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1 # Costs and benefits of plant allelochemicals in herbivore diet in a
2 multi enemy world

3

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Abstract

Sequestration of plant defensive chemicals by herbivorous insects is a way to defend themselves against their natural enemies. Such herbivores have repeatedly evolved bright colours to advertise their unpalatability to predators, i.e. they are aposematic. This often comes with a cost. In this study we examined the costs and benefits of sequestration of iridoid glycosides (IGs) by the generalist aposematic herbivore, the wood tiger moth, *Parasemia plantaginis*. We also asked whether the defence against one enemy (a predator) is also effective against another (a parasitoid). We found that the larvae excrete most of the IGs and only small amounts are found in the larvae. Nevertheless, the amounts present in the larvae are sufficient to deter ant predators and play a role in defence against parasitoids too. However, excreting and handling these defensive plant compounds is costly, leading to longer development time and lower pupal mass. Interestingly, the warning signal efficiency and the amount of IGs in the larvae of *P. plantaginis* are negatively correlated; larvae with less efficient warning signals contain higher levels of chemical defence compounds. Our results may imply that there is a trade-off between production and maintenance of coloration and chemical defence. Although feeding on a diet containing IGs can have life-history costs, it offers multiple benefits in the defence against predators and parasitoids.

Key words bio assay, *Cotesia villana*, iridoid glycosides, *Plantago lanceolata*, warning signal

Introduction

Insect herbivores in several different orders have the ability to sequester defensive compounds from their host plants and use these compounds for their own defence (Duffey 1980; Nishida 2002). Price et al. (1980) already stated that plant-herbivore interactions both affect and are affected by their relationships with the third trophic level, their natural enemies (predators, parasitoids, or parasites). The interaction of plants, herbivores, and natural enemies is an active field in evolutionary ecology, the role of sequestered plant defence chemicals (allelochemicals) on herbivore susceptibility to parasitoids and parasitoid success has been explored in several studies (e.g. Barbosa et al. 1986; Gunasena et al. 1990; Singer et al. 2004). These interactions are often mediated through the sequestration of plant defence chemicals by herbivores.

Insects that are unpalatable do not only use their bad taste or unpleasant odour as a defence, but they can advertise this defence to potential enemies, e.g. by conspicuous coloration (Bowers 1993), however the cost of being conspicuous to predators can only be borne by sufficiently-defended individuals (Guilford and Dawkins 1993; Lee et al. 2010; Sherratt 2002). This phenomenon, known as aposematism, is widely observed across taxa and habitats, but it is especially common in herbivorous species (e.g. Nishida 2002). Possession of unpalatable qualities, coupled with advertisement of those qualities, has many consequences for the life history features, population biology, physiology and foraging behaviour of aposematic herbivores (Bowers 1993; Lindstedt et al. 2010). If both conspicuous warning signals and higher levels of chemical defence are expensive to produce and they compete for the same resources, such as antioxidants (e.g. Blount et al. 2009; Leimar et al. 1986), these two traits can be negatively correlated.

53 Sequestration of defence compounds from larval host plants may require particular
54 physiological adaptations by larvae to ingest, accumulate and store those compounds (Bowers
55 1992; Brattsten 1988) and this may come at a cost (e.g. lower mass, longer development time,
56 lower fecundity) (Bowers and Collinge 1992; Higginson 2011; Lindstedt et al. 2010; Ojala et al.
57 2007). Therefore, most insects that sequestrate plant secondary compounds are specialists,
58 feeding on one or a few plant species from which they acquire their defensive compounds
59 (Bowers 1988), only needing physiological mechanisms for handling specific compounds. In
60 addition to these specialists there are a few generalists able to sequester host-plant secondary
61 compounds as well. The lubber grasshopper (*Romalea guttata*) is a generalist species and has a
62 defensive secretion normally containing phenolics and quinones synthesized by the insect.
63 However, when insects are reared on a restricted diet of wild onion, they sequester sulfur
64 volatiles from the plant into their defensive secretions (Jones et al. 1989). A group of generalist
65 moths, the Arctiinae (Family Erebidae) (Rothschild 1985) are also able to sequester plant
66 compounds (Bowers and Stamp 1997; Rothschild et al. 1979; Singer et al. 2004; Weller et al.
67 1999).

68 Iridoid glycosides (IGs) are one group of plant secondary compounds that are sequestered
69 by several groups of insects (Bowers 1991). In some of these species, e.g. the checkerspot
70 butterflies (*Euphydryas* spp., Nymphalinae), the IGs are retained through the adult stage (Bowers
71 1991; Rimpler 1991; Stermitz et al. 1994; Suomi et al. 2003). This is in contrast to the buckeye
72 butterfly, *Junonia coenia* (Nymphalinae), which stores IGs during larval and pupal stages but
73 eliminates them at adult eclosion (Bowers and Collinge 1992). It is known that some Arctiine
74 moth larvae, which are generalist feeders, also concentrate IGs (Bowers and Stamp 1997;
75 Lampert and Bowers 2010). This suggests that the physiology of generalist Arctiine moths that
76 sequester secondary compounds may allow them to sequester a wide variety of compounds (e.g.

77 alkaloids Von Nickisch-Rosenegk and Wink 1993), although they may be less efficient than
78 species that sequester a single class of plant compounds (Johnson 1999).
79 A diversity of natural enemies (e.g. pathogens, predators and parasitoids) are the primary
80 source of mortality for larvae (Cornell and Hawkins 1995; Dempster 1983), increasing in
81 importance in older larvae and the pupal stage (Cornell et al. 1998). Studies of parasitoid-arctiine
82 larvae interaction indicate that the features that appear to be important in protecting larvae from
83 predators, such as sequestration of plant chemical compounds, bright coloration and the presence
84 of hairs or spines (Dyer 1995; Dyer 1997; Lindstedt et al. 2008), may not be as effective against
85 parasitoids (Gentry and Dyer 2002; but see Singer et al. 2009). Sequestration of plant chemicals
86 may even be beneficial for parasitoids, because they live in a chemically protected larva and are
87 thus protected themselves as well (Gauld and Gaston 1994). It has also been shown that
88 allelochemicals in the host diet weaken the immune system of the herbivore (Smilanich et al.
89 2009), this would benefit the developing parasitoids by enabling them to reallocate metabolic
90 energy from immunosuppression or avoidance to growth and development (Kraaijeveld and
91 Godfray 1997; Ojala et al. 2005). On the other hand, host plant secondary metabolites can be
92 harmful for insects, and detoxification can be energetically costly (Berenbaum and Zangerl 1993;
93 Després et al. 2007), which results in lower host quality for the parasitoid (Smilanich et al. 2009).
94 Furthermore, if the parasitoid cannot avoid or tolerate the sequestered plant chemicals, they may
95 have detrimental effects for them (Gauld and Gaston 1994).
96 In this study we tested the effects of plant defence chemicals in a multitrophic system,
97 starting with differences in the levels of defence chemicals in the plant, sequestered via the
98 aposematic larva. We examined the ability of the generalist Arctiine moth (*P. plantaginis*) to
99 sequester IGs from a diet containing only *Plantago lanceolata* and the effects of different
100 amounts of IGs in this diet on its performance. We knew from a previous study that these

101 caterpillars are able to sequester small amounts of IGs (Lindstedt et al. 2010) and it is know from
102 other Arctiine moths that they can sequester plant defence chemicals (Bowers and Stamp 1997;
103 Rothschild et al. 1979; Weller et al. 1999). Further we assumed that higher levels of IGs in their
104 diet would have more negative effects on their performance. Second we tested if chemical
105 defence (sequestered IGs) was affective against predators (ants): we performed a bioassay
106 experiment to determine the deterrent effects of the IGs aucubin and catalpol on ants, both
107 compounds separate and combined, and tested whether extracts from larvae that had eaten a diet
108 that contained IGs were less palatable for ants than larvae with an IG-free diet. Last we measured
109 the parasitism rate of larvae in their natural environment in a field experiment. The parasitoid
110 wasps from the field experiment were used under lab conditions to examine the effect of host
111 plant species on parasitoid performance.
112
113 **Material and Methods**
114 *Study Organism*. The generalist *P. plantaginis* (family Erebidae, subfamily Arctiinae; formerly
115 Arctiidae) has warningly coloured larvae and adults and is unpalatable to several vertebrate and
116 invertebrate predators (Lindstedt et al. 2008; Nokelainen et al. 2012). Their natural diet consists
117 mainly of the host plants *Plantago lanceolata* (narrow leaf plantain), *Plantago major* (broad leaf
118 plantain), *Rumex oblongifolius* (bluntleaf dock), *Senecio vulgaris* (Groundsel), *Hieracium*
119 *pilosella* (mouse-ear hawkweed), *Vaccinium myrtillus* (myrtle blueberry), *Taraxacum*
120 *campylodes* and *T. officinale* (dandelion) (Bellman 2007; Lindsey 2006; Robinson et al. 2010 and
121 own obeservation). The larvae are hairy in all instars, but their colouration changes from (cryptic)
122 greenish grey in the first two instars to orange-black in the third instar and onwards. They pupate
123 after five to seven instars (Ojala et al. 2007). In Finland this species has one generation per year,

124 and overwinters as a larva. In the laboratory, *P. plantaginis* can produce two to three generations
125 of which the second or third generation overwinters.

126 Larval signal size (the orange patch on the black body) varies continuously in *P.*
127 *plantaginis* larvae (Ojala et al. 2007) and is highly heritable (Lindstedt et al. 2009). In the
128 laboratory we have artificially selected for the extremes of this signal size continuum and created
129 two selection lines, small and large signal (for details see Lindstedt et al. 2009). For our
130 experiments we used larvae derived from these two selection lines with small (3 or less body
131 segments orange) and large orange signal (5 or more body segments orange). A previous study by
132 Lindstedt et al. (2008) showed that the predator *Parus major* (great tit) learns to avoid larvae with
133 a large signal faster than larvae with a small signal. The orange patch against a black body had a
134 high signal value for predators. The avoidance learning rate was higher when larvae had an
135 orange patch than when larvae were without one. The size of the orange signal also mattered. A
136 large patch enhanced the avoidance learning rate of avian predators, as shown by a longer latency
137 to attack (Lindstedt et al. 2008).

138 *Plantago lanceolata* is a perennial herb with a worldwide distribution and high ecological
139 amplitude (Sagar and Harper 1964). The distribution ranges of *P. lanceolata* and *P. plantaginis*
140 overlap, and *P. lanceolata* is a known host plant of *P. plantaginis* larvae (Bellman 2007; Pabis
141 2007). The main defence compounds of *P. lanceolata* are the IGs, aucubin and catalpol (Duff et
142 al. 1965), they play an important role in plant-insect interactions as chemical defence compounds.
143 Many herbivores are known to sequester them and use them for their own defence against natural
144 enemies (Camara 1997; Dyer and Bowers 1996; Nieminen et al. 2003; Willinger and Dobler
145 2001).

146 In wild populations, the IG levels of *P. lanceolata* range from undetectable to 12% of
147 their dry weight in the youngest leaves (Bowers et al. 1992; Bowers and Stamp 1992). Previous

148 studies have identified plant age as an important intrinsic factor affecting *P. lanceolata* chemistry
149 (e.g. Barton 2007; Bowers and Stamp 1993; Fuchs and Bowers 2004); and these ontogenetic
150 trajectories in IG production significantly vary among both maternal families and populations
151 (Barton 2007; Bowers and Stamp 1993) and are highly heritable (Marak et al. 2000). The plants
152 used for this study were offspring from plants derived from an artificial selection experiment in
153 which plants were selected on the basis of high and low concentration of total leaf IGs for four
154 generations (for details see Marak et al. 2000).
155
156 *Sequestration and performance experiment*
157 To determine if *P. plantaginis* larvae are able to take up IGs from their diet, and to see if different
158 levels of IGs in their diet have on effect on their performance, we conducted a sequestration
159 experiment. For this experiment we used larvae from both selection lines, due to availability only
160 10 families from the small signal selection line and 20 families from the large signal selection
161 line were used. The larvae were fed six different diets. Each diet consisted of plant clones from
162 one *P. lanceolata* genotype. All different genotypes used differed significantly in their IG levels
163 (Table 1). In total we used 60 larvae from the small selection line (10 larvae per diet treatment)
164 and 120 larvae from the large selection line (20 larvae per diet treatment). The plants used for the
165 diets were grown in a greenhouse at the University of Jyväskylä. Larvae were reared individually
166 in a petri-dish and given fresh leaves every day. We measured their development time, signal size
167 (number of orange segments) and pupal mass. We froze 3-8 larvae per diet for analyses with high
168 performance anion exchange chromatograph with pulsed amperometric detection (HPAEC-PAD).
169 Furthermore, we collected samples of their diet and their droppings during the experiment for
170 HPAEC-PAD analyses (see chemical analyses). The IGs (aucubin and catalpol) of three random
171 samples per diet were measured at two points in time. We also collected the droppings of each

172 larva for IG analyses at these two time points (one at the start of the experiment and the second
173 one month later).
174
175 *Bioassay experiment with ants*
176 In a previous study (Lindstedt 2008) *P. plantaginis* larvae were attacked by ants, but not
177 preferred and none of the larvae were killed. With the bioassay we wanted to exclude all other
178 factors to separate the chemical defence from other defences (e.g. hairiness, warning coloration).
179 One common bioassay method for assessing deterrence of specific toxins is to offer sugar
180 solutions laced with animal or plant extracts to predaceous ants (Dyer et al. 2003a; Hare and
181 Eisner 1993; Molleman et al. 2012). Two different bioassay experiments were conducted, one
182 experiment with pure IG assays (aucubin and catalpol), and one with bioassays made from larvae
183 that either had IGs in their diet or not (non IG larvae). Both experiments were performed in the
184 vicinity of ant nests in Kuokkala, Jyväskylä (E3435985, N 6903199). The test site was pine
185 dominated forest with some deciduous trees. All tests were performed in August 2008 (pure IGs)
186 and August 2009 (larval extractions), in sunny to half cloudy weather when ants were active.
187 For the pure IG experiment we made three IG solution of 0.02% (which is comparable
188 with the amount of catalpol found previously in *P. plantaginis* larvae (Lindstedt et al. 2010),
189 aucubin, catalpol or both compounds present. All solutions were made in a 0.1M sugar water
190 solution. As control we used a 0.1M sugar water solution without adding any IGs. The study was
191 conducted at 5 different ant nests which were not connected to each other. A drop of 200 µl test
192 solution was put on a leaf at an ant path (at least 1.5m from the nest). We measured how long an
193 individual ant drank from the solution. When it finished drinking it was taken away in a petri-
194 dish, so we would not measure the same individual twice, and the next ant to observe was chosen.

195 We continued this until we had measurements 20 ants per treatment per nest, including the
196 control treatment.

197 For the larva IG experiment we fed *P. plantaginis* larvae artificial diet (modified from
198 Poitout and Bues (1974) by leaving out ascorbic acid and formaldehyde and adding 0.5 %
199 Vanderzant vitamin mixture for insects). Half of the larvae ate diet that contained dried and
200 ground *P. lanceolata* leaves (IG diet) and the other half ate the same diet without the added
201 leaves (non IG). After the larvae reached 100mg they were starved for 24 hours, to make sure
202 their gut was empty, after which they were frozen and freeze dried. The ground larvae were
203 extracted in methanol (1ml methanol/60mg larva) at 15°C overnight. The solution was filtered
204 over a Whatmann #4 filter paper and 0.2 ml solution was added to 4.5 ml 20 % sugar water. As a
205 control we had 0.2 ml pure methanol with 4.5 ml 20 % sugar water solution. The solutions were
206 also tested for free amino acids (see chemical analyses).

207 In this experiment we tested 10 ant nests. Every nest was tested three times per test-
208 solution. At the same time 200 µl of test solution (IG or non IG) and 200 µl control solution
209 (sugar water) were put on a leaf collected from the surroundings of the ant nest. Because of the
210 high abundance of ants, instead of following one individual ant the total number of ants drinking
211 from a solution was measured. Timing was started when the first ant arrived and drank from the
212 solution. The duration of each test was eight minutes. After every minute the leaf was
213 photographed, so the number of ants drinking from each solution could be counted. After eight
214 minutes the ants and leaf were removed from the test spot. As a measurement we took the
215 average amount of ants drinking from the solution during these eight minutes. We also measured
216 the ant activity per nest, for one minute all ants passing by on the test route were counted, prior to
217 each experiment.

218

219 *Parasitism experiments*

220 To examine larval parasitism rate of *P. plantaginis* in the field, we conducted a field experiment
221 on the Åland islands where this species naturally occurs (Leraut 2006). The experiment was
222 conducted in summer 2008 (end of May to first week of June) when *P. plantaginis* larvae are
223 naturally occurring in the field. We put mass-reared (50-100 larvae per bucket) post diapause
224 larvae from both signal selection lines on 48 potted *P. lanceolata* plants outside in the field (10
225 larvae per plant), on six different locations (total of 480 larvae). The plants were covered with a
226 mesh cloth that prevented the larvae from leaving the plant, but allowed parasitoids to parasitize
227 the larvae. They were left in the field until the first larva started to pupate (after 13-14 days), to
228 obtain only the larval parasitoids. We reared all larvae until an adult butterfly or parasitoid
229 egressed. We noted the signal size of the larva, development time, sex and parasitism success.

230 The emerged parasitoid wasps were mated and used in the laboratory to parasitize 33
231 randomly selected laboratory reared *P. plantaginis* larvae from both selection lines. The larvae
232 were fed *Taraxcum officinale*, one of their natural host plants (Bellman 2007), from the field. We
233 measured the clutch size, development time and sex-ratio of the parasitoids and used them again
234 to parasitize 139 larvae (from 11 available families, 2 from the large signal selection line, and 9
235 from the small signal selection line) that were fed *P. lanceolata* leaves. The same parameters
236 were measured as in the previous generation.

237

238 *Chemical analyses*

239 *HPAEC-PAD*- All larvae were frozen at -80°C and freeze dried, weighed, then ground by hand in
240 an Eppendorf tube. Ground material was extracted in 5 ml 7 % MeOH and left overnight. The
241 crude extract was filtered on a Whatman #4 filter paper and the filtrate was diluted in 1:5 ratio
242 with 7 % MeOH. The concentrations of aucubin and catalpol were analysed by HPLC using a

243 Bio-LC (Dionex Corp., Sunnyvale, USA) equipped with a GS50 gradient pump, a CarboPac PA
244 20 guard (3 x 30mm) and analytical column (3 x 150 mm). Detection was performed with an ED
245 50 PAD equipped with a disposable gold electrode using carbohydrate Waveform A. Isocratic
246 elution with 70 mM NaOH (flow rate 0.25 ml/min) was used for the separation. Columns and
247 disposable gold electrode were cleaned after each sample with alkaline (100 mM NaOH) 300
248 mM sodium acetate solution. Retention times were 4.1 min. and 6.3 min. for aucubin and catalpol,
249 respectively. Concentrations were analysed using Chromeleon Client version 6.50 SP10a Build
250 1065 (Dionex Corp., Sunnyvale, USA).

251 For the droppings we used the same method as for the larvae, except that we did the
252 extraction in 10 ml 70% MeOH and diluted the samples in 1:10 ratio with Milli-Q water (internal
253 resistance ≥18.2MΩ·cm; Milli-Q Plus, Millipore, Bedford, MA, USA) and did not grind the
254 samples beforehand. For the measurements of the diets, we took randomly selected leaves from
255 several plants of the same genotype per diet, and froze them at -80°C. After freeze drying the
256 leaves were ground with a Mikro-dismembrator U (B.Braun Biotech International, Allentown,
257 PA). For the extraction we followed the same method as for the droppings, only we took 25 mg
258 of ground leaf material.

259

260 *UV-spectrophotometry*- The bioassay larva solutions were analysed spectrophotometrically to
261 determine the amount of free amino acids in the solutions. An OPA-reagent solution was made
262 by combining 25 ml of 100mM sodium tetraborate solution, 2.5 ml 20% sodium lauryl sulphate
263 water solution, 40 mg *o*-phtalaldehyde (dissolved in 1 ml of MeOH) and 100 µl β-
264 mercaptoethanol and the mixture was diluted to a final volume of 50 ml with Milli-Q. The larva
265 test solutions were mixed in 1:20 ratio with OPA solution in 3 ml glass tubes. The absorbance of
266 the solutions was measured at 340 nm by a Beckman DU 640 (Beckman Instruments Inc.,

267 Fullerton, CA, USA) spectrophotometer. Sample solutions were compared with the control
268 solution (MeOH with sugar water), and solutions with known amount of L-leucine (a standard
269 amino acid with two concentration levels).
270
271 *Statistical analyses*
272 All statistical analyses were performed using the statistical program PASW Statistics 18 (SPSS
273 Inc., Chicago, Illinois, USA). Data were controlled for homogeneity and normality.
274 We used analysis of variance (ANOVA) with Post Hoc Tukey to test for differences in IG
275 content (dependent variable) between the six diets/genotypes (fixed factor) at both measuring
276 times, used in the performance and sequestration experiment. For the sequestration and
277 performance experiment we used independent *t*-tests to compare between the diets in time, and to
278 test for differences between larval selection line (small signal vs large signal) and the amount of
279 IGs sequestered by the larvae, a paired *t*-test was used to test for differences between the
280 compounds (aucubin vs catalpol) sequestered by the larvae. A bivariate correlation was used to
281 analyse if the amounts of IGs in the diets were related to the amounts in the larval droppings and
282 to analyse the relation of signal size of the larvae to their level of sequestered catalpol.
283 For the bioassay experiment, ANOVA's were used to test for differences between the
284 treatments and ant nests. Drinking time was the dependent factor, treatment the fixed factor and
285 nest was included as random factor.
286 In the parasitism experiments, independent *t*-tests were used to look at the differences in
287 development time between male and female wasps. The analyses of the larvae that were not
288 successfully parasitized, reared on *T. officinale* and the *P. lanceolata* diet, were performed
289 separately for males and females, because there was an interaction of sex × diet. We used

290 independent *t*-test to determine the differences of the performance parameters between the two
291 diets.
292
293 **Results**
294 *Sequestration and performance*
295 There was a significant interaction between the IGs in the diets and the time of harvest ($F_{5,25}$ =
296 61.037, P < 0.001), therefore we analysed the diets separately per harvest time (start of the
297 experiment and one month later). The six diets differed significantly in their amounts of aucubin,
298 catalpol and total amount of IGs (aucubin+catalpol) at the start of the experiment and also one
299 month later (Table 1). The average amount of total IGs in the diets at the start of the experiment
300 (1.35 % of the dry weight) was significantly lower than the amount measured one month later
301 (3.36 % of the dry weight; *t*-test: t = -2.790, df = 35, P = 0.008).
302 Trace amounts of IGs were detected in the larvae and the amount of catalpol was
303 significantly higher than the amount of aucubin (0.047 vs 0.012 % of dry weight; *t*-test t = 5.311,
304 df = 27, P < 0.001). These trace amounts were not correlated (P > 0.05) with the amount of IGs
305 in the different diets at both measuring times (Fig. 1). However, larvae from the small signal
306 selection line had significantly higher amounts of catalpol in their bodies, than larvae from the
307 large signal selection line (*t*-test t = 2.297, df = 26, P = 0.030; Fig 2A). This amount of catalpol
308 was also significantly negatively correlated with the absolute signal size of the larvae (r = 0.517,
309 P = 0.004), significantly more catalpol was detected in larvae with a smaller orange patch (Figure
310 2B).
311 To see if there was a correlation between the amount of IGs in the larval diet, and the
312 amount larvae would excrete directly, we measured the amount of IGs in their droppings. The
313 amount of IGs in the droppings was positively correlated with the amount of IGs in the diets in

314 the first month of the experiment (r = 0.417, $P < 0.001$; Figure 3A), however this association
315 disappeared in the second month of the experiment, and also the total amount of IGs that was
316 excreted decreased (0.38 % vs. 0.056 % of the dry weight; t-test $t = 5.812$, df = 113, $P < 0.001$,
317 Figure 3B), while the total amount of IGs in the diet increased (see above).

318 The diet had no effect on the size of the orange patch of the larvae or on the
319 developmental time or their pupal weight. However, selection line did have an effect on the
320 performance measurements of the larvae. Larvae from the small selection line had a longer larval
321 development time ($F_{1,22} = 4.324$, $P = 0.049$) than larvae from the large selection line, there was
322 no effect of selection line on the pupal development time. There was no difference in the other
323 performance measurements between the selection lines.

324

325 *Bioassay experiment*

326 In the pure IG-treatment there was a significant difference between the treatments ($F_{3,424} = 4.016$,
327 $P = 0.008$). Ants drank longer from the pure sugar solution (86.14 ± 7.27 sec) than from the test
328 solutions (57.85 ± 6.46 sec, 60.75 ± 8.32 sec, 58.59 ± 8.32 sec, aucubin, catalpol or both IGs
329 respectively, Fig. 4). The compounds of the test solution had no effect on the drinking time,
330 neither was there a combined effect of the compounds (Figure 4). There was a significant
331 difference in the time ants drank from the solutions among the different ant nests ($F_{4,424} = 5.738$,
332 $P < 0.001$), but no interaction between nests and treatment ($F_{12,412} = 1.44$, $P = 0.158$).

333 In the larva IG-treatment there was a significant effect of treatment ($F_{1,49} = 8.299$, P =
334 0.006) and ant nest ($F_{9,49} = 14.378$; $P < 0.001$) on the total number of visits (corrected for the
335 amount of ants walking on the ant path): more ants visited the IG-free solution (96.97 ± 11.4 ants)
336 than the IG-solution extracted from the larvae (78.10 ± 9.9 ants). However, ants preferred the
337 solution extracted from the larvae instead of sugar water since significantly more ants drank from

338 the treatment solutions (87.53 ± 7.59 ants), than from the control sugar solutions (36.72 ± 3.69
339 ants; *t*-test t= 10.306, df = 59, P < 0,001). Because this result was not expected we hypothesized
340 that the larvae contain nutrients (e.g. proteins) that are favourable for ants and thus we measured
341 the free amino acids present in the different solutions. There were significantly fewer amino acids
342 present in the control sugar solution than in the test solutions ($F_{2,10}$ = 12.593, P = 0.002), there
343 was no difference between the two larval test solutions.
344
345 *Parasitism experiments*
346 *Field* – Of the 480 larva that were placed in the field, 398 were brought back to the laboratory,
347 the other ones died or were lost in the field. Of these 398 only two larvae were parasitized. One
348 (large selection line, signal size 7) was parasitized by the Tachinid, *Carcelia lucorum* (Identified
349 by Liekele Sijstermans), one fly emerged from this larva. The other larva (small selection line,
350 signal size 3) was parasitized by the gregarious Braconid endoparasitoid *Cotesia villana* (Reinard)
351 (Identified by Mark Shaw). Twenty one wasps emerged from this larva, 16 females and 5 males.
352 These offspring were used to artificially parasitize *P. plantaginis* larvae in the laboratory.
353 *Laboratory* – There was a difference in the successful parasitism rate between the larval diets (3
354 % *P. lanceolata* vs 28 % *T. officinale*) and more wasps hatched when larvae were fed *T.*
355 *officinale* as diet compared to *P. lanceolata* diet. From the 33 larvae reared on *Taraxacum*
356 *officinale* diet 8 died, 18 pupated and 7 were successfully parasitized by *C. villana*. The
357 parasitoids had an average clutch size of 16 (± 4.61) per larva, with a hatching success of 88.9 %
358 and a sex ratio (males/total emerged) of 69.32 % (± 13.32). The males had a significantly longer
359 larval development time than female wasps (*t*-test t = -3.283, df = 93, P = 0.002). However,
360 males had a shorter pupal development time (*t*-test t = -6.508, df = 93, P < 0.001), therefore there
361 was no difference in the total development time between males and females, nor in the weight of

362 their cocoons. From the 139 larvae reared on *P. lanceolata*, 44 died, 92 pupated and only 3 larvae
363 were successfully parasitized, with an average clutch size of 10 (± 2.65) and a hatching success
364 of 43.3 % (only females emerged).

365 When we compared the larvae that were not successfully parasitized and developed into
366 adults on the two different diets, we found that pupa from the *T. officinale* diet were significantly
367 heavier than pupa reared on *P. lanceolata* both for females and males (*t*-test ♀ $t = 7.372$, df = 45,
368 $P < 0.001$; ♂ $t = 2.925$, df = 51, $P = 0.005$), the same was true for the adult weight of the moths
369 (*t*-test ♀ $t = 6.700$, df = 45, $P < 0.001$; ♂ $t = 4.281$, df = 53, $P = 0.003$). Also larvae reared on *T.*
370 *officinale* diet developed faster from pupa to adult in both females and males, compared to larvae
371 reared on *P. lanceolata* diet (*t*-test ♀ $t = -5.668$, df = 45, $P < 0.001$; ♂ $t = -11.563$, df = 51, P <
372 0.001).

373

374 # Discussion

375 The role of plant chemistry is important in many ecological and evolutionary hypotheses
376 addressing plant-insect-natural enemy relationships, species diversity and community dynamics
377 (Price et al. 1980; Speed et al. 2013). Especially in herbivorous insects that are capable of
378 sequestering or storing plant defence chemicals, plant chemistry plays a role in all trophic
379 interactions (Ode 2006).

380 In this study we only found trace amounts of the plant allelochemicals present in the
381 larvae. This suggest that *P. plantaginis* detoxifies or excretes the plant defence chemicals rather
382 than sequestrates them (see also Lindstedt et al. 2010). This was also confirmed by the positive
383 correlation between the amount of IGs present in the diets of the larvae and in their droppings.
384 However, this association was not present anymore later in the experiment, when there were less

385 IGs present in the excretion products of the larvae, but more in their diets. This could suggest a
386 higher uptake of the plant chemicals by the larvae later in their development. Unfortunately, we
387 only measured the IGs present in the larvae at one time point, so we cannot determine if there
388 would be a more efficient uptake of IGs by the larvae later in time. Development-dependent
389 sequestration of IGs was found for the sawfly species *Athalia cordata* and *A. circularis* (Opitz et
390 al. 2010). The concentration of IGs increased significantly over time in the hemolymph of *A.*
391 *cordata* larvae reared on *P. lanceolata* and in the hemolymph of *A. circularis* fed on *Veronica*
392 *beccabunga.*

393 *Parasemia plantaginis* seems to take up IGs selectively. We detected significantly more
394 catalpol than aucubin in the larvae, although this ratio was the opposite in the diets fed to the
395 larvae. Either there was a higher sequestration efficiency of catalpol or a more efficient excretion
396 of aucubin. This same result has been found for another Arctiine moth (*Spilosoma congrua*)
397 feeding on *P. lanceolata* (Bowers and Stamp 1997). The higher ratio of catalpol to aucubin in the
398 larval samples, may also be due to an epoxidation of the precursor aucubin into catalpol (Opitz et
399 al. 2010).

400 That larvae from the small signal selection line had significantly higher amounts of
401 catalpol present than larvae from the large signal selection line, may be due to their prolonged
402 larval development time. They just had more time to accumulate catalpol from their diet, or they
403 ingested more because they ate more food. Another explanation could be that the higher level of
404 catalpol present reflects the trade-off between the warning signal and chemical defence, because
405 the production of more melanin coloration (small signal selection larvae) is costly (Ojala et al.
406 2007) and larvae with more melanin synthesis may have fewer resources left for excretion of IGs.
407 On the other hand, it is possible that more melanic larvae are able to compensate their less
408 efficient warning signal by containing more defensive toxins (Darst et al. 2006; Leimar et al.

409 1986; Speed and Ruxton 2005). In earlier study by Friman et al. (2009) more melanic *P.*
410 *plantaginis* larvae had better immunological defence against certain pathogens. Zhang et al.
411 (2012) found that more melanin larvae were able to recover from infections of *Serratia*
412 *marcescense*. It is possible that melanic forms are also better in tolerating the auto toxicity costs
413 of IG compounds.

414 Although only relatively small amounts of IGs were present in the larvae, these seem to
415 be sufficient to be a deterrent for a generalist ant predator. It is know from field experiments with
416 six different species of ants (Hymenoptera, Formicida) that they drink less *Catalpa speciosa*
417 nectar, which contains IGs (e.g. catalpol), than a sucrose solution of identical sugar concentration.
418 The ants that did drink for the *C. speciosa* nectar, developed behavioural abnormalities, such as
419 erratic movements, loss of balance or loss of locomotion (Stephenson 1981). In our study fewer
420 ants drank from the solutions made from larvae fed diets that contained IGs than from the
421 solution of the control diet larvae. An unexpected result was that the ants preferred the larva
422 solutions above the control sugar solutions. This was probably due to the extra nutrients available
423 in the larva's solutions (Lanza 1988). The test solutions contained significant more free amino
424 acids than the control sugar solution. The pure IG solutions clearly show a deterrent effect of both
425 IGs for ants. There was no significant difference between the deterrence of aucubin or catalpol,
426 and there was no synergistic effect when both IGs were present in the same solution (in contrast
427 to Dyer et al. 2003b; Richards et al. 2010).

428 Parasitism rate of *P. plantaginis* in the field was very low, only two out of the 480 larvae
429 that were put in the field were parasitized. This can be due to low density of parasitoids in the
430 field, an effective larval defence or low host suitability. In the laboratory we found that the
431 endoparasitoid wasp *C. villana* was capable of parasitizing *P. plantaginis* larvae successfully.

432 The difference we find between the two diet treatments could also be caused by a difference in
433 the quality of the parasitoids due to inbreeding. All wasps used in these experiments came from a
434 single brood. The F1 generation was used for the *T. officinale* diet and the F2 generation for the *P.*
435 *lanceolata* diet, therefore we cannot distinguish between diet or generation effects. However,
436 larvae reared on a diet of *T. officinale* were parasitized significantly more successfully than the
437 larvae reared on a diet of *P. lanceolata*. Another study of *P. plantaginis* found that survival of
438 *Serratia marcescense* infected larvae was significantly higher among larvae that fed *Plantago*
439 *major* than *T. officinale* (Zang et al. 2012). *Plantago major* also contains IGs although their
440 concentrations are lower than in *P. lanceolata* (Reudler Talsma et al. 2008). In the present study,
441 the successful egression was much higher on the non-IG diet. This could be due to the uptake of
442 IGs from the *P. lanceolata* diet, which could work as a defence for the *P. plantaginis* larvae
443 against the parasitoids. Another possible explanation is that the quality of the host is much lower,
444 due to costs of the chemically defended diet (Lindstedt et al. 2010; Ode 2006), and does not
445 support the development of the parasitoids. Studies in other Arctiinae show diet affecting
446 parasitoid success. Larvae of *Platyprepia virginali* that feed on lupine have a reduced chance of
447 being parasitized (indicated by dissection results), but a greater chance of surviving parasitism
448 when feeding on hemlock. Therefore, selection should favour multiple host use by woolly bears
449 (English-Loeb et al. 1993). Singer et al. (2004) found that for the Arctiinae caterpillar *Estigmene*
450 *acrea* a pure *Viguiera dentata* diet provides superior growth performance over a pure *Senecio*
451 *longilobus* or mixed diet in the absence of parasitism. However, when parasitism risk is at least
452 moderate, the mixed diet provides a survival advantage over the pure diets of *Viguiera* or
453 *Senecio*, showing trade-offs between growth and enemy-free space. Recently, it has been found
454 that many herbivores can adaptively modify their food intake and choice of diet when risk of

455 infection is high (prophylactic consumption) or infection has already happened (therapeutic self-
456 medication) and improve their chance of survive (see rev. Abbott 2014).

457 Further, we did find life history costs for *P. lantaginis* larvae that were not successfully
458 parasitized reared on *P. lanceolata* diet compared to these larvae reared on *T. officinale* diet; they
459 had lower pupal and adult mass, which is correlated with their fecundity (Honek 1993), and a
460 longer development time. Besides differences in plant defence chemicals, it is also possible that
461 the two host plant species differ nutritionally, which can affect the performance of the larvae and
462 also their immunological defence (Singer et al. 2014)

463 Overall, this study shows that *P. plantaginis* larvae are able to take up some amount of the
464 defensive compounds from their diet, which is sufficient as a deterrent against generalist
465 predators and might be a good defence against parasitoids as well. It is worthwhile to note that
466 because *P. plantaginis* is a polyphagous species (Marttila et al. 1996), they probably exploit other
467 defence chemicals in their diet as well as IGs. If there are differences in the sequestration and
468 excretion efficacy among these chemicals, defence and its efficacy can vary as well. Different
469 chemical resources may provide defence during different stages of development, and may be
470 effective against different enemies or may work additively or synergistically against a given
471 enemy at a given developmental stage (Mason and Singer 2015). In this study the consumption of
472 a diet which contains defensive chemicals comes at a cost, by decreasing the performance of the
473 herbivores. Finally, it is possible that variation in the warning signal efficiency in this species
474 could be maintained due to compensating effects of higher amount of defensive toxins in larvae
475 with less efficient signals. Further studies are needed to investigate the development-dependent
476 efficiency of sequestration of *P. plantaginis* and the effect of host plant use in the field to their
477 rate of predation and parasitism.

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674

675 **Table 1a.** Average amounts of aucubin, catalpol and both iridoid glycosides (% dry weight)
676 present in the different diets, measured at two time points. Significant differences ($P < 0.005$)
677 between the diets are indicated with different letter (Tukey Post Hoc test).

	Start of experiment			After one month		
Diet	aucubin	catalpol	total	aucubin	Catalpol	Total
1	0.60^{b}	0.48^{b}	1.08^{b}	0.24^{a}	0.20^{a}	0.44^{a}
2	0.19^{a}	0.20^{a}	0.39^{a}	2.09^{ab}	1.04^{a}	3.13^{c}
3	0.68^{b}	0.34^{ab}	1.02^{b}	0.36^{a}	0.03^{a}	0.39^{a}
4	0.89^{c}	0.86^{c}	1.75^{c}	1.25^{ab}	0.81^{a}	2.06^{b}
5	0.27^{a}	0.37^{b}	0.63^{a}	0.63^{ab}	0.36^{a}	0.99^{a}
6	1.78^{d}	1.45^{d}	3.24^{d}	2.85^{b}	4.45^{b}	7.29^{d}

678 **Table 1b.** Differences in amounts of IGs (aucubin, catalpol and total IGs) between the different
679 diets, measured at two time points (start of the experiment and one month later).

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Source	Dependent Variable		Type III Sum of Squares	df	Mean Square	F	P
diet	Start experiment	Auc	4.988	5	0.998	186.270	<0.001
		cat	3.291	5	0.658	174.244	<0.001
		iri	16.072	5	3.214	212.711	<0.001
Error		auc	0.064	12	0.005		
		cat	0.045	12	0.004		
		iri	0.181	12	0.015		
diet	After one month	auc	19.781	5	3.956	4.318	0.016
		cat	64.835	5	12.967	9.965	<0.001
		iri	149.890	5	29.978	174.229	<0.001
Error		auc	11.910	13	0.916		
		cat	16.915	13	1.301		
		iri	2.237	13	0.172		

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Figure legends

Figure 1 The mean amounts of iridoid glycosides (IGs) present in the diet at the start of the experiment (A) and one month later (B) and the mean amount of IGs found in the larvae. Black dots represent the larvae from the small signal selection line; white dots represent the larvae from the large signal selection line. Bars indicate standard error.

Figure 2. Average percentage of catalpol present in the larvae plotted against their selection line (small signal or large signal; categorical variable) (A) or the number of orange body segments (signal size; count varialbe) (B). Bars indicate standard error.

Figure 3. Average percentage of IGs present in the diets plotted against the average percentage of IGs present in the droppings of the larvae, at the start of the experiment (A) and after one month (B). Bars indicate standard error.

Figure 4. Average drinking time of the ants (in sec) on the four different solutions: sugar control, aucubin, catalpol and the combined effect of aucubin and catalpol. The different letters indicate significant differences between the treatments ($P < 0.05$). Bars indicate standard error.

699 Figure 1.

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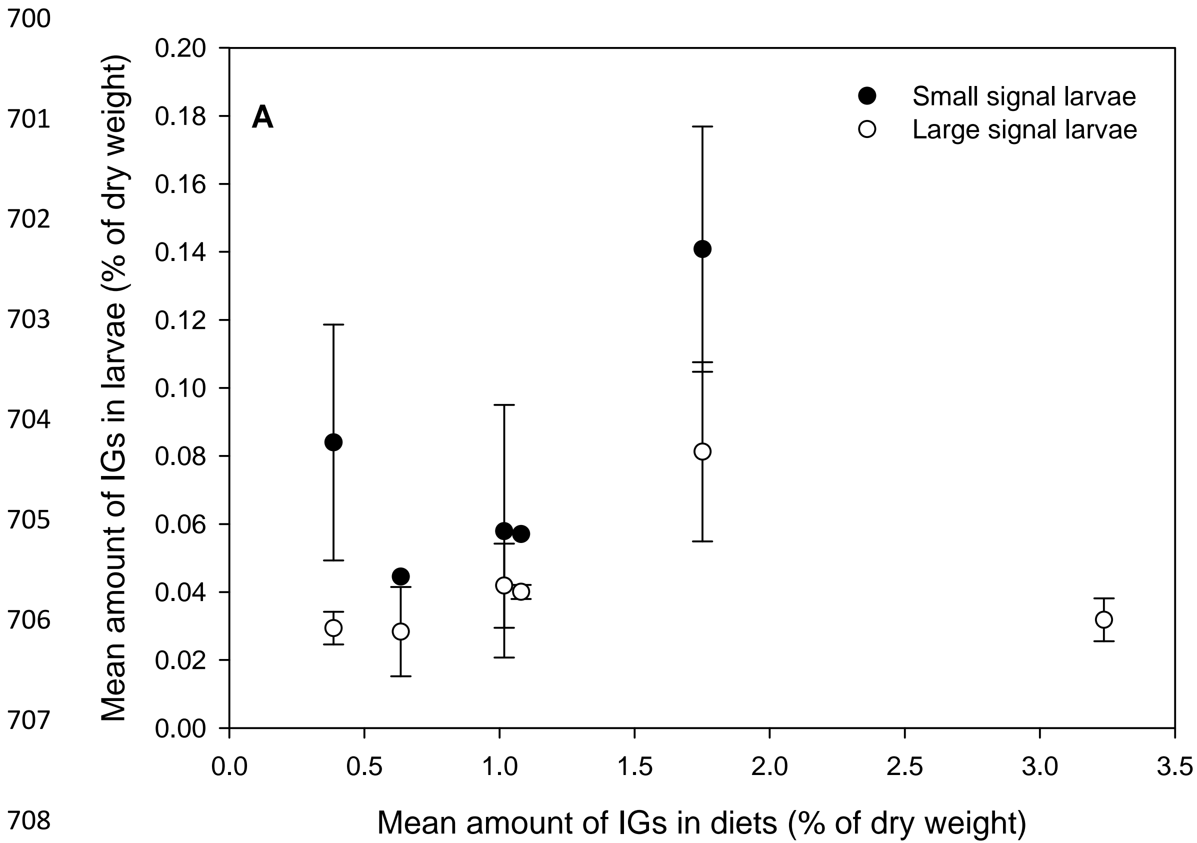

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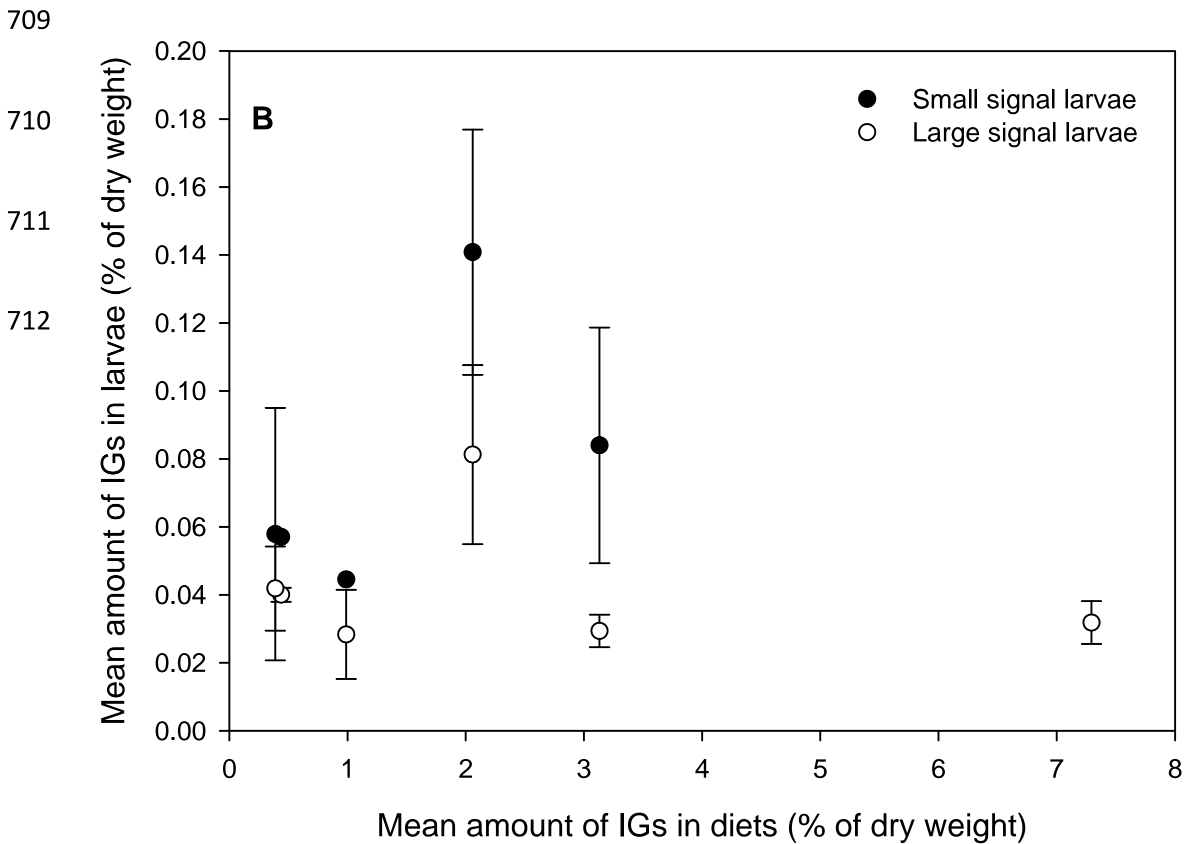

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713 Figure 2

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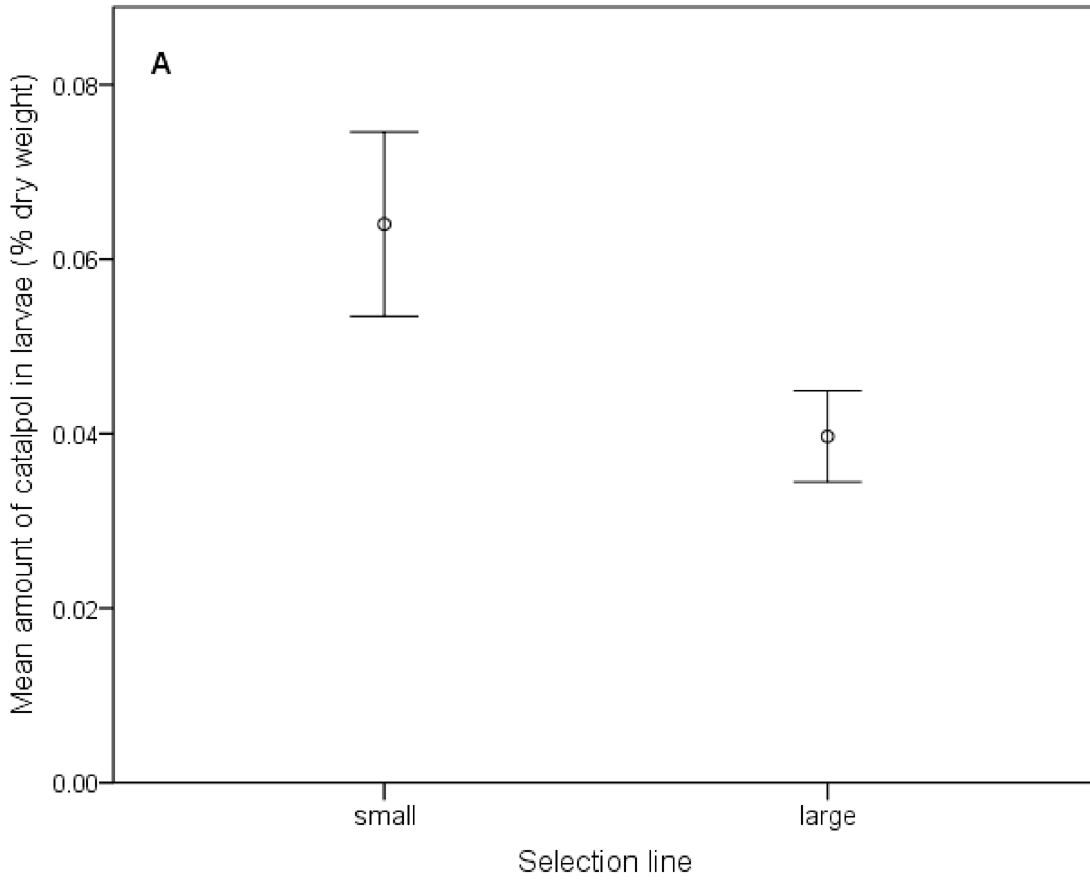

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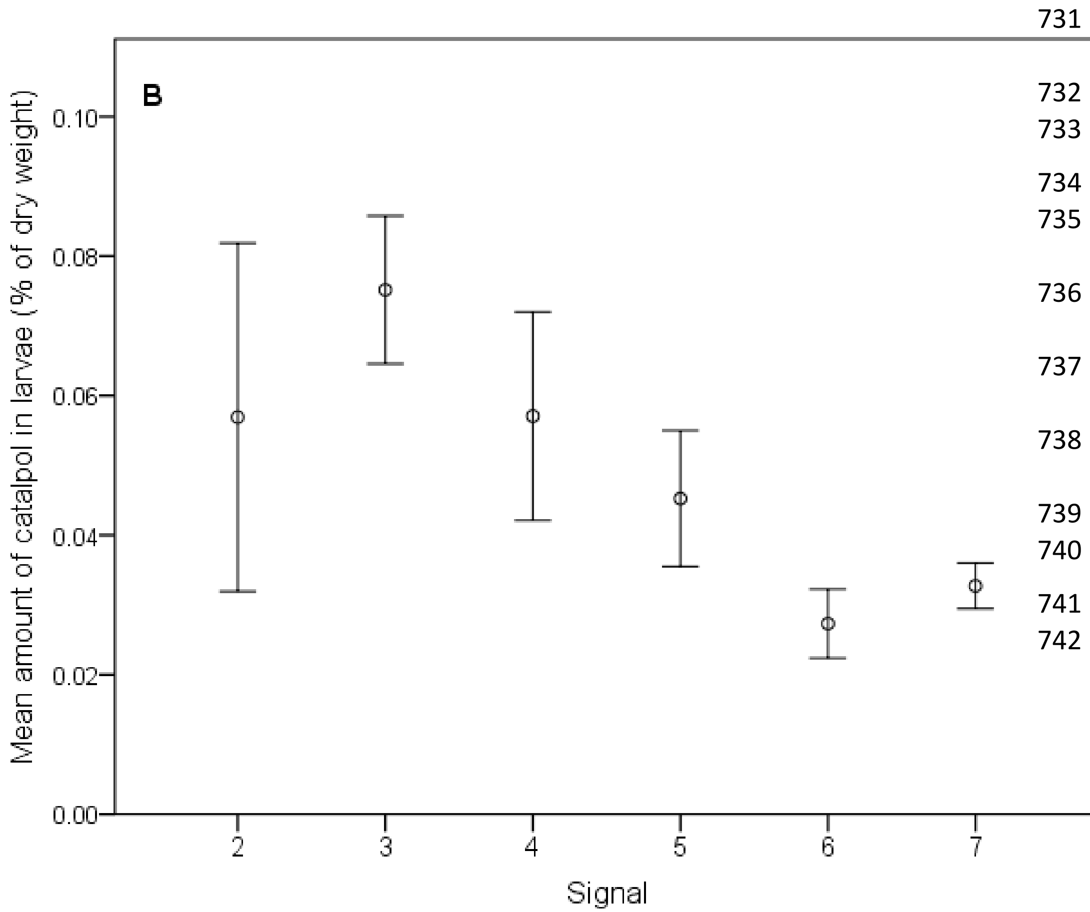

743 Figure 3

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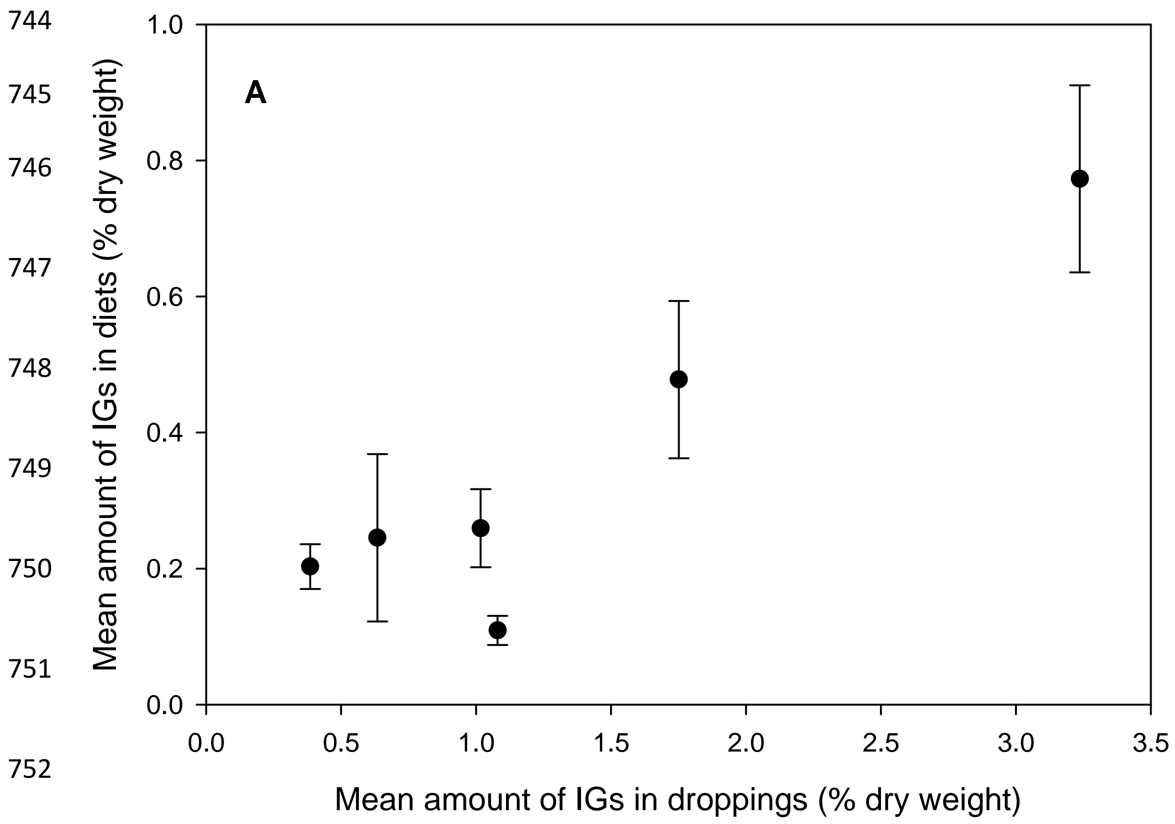

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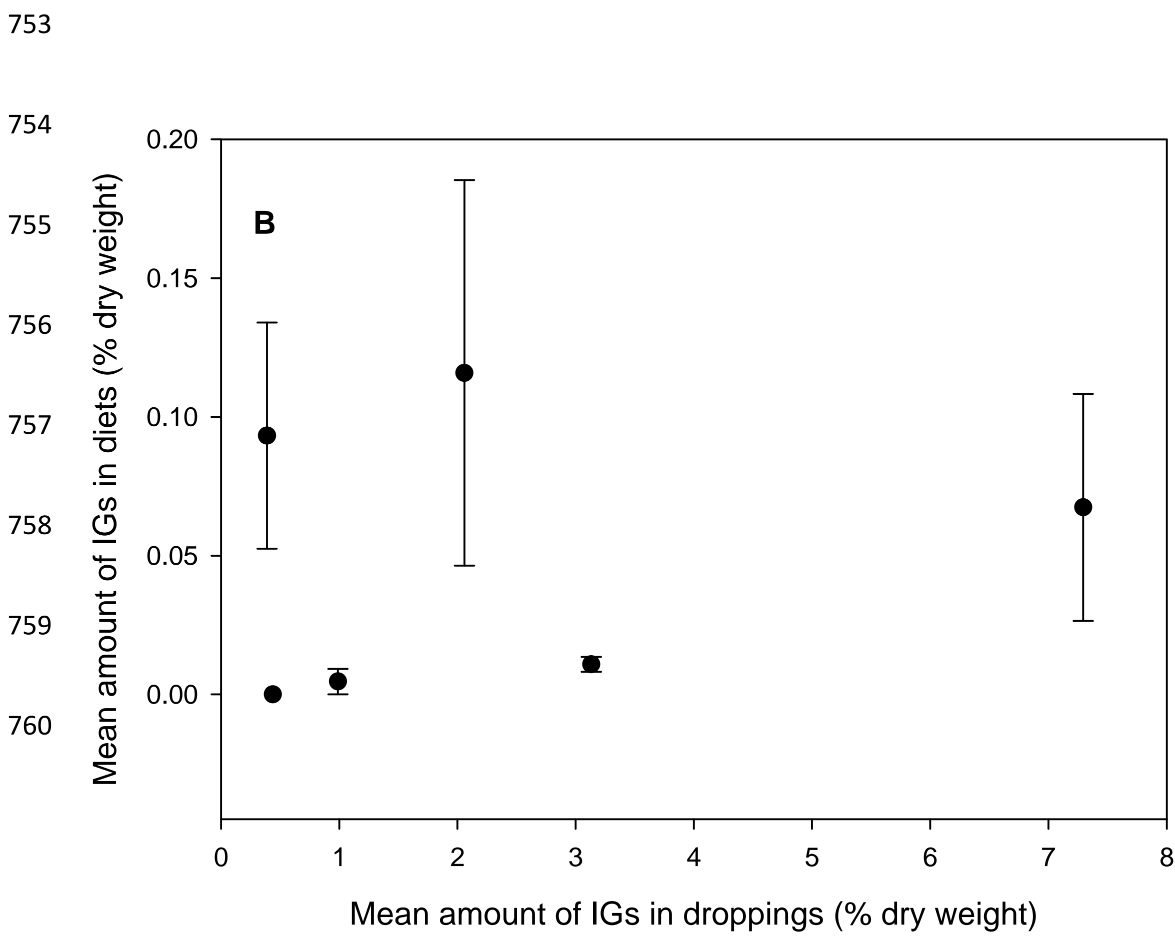

761 Figure 4