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

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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.

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

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

alkaloids Von Nickisch-Rosenegk and Wink 1993), although they may be less efficient than species that sequester a single class of plant compounds (Johnson 1999).

A diversity of natural enemies (e.g. pathogens, predators and parasitoids) are the primary source of mortality for larvae (Cornell and Hawkins 1995; Dempster 1983), increasing in importance in older larvae and the pupal stage (Cornell et al. 1998). Studies of parasitoid-arctiine larvae interaction indicate that the features that appear to be important in protecting larvae from predators, such as sequestration of plant chemical compounds, bright coloration and the presence of hairs or spines (Dyer 1995; Dyer 1997; Lindstedt et al. 2008), may not be as effective against parasitoids (Gentry and Dyer 2002; but see Singer et al. 2009). Sequestration of plant chemicals may even be beneficial for parasitoids, because they live in a chemically protected larva and are thus protected themselves as well (Gauld and Gaston 1994). It has also been shown that allelochemicals in the host diet weaken the immune system of the herbivore (Smilanich et al. 2009), this would benefit the developing parasitoids by enabling them to reallocate metabolic energy from immunosuppression or avoidance to growth and development (Kraaijeveld and Godfray 1997; Ojala et al. 2005). On the other hand, host plant secondary metabolites can be harmful for insects, and detoxification can be energetically costly (Berenbaum and Zangerl 1993; Després et al. 2007), which results in lower host quality for the parasitoid (Smilanich et al. 2009). Furthermore, if the parasitoid cannot avoid or tolerate the sequestered plant chemicals, they may have detrimental effects for them (Gauld and Gaston 1994).

In this study we tested the effects of plant defence chemicals in a multitrophic system, starting with differences in the levels of defence chemicals in the plant, sequestered via the aposematic larva. We examined the ability of the generalist Arctiine moth (*P. plantaginis*) to sequester IGs from a diet containing only *Plantago lanceolata* and the effects of different amounts of IGs in this diet on its performance. We knew from a previous study that these

caterpillars are able to sequester small amounts of IGs (Lindstedt et al. 2010) and it is know from other Arctiine moths that they can sequester plant defence chemicals (Bowers and Stamp 1997; Rothschild et al. 1979; Weller et al. 1999). Further we assumed that higher levels of IGs in their diet would have more negative effects on their performance. Second we tested if chemical defence (sequestered IGs) was affective against predators (ants): we performed a bioassay experiment to determine the deterrent effects of the IGs aucubin and catalpol on ants, both compounds separate and combined, and tested whether extracts from larvae that had eaten a diet that contained IGs were less palatable for ants than larvae with an IG-free diet. Last we measured the parasitism rate of larvae in their natural environment in a field experiment. The parasitoid wasps from the field experiment were used under lab conditions to examine the effect of host plant species on parasitoid performance.

Material and Methods

Study Organism. The generalist *P. plantaginis* (family Erebidae, subfamily Arctiinae; formerly Arctiidae) has warningly coloured larvae and adults and is unpalatable to several vertebrate and invertebrate predators (Lindstedt et al. 2008; Nokelainen et al. 2012). Their natural diet consists mainly of the host plants *Plantago lanceolata* (narrow leaf plantain), *Plantago major* (broad leaf plantain), *Rumex oblongifolius* (bluntleaf dock), *Senecio vulgaris* (Groundsel), *Hieracium pilosella* (mouse-ear hawkweed), *Vaccinium myrtillus* (myrtle blueberry), *Taraxacum campylodes* and *T. officinale* (dandelion) (Bellman 2007; Lindsey 2006; Robinson et al. 2010 and own obeservation). The larvae are hairy in all instars, but their colouration changes from (cryptic) greenish grey in the first two instars to orange-black in the third instar and onwards. They pupate after five to seven instars (Ojala et al. 2007). In Finland this species has one generation per year,

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

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

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

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

studies have identified plant age as an important intrinsic factor affecting *P. lanceolata* chemistry (e.g. Barton 2007; Bowers and Stamp 1993; Fuchs and Bowers 2004); and these ontogenetic trajectories in IG production significantly vary among both maternal families and populations (Barton 2007; Bowers and Stamp 1993) and are highly heritable (Marak et al. 2000). The plants used for this study were offspring from plants derived from an artificial selection experiment in which plants were selected on the basis of high and low concentration of total leaf IGs for four generations (for details see Marak et al. 2000).

Sequestration and performance experiment

To determine if *P. plantaginis* larvae are able to take up IGs from their diet, and to see if different levels of IGs in their diet have on effect on their performance, we conducted a sequestration experiment. For this experiment we used larvae from both selection lines, due to availability only 10 families from the small signal selection line and 20 families from the large signal selection line were used. The larvae were fed six different diets. Each diet consisted of plant clones from one *P. lanceolata* genotype. All different genotypes used differed significantly in their IG levels (Table 1). In total we used 60 larvae from the small selection line (10 larvae per diet treatment) and 120 larvae from the large selection line (20 larvae per diet treatment). The plants used for the diets were grown in a greenhouse at the University of Jyväskylä. Larvae were reared individually in a petri-dish and given fresh leaves every day. We measured their development time, signal size (number of orange segments) and pupal mass. We froze 3-8 larvae per diet for analyses with high performance anion exchange chromatograph with pulsed amperometric detection (HPAEC-PAD). Furthermore, we collected samples of their diet and their droppings during the experiment for HPAEC-PAD analyses (see chemical analyses). The IGs (aucubin and catalpol) of three random samples per diet were measured at two points in time. We also collected the droppings of each

larva for IG analyses at these two time points (one at the start of the experiment and the second one month later).

Bioassay experiment with ants

In a previous study (Lindstedt 2008) *P. plantaginis* larvae were attacked by ants, but not preferred and none of the larvae were killed. With the bioassay we wanted to exclude all other factors to separate the chemical defence from other defences (e.g. hairiness, warning coloration). One common bioassay method for assessing deterrence of specific toxins is to offer sugar solutions laced with animal or plant extracts to predaceous ants (Dyer et al. 2003a; Hare and Eisner 1993; Molleman et al. 2012). Two different bioassay experiments were conducted, one experiment with pure IG assays (aucubin and catalpol), and one with bioassays made from larvae that either had IGs in their diet or not (non IG larvae). Both experiments were performed in the vicinity of ant nests in Kuokkala, Jyväskylä (E3435985, N 6903199). The test site was pine dominated forest with some deciduous trees. All tests were performed in August 2008 (pure IGs) and August 2009 (larval extractions), in sunny to half cloudy weather when ants were active.

For the pure IG experiment we made three IG solution of 0.02% (which is comparable with the amount of catalpol found previously in *P. plantaginis* larvae (Lindstedt et al. 2010), aucubin, catalpol or both compounds present. All solutions were made in a 0.1M sugar water solution. As control we used a 0.1M sugar water solution without adding any IGs. The study was conducted at 5 different ant nests which were not connected to each other. A drop of 200 µl test solution was put on a leaf at an ant path (at least 1.5m from the nest). We measured how long an individual ant drank from the solution. When it finished drinking it was taken away in a petri-dish, so we would not measure the same individual twice, and the next ant to observe was chosen.

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

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

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

Parasitism experiments

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

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

Chemical analyses

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

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

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

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

Fullerton, CA, USA) spectrophotometer. Sample solutions were compared with the control solution (MeOH with sugar water), and solutions with known amount of L-leucine (a standard amino acid with two concentration levels).

Statistical analyses

All statistical analyses were performed using the statistical program PASW Statistics 18 (SPSS Inc., Chicago, Illinois, USA). Data were controlled for homogeneity and normality.

We used analysis of variance (ANOVA) with Post Hoc Tukey to test for differences in IG content (dependent variable) between the six diets/genotypes (fixed factor) at both measuring times, used in the performance and sequestration experiment. For the sequestration and performance experiment we used independent *t*-tests to compare between the diets in time, and to test for differences between larval selection line (small signal vs large signal) and the amount of IGs sequestered by the larvae, a paired *t*-test was used to test for differences between the compounds (aucubin vs catalpol) sequestered by the larvae. A bivariate correlation was used to analyse if the amounts of IGs in the diets were related to the amounts in the larval droppings and to analyse the relation of signal size of the larvae to their level of sequestered catalpol.

For the bioassay experiment, ANOVA's were used to test for differences between the treatments and ant nests. Drinking time was the dependent factor, treatment the fixed factor and nest was included as random factor.

In the parasitism experiments, independent *t*-tests were used to look at the differences in development time between male and female wasps. The analyses of the larvae that were not successfully parasitized, reared on *T. officinale* and the *P. lanceolata* diet, were performed separately for males and females, because there was an interaction of sex × diet. We used

independent *t*-test to determine the differences of the performance parameters between the two diets.

Results

Sequestration and performance

There was a significant interaction between the IGs in the diets and the time of harvest ($F_{5,25}$ = 61.037, $P < 0.001$), therefore we analysed the diets separately per harvest time (start of the experiment and one month later). The six diets differed significantly in their amounts of aucubin, catalpol and total amount of IGs (aucubin+catalpol) at the start of the experiment and also one month later (Table 1). The average amount of total IGs in the diets at the start of the experiment (1.35 % of the dry weight) was significantly lower than the amount measured one month later (3.36 % of the dry weight; *t*-test: $t = -2.790$, df = 35, $P = 0.008$).

Trace amounts of IGs were detected in the larvae and the amount of catalpol was significantly higher than the amount of aucubin (0.047 vs 0.012 % of dry weight; *t*-test $t = 5.311$, df = 27, $P < 0.001$). These trace amounts were not correlated ($P > 0.05$) with the amount of IGs in the different diets at both measuring times (Fig. 1). However, larvae from the small signal selection line had significantly higher amounts of catalpol in their bodies, than larvae from the large signal selection line (*t*-test $t = 2.297$, df = 26, $P = 0.030$; Fig 2A). This amount of catalpol was also significantly negatively correlated with the absolute signal size of the larvae ($r = 0.517$, $P = 0.004$), significantly more catalpol was detected in larvae with a smaller orange patch (Figure 2B).

To see if there was a correlation between the amount of IGs in the larval diet, and the amount larvae would excrete directly, we measured the amount of IGs in their droppings. The amount of IGs in the droppings was positively correlated with the amount of IGs in the diets in

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

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

Bioassay experiment

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

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

the treatment solutions (87.53 ± 7.59 ants), than from the control sugar solutions (36.72 ± 3.69 ants; *t*-test *t*= 10.306, df = 59, *P* < 0,001). Because this result was not expected we hypothesized that the larvae contain nutrients (e.g. proteins) that are favourable for ants and thus we measured the free amino acids present in the different solutions. There were significantly fewer amino acids present in the control sugar solution than in the test solutions ($F_{2,10}$ = 12.593, *P* = 0.002), there was no difference between the two larval test solutions.

Parasitism experiments

Field – Of the 480 larva that were placed in the field, 398 were brought back to the laboratory, the other ones died or were lost in the field. Of these 398 only two larvae were parasitized. One (large selection line, signal size 7) was parasitized by the Tachinid, *Carcelia lucorum* (Identified by Liekele Sijstermans), one fly emerged from this larva. The other larva (small selection line, signal size 3) was parasitized by the gregarious Braconid endoparasitoid *Cotesia villana* (Reinard) (Identified by Mark Shaw). Twenty one wasps emerged from this larva, 16 females and 5 males. These offspring were used to artificially parasitize *P. plantaginis* larvae in the laboratory.

Laboratory – There was a difference in the successful parasitism rate between the larval diets (3 % *P. lanceolata* vs 28 % *T. officinale*) and more wasps hatched when larvae were fed *T. officinale* as diet compared to *P. lanceolata* diet. From the 33 larvae reared on *Taraxacum officinale* diet 8 died, 18 pupated and 7 were successfully parasitized by *C. villana*. The parasitoids had an average clutch size of 16 (± 4.61) per larva, with a hatching success of 88.9 % and a sex ratio (males/total emerged) of 69.32 % (± 13.32). The males had a significantly longer larval development time than female wasps (*t*-test *t* = -3.283, df = 93, *P* = 0.002). However, males had a shorter pupal development time (*t*-test *t* = -6.508, df = 93, *P* < 0.001), therefore there was no difference in the total development time between males and females, nor in the weight of

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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674

Table 1a. Average amounts of aucubin, catalpol and both iridoid glycosides (% dry weight) present in the different diets, measured at two time points. Significant differences ($P < 0.005$) 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}

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

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		

Figure legends

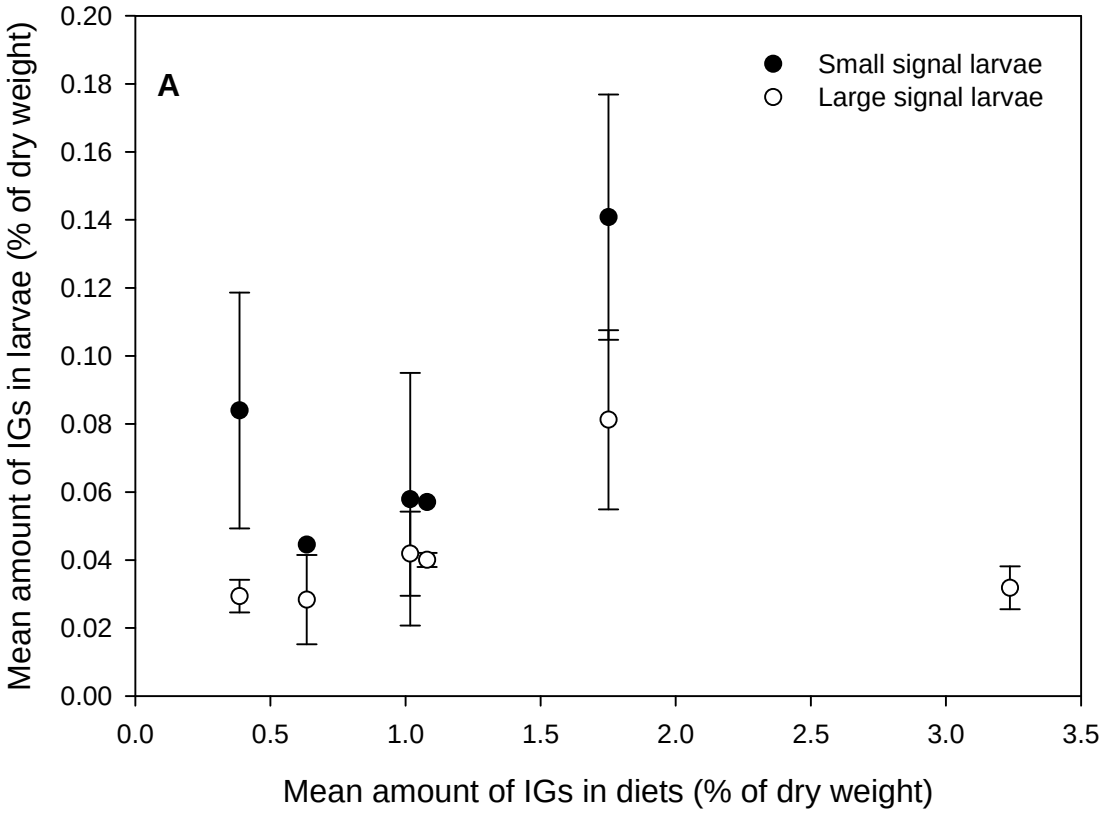
Figure 1 The mean amounts of iridoid glycosides (IGs) present in the diet at the start of the experiment (A) and one month later (B) and the mean amount of IGs found in the larvae. Black dots represent the larvae from the small signal selection line; white dots represent the larvae from the large signal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

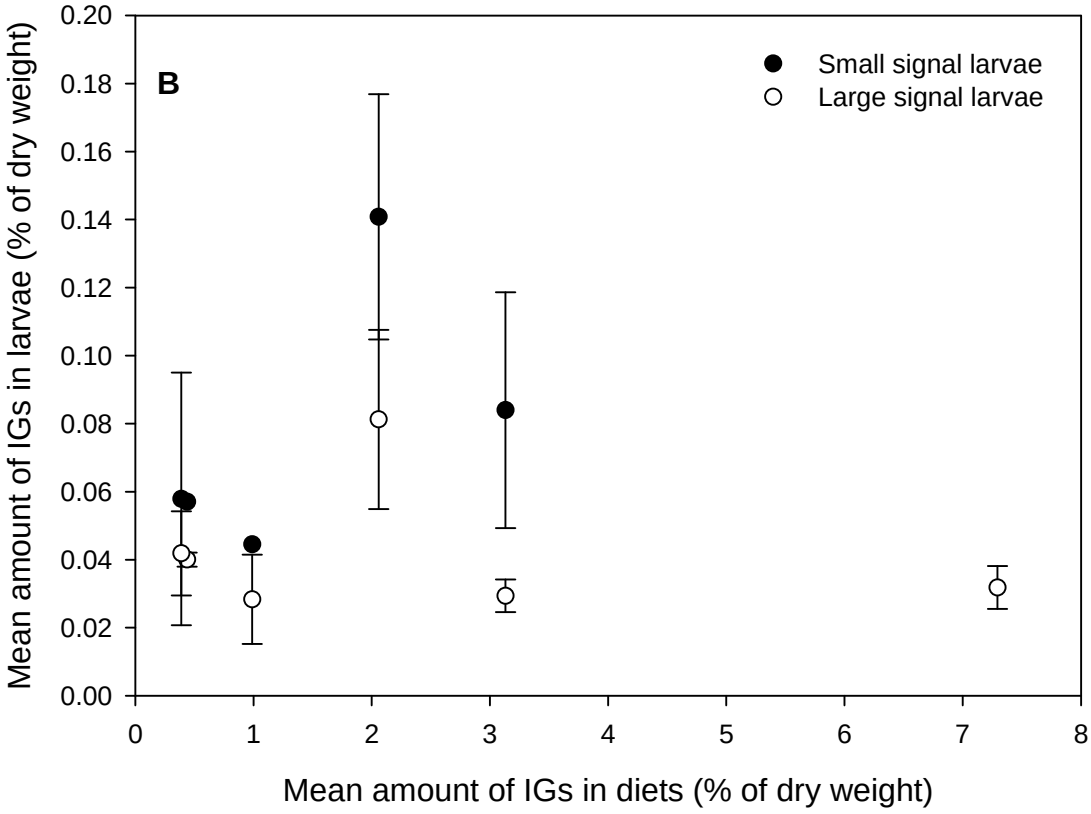
Figure 2. Average percentage of catalpol present in the larvae plotted against their selection line (small signal or large signal; categorical variable) (A) or the number of orange body segments (signal size; count varialbe) (B). Bars indicate standard error.

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.

Figure 1.


A
Small signal larvae
Large signal larvae
Mean amount of IGs in larvae (% of dry weight)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Mean amount of IGs in diets (% of dry weight)


B
Small signal larvae
Large signal larvae
Mean amount of IGs in larvae (% of dry weight)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0
1
2
3
4
5
6
7
8
Mean amount of IGs in diets (% of dry weight)

713 Figure 2

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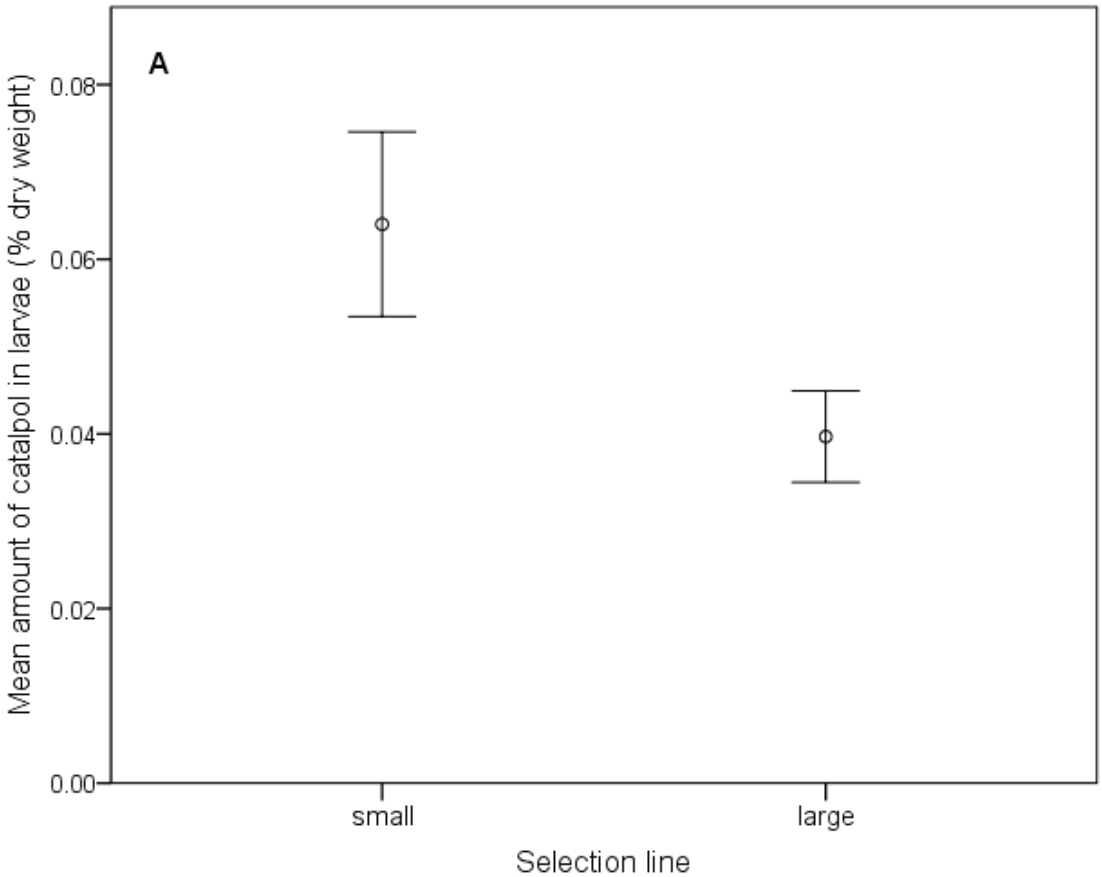

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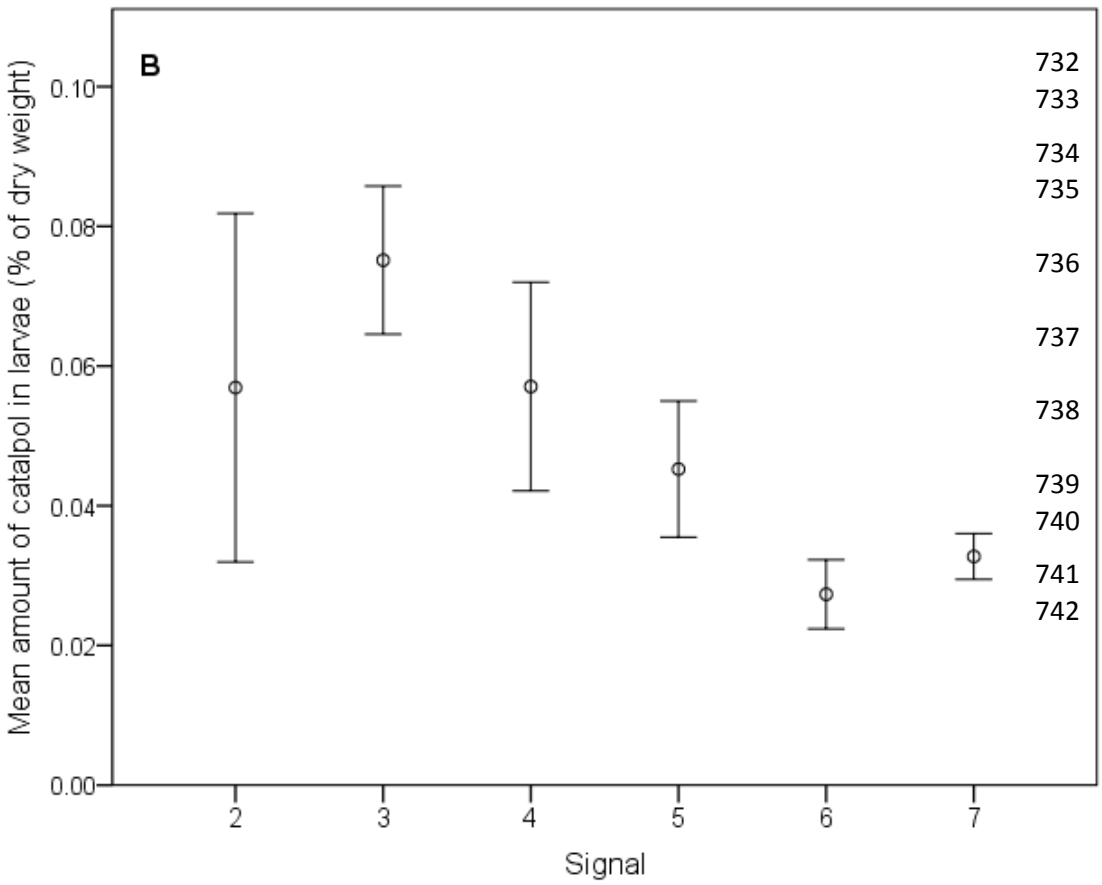
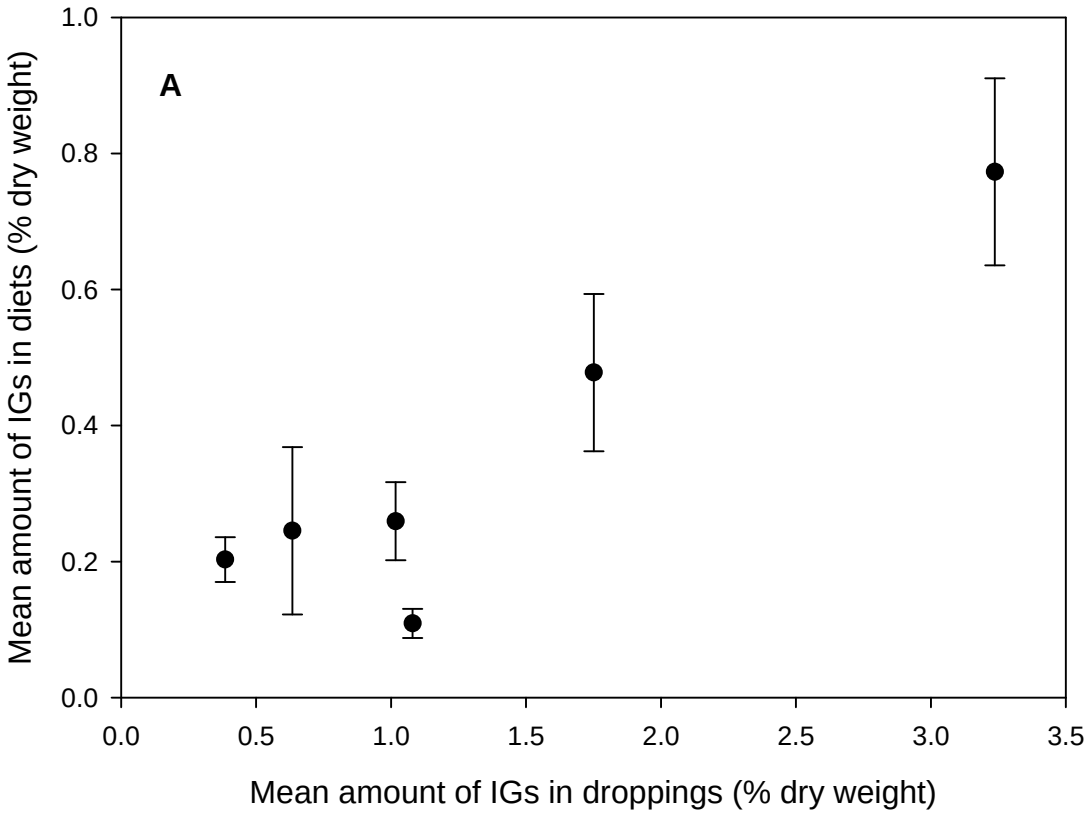
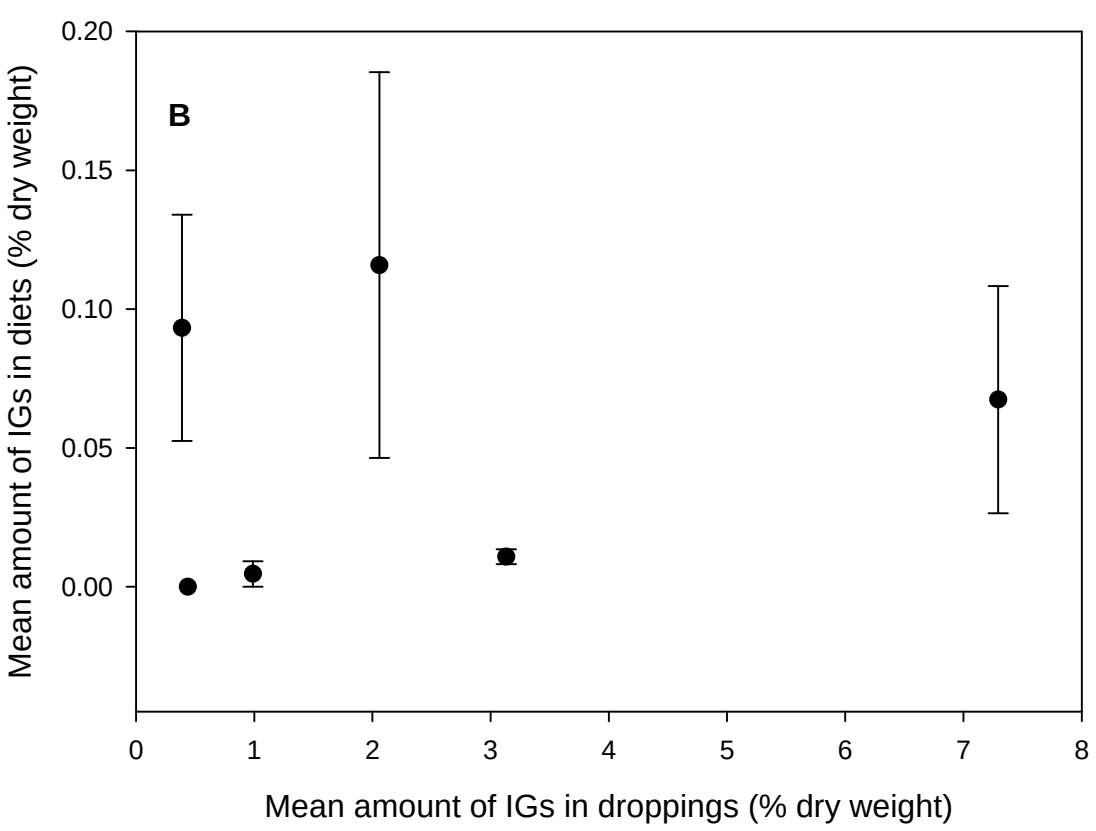
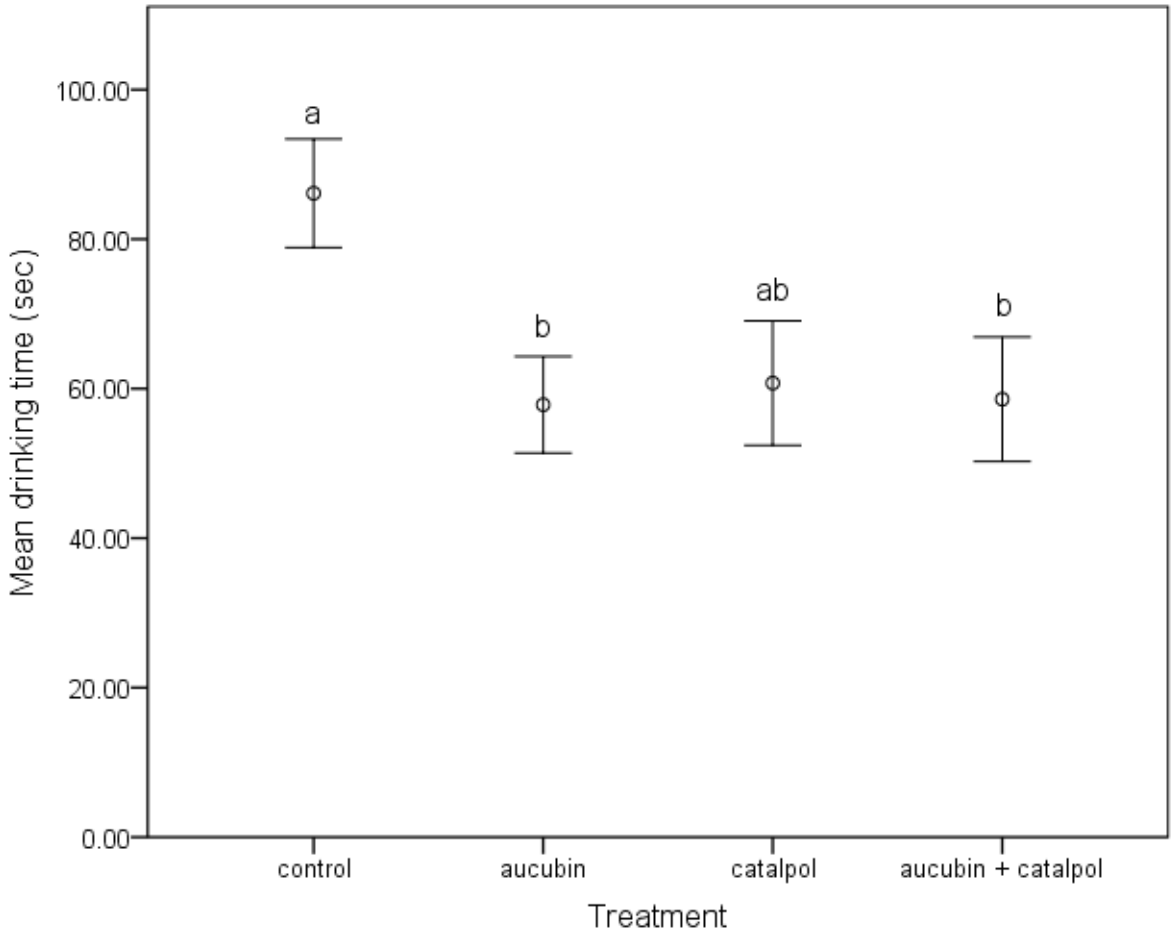


Figure 3


A
Mean amount of IGs in diets (% dry weight)
Mean amount of IGs in droppings (% dry weight)
0.0
0.2
0.4
0.6
0.8
1.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5


B
Mean amount of IGs in diets (% dry weight)
Mean amount of IGs in droppings (% dry weight)
0.00
0.05
0.10
0.15
0.20
0
1
2
3
4
5
6
7
8

761 Figure 4


100.00
80.00
60.00
40.00
20.00
0.00
Mean drinking time (sec)
a
b
ab
b
control
aucubin
catalpol
aucubin + catalpol
Treatment