *6-tox* and *Galiomicin* followed the
269 implantation procedure.

270 In *Drosophila melanogaster*, *18-Wheeler* has been proposed to be directed against
271 Gram-negative and Gram-positive bacteria (Ligoxygakis et al., 2002), while in *G. mellonella* it
272 was found to be facultative for immune responses (Dubovskiy et al., 2013b). Our results reveal
273 that *18-Wheeler* is not involved in recognition of such a foreign body as the nylon
274 monofilament. Besides *Gallerimycin* (Langen et al., 2006), *Galiomicin* is the defensin-like
275 antifungal peptide (Lee et al., 2004) which is not used by *G. mellonella* larvae in their responses
276 against the nylon inserts. Although the encapsulation response begins as soon as the cuticle of
277 an insect is pierced, the response may last for hours (Dubovskiy et al., 2010) or days
278 (Eggenberger et al., 1990; Schmit & Ratcliffe, 1977; Krams et al., 2013). Hence, it would be
279 important to study the kinetics of AMP gene expression because elevated expressions of
280 *Gallerimycin*, *Cecropin-D* and *Gloverin* were highly upregulated by 48 h after fungal infection;
281 in contrast, by 24 h after infection, expression of these AMPs was found to be either slightly

282 upregulated or not affected at all (Dubovskiy et al., 2013b), a finding replicated in the current
283 study.

284 Food diversity did not affect the expression of *18-Weeler*, *Galiomicin*, *Gloverin*, while
285 the expression of *6-tox*, *Cecropin-D*, *Gallerimycin* significantly increased from the 'low-quality
286 diet / control' group to the 'high-quality diet / control' group. The composition of gut
287 microbiomes is known to be structured through diet (Muegge et al., 2011) and the increase in
288 the diversity of nutrients positively affects symbiont numbers and microbiota diversity (David et
289 al., 2014; Carmody et al., 2015; Sonnenburg et al., 2016). It is known that the microbiome is of
290 high importance in maintaining homeostasis of the host's body (Russell & Dunn, 1996;
291 Chatelier et al., 2013). A recent study showed that host and symbiont communities
292 cooperatively interact to maintain the midgut microbiota in a symbiotic balance (Johnston &
293 Rolff, 2015), suggesting that the host needs more control over symbionts by means of AMP
294 proteins. Symbionts may become pathogenic if they grow and reproduce uncontrollably,
295 diverting resources away from growth and other needs of the host if not controlled by the host's
296 immune system (Erdogan & Rao, 2015; Fujimori, 2015). Importantly, the food of *G. mellonella*
297 was not sterilized in this study, which makes it possible that resident microbes in the gut may be
298 flushed away by a downstream flow of ingested content (Nyholm & McFall-Ngai, 2004; Blum
299 et al., 2013) and replaced by opportunistic or pathogenic bacteria (Jones et al., 2013; Cariveau et
300 al., 2014). This could also be a reason behind the increased expressions of *6-tox*, *Cecropin-D*
301 and *Gallerimycin*. One more possibility is that a high-quality diet results in a higher probability
302 of opportunistic infections entering the midgut of the larvae, while the upregulation of AMP
303 gene expression may indicate a prophylactic response by the host (Barnes & Siva-Jothy, 2000;
304 Krams et al., 2016).

305 In conclusion, the knowledge about antibacterial and antifungal properties of AMPs was
306 not helpful in predicting their expression in response to the insertion of a nylon monofilament –

307 a ‘synthetic parasite’. This may be partly explained by the elevated expression of certain
308 immunity-related AMP in response to more diverse diet. Our results suggest that not only food
309 quantity (Adamo et al., 2016) but also food *quality* affects immune responses of *G. mellonella*
310 larvae. In future research it is necessary to test whether the heightened expression of some
311 AMPs represents a surveillance system that recognizes and attacks the intruders entering the
312 host’s body with more diverse food, or whether this is a response to pathogens that have already
313 breached the host’s defense system. In this study, however, we did not observe any increased
314 mortality of the larvae associated with food of higher quality. *G. mellonella* is often used as a
315 model host to study interactions between human pathogens and microbiota (e.g., Glavis-Bloom
316 et al., 2012; Mukherjee et al., 2013), and so future research could combine different types of
317 food and bacterial/fungal infections to see possible effects of food on pathogen virulence, the
318 variation of the host’s immune responses and tolerance against infections. Manipulation of
319 specific nutrients provides better control for isolating the dietary causes of immunological
320 responses (Ponton et al., 2011, 2013; Povey et al., 2013). This is especially important in *G.*
321 *mellonella* because the larval food in this species consists of bee-wax and honey, both
322 possessing substantial antibacterial properties (Fratini et al., 2016). Most likely the antibacterial
323 properties of food explain the dominance of *Enterococci mundtii* (syn. *Streptococcus faecalis*
324 Andrewes and Horder) in the midgut of *G. mellonella* (Jarosz, 1979; Johnston & Rolff, 2015).
325 This microbe is a heritable nutrient-providing symbiont of *G. mellonella* (Bucher, 1963;
326 Johnston & Rolff, 2015) that is transmitted vertically − from mother to offspring (Chen et al.,
327 2016). It is probably among those rare microorganisms that can survive under the antibacterial
328 properties of the *G. mellonella* diet.

329 Finally, we would like to suggest an approach based on a combination of diet-level
330 studies like the present one and nutrient-level experiments done earlier (Ponton et al., 2011,
331 2013; Povey et al. 2013). Food manipulation experiments such as those performed in the current

332 study are effective for establishing the overall impacts of different types of food resources and
333 to find specific pointers of which nutrients are likely to be involved in organismal growth and
334 which to manipulate in nutrient-level experiments. The nutrient-level analysis would be the next
335 important step in order to analyze the specific actions and roles of each nutrient separately.
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337
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557

558

559 **FIGURE LEGENDS**

560

561 **Figure 1** The experimental protocol used to study effects of diet diversity on the expression of

562 AMP genes of greater wax moth larvae.

563

564 **Figure 2** Mean (± SEM) fold mRNA expression levels of (A) Gallerimycin, (B) Gloverin, (C)

565 Cecropin-D, (D) *6-tox* gene in the whole body samples of the greater wax moth larvae grown on

566 high-quality and low-quality diets that received the nylon implant or did not receive the implant.

567 Lower-case letters 'a', 'b' and 'c' denote significant differences by post hoc tests at $P < 0.05$.

568 For instance, the 'ab' bar significantly differs from the 'c' bar, while the 'ab' bar does not

569 significantly differ from the 'a' and 'b' bars.

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