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Diet Quality Can Play a Critical Role in Defense Efficacy against Parasitoids and Pathogens in the Glanville Fritillary (*Melitaea cinxia*)

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Abstract Numerous herbivorous insect species sequester noxious chemicals from host plants that effectively defend against predators, and against parasitoids and pathogens. Sequestration of these chemicals may be expensive and involve a trade off with other fitness traits. Here, we tested this hypothesis. We reared Glanville fritillary butterfly (*Melitaea cinxia* L.) larvae on plant diets containing low- and high-levels of iridoid glycosides (IGs) (mainly aucubin and catalpol) and tested: 1) whether IGs affect the herbivore's defense against parasitoids (measured as encapsulation rate) and bacterial pathogens (measured as herbivore survival); 2) whether parasitoid and bacterial defenses interact; and 3) whether sequestration of the plant's defense chemicals incurs any life history costs. Encapsulation rates were stronger when there were higher percentages of catalpol in the diet. Implanted individuals had greater amounts of IGs in their bodies as adults. This suggests that parasitized individuals may sequester more IGs, increase their feeding rate after parasitism, or that there is a trade off between detoxification efficiency and encapsulation rate. Larval survival after bacterial infection was influenced by diet, but probably not by diet IG content, as changes in survival did not correlate linearly with the levels of IGs in the diet. However, *M. cinxia* larvae with good encapsulation abilities were better defended against bacteria. We did not find any life history costs of diet IG concentration for larvae. These results suggest that the sequestering of plant defense chemicals can help herbivorous insects to defend against parasitoids.

Keywords Aucubin · Catalpol · Chemical defense · Encapsulation rate · Immunological defense · Iridoid glycosides · *Plantago lanceolata* · *Serratia marcescens* · Tritrophic interactions

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Introduction

Many plants produce secondary metabolites that can be harmful to insects eating the plant, and detoxification can be energetically costly (Berenbaum and Zangerl, 1993; Després et al., 2007; but see Cohen, 1985; Camara, 1997). However, herbivorous insects can sequester plant secondary metabolites (PSM) that are used for defense against natural

enemies such as predators (de la Fuente et al., 1994/1995; Theodoratus and Bowers, 1999; Nishida, 2002), parasitoids (Nieminen et al., 2003; Harvey et al., 2005; Singer et al., 2009), and pathogens (Baden and Dobler, 2009). Thus, the fitness of herbivorous species is affected by the quality of their diet (Ojala et al., 2005; Mody et al., 2007; Lindstedt et al., 2010). Poor diet, in terms of quality and/or quantity, can have detrimental consequences for many life history traits of herbivorous insects including growth rate, development time, pupal mass, fecundity (Ojala et al., 2005; Mody et al., 2007, Lindstedt et al., 2010), immunological defense capacity (Ojala et al., 2005; Klemola et al., 2007), and anti-predator traits (Codella and Raffa, 1995, Grill and Moore, 1998, Lindstedt et al., 2006, 2010).

Insects are defended against parasitoids with encapsulation. Layers of haemocytes and/or melanin encapsulate the parasitoid egg causing the parasitoid to die (Gillespie et al., 1997). Plant chemicals provide the substances necessary to mount the encapsulation reaction (Ojala et al., 2005). Diet protein content, for example, enhances lysozyme-like anti-bacterial activity and phenoloxidase activity in insects (Lee et al., 2008). The chemical composition of the diet may affect the health of the herbivore also, which then indirectly affects the success of the encapsulation reaction positively (Ojala et al., 2005; Kapari et al., 2006) or negatively (Klemola et al., 2007). The effects of PSM on insects' defense capacity against parasitoids and pathogens are widely recognized (Karban and English-Loeb, 1997; Nieminen et al., 2003; Harvey et al., 2005; Smilanich et al., 2009) However, most published studies have been comparative, and only a few have directly tested the effect of secondary metabolites on insect immunological defenses (Nieminen et al., 2003; Harvey et al., 2005).

Maintaining and mounting immunological responses is costly and involves a trade off with other traits (Cotter et al., 2004a; Rantala and Roff, 2005), such as detoxification and sequestration of chemicals from the diet (Smilanich et al., 2009) and antipredation (Rigby and Jokela, 2000). Thus, the effects of PSM on the efficacy of an immune defense are not necessarily additive. Maximization of immune defense can be detrimental to the herbivore through an increase in the risk of autoimmunity (attack against the herbivore's own tissues) (Rolff and Siva-Jothy, 2003) and thus is likely to be under stabilizing selection (Rolff and Siva-Jothy, 2004). The optimal diet choice is likely to be determined by multiple factors that include the composition of the natural enemy community, diet quality and quantity, and time limitations for development.

We conducted a factorial rearing experiment to test the costs and benefits of host PSM on the performance of the herbivorous Glanville fritillary (*Melitaea cinxia* L.). *Melitaea cinxia* is a specialist and can benefit from PSM as its larvae are heavier and develop faster on diets high in iridoid glycoside (IG) content than larvae reared on low IG diets (Suomi et al., 2002; Harvey et al., 2005; Saastamoinen et al., 2007). The high defense chemical content of the host plant also decreases the parasitism risk of caterpillars in the field: Nieminen et al. (2003) found that larval groups of *M. cinxia* that were feeding on plants with lower catalpol concentrations suffered higher parasitism by the specialist parasitoid *Cotesia melitaearum* (Braconidae: Microgastrinae) (Wilkinson, 1937) than those on high-catalpol plants. In addition to defense against predators and parasitoids, diet quality influences defense against pathogens (Bauce et al., 2002; McVean et al., 2002; Cotter et al., 2011). Baden and Dobler (2009) found that IGs had an inhibitory effect on the growth of *Bacillus thuringiensis*.

We divided *M. cinxia* larvae into five groups that were fed on different diets. The five diets differed significantly in IG-levels. Total IG concentration was lowest in diet 1 and highest in diet 5. Larvae were divided between three different immunological treatments within each diet treatment. In this way, we were able to test: 1) how diet quality (specifically iridoid glycosides) affects larval encapsulation activity (defense against an artificial parasitoid) and defense against the bacterial pathogen *Serratia marcescens* (measured as survival time after infection); 2) whether defenses against parasitoids and pathogens interact (both encapsulation and survival after bacterial infection were measured); and 3) how the IG concentration of host plants affects the life history (growth rate, pupal mass) and anti-predatory (chemical defense) traits of individuals. If IGs enhance defense against parasitoids and pathogens (Nieminen et al., 2003), a diet high in IGs could increase larval encapsulation rate and survival time after bacterial infection. However, if handling IGs is costly, the relative benefits of sequestering IGs would decrease with increasing diet IG content, or a high diet IG content would trade off with other life history traits such as fecundity (Lindstedt et al., 2010).

Methods and Materials

Study Species Melitaea cinxia (Nymphalidae) is found throughout Europe, northern Africa, and in the east from Russia to West Asia. Numbers have declined and distribution has become fragmented in northern Europe in the last decades. *Melitaea cinxia* overwinter gregariously as larvae in winter nests. In spring, the larvae feed gregariously on two host plants: ribwort plantain (*Plantago lanceolata*) (Plantaginaceae) and spiked speedwell (*Veronica spicata* L.) (Plantaginaceae) (Marttila, 2005). Both produce IGs, mainly aucubin and catalpol (Duff et al., 1965; Suomi et al., 2002). Glanville fritillary sequester IGs as larvae, and retain these chemicals later as adults (Suomi et al., 2001, 2003). Iridoid glycosides also are used as oviposition cues (Nieminen et al., 2003; Reudler Talsma et al., 2008).

The caterpillars are parasitized by two specialist parasitoids: the solitary endoparasitoid *Hyposoter horticola* (Ichneumonidae) and the gregarious endoparasitoid *Cotesia melitaearum* (Hanski et al., 1995). *Hyposoter horticola* avoid the larval behavioral defenses of *M. cinxia* by parasitizing first instars while they are still within the eggshell (van Nouhuys and Hanski, 2002). Most individuals of *M. cinxia* individuals encapsulate some of the parasitising eggs and larvae of *C. melitaearum*, and some manage to kill all of them (Saskya van Nouhuys, personal communication).

Rearing of Larvae The *M. cinxia* larvae used were descendants of 23 families collected from the Åland Islands in the autumn of 2006. Three generations were maintained as laboratory stock (Metapopulation Research Group, University of Helsinki).We used post-diapausing fifth instars for the experiment because they grow well individually. All larvae were reared on *P. lanceolata*. The experiment was carried out in the spring of 2008 at the University of Jyväskylä.

Larvae from the different families were divided randomly among the diets to obtain genetically similar groups for the 5 diet treatments. Larvae were placed in group containers for the first 5 day (85–108 larvae per container), and fresh *P. lanceolata* leaves were offered *ad libitum*. Each diet group consisted of 4 group containers. Twenty-eight percent of the larvae woke from diapause, and after 5 day, larvae were reared individually in Petri dishes lined with a filter paper (General Purpose Filter Paper, ø 70 mm, Munktell Filter AB). Fresh food was added *ad libitum*. During the experiment, larvae were kept in an environmental chamber with regulated temperature and light conditions (see supplemental material, Table S1).

Larval growth was monitored during the experiment. Larvae were first weighed 6 day after diapause (i.e., initial weight: mean 13.65 mg±SD 3.93 mg) and once a week after that. Immune defense treatments were performed when larvae reached the weight of 100 mg. Mortality, moulting, pupation, and adult eclosion were recorded every day. Pupae were weighed on the day of pupation.

Diet Treatments The plants used were derived from an artificial selection experiment in which lines of Dutch *P. lanceolata* were selected for high and low leaf IG concentration for four generations: low-IG genotypes were crossed with low-IG genotypes and high-IG genotypes, with high-IG genotypes (Marak et al., 2000). A fifth generation of 16 different crosses then was grown in our greenhouse, and the IG content of each cross was measured at two time points and then ranked. Four low-IG, two intermediate-IG and four high-IG genotype crosses were selected to create five distinct diets for the experiment. Each diet consisted of two, consecutively ranked, plant crosses to ensure enough food per diet. Plants were grown under greenhouse conditions. Larvae were fed with a mixture of leaves of both plant crosses that made up their diet every day.

The five diet groups varied significantly in IG content (see results). Plants were measured before the experiment (twice: to rank the crosses by their IG levels), four and 9 week after the experiment began, and twice 1 year after the experiment. A subset of the plants was used in a different experiment and also was measured. The six and, for some, seven measuring points that we have for all diets show that the rank order was constant over time, and that there was no correlation between diet IG-content and nutritional quality (see results). We used the IG measurements that were conducted during the experiment (4 and 9 week after the start of the experiment) for all statistical analyses with diet treatments (Table 1A).

Random leaf samples per cross (before the experiment) or per diet treatment were used for IG measurements, which were done with high-performance liquid chromatography (HPLC).

HPLC Analyses Storage of sequestered chemicals, in the body or wings, could have different anti-predator functions. We, therefore, analyzed wings and bodies of adults separately. We used total sequestered chemicals per individual (wings and body) in our final analyses. Butterflies were freeze-dried and individually weighed to measure dry weight. The manually-ground wings and bodies were extracted in 5 ml 70% methanol and left to sit overnight. The crude extract was filtered on Whatman filter papers (no. 4, diam. 90 mm). The filtrate was diluted 10 times with Milli-Q water. We used a Dionex (Sunnyvale, CA) Bio-Lc equipped with a GP40 gradient pump, a CarboPac PA20 3× 30 mm guard column, a CarboPac PA20 3×150 mm analytical column and an ED40 electrochemical detector for pulsed amperimetric detection (PAD) for HPLC analyses. The eluent was NaOH 7%, and the flow rate 0.25 ml/min. A standardization curve for aucubin, catalpol, and glucose of 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 ppm was used to calibrate samples.

For diet analyses, all leaves were freeze-dried and ground to a fine powder with a mikro-dismembrator U (B.Braun Biotech international). Finely ground and dry leaf material (25 mg) was extracted in 10 ml of 70% MeOH and shaken overnight (15°C/110 RPM). Filtration and other methods were the same as above.

Nutrient Analyses A subset of plants from all diets *(N*=16) was used for the nutrient analyses. For P and K analysis, ground leaf samples (250 mg) were digested with a microwave-accelerated method in closed PTFE-vessels. Nine ml of 3 M HNO_3 and 1 ml of 30% H_2O_2 were added to the samples, which were heated using a CEM (Matthews, NC, USA) Mars 5 microwave oven. Total time of the digestion

Table 1 Experimental conditions: A) average concentrations (% dry weight) of aucubin, catalpol, and total iridoid glycosides (aucubin + catalpol) in each diets and B) number of individuals of each diet group in each treatment

A		Average concentrations ± SE		
Diet	No. of larvae	Aucubin	Catalpol	Total IGs
1	108	0.84%±0.65	0.78%±0.56	1.62%±1.20
2	102	1.58%±0.65	1.20%±0.68	2.77%±1.34
3	118	1.72%±0.35	1.23%±0.47	2.96%±0.12
4	115	1.36%±0.18	1.86%±0.25	3.22%±0.43
5	108	1.57%±0.56	1.84%±0.93	3.42%±0.36

B						
Treatment	Diet					
	1	2	3	4	5	Total
Implant	14	15	17	18	16	80
Bacterial infection	16	13	14	14	16	73
Implant + bacterial infection	11	14	15	14	15	69
Control	13	15	15	16	18	77
Water control	13	13	18	14	21	79
Total	67	70	79	76	86	378

program was 33 min, and a maximum pressure of 15 bars for 10 min was used. Digested samples were analyzed by a Perkin-Elmer (Norwalk, CT, USA) model Optima 4300 DV inductively-coupled plasma optical emission spectrometer. For the P analyses, samples were measured at a wavelength of 213 nm. Samples were diluted 10 times for the K analyses, and measured at a wavelength of 766 nm.

The same ground plant samples (5 mg) were weighed in a tin container, with an Elementar (Hanau, Germany) Vario EL III CHN analyser. The C, H, and N amounts of the samples were determined.

Immune Defense Treatments Larvae were divided equally among the five diet treatments according to their weight (mean 13.65 mg±SD 3.93 mg) at the start of the experiment. Mortality over the experiment was high, and some larvae were added to treatments to increase the sample size and ensure equal weight distribution among the treatments. Treatments were implemented after larvae reached a weight of around 100 mg (mean weight=121 mg±17.7 mg (±SD). We investigated: 1) the effects of diet on the life history traits and IG content of the insects (control treatment); 2) the effects of diet on encapsulation activity (implant treatment); 3) the effects of diet on larval survival after pathogen infection (bacterial infection treatment); and 4) the effects of diet on the interaction of pathogen and parasitoid infection (implant + bacterial infection treatment). Survival of larvae was checked once a day in all treatment groups. Individuals from all diet groups participated in the immune defense treatments (Table 1B).

Diet Quality and Life history Traits (control treatment). All larvae were monitored for growth rate, pupal mass, and survival to pupal and adult stages. We also measured the IG concentrations of all adults.

Diet Quality and Encapsulation Activity (Implant treatment). The degree of darkening (melanization) of artificial implants has been used previously as an assay of immunity (König and Schmid-Hempel, 1995; Baer and Schmid-Hempel, 2003; Cotter et al., 2004b; Ojala et al., 2005; Rantala and Roff, 2005; Klemola et al., 2007, 2008). This method measures the strength of the insect host's neutral encapsulation reaction without the added effects of a real parasitoid (Siva-Jothy et al., 2005), giving a relevant quantitative measure of the host's ability to defend against parasitoids

We used a nylon implant (artificial parasitoid; length 4 mm, diam. 0.11 mm) to measure the strength of the encapsulation reaction. Larvae were anesthetized with carbon dioxide before implantation. A small cut was made with a sterilized needle, and two thirds of the implant was inserted into the 2nd segment of the larva. The implant was removed after 6 h, air dried, and photographed. After 6 h, most implants showed detectable encapsulation, and detectable variation in encapsulation rate.

All implants were photographed against white paper from three different angles with 10× magnification, by the same person. The black and white photographs were analyzed with ImagePro Plus 4.0 (Media Cybernetics). One third of each implant was analyzed using the mean grey value of the measured area. The grey value of the background was used to control differences in lighting conditions between photographs. The grey values of implants were corrected by subtracting the grey values of the backgrounds. Encapsulation rate was calculated as the mean difference between the

background and the mean of the three implant images. Higher values (a greater contrast between the dark implant and light background) indicated a stronger encapsulation reaction (i.e., more black melanin around the implant). For more detailed methods of measuring encapsulation see, e.g., Ojala et al. (2005).

Diet Quality and Defense against Pathogens (Bacterial infection treatment). *Serratia marcescens* (Enterobacteriaceae) bacteria were used in the bacterial infection treatment. The *S. marcescens* strain was obtained from the American Type Culture Collection (#13880). *Serratia marcescens* is a cosmopolitan bacterium and a common, opportunistic pathogen with a broad host range that includes plants, nematodes, insects, fishes, and mammals (Grimont and Grimont, 1978). *Serratia marcescens* is present in soil and can invade the haemocoel of insects through wounds (Daly et al., 1998). It is pathogenic to *Drosophila melanogaster* (Flyg et al., 1980) and *Parasemia plantaginis* (Arctiidae) (Friman et al., 2009) when injected into the haemocoel.

Melitaea cinxia larvae were infected by injecting 5 µl of bacterial solution (approx. $1.66*10^6$ bacterial cells) between the 2nd and 3rd segments. The syringe was cleaned with sterilized water between injections. The syringe was filled with pure ethanol between treatment days to ensure sterility. After bacterial infection, infected larvae were kept in a separate environmental chamber (under the same temperature and light conditions) to prevent the spread of infection to control larvae. Survival of the infected larvae was checked four times per day (at 8.00 and 11.00a.m., 2.00 and 5.00p.m.) for the next 5 day and once per day after that.

As a control we injected 5 µl sterile water into 79 larvae. Previous experiments with similar sized larvae show that this amount of water does not cause any hypotonic reactions for the cells of *P. plantaginis* (Friman et al., 2009, 2011). This has been confirmed for *M. cinxia.* Mortality in the water control treatment in 2009 was only 7.9%. The methods were the same as for bacterial infection. Due to a small sample size in 2008, more water control larvae were treated the following summer (2009) under similar experimental conditions (diet, rearing conditions, and methods). Survival of the water control larvae to pupal stage was significantly lower in 2008 than in 2009 (Fisher's exact test: $P=0.002$). However, in both years, the survival in the water control group was significantly higher than that of bacteria-treated larvae (in 2008: all P-values $<$ 0.019, and in 2009: all P-values <0.001). The data sets from both years were pooled to increase the power of the test.

Diet Quality and the Interaction of Pathogen and Parasitoid Infection (implant + bacterial infection treatment). Larvae were implanted and, subsequently, exposed to bacterial pathogens. Encapsulation rate was measured first (for details see implant treatment). Larvae were infected with bacteria within 2 h of implant removal (for details see bacterial infection). We tested the correlation between encapsulation rate and survival after bacterial infection.

Statistical Analyses All statistical analyses were performed with SPSS 15.0 (SPSS Inc.). Differences between the diets were tested with one-way ANOVA using aucubin, catalpol, and total IG as dependent variables, and diet as the factor. The same test was used to test differences in nutritional quality between the diets, with P, K, N, and C as dependent variables, and diet as the factor. Correlations between leaf IG measurements and leaf nutrients were tested with a Spearman correlation. Differences in encapsulation rate between diets were tested with ANCOVA using diet as a fixed factor and larval weight and the time elapsed after moulting as covariates. Linear regression (with stepwise deletion) was used to analyze the effects of diet catalpol and aucubin concentrations on the encapsulation rate. The aucubin concentration of the diet was excluded from the model because it had no significant effect on encapsulation rate ($t=-0.090$, $P=0.929$). Differences in IG concentrations (% from dry weight measured from adults) between implanted (implant treatment) and non-implanted (control) individuals were tested with two-way ANOVA using diet and implant treatment as fixed factors. Spearman's correlation coefficients were used to analyze correlations between encapsulation rate and individual IG concentrations. Correlations between adult IG concentrations and encapsulation rate were analyzed using individuals of the implant treatment only. Implantation had no significant effect on survival after bacterial infection (two-way ANOVA, diet and implantation as fixed factors; implantation: $F_{1,132}=2.113$, $P=0.148$). Therefore, both infection groups (implanted and non-implanted) were pooled for final analyses. Kaplan Meier analysis with log-rank statistics was used to analyze differences in larval survival between diet groups 72 h after bacterial infection. The formula ln pupal mass (mg) / larval development time to pupal stage in days was used to calculate growth (Ojala et al., 2005). Two-way ANOVA was used to test differences in pupal mass and growth rate of control and implanted individuals (no infection) using diet, sex, and implant treatment as fixed factors. All interactions were kept in the model because deleting them did not change the results. Sequestration data were analyzed using non-parametric Spearman's correlation coefficients because IG concentrations were not normally distributed.

Results

Diet Treatments The five diet groups differed significantly in amounts of total IGs and catalpol, based on long-term

measurements of the plants (ANOVA: $F_{total\ IG\ 4,28}$=3.346, P=0.023; $F_{catalpol\ 4,28}$=2.996, P=0.035; $F_{aucubin\ 4,28}$= 2.151, P=0.101). Diet ranking was constant before, during and after the experiment (total IGs as % dry weight given as means of all measurements ± SE; diet 1: 1.26%±0.31, diet 2: 1.87%±0.46, diet 3: 2.02%±0.30, diet 4: 2.62%±0.27, diet 5: 2.76%±0.33). The diets did not differ significantly in their nutritional quality (ANOVA: $F_{P\ 4,15}$=0.732, P=0.589; $F_{K\ 4,15}$=0.650, P=0.639; $F_{N\ 4,12}$=0.360, P=0.830; $F_{C\ 4,12}$= 0.831, P=0.542). Total IGs (all P values>0.491), catalpol (all P values>0.479) and aucubin (all P values>0.714) content were not correlated with the nutritional values of the plants (Table S2, supplemental material).

Diet Quality and Life History Traits Diet type (1–5) did not have significant effects on growth rate (Table 2) or pupal weight. However, pupal weight was higher in females than in males and females had significantly lower growth rates than males. Implanted individuals reached lower pupal masses but implantation did not affect growth rate (Table 2). There were no significant two- or three-way interactions between diet, implant treatment, and sex for pupal mass or growth rate (Table 2). Adult catalpol concentrations correlated positively with pupal mass (r_s=0.336, N=41, P= 0.032), but not with growth rate (r_s=-0.261, N=41, P= 0.099). Adult aucubin concentrations did not correlate with pupal mass (r_s=0.067, N=41, P=0.679) or growth rate (r_s= 0.213, N=41, P=0.180).

Table 2 The effects of diet and gender on *melitaea cinxia* pupal mass and growth rate

Source of variation	*df*	*MS*	*F*	*P*
Pupal mass				
Diet	4	529.159	1.000.	0.414
Implantation	1	4699.981	8.878	0.004
Sex	1	15229.015	28.766	0.000
Diet x implantation	4	787.095	1.487	0.216
Diet x sex	4	915.784	1.730	0.154
Implantation x sex	1	63.309	0.120	0.731
Diet x implantation x sex	4	200.342	0.378	0.823
Error	68	529.414		
Growth rate				
Diet	4	<0.001	0.548	0.701
Implantation	1	<0.001	0.266	0.608
Sex	1	0.007	14.917	0.000
Diet x implantation	4	<0.001	0.703	0.592
Diet x sex	4	<0.001	0.820	0.517
Implantation x sex	1	0.001	1.714	0.195
Diet x implantation x sex	4	<0.001	0.610	0.657
Error	68	<0.001		

The number of larvae that survived to the pupal stage (χ^2=2.169, df=4, P=0.705) or that emerged as adults (χ^2= 1.696, df=4, P=0.791) did not differ among the diets. The immune defense treatments reduced larval survival. Fewer implanted or infected individuals pupated compared to the control group (implanted vs. control: Pearson χ^2=4.866, N= 157, P=0.027, infected vs. control: Pearson χ^2=68.983, N= 150, P<0.001).

Diet Quality and Encapsulation Activity The encapsulation rate (i.e., defense against parasitoids) did not differ among the different diets (ANCOVA: $F_{4,133}$=1.733, P=0.146). Encapsulation rate did not correlate with adult IG concentrations (total IG: r_s=0.275, N=35, P=0.109; catalpol: r_s= 0.250, N=35, P=0.147; or aucubin: r_s=0.220, N=35, P= 0.204). However, the encapsulation rate did increase with increasing diet catalpol concentrations (linear regression, model: R^2=0.043, $F_{1,138}$=6.193, P=0.014, y=30.164+ 8.789× ; catalpol concentration in the diet: t=2.489, P= 0.014) (Fig. 1).

Adult catalpol and total IG concentrations were higher in implanted individuals than in non-implanted individuals (independent t-test catalpol: t=-3.00, df=89, P=0.003; total IG: t=-3.07, df=89, P=0.003) irrespective of variations in their diet catalpol and IG content (Tables 3 and 4). However, diet did have a significant effect on adults' aucubin content (Table 4). Pairwise comparisons with Tukey HSD adjustment showed that adult aucubin concentrations were higher on diet 2 than on diet 4 (P=0.046). There were no significant among-diet differences in the aucubin concentrations of the adults on the other diets (all P-values>0.180).

Diet Quality and Defense against Pathogens Diet affected the survival of larvae infected with *Serratia marcescens* (Kaplan-Meier survival analysis, Log-rank statistics, χ^2= 10.173, df=4, P=0.038). Although there was slightly more

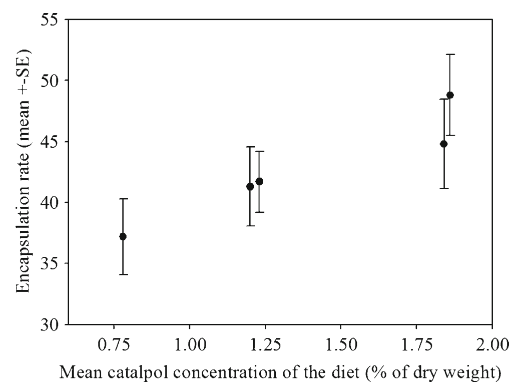

Fig. 1 Catalpol concentrations of the five experimental diets and encapsulation rates of *Melitaea cinxia* larvae after artificial parasitation Error bars show the individuals' mean rate of implant encapsulation (± SE). A higher encapsulation rate indicates a stronger response against an artificial parasitoid. *N=149;* for statistical information see text

Table 3 Average concentrations (±se) (% of dry weights) of total iridoid glycosides (ig) (aucubin and catalpol) in adults according to diet and immune defense treatment

	Diet				
	1	2	3	4	5
Total IG of adults					
Implanted	3.39±0.72	3.47±0.36	3.53±0.39	4.22±0.38	3.40±0.55
Control	4.30±0.48	2.53±0.22	2.60±0.38	2.87±0.40	2.67±0.32
Catalpol of adults					
Implanted	3.30±0.72	3.26±0.35	3.44±0.384	4.15±0.38	3.30±0.53
Control	4.13±0.45	2.44±0.22	2.52±0.36	2.83±0.40	2.59±0.31
Aucubin of adults					
Implanted	0.10±0.02	0.21±0.08	0.10±0.01	0.06±0.01	0.10±0.02
Control	0.17±0.06	0.08±0.02	0.08±0.02	0.04±0.00	0.08±0.02

aucubin in diets 2 and 3 (Table 1A), on which survival was lower, there was no clear trend relating to diet IG concentration (Fig. 2). Pairwise comparisons showed that larval survival was lower on diet 2 than on diets 1, 4, and 5 (all P values<0.032). There were no differences in larval survival among the other diets (all P values>0.072, Fig. 2). Survival time after infection did not correlate with aucubin (r_s=-0.139, N=142, P=0.099) or catalpol (r_s=0.132, N=142, P=0.117) levels in the diet, although the positive correlation between survival time and total diet IG concentration was nearly significant (r_s=0.159, N=142, P=0.058).

The injection method itself did not cause a significant increase in larval mortality. The mortality of larvae injected with water was lower in all diet groups (Pearson χ^2 test for diet 1: P=0.004, 2: P<0.001, 3: P<0.001, 4: P<0.001, 5: P<0.001) than that of larvae injected with bacterial cells.

Table 4 The effects of diet and implant treatment on iridoid glycdoside (ig) (aucubin and catalpol) concentrations of *melitaea cinxia* adults

Source of variation	*df*	*MS*	*F*	*P*
Total IG concentration of adults				
Diet	4	1.925	1.490	0.215
Implant	1	6.845	5.299	0.024
Diet x implant	4	2.334	1.807	0.138
Error	67	1.292		
Catalpol concentration of adults				
Diet	4	2.046	1.660	0.170
Implant	1	6.349	5.149	0.026
Diet x implant	4	2.102	1.705	0.159
Error	67	1.233		
Aucubin concentration of adults				
Diet	4	0.024	2.581	0.045
Implant	1	0.010	1.076	0.303
Diet x implant	4	0.017	1.801	0.139
Error	67	0.009		

Diet Quality and the Interaction of Pathogen and Parasitoid Infection There was a positive correlation between encapsulation rate and survival time after bacterial infection (r_s=0.331, N=65, P=0.007). Larvae with higher encapsulation rates survived longer after bacterial infection.

Discussion

Host immune defense evolves not only based on direct interaction with pathogens but also in response to interaction with other species, such as predators (e.g., Friman et al., 2009), or in relation to diet quality and quantity (Lill et al., 2002). Here, we showed that diet quality affects the ability of *M. cinxia* to defend against parasitoids (*via* encapsulation activity) and pathogens. We found that encapsulation activity (defense against parasitoids) was positively correlated with defense against pathogens. In contrast to previous studies (Harvey et al., 2005; Saastamoinen et al., 2007), we did not find effects of diet on life history traits in *M. cinxia*. This may be due to the relatively small differences in IG concentration between our diets compared to natural values (Bowers et al., 1992; Fajer et al., 1992). Our results

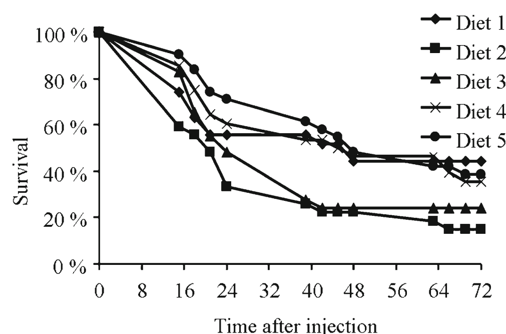

Fig. 2 Survival rates (%) of larvae recorded at different periods after bacterial injection (hours) according to diet (1–5). The concentrations of iridoid glycosides were lowest in diet 1 and the highest in diet 5

suggest that the immunological defense of *M. cinxia* increases with increasing IG concentrations in the diet. These diet-dependent immunological changes are without any life history costs.

Diet catalpol concentration was positively correlated with encapsulation rate. This is consistent with the results of Nieminen et al. (2003) and Reudler Talsma et al. (2011) (but see Harvey et al., 2005) which showed that *M. cinxia* parasitism by the specialist parasitoid wasp *C. melitaearum* was reduced when host-plant catalpol concentrations were high. Nieminen et al. (2003) hypothesized that *C. melitaearum* parasitoids could avoid oviposition of hosts that have high catalpol concentrations in their bodies. Alternatively, the parasitoid larvae could suffer higher mortality when the parasitized hosts have high catalpol concentrations. Our results offer an alternative mechanism: high catalpol concentrations in the diet could directly enhance host immune defense by increasing the larval encapsulation rate. This could happen if catalpol has an antioxidant function (Tundis et al., 2008). Antioxidants can reduce the costs of encapsulation and enable higher encapsulation activity by preventing the damage to the cell that free radicals (produced during encapsulation reactions) can cause (Nappi et al., 1995; von Schantz et al., 1999).

While artificial implants mimicking parasitoids have been widely used as a controlled measurement of the encapsulation reaction (Ojala et al., 2005; Rantala and Roff, 2005; Siva-Jothy et al., 2005; Klemola et al., 2007, 2008), the encapsulation of an artificial parasitoid may not fully correspond to the infection of a natural parasitoid (Klemola et al., 2008). The effects of IGs in the diet of *M. cinxia* on its specialist parasitoids *C. melitaearum* and *H. horticola* have been studied by Harvey et al. (2005) who found that IGs in the host's diet did not affect the development time or adult body mass of *C. melitaearum*. However, increasing the IG content of the hosts' diet shortened the development time of *H. horticola*. Thus, IGs are not necessarily harmful to all parasitoids, and specialist parasitoids have different mechanisms to overcome the host's immune defense system (Poirié et al., 2009). Nevertheless, host encapsulation rate is likely to show the host's defense capacity against generalist parasitoids as well as other foreign objects detected by the individuals' immunological system.

Interestingly, implanted (artificially parasitized) adult individuals had higher amounts of catalpol in their bodies than did non-implanted individuals. Implanted individuals may have sequestered more IGs from their food plants for medicinal purposes (i.e., for self-medication, see, e.g., Singer et al., 2009). Alternatively, implanted individuals may have increased their food consumption leading to a higher accumulation of IGs in their bodies. The higher PSM in implanted individuals also may indicate an energetic trade-off between IG excretion and parasitoid encapsulation rate. Allocation of energy for encapsulation may trade off with excretion of IGs from the body and explain why IG concentrations were higher in implanted individuals. This seems a likely possibility because implanted individuals reached smaller body sizes than non-implanted individuals, suggesting that implantation had costs for developing larvae. Smilanich et al. (2009) found that *Junonia coenia* larvae (specialist herbivores on IG plants) had weaker encapsulation rates when they sequestered higher concentrations of catalpol, indicating negative effects of IGs on encapsulation (Smilanich et al., 2009). Further study, concentrating on the costs of sequestering IGs and the relative importance of different natural enemies on the immune defense of *M. cinxia*, is needed to understand these effects.

Diet also had an effect on the survival of larvae after bacterial infection. Survival was lower on diets 2 and 3 that contained slightly more aucubin. While this difference was not significant, it suggests a detoxification cost for aucubin, which trades off with pathogen defense. With a larger sample size and greater chemical differences among the diet treatments we might have detected these costs. It is also possible that other factors, such as water concentration, nutritional value, or antioxidant content, could have affected larval survival. These have all been shown to affect herbivores' immunological mechanisms (Bauce et al., 2002; Ojala et al. 2005). However, we did not find any nutritional differences between the diets or effects of diet on life history traits, suggesting that the plants were similar in their quality. Variation among diet treatments in our experiment was lower than, for example, in Ojala et al. (2005) where larvae were reared on different plant species.

The encapsulation rate did not trade off with larval survival after bacterial infection. Instead, resistance against both types of enemies correlated positively. A similar result was found in a 2004 study by Rantala and Kortet in which a high encapsulation rate correlated positively with high lytic activity (indicating antibacterial defense) in field crickets (*Gryllus bimaculatus*). Those authors concluded that this result was probably due to the good condition of individuals that allowed them to invest more resources in immune defense (Rantala and Kortet, 2004). We provided larvae with food *ad libitum*, which could offer one explanation for the positive correlation between the two different defenses (encapsulation and antibacterial defense). However, we did not find any significant correlations between our immunological measurements and individual performance (growth rate, pupal weight, or development time) suggesting that good body condition was not the only explanatory factor. It may be that defense against parasitoids and bacteria are genetically linked in *M. cinxia*. Lambrechts et al. (2004) found that high encapsulation rate correlated positively with antibacterial response in mosquito (*Anopheles gambiae*) families, indicating a genetic correlation between these

defenses. Cotter et al. (2004a) found, in contrast, a negative genetic correlation between antibacterial activity and haemocyte density, suggesting a trade off within the immune system in the generalist herbivore *Spodoptera litteralis*, the Egyptian cotton leafworm. Thus, the outcome could depend on insect and pathogen species (Lambrechts et al., 2004). For simplicity, the family effect of immune defense was not tested in this study and, therefore, the possible genetic basis behind the positive correlation remains a topic for future studies.

To understand the evolution of a host's ability to resist infections and parasitoids, it is important to take into account selection from multiple trophic levels as our results emphasize. We conclude that IGs likely benefit *M. cinxia* in the defense against parasitoids because of the positive effect on their encapsulation rate. Moreover, effective defense against parasitoids correlated positively with defense against bacterial pathogens, which could be due to a genetic linkage between these two traits or result from condition-dependent effects. Nevertheless, our results show that the availability of host plants with high IG concentration can be critical for the *M. cinxia* fitness, especially when the risk of parasitism is high. Interestingly, IGs did not cause any life -history costs for the larvae, which suggest that plant defense chemicals can be used to enhance insect immunity without necessarily paying energetic costs. In the future, more studies are needed to understand how other properties of the herbivore diet (e.g., nutritional and antioxidant content) are linked to different immune defense and what role the genetic variation of host plants, insects and parasitoids play in this tritrophic system are essential.

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References

BADEN, C. U. and DOBLER, S. 2009. Potential benefits of iridoid glycoside sequestration in *Longitarsus melanocephalus* (Coleoptera, Chrysomelidae). *Basic Appl. Ecol.* 10: 27-33.

BAER, B. and SCHMID-HEMPEL, P. 2003. Effects of selective episodes in the field on life history traits in the bumblebee *Bombus terrestris*. *Oikos* 101: 563-568.

BAUCE, E., BIDON, Y., and BERTHIAUME, R. 2002. Effects of food nutritive quality and *Bacillus thuringiensis* on feeding behaviour, food utilization and larval growth of spruce budworm *Choristoneura fumiferana* (Clem.) when exposed as fourth- and sixth-instar larvae. *Agric. For. Entomol.* 4: 57-70.

BERENBAUM, M. R. and ZANGERL, A. R. 1993. Furanocoumarin metabolism in *Papilio polyxenes*: biochemistry, genetic variability, and ecological significance. *Oecologia* 95: 370–375.

BOWERS, M. D., COLLINGE, S. K., GAMBLE, S. E., and SCHMITT, J. 1992. Effects of genotype, habitat, and seasonal variation on iridoid glycoside content of *Plantago lanceolata* (Plantaginaceae) and the implications for insect herbivores. *Oecologia* 91: 201-207.

CAMARA, M. D. 1997. Physiological mechanisms underlying the costs of chemical defence in *Junonia coenia* Hubner (Nymphalidae): A gravimetric and quantitative genetic analysis. *Evol. Ecol.* 11: 451-469.

CODELLA, S. G., and RAFFA, K. F. 1995. Host plant influence on chemical defense in conifer sawflies (Hymenoptera: Diprionidae). *Oecologia* 104: 1-11.

COHEN, J. A. 1985. Differences and similarities in cardenolide contents of queen and monarch butterflies in Florida and their ecological and evolutionary implications. *J. Chem. Ecol.* 11: 85-103.

COTTER, S. C., KRUUK, L. E. B., and WILSON, K. 2004a. Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *J. Evol. Biol.* 17: 421-429.

COTTER, S. C., HAILS, R. S., CORY, J. S., and WILSON, K. 2004b. Density-dependent prophylaxis and condition-dependent immune function in Lepidopteran larvae: a multivariate approach. *J. Anim. Ecol.* 73: 283-293.

COTTER, S. C., SIMPSON S. J., RAUBENHEIMER, D., and WILSON, K. 2011. Macronutrient balance mediates trade-offs between immune function and life history traits. *Funct. Ecol.* doi:10.1111/j.1365-2435.2010.01766.x.

DALY, H. W, DOYEN, J. T., and PURCELL, A. H. 1998. Introduction to Insect Biology and Diversity. 2nd edn. Oxford University Press, 696 p.

DE LA FUENTE, M.-A., DYER, L. A., and BOWERS, M. D. 1994/1995. The iridoid glycoside, catalpol, as a deterrent to the predator *Camponotus floridanus* (Formicidae). *Chemoecology* 5/6: 13–18.

DESPRÉS, L., DAVID, J.-P., and GALLET C. 2007. The evolutionary ecology of insect resistance to plant chemicals. *Trends Ecol. Evol.* 22: 298-307.

DUFF, R. B., BACON, J. S. D., MUNDIE, C. M., FARMER, V. C., RUSSELL, J. D., and FORRESTER, A. R. 1965. Catalpol and methylcatalpol: naturally occurring glycosides in *Plantago* and *Buddleia* species. *Biochem. J.* 96: 1-5.

FAJER, E. D., BOWERS, M. D., and BAZZAZ, F. A. 1992. The effect of nutrients and enriched CO_2 environments on production of carbon-based allelochemicals in *Plantago*: a test of the carbon/nutrient balance hypothesis. *Am. Nat.* 140: 707-723.

FLYG, C., KENNE, K., and BOMAN, H. G. 1980. Insect pathogenic properties of *Serratia marcescens*: phage-resistant mutants with a decreased resistance to *Cecropia* immunity and a decreased virulence to *Drosophila*. *J. Gen. Microbiol.* 120: 173-181.

FRIMAN, V.-P., LINDSTEDT, C., HILTUNEN, T., LAAKSO, J., and MAPPES, J. 2009. Predation on multiple trophic levels shapes the evolution of pathogen virulence. *PLoS ONE* 4: e6761.

FRIMAN, V.-P., HILTUNEN, T., JALASVUORI, M., LINDSTEDT, C., LAANTO, E., ÖRMÄLÄ, A.-M., LAAKSO, J., MAPPES, J., and BAMFORD, J. K. H. 2011. High temperature and bacteriophages can indirectly select for bacterial pathogenicity in environmental reservoirs. *PLoS ONE* 6: e17651.

GILLESPIE, J. P., KANOST, M. R., and TRENCZEK, T. 1997. Biological mediators of insect immunity. *Annu. Rev. Entomol.* 42: 611–43.

GRILL, C. P. and MOORE, A. J. 1998. Effects of a larval antipredator response and larval diet on adult phenotype in an aposematic ladybird beetle. *Oecologia* 114: 274-282.

GRIMONT, P. A. D. and GRIMONT, F. 1978. The genus *Serratia*. *Annu. Rev. Microbiol.* 32: 221-248.

HANSKI, I., PAKKALA, T., KUUSSAARI, M., and LEI, G. 1995. Metapopulation persistence of an endangered butterfly in a fragmented landscape. *Oikos* 72: 21-28.

HARVEY, J. A., VAN NOUHUYS, S., and BIERE, A. 2005. Effects of quantitative variation in allelochemicals in *Plantago lanceolata*

on development of a generalist and a specialist herbivore and their endoparasitoids. *J. Chem. Ecol.* 31: 287-302.

Kapari, L., Haukioja, E., Rantala, M. J., and Ruuhola, T. 2006. Defoliating insect immune defense interacts with induced plant defense during a population outbreak. *Ecology* 87: 291-296.

Karban, R. and English-Loeb, G. 1997. Tachinid parasitoids affect host plant choice by caterpillars to increase caterpillar survival. *Ecology* 78: 603-611.

Klemola, N., Kapari, L., and Klemola, T. 2008. Host plant quality and defence against parasitoids: no relationship between levels of parasitism and a geometrid defoliator immunoassay. *Oikos* 117: 926-934.

Klemola, N., Klemola, T., Rantala, M. J., and Ruuhola, T. 2007. Natural host-plant quality affects immune defence of an insect herbivore. *Entomol. Exp. Appl.* 123: 167-176.

König, C. and Schmid-Hempel, P. 1995. Foraging activity and immunocompetence in workers of the bumble bee *Bombus terrestris* L. *Proc. R. Soc. Lond., B, Biol. Sci.* 260: 225-227.

Lambrechts, L., Vulule, J. M., and Koella, J. C. 2004. Genetic correlation between melanization and antibacterial immune responses in a natural population of the malaria vector *Anopheles gambiae*. *Evolution* 58: 2377-2381.

Lee, K. P., Simpson, S. J., and Wilson, K. 2008. Dietary protein-quality influences melanization and immune function in an insect. *Funct. Ecol.* 22: 1052-1061.

Lill, J. T., Marquis, R. J., and Ricklefs, R. E. 2002. Host plants influence parasitism of forest caterpillars. *Nature* 417: 170-173.

Lindstedt, C., Mappes, J., Päivinen, J., and Varama, M. 2006: Effects of group size and pine defence chemicals on diprionid sawfly survival against ant predation. *Oecologia* 150: 519-526.

Lindstedt, C., Reudler Talsma, J. H., Ihalainen, E., Lindström, L., and Mappes, J. 2010. Diet quality affects warning coloration indirectly: excretion costs in a generalist herbivore. *Evolution* 64: 68-78.

Marak, H. B., Biere, A., and Van Damme, J. M. M. 2000. Direct and correlated responses to selection on iridoid glycosides in *Plantago lanceolata* L. *J. Evol. Biol.* 13: 985-996.

Marttila, O. 2005. Suomen päiväperhoset elinympäristössään. Käsikirja. Auris, Joutseno, 272 p.

McVean, R. I. K., Sait, S. M., Thompson, D. J., and Begon, M. 2002. Dietary stress reduces the susceptibility of *Plodia interpunctella* to infection by granulovirus. *Biol. Control* 25: 81-84.

Mody, K., Unsicker, S. B., and Linsenmair, K. E. 2007. Fitness related diet-mixing by intraspecific host-plant-switching of specialist insect herbivores. *Ecology* 88: 1012-1020.

Nappi, A. J., Vass E., Frey, F., and Carton, Y. 1995. Superoxide anion generation in *Drosophila* during melanotic encapsulation of parasites. *Eur. J. Cell Biol.* 68: 450-456.

Nieminen, M., Suomi, J., Van Nouhyus, S., Sauri, P., and Riekkola, M.-L. 2003. Effect of iridoid glycoside content on oviposition host plant choice and parasitism in a specialist herbivore. *J. Chem. Ecol.* 29: 823-844.

Nishida, R. 2002. Sequestration of defensive substances from plants by Lepidoptera. *Annu. Rev. Entomol.* 47: 57-92.

Ojala, K., Julkunen-Tiitto, R., Lindström, L., and Mappes, J. 2005. Diet affects the immune defence and life-history traits of an Arctiid moth *Parasemia plantaginis*. *Evol. Ecol. Res*. 7: 1153-1170.

Poirié, M., Carton, Y., and Dubuffet, A. 2009. Virulence strategies in parasitoid Hymenoptera as an example of adaptive diversity. *C. R. Biol.* 332: 311-320.

Rantala, M. J. and Kortet, R. 2004. Male dominance and immunocompetence in a field cricket. *Behav. Ecol.* 15: 187-191.

Rantala, M. J., and Roff, D. A. 2005. An analysis of trade-offs in immune function, body size and development time in the Mediterranean field cricket, *Gryllus bimaculatus*. *Funct. Ecol.* 19: 323-330.

Reudler Talsma, J. H., Biere, A., Harvey, J. A., and Van Nouhuys, S. 2008. Oviposition cues for a specialist butterfly–plant chemistry and size *J. Chem. Ecol.* 34: 1202-1212.

Reudler, J. H., Biere, A., Harvey, J. A., and Van Nouhuys, S. 2011. Differential performance of specialist and generalist herbivores and their parasitoids on *Plantago lanceolata*. *J. Chem. Ecol.* 37: 765-778.

Rigby, M. C. and Jokela, J. 2000. Predator avoidance and immune defence: costs and trade-offs in snails. *Proc. R. Soc. Lond. B* 267: 171-176.

Rolff, J. and Siva-Jothy, M. T. 2003. Invertebrate ecological immunology. *Science* 301: 472-475.

Rolff, J. and Siva-Jothy, M. T. 2004. Selection on insect immunity in the wild. *Proc. R. Soc. Lond. B* 271: 2157-2160.

Saastamoinen, M., Van Nouhuys, S., Nieminen, M., O'Hara, B., and Suomi, J. 2007. Development and survival of a specialist herbivore, *Melitaea cinxia*, on host plants producing high and low concentrations of iridoid glycosides. *Ann. Zool. Fenn.* 44: 70-80.

Singer, M. S., Mace, K. C., and Bernays, E. A. 2009. Self-medication as adaptive plasticity: increased ingestion of plant toxins by parasitized caterpillars. *PLoS ONE* 4: e4796.

Siva-Jothy, M. T., Moret, Y., and Rolff, J. 2005. Insect immunity: an evolutionary ecology perspective. *Adv. Insect Physiol.* 32: 1-48.

Smilanich, A. M., Dyer, L. A., Chambers, J. Q., and Bowers, M. D. 2009. Immunological cost of chemical defence and the evolution of herbivore diet breadth. *Ecol. Lett.* 12: 612-621.

Suomi, J., Sirén, H., Wiedmer, S. K., and Riekkola, M.-L. 2001. Isolation of aucubin and catalpol from *Melitaea cinxia* larvae and quantification by micellar electrokinetic capillary chromatography. *Anal. Chim. Acta* 429: 91-99.

Suomi, J., Wiedmer, S. K., Jussila, M., and Riekkola, M.-L. 2002. Analysis of eleven iridoid glycosides by micellar electrokinetic capillary chromatography (MECC) and screening of plant samples by partial filling (MECC)-electrospray ionisation mass spectrometry. *J. Chromatogr.* 970: 287-296.

Suomi, J., Sirén, H., Jussila, M., Wiedmer, S. K., and Riekkola, M.-L. 2003. Determination of iridoid glycosides in larvae and adults of butterfly *Melitaea cinxia* by partial filling micellar electrokinetic capillary chromatography–electrospray ionisation mass spectrometry. *Anal. Bioanal. Chem.* 376: 884-889.

Theodoratus, D. H. and Bowers, M. D. 1999. Effects of sequestered iridoid glycosides on prey choice of the prairie wolf spider, *Lycosa carolinensis*. *J. Chem. Ecol.* 25: 283-295.

Tundis R., Loizzo M. R., Menichini F., Statti G. A., and Menichini F. 2008 Biological and pharmacological activities of iridoids: recent developments. *Mini Rev. Med. Chem.* 8: 399-420.

Van Nouhuys, S. and Hanski, I. 2002. Colonization rates and distances of a host butterfly and two specific parasitoids in a fragmented landscape. *J. Anim. Ecol.* 71: 639-650.

Von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D., and Wittzell, H. 1999. Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. Lond.* B 266: 1-12.