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167

Katja Ojala

Variation in Defence and its Fitness Consequences in Aposematic Animals

Interactions among Diet, Parasites and Predators

JYVÄSKYLÄN YLIOPISTO

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ABSTRACT

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All animals face a challenge when defending themselves against several different enemies, e.g. predators and parasites. This has resulted in an enormous variation in defence strategies. Individuals have a limited amount of resources to allocate to defence, and thus there can be trade-offs among different strategies. Aposematism is a defence strategy in which an unprofitable animal advertises to predators that it is defended. The most effective warning signals maximize conspicuousness, as this is easier for predators to recognize, learn and remember. The theory of aposematism predicts reduced variation in the warning signal and chemical defence since variation may slow down learning. I have studied causes and consequences of variation in defence strategies against predators and parasites and possible trade-offs among them. My studies with aposematic arctiid moths demonstrate that the size of the signal has a genetic basis, but that it is also affected by diet. Variation in warning signals and chemical defence had surprisingly little effect on predator learning in laboratory experiment with artificial prey items. However, the efficacy of warning colours varied according to changes in the predator community age structure. When there were mainly young, naive predators present, the conspicuousness of a warning signal places the bearer at an increased risk of predation, whereas when most predators are educated about warning signals, the signals protect the bearer. Therefore, the benefit of aposematism as an antipredator strategy varies according to time and place. Genetic and environmental factors, like diet, can constrain the signal expression and defence ability of aposematic animals against predators and parasites partly explaining the large variation in signal design and defence.

Key words: Aposematism; predator-prey interactions; warning colours; immunocompetence; Müllerian mimicry; Quasi-Batesian mimicry; *Parasemia plantaginis*; *Diacrisia sannio*; plant secondary metabolites.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on five original papers, which will be referred to in the text by their Roman numerals I-V. I am the main author of papers I and III-V, and I carried out a large part of the planning and data collection for each paper.

I Ojala, K., Lindström, L. & Mappes, J. Considerable phenotypic and genetic variation in the warning signal expression of *Parasemia plantaginis* (Arctiidae). Manuscript.

II Lindström, L., Lyytinen, A., Mappes, J. & Ojala, K. 2006. Relative importance of taste and visual appearance for predator education in Müllerian mimicry. Animal behaviour 72: 323-333.

III Ojala, K., Kokko, H., Lindström, L. & Mappes, J. Changes in predator community generate seasonally varying selection for warning signals. Manuscript.

IV Ojala, K., Lindström, L. & Mappes, J. The effects of qualitative versus quantitative plant secondary metabolite on growth and defence against predators and parasites in an arctiid moth *Diacrisia sannio.* Manuscript.

V Ojala, K., Julkunen-Tiitto, R., Lindström, L. & Mappes, J. 2005. Diet affects immune defence and life-history traits of an Arctiid moth *Parasemia plantaginis*. Evolutionary Ecology Research 7: 1153-1170.

1 INTRODUCTION

1.1 Variation in defence against natural enemies

Attacks by predators and parasitoids are extremely important selection pressure to all organisms: if the attack occurs before reproduction, the fitness of an individual can be reduced to zero. Consequently, animals have evolved several different strategies to defend themselves against attacks by natural enemies, with the specific style of defence depending on the specific abilities of each species. Endler (1991) has classified antipredator defences based on the corresponding stages of predation. A species and an individual can use several mechanisms that occur at different stages of predation, especially if a previous attempt to avoid predation has failed. However, adaptations to use various different antipredator mechanisms can be limited, and thus, a certain species is likely able to use only a few of these mechanisms, depending on the specific ecology of the species (Ruxton et al. 2004).

Variation within and among defence mechanisms can sometimes be puzzling; for example, if it is beneficial for an individual to be able to fight parasites efficiently, why aren't all individuals maximally defended? Also, why do some species use one type of defence and other species use another type of defence? And further, does variation within and among defence mechanisms always produce variation in the fitness of an organism?

There are many possible reasons for the existence of variation in defence ability. New mutations occur and produce variation and, if there is no selection against a new allele, neutral variation is possible (Ridley 2004). Also, variation promotes variation: if there is spatial or temporal variation e.g. in the parasite community, one kind of defence against parasites is not likely to be optimal in all environments and all moments in time (Mappes et al. 2005). Further, resources are limited in nature. It is possible that only some of the individuals in a population are able to produce and maintain an effective defensive arsenal against parasites (Roff 2002). Therefore, if environmental conditions vary either in time or space and especially if animals must trade off their defensive ability with some other trait that also influences their fitness, individuals are likely to evolve differences in defensive ability.

1.2 Variation in defence strategies against predators

1.2.1 Aposematism

One striking anti-predatory strategy common in insects is when some species advertise their existence to predators with conspicuous colours and colour patterns (Poulton 1890, Cott 1940). These animals escape predation because the conspicuous warning colour is a sign to the predator that animals possessing these signals are unprofitable as prey. Only when signal and unprofitability occur together is the prey species said to be aposematic (see examples in Poulton 1890, Cott 1940, Edmunds 1974, but see Guilford 1988, 1990). Typically aposematic prey have poisons (e.g. coral snakes, Greene & McDiarmid 1981, Brodie III 1993) or are inedible (e.g. desert locusts, Sword 1999), or even difficult to catch (Brodie Jr. et al. 1998). Studies on predator psychology have revealed that conspicuousness of a warning signal makes aposematic prey easily identifiable and thus avoidable but also easily detectable (Gittleman & Harvey 1980, Alatalo & Mappes 1996, Lindström et al 1999a, Riipi et al. 2001). Because this is so, the evolution of aposematism has been difficult to understand since while the defence is very advantageous against educated predators it simultaneously makes the prey vulnerable to naïve predators (Gittleman & Harvey 1980, Alatalo & Mappes 1996, Lindström et al. 1999a, 2001a, b, Riipi et al. 2001). Conspicuous signalling also seems to be beneficial only when it is relatively common (Sword 1999, Servedio 2000, Lindström et al. 2001b; but see Mappes et al. 2005).

However, many unprofitable species do not have conspicuous warning signals, (e.g. toads, several species of snakes); on the contrary they can be cryptic in appearance. Also, there are species that are unpalatable, but have a relatively inconspicuous warning colouration (e.g. some morphs of the poison arrow frog *Dendrobates pumilio*) (rev. in Endler & Mappes 2004), and some species can appear conspicuous in some environments but are not aposematic. Thus, there is variation in the presence of a warning signal in an unpalatable species, and also variation in the strength of a warning signal among aposematic species.

1.2.2 Müllerian mimicry

Müllerian mimicry (Müller 1879) is a classical example of an anti-predatory defence in which two or more aposematic species exhibit sometimes strikingly similar warning patterns. The benefit of this resemblance is that if predators learn to avoid the warningly coloured prey from a fixed experience (Cott 1940, Edmunds 1974, but see discussion in Sherratt 2002a, Sherratt & Beatty 2003), mimetic species will have lower per capita mortality rates than dissimilar species for which predators have to learn each pattern separately (Müller 1879, but see Rowe et al. 2004). Thus, predator education via Müllerian mimicry is a mechanism which reduces variation among warning signals of aposematic species.

Müllerian mimicry is a strategy that is more beneficial the more common it is (Greenwood et al. 1989, Lindström et al. 2001a, Kapan 2001), thus Müllerian mimicry is considered an example of a mutualism where the cost of educating a predator is shared between similar prey. This expected positive density dependence creates a selection against dissimilar patterns, reducing variation in a warning pattern (Benson 1972, Mallet & Barton 1989, Kapan 2001). However, often Müllerian mimicry is thought to include also imperfect mimicry such as the crude yellow and black colour pattern shared by several species (see literature in Gilbert 2005). It seems that in the literature there is no consensus of how similar warning signals are expected to evolve; sometimes monomorphism is expected, because it is expected to enhance predator learning (e.g. Edmunds 1974, Turner 1984, Endler 1991, Gamberale & Tullberg 1996), but also generalization by predators is expected to be wide, since all species in a Müllerian mimicry ring are protected by a secondary defence, and thus, even crude resemblance can be beneficial (Fisher 1930).

1.2.3 Batesian mimicry and variation in Müllerian mimics

In Batesian mimicry (Bates 1862), only one of the mimetic species is defended against predation (the model). The mimic is not defended but receives an advantage of the warning signal because it resembles the unpalatable model. Thus, only the mimic benefits from mimicry and the model is likely to suffer from increased predation, because the existence of the palatable mimic slows down predator learning (Lindström et al. 1997, Pfennig et al. 2001). The mimic is expected to evolve to resemble the model very closely (Mappes & Alatalo 1997), since the predators would then not be able to distinguish between the inedible model and the edible mimic and resulting predation pressure on the mimic would be reduced.

The evolutionary dynamics of Batesian mimicry, in which the frequency of the mimic is crucial (Lindström et al. 1997, Pfennig et al. 2001), is very different from number-dependent Müllerian mimicry. However, there is enormous intra- and interspecific variation in defensive compounds present in aposematic species and Müllerian co-mimics (Brower 1958, Brower et al. 1963, 1968, 1972, Ritland & Brower 1991); and we could ask, does this variation affects the evolutionary dynamics of these species?

Palatability or defence differences have an impact on the theory of aposematic defence. Predators learn to avoid highly unpalatable prey more quickly than moderately unpalatable prey (Duncan & Sheppard 1965; Lindström et al. 1997). Thus, it is logical to assume that variation in the levels of unpalatability of Müllerian co-mimics interferes with predators' avoidance learning. If the less toxic mimic slows down the avoidance learning of the predator, this should increase the required number of mimetic aposematic prey eaten to produce avoidance by predators (Speed et al. 2000). It is expected that the more toxic species pays the cost of this increase in total number of aposematic eaten (Speed 1993, 1999, Speed et al. 2000) as its mortality increases compared to a situation without mimicry. If this were the case, then the less defended prey would benefit from the mimicry in the same way that Batesian mimics benefit. Thus, it is suggested that mimicry between two unpalatable species resembling each other without mutual benefits should be termed quasi-Batesian mimicry (Speed 1993, 1999). Despite the wide interest in the dynamics of Müllerian mimicry (e.g. Huheey 1976, Turner et al. 1984, Speed 1993, Turner & Speed 1996, Joron & Mallet 1998, Mallet 1999, Mallet & Joron 1999, Speed & Turner 1999), there are only a few experimental studies that test how the palatability difference between Müllerian co-mimics affects the attack rates and the learning performance of wild predators (Speed et al. 2000, Ihalainen & Suutari 2003, but see the overview of psychological literature on learning e.g. Mackintosh 1994). Also, most models and experimental studies have concentrated on the role of palatability differences and reasoned that the less defended species should evolve to resemble the more strongly defended; the effect of variation in signal efficacy has not been considered in this context to my knowledge.

1.3 Variation within the defence strategies against predators

1.3.1 Variation in signal efficacy

Not all aposematic species are Müllerian mimics even though mimicry could potentially reduce predation on them. Also, in many aposematic species there are differences in the warning signals among individuals. Further, many aposematic species do not have maximally effective (large, conspicuous and thus memorable; Gamberale & Tullberg 1996, Forsman & Merilaita 1999, Lindström et al. 1999, Wuester et al. 2004, Jansson & Enquist 2005) warning signals, even though this is what the theory predicts if the species is unpalatable and predation is an important selection pressure for colour. One explanation for the deviation from the theoretical expectations is that there is no perfect defence: often there are some predators that can overcome the defence, e.g. some predators are able to feed on poisonous coral snakes (Beckers et al. 1996, Endler & Mappes 2004). Indeed highly toxic prey are more likely to evolve aposematism than are weakly defended prey (Sherratt 2003). Also, a warning signal which is not maximally conspicuous but combines the benefits of crypsis with a warning signal can be more beneficial, especially if the animal is not strongly protected e.g. by poisons (Tullberg et al 2005).

Aposematism is an example of a strategy where we sometimes do not understand the causes of variation (Gordon & Smith 1999, Mallet & Joron 1999, Joron & Mallet 1998); theoretical considerations suggest that the warning signals of aposematic organisms should evolve towards reduced variation at least within a location (Endler 1991, Mallet & Joron 1999, Speed & Ruxton 2005, but see Mappes et al. 2005). This is because aposematism as a defence against predation is based on predators learning to associate a certain signal to a defence, and learning and remembering is expected to be easier if there is only one warning signal, as opposed to many signals (e.g. Beatty et al. 2004, but see Mappes et al 2005). However, sometimes there can be considerable variation in the appearance of aposematic organisms, even within species and locality (Fields & McNeill 1988, Liebert & Brakefield 1990). Can this variation be caused by differences in the environment, e.g. in diet? Or can it be neutral variation, not affecting the fitness of an individual? Or have we not taken into account all important selection pressures affecting the colouration of an organism, and thus, also warning signal (Endler & Mappes 2004, Mappes et al. 2005)?

A factor which potentially causes variation in the efficacy of aposematism as a defence strategy is seasonality. Since predator community age structure, and thus also their experience level about warning signal changes predictably, warning colouration is likely more efficient at times when most predators are not juveniles and already educated to avoid warning signalled prey. Seasonal effects in mimicry have been studied and found in aposematic bumblebees and their Batesian mimics (Waldbauer & LaBerge 1984). However, since also Müllerian mimicry is based on predator learning, it seems likely that the benefits from mimicry are not symmetrical if the co-mimics differ in their phenology, since the earlier individuals are likely to encounter more naïve predators than later ones. Also, it is possible that this applies to aposematic species in general, since their warning signals usually share some common elements such as conspicuous colouration, and predators that have encountered unprofitable prey might be more cautious in approaching even distantly similar prey later.

1.3.2 Variation in secondary defences

Within and among aposematic species, there is also variation in the efficacy of secondary defences (e.g. chemical defence; warning signal being the primary defence). Aposematic animals often sequester defence chemicals from their diet by eating plants which contain secondary compounds that make the plants inedible to many herbivores (Rothschild 1973, Dobler & Rowell-Rahier 1994, Camara 1997, Stamp 2001). In such cases, aposematic animals are necessarily adapted to eating these potentially harmful chemicals without suffering much adverse effect themselves. However, different plant species have different chemical composition, and this is a likely reason for the differences in the subsequent defensive ability of the herbivores that eat them. To make things more complicated, plant secondary metabolites also affect herbivores' ability to grow and defend themselves against parasites. Therefore, aposematic herbivores have to face selection pressures from the plant species they consume and also from their natural enemies, mainly predators and parasites. Thus they can be thought of as being "between plants and predators" (Olff et al. 1999). Variation among individuals, as well as among species, of aposematic herbivores can be caused by variation in their diet or by variation in the selection pressures caused by predators and parasitoids.

1.4 The effect of diet on variation in growth and defence against parasites

Insects, and especially order Lepidoptera, are the most studied group of aposematic organisms (Ruxton et al. 2004). One of the most important factors affecting the fitness of insect herbivores is their diet, i.e., the quality of the plant species they eat. Eating different host plant species can result in differences in life-history traits, e.g. in growth, development time and fecundity (e.g. Price et al. 1980, Bernays & Chapman 1994). Much of these differences are caused by differences in secondary metabolite contents among plant species, which can, e.g., make plants inedible to most herbivores or reduce the digestibility of plant material (Rosenthal & Janzen 1979). However, plant-derived compounds in herbivores can also make them inedible and thus provide protection from predators (Rothschild 1973). Therefore, for aposematic herbivores relying on plant-derived chemicals to make them unpalatable, plant secondary metabolites can potentially be both harmful and beneficial (Rosenthal & Janzen 1979). For polyphagous (i.e. eating several host plant species), aposematic herbivores the choice among host plant species can be especially crucial, since eating a host plant that makes them protected can also be detrimental for their growth and survival (Singer 2001). Variation in the chemical composition among different host-plant species can produce variation in the herbivore's defensive ability against natural enemies (Singer & Stireman 2003).

Plant secondary compounds can also protect herbivores from parasites and disease. It has been well established that insects' host plant quality affects their probability of being parasitized as well as the fitness of the parasite (Fox et al. 1990, Farrar & Kennedy 1993, Lill et al. 2002, Teder & Tammaru 2002, Mira & Bernays 2002). Also, some plant secondary metabolites are beneficial for growth; carotenoids, for example, are important antioxidants and reduce the harmful effects of stress caused by, UV-radiation or infection (Demming-Adams & Adams, 1996, Ouchane et al. 1997). Therefore, it seems likely that the optimal diet choice for polyphagous insect herbivores varies in time and place, depending on many factors, for example, the enemy community and time available for development.

1.5 Aims of the study

The aim of this thesis was to study how aposematism functions as an anti-predator strategy and how it is affected by variation at three different levels: in diet, in aposematic herbivores and in their predators. The need for studies focusing on the tri-trophic interactions has been recognised (Singer & Stireman 2005), since only by studying all three levels can we understand the evolutionary and ecological forces that affect all the organisms involved. Since aposematic herbivores face selection pressures from both having to feed on variable diets and needing to avoid being fed upon by their natural enemies, I used a combined approach to study the selection pressures from both directions.

I have been interested especially in whether there are trade-offs and constraints in defence strategies against different enemies. I was particularly interested in whether genetic and environmental factors can explain the tremendous variation in the defence of aposematic herbivores against predators and parasites. Finally, I tested how the variation in warning signal and unprofitability affect the fitness of an aposematic prey. In paper I, I studied the genetic and environmental causes of variation in a warning signal of an aposematic organism. Abundant variation in all of the above was found, and in papers II and III I examined what type of selection on this variation in aposematic organisms can be created by predators. Paper II examines the variation from the predators' point of view, how variation in warning signal and unpalatability affects the learning and remembering of the predator. Paper IV examines how changes in predator community can result in changes in the selection pressures and efficacy of warning signals and how this has affected the variation in the appearance of lepidopteran species as larvae. In papers I, II and V, I examined the causes of variation: the effect of diet of an aposematic herbivore on growth, survival, as well as defence against predators and parasitoids and the possible trade-offs between different defences.

2 MATERIAL AND METHODS

2.1 Study species and their maintenance

To study the effect of diet on growth, survival and immunocompetence of a polyphagous herbivore, I used two species of moths belonging to the family Arctiidae; *Parasemia plantaginis* (I & V) and *Diacrisia sannio* (IV). Because the larval colouration of *P. plantaginis* varies, this species also was used to study variation in the warning signal (I). Since aposematism is a defence strategy against predators, I also studied how variation in the warning signal and chemical defence affects predator (wild great tits, *Parus major*) learning and the selection pressures imposed by predators (II & III).

2.1.1 Arctiid moths (I, IV & V)

Both *Parasemia plantaginis* and *Diacrisia sannio* are polyphagous and feed on numerous herbaceous and arborescent plant species (Marttila et al. 1996). They live in open and semi-open environments, and despite their relatively conspicuous colouration, they are usually somewhat inconspicuous and difficult to find because they spend most of their time near the ground (K. Ojala personal observation). Arctiid moths are capital breeders, i.e. the adults do not feed. The larvae have 5-7 instars and have to collect all the nutrients and other substances the moths need to survive and reproduce, making the larval diet critical to the fitness of the individual. In Finland, these species usually have only one generation per year with larvae in 3rd-4th instar overwintering, but during warm summers they can have a second generation within a year. However, in laboratory conditions larvae keep growing if kept in warm and light conditions and can produce several generations per year.

These species were selected because they are likely to be aposematic and because they are polyphagous, I was able to manipulate their diet easily. Also, rearing them in laboratory conditions is relatively easy. Most studies on aposematic insects have been done on species that are specialists to their host plants. This is because specialists often have overcome the defence of a chemically defended plant and are able to use plant-derived compounds for their own chemical defence. Furthermore, although generalists may use variety of plants as hosts, they might not receive as efficient defence (Dyer 1995) and thus not be considered strongly aposematic. However, generalists may incorporate different plant secondary metabolites into their defence and thus increase their defence arsenal. Nevertheless, maintaining several detoxification mechanisms simultaneously might be costly (e.g. Hatle & Spring 1998) and therefore even generalist herbivores often remain conservative to their larval host plant. Given these we can assume that underlying costs of defence might be easier to detect from a generalist herbivore that has not co-adapted to use a specific host plant but has to maintain a broad detoxification mechanism.

P. plantaginis is not eaten by blue tits, pied flycatchers or ants, and is an unpreferred prey of great tits (Lindstedt et al. unpublished manuscript). The species is warningly coloured both as a larva and an adult, and therefore, it can be considered aposematic. The hairy larvae have a patch of orange hair on their dorsal side, and in combination with an otherwise black body, this orange patch is a visual warning signal to potential predators (Lindstedt et al. unpublished manuscript, IV). The adults are diurnal and extremely conspicuous: males are either black and white or black with yellow and white; females are usually black, red and white but sometimes red is replaced with orange. The colouring of the larvae varies also; the size and brightness of the orange patch varies. Variation in colouration is typical of Arctiid moths (Fields & McNeill 1988, Liebert & Brakefield 1990). This variation, combined with the relative abundance of the species makes it an excellent species for studying what maintains variation in warning signals and how this variation influences fitness.

D. sannio has aposematic larvae that are mostly black and hairy. They have a narrow strip of white, yellow or orange on their dorsal side. The adults of this species are sexually dimorphic: females are brownish orange and males are bright yellow and pink. It is not known whether the adults are unpalatable. *D. sannio* is more abundant than *P. plantaginis*, and I chose to use both species so I could generalise the results of the studies and also so I could get sufficient sample sizes for the studies.

Adult females of these two species were caught with a butterfly net in late June and early July from Southern Finland, Jomala in the Åland Islands and near Tartu in Estonia. The females had already mated in nature and laid eggs in the laboratory. The studies on the effect of diet on these two species were done as a full-sib, common garden design. Larvae were checked every day and fresh food was added *ad libitum* if necessary or at least every second day.

To assess the effect of diet on the growth, survival, immunocompetence and warning colour of the larvae, I reared larvae of *P. plantaginis* on their natural host plants of *Taraxacum* (poor in plant secondary metabolites), *Rumex* (rich in plant secondary metabolites) and *Lactuca* (plant secondary metabolites are nearly absent), and on an artificial diet which did not contain plant secondary metabolites. To study how novel and common plant secondary metabolites affect the growth, survival and immunocompetence of *D. sannio,* the larvae were fed with four diets of different quality: *Taraxacum*, which is a natural host plant and expected to be beneficial for them, and three different kinds of artificial diet; one with a quantitative plant secondary metabolite (chlorogenic acid); one with a qualitative secondary metabolite (monocrotaline); and one with no added secondary metabolites (control). Chlorogenic acid is a common plant phenolic. *D. sannio* was expected to be somewhat adapted to feeding on it, but detrimental effects were also expected since chlorogenic acid likely reduces the growth rate of herbivores (Stamp & Yang 1996). Monocrotaline is a toxic pyrrolizidine alkaloid which occurs in the tropical plant genus *Crotalaria*, and was expected to cause adverse effects on the growth and survival of *D. sannio*. Both of these chemicals can be used for chemical defence by insects (Cardoso 1997, Traugott & Stamp 1997).

2.1.2 Great tits (II & III)

The predation experiments with great tits (*Parus major*) were carried out at Konnevesi Research Station (Central Finland). I used adult wild birds which were captured from feeding sites around the station. Central Finland Regional Environment Centre gave us permission to capture and keep the birds and Experimental Animal Committee of the University of Jyväskylä gave the permission to do the experiments. Birds were kept individually in illuminated plywood cages on a diet of sunflower seeds and tallow. Experiments were done in the winter to avoid disturbing the birds' breeding. After the experiment, all birds were released to the sites from which they were originally captured.

2.2 Testing the efficiency of defence strategies in Arctiid moths

2.2.1 Growth rate and survival (I, IV & V)

Arctiid moths were weighed the day after pupation and the adults were sexed after their emergence. Both rapid development and large size are likely beneficial for the fitness of the arctiid moths, and I used larval growth rate as a measure of growth, since it combines development time and pupal weight in a single variable (weight gained per milligram per day). Also, by using growth rate, which does not differ between males and females, we were able to pool the sexes for analyses. I also assessed the survival of the moths on different diets, from neonate larva to adulthood for *P. plantaginis* and from the beginning of the experiment to pupation for *D. sannio*.

2.2.2 Encapsulation ability (IV & V)

Encapsulation is the primary insect defence against hymenopteran and dipteran parasitoids (Nappi 1975, Godfray 1994) as well as against nematodes (Stoffolano 1986) and fungi (Vey & Götz 1986). Encapsulation reaction is a general response to foreign intrusions: all small inert particles inside an insect are encapsulated (Nappi 1975, Lackie 1988). Encapsulation reaction can be quantitatively measured by using novel and standardized "parasites" such as nylon monofilament implants (König & Schmid-Hempel 1995, Ryder and Siva-Jothy 2000) that mimic a parasitoid inside the insect. The advantage of the implant method is that the "parasite" is neutral and does not have a means to overcome the host's defences. Thus, the outcome of encapsulation is solely dependent on the host, giving a relevant quantitative measure of the insect's ability to fight parasitoids. Moreover, lepidopteran larvae survive the implantation (however the pupae often do not) and develop into adults, enabling measurement of life history parameters of the same individuals.

For *P. plantaginis*, 5th – 6th instar larvae were anesthetized with CO_2 and a small cut was made in the dorsal side of the larva. For *D. sannio* cuts were made into the sixth abdominal segment of a pupa. A part of a nylon implant was inserted inside the larva or pupa and the immune system was allowed to react to the implant for 5 hours. The implant was then removed, dried and photographed. The mean grey value of the implant was measured from the end of the implant that had been inside the individual; the darker the implant, the stronger the encapsulation reaction.

2.2.3 Warning signal size (I)

The orange patch on the dorsal side of the otherwise black *P. plantaginis* larvae functions as a warning signal but varies greatly in size. The patch can be very small or the larvae can even be mostly orange with only the two anterior body segments and head being black. To study the effect of diet and genetic factors on the size of the warning signal, I reared *P. plantaginis* larvae on two diets of different quality and measured the development of the size of the warning signal. Larvae gained their dorsal orange patch in their fourth instar. After gaining an orange patch, the larvae were photographed at each instar. The number of larval instars per individual varied; therefore we had 2-4 digital images of each larva. Both the total length of the larva and the length of the orange patch were measured from the photos. The length of the orange patch was divided by the length of the larva, thereby giving us a measure of the relative size of the orange warning signal.

2.3 Testing the predation pressure in a novel world setup (II)

Since predators and aposematic prey have had a long history of coexistence, the predators have evolved preferences and aversions towards prey of different appearance (Roper 1990, Mastrota 1995). Therefore, to study the initial evolution of aposematism as well as the dynamics of predator learning and memory, it is necessary to use novel warning signals of which the predators have no prior experience (Alatalo & Mappes 1996). Using novel warning signals in a black-and-white environment that can be easily manipulated, I was able to test the effects of variation in a warning signal as well as in the level of variation in unpalatability. This set-up gives me confidence that the results are not skewed by experienced predators and unlearned biases, e.g. for certain colours.

Predation experiments were run in aviaries where a novel world with a landscape of black-and-white symbols (Alatalo and Mappes 1996, Lindström et al. 1999) was set up. The floor was covered with white paper sheets, and there were crosses in random positions printed on each sheet. The prey items were then placed on the sheets: I used pieces of almond glued between pieces of paper with a symbol printed on them. The cryptic, palatable prey items had a cross so that they would blend them into the background of crosses, and the aposematic, distasteful prey items (first soaked in chloroquinine) had either a conspicuous black square or an equally conspicuous black irregular star on them.

I studied the predation pressure that inexperienced predators create on different Müllerian mimicry complexes. In nine different treatments, I tested the effect of palatability level, signal level and combinations of these. The birds were presented prey with irregular stars, prey with squares, or prey with a mixture of both symbols in three different configurations of taste. Predators encountered aposematic prey as mildly unpalatable, strongly unpalatable, or as a mixture of these two levels of unpalatability. By keeping the level of unpalatability constant, I could assess the importance of the signal and when the signal was kept constant, I could assess the importance of palatability to bird predation on a mimicry complex.

2.4 Study on seasonal effects on warning colour efficacy (III)

Aposematism works because predators learn to avoid defended prey with a warning signal (Poulton 1890, Cott 1940, Edmunds 1974). Therefore, aposematism is not likely to be as successful when there are numerous naïve predators in the environment as when most of the predators are experienced about warning signals. However, even naïve predators have preferences to certain colours and they avoid eating prey with certain colours (Roper 1990, Mastrota 1995). To test the idea that warning colours might be more advantageous at times when there are many experienced predators in the environment, I conducted an experiment with artificial larvae either with a warning signal or without a signal placed in nature for wild birds to prey upon. I also studied if there is proportionally more warningly coloured lepidopteran species when there are mainly experienced predators in the environment, because this would indicate different pay-offs from warning colours at different times of the year, when the predator community is also different.

2.4.1 Predation experiment with artificial larvae

To test the effectiveness of warning signals in the seasonal environment I conducted experiments with artificial larvae that mimic lepidopteran larvae. The model species for the warning color patterns was *Parasemia plantaginis*, which has an orange warning signal on the dorsal side on an otherwise black body. Some of the artificial larvae had an orange patch (=warning signal) on the dorsal side of an otherwise black body, and some of the larvae were completely black, thus without conspicuous warning colouration. Therefore, I was able to compare the predation on warningly coloured larvae with predation on larvae without warning colours. Because the size of the orange patch of *P. plantaginis* varies greatly and since we were also interested in if warning signals of different sizes have different qualities, I made two types of warningly coloured artificial larvae that mimic the extremes of this variation: either with a small orange patch or a large orange patch on an otherwise black body. With these two warning signal types, I was able to compare the attack rate on larvae with warning signals of different sizes.

2.4.2 Predator community age structure

To study how the predator community age structure changes along the season in Southern Finland, I estimated when the young birds leave their nest and start feeding on their own. I used data of insectivorous passerine birds ringed as nestlings in continental Southern Finland in 2005 provided by the Finnish ringing centre and a dataset of mist-netted juvenile and adult birds throughout the summer 2005 in Southern Finland.

2.4.3 Warning colours of macrolepidopteran species

To test the seasonal occurrence of warningly coloured species I scored nearly all species of Finnish (excluding Lapland) macrolepidopteran larvae (745 out of 759 species) for warning colours in their last larval instar from colour slides taken by Kimmo Silvonen. Species were assigned to one of four categories: 0) no typical warning colours, 1) some features of warning colouration, 2) moderate warning colours and 3) very strong, conspicuous warning colours (Fig. 1). Unfortunately there is no data available for determining whether a given species has a secondary defence (toxic compounds etc.) against predation or not. Therefore, my classification pools both aposematic species and possible

Batesian mimics. I also recorded the time when each of the species occurs as last-instar larvae in Southern Finland (Mikkola & Jalas 1977, 1979, 1983, 1989, Marttila et al. 1991, 1996).

FIGURE 1 Examples of the types of colouration in Finnish macrolepidopteran larvae as assessed in article III. a) category 0, no typical warning colours, *Macaria carbonaria* , b) category 1, some features of warning colouration, *Mesoleuca albicillata*, c) category 2, moderate warning colouration, *Diacrisia sannio*, and d) category 3, strong warning colouration, *Nymphales antiopa*.

3 RESULTS AND DISCUSSION

3.1 Effects of diet on aposematic animals

3.1.1 Diet and immunocompetence

I found that the ability of *P. plantaginis* to encapsulate a foreign object varies depending on the host plant species and that the ability corresponds with the amount of antioxidants in the diet (V, Ojala et al. 2005). However, the other fitness measures (growth and fecundity) did not correlate with encapsulation ability even though they were also affected by diet. This suggests that diet *per se* affects encapsulation and the high scores for encapsulation reactions are not attributable to the better condition of the larvae. The lack of correlation between developmental rate and encapsulation ability suggests that *P. plantaginis* larvae do not directly trade off growth rate for encapsulation ability. This suggests that, in *P. plantaginis,* the host plant species affects the encapsulation ability via plant secondary metabolites that affect herbivores' growth but also enable the larva to produce successful encapsulation. In this study the amounts of digestibility-reducing tannins and potentially beneficial flavonoids and carotenoids of the diets co-varied positively. Thus, eating the non-tannin diet meant that the moth larva received much less antioxidants and possibly also much less other substances beneficial to successful immunodefense.

Surprisingly, among *D. sannio* larvae that were fed with four diets of different quality, the encapsulation ability of the pupa did not differ, even though the diets affected the growth and survival of the larvae. Contrary to the experiment with *P. plantaginis,* individuals on a diet high in antioxidants (*Taraxacum*) did not fare better than those on a diet without antioxidants. Therefore, the effect of diet on immunocompetence seems to be different for different species. Alternatively, the result could be because the encapsulation ability was measured from pupae and not from larvae as in the experiment with *P. plantaginis,* since allocation to defence against parasitoids can be different at different developmental stages.

It seems likely that the optimal diet choice for a polyphagous insect herbivore can vary in time and place depending on, for example, the enemy community and time available for development (Singer et al. 2004). When parasitoids are common, it is beneficial to eat relatively more plants that have a high concentration of plant secondary metabolites, which might slow down development, but give greater potential to defend against parasites. However, in aposematic insects, the choice of diet can also affect the defence against predators (Rothschild 1973); the warning signal as well as chemical defence efficacy (see next chapter). Therefore, the choice of a host plant can be extremely complicated to an aposematic, polyphagous herbivore species.

3.1.2 Diet and aposematic defence

I also studied if eating artificial diets with different plant secondary compound would change the edibility of *D. sannio* larvae to their potential predators. Somewhat surprisingly, even monocrotaline, which is highly toxic to vertebrates and is used for chemical defence in many species of arctiid moths (Wink & van Nickisch-Rosenegk 1997, Weller et al. 1999), did not make *D. sannio* larvae completely unpalatable to great tits: even though they preferred other prey, the birds also ate some *D. sannio* larvae (IV). Therefore, *D. sannio* seems to be an example of a species which is not strongly defended against predators and possibly can not sequester monocrotaline. In northern latitude summers, there is plenty of alternative prey available for the predators and per capita mortality is low (III). Thus, even a suboptimal chemical defence can possibly protect the larvae from suffering much mortality from predation by birds.

Diet can also affect the size of the warning signal of an aposematic organism, as demonstrated by the study in which I fed *P. plantaginis* larvae with two diets of different quality (I). The warning signal size, which possibly affects its effectiveness as a signal, was larger in the larvae with a high growth rate. It is possible that larvae with a longer development time (and thus lower growth rate) could benefit from darker colouration, since these larvae are more likely to overwinter than those with fast development, and because during the autumn and spring they need to warm up quickly to be able to feed. In the warmer summer months the dark colouration would probably not be so important to the growth of the larvae, or it could even be selected against, since it exposes the larva to a risk of overheating (Fields & McNeill 1988). Under such a scenario, if some diets produce slower growth, the frequency of small signals would increase in the population eating this diet although small signal *per se* would not be favoured. Thus, it is possible that the colouration of *P. plantaginis* is a compromise between two different functions; aposematism and thermoregulation (Lindstedt et al. unpubl.).

3.2 Variation in aposematic defence

3.2.1 Aposematic animal perspective

In the study of the warning colour pattern of *P. plantaginis* larvae, I found considerable continuous variation, which has genetic as well as environmental basis (I). Variation of this scale seems counter-intuitive, since variation in warning signals is usually expected to slow down predator learning (Müller 1879, Mallet & Singer 1987, Turner 1987, Endler 1991, Beatty et al. 2004). The efficacy of the warning signals in predator education is essential for the warningly coloured organisms, since the theory of aposematism is based on predators having to learn to avoid individuals with warning signals (see e. g. Cott 1940, Edmunds 1974, Guilford 1990). Based on experiments with artificial prey items and bird predators, an aposematic signal with large pattern elements is easier for predators to recognise and learn than a signal with small pattern elements (Gamberale & Tullberg 1996, Forsman & Merilaita 1999, Lindström et al. 1999, Jansson & Enquist 2005). Therefore, a large orange signal could be more beneficial than a small one for *P. plantaginis* surviving predation by birds.

My experiments give some answers to the question: why don't all individuals have a similar, large signal expression if it would help them avoid predation? I can suggest four hypotheses: 1) trade-offs between signal size and life-history traits exist 2) genetic correlations between signal size and life-history traits exist 3) selection pressures on colour vary in space or time, and 4) selection by predation for signal size is weak, which allows high variability in signal size.

First, it may be costly for an insect to produce melanin-based black pigments (Windig 1999, Talloen et al. 2004). Thus, it is possible that it is costly for *P. plantaginis* larvae to express black colour (which contains a large amount of melanin) instead of orange colour (which contains very little melanin). However, if melanin is costly, the larvae on the poorer artificial diet should have had more inexpensive orange colour than the larvae on *Rumex,* especially since the artificial diet had slightly less nitrogen than *Rumex* (Ojala et al. 2005). On the contrary, these individuals had more black and a smaller orange signal, and thus, had to use more melanin for their colouration. Therefore, it seems unlikely that *P. plantaginis* larvae would trade-off growth with melanin production. Instead, it is possible that excretion of excess melanin from their orange hairs is costly to the larvae. According to the "costly orange" hypothesis, larvae in good condition were able to produce an effective orange signal, whereas larvae in poorer condition (such as the larvae on artificial diet) were not. Also, the larvae that died before adulthood were possibly already infected or weak for some other reason in the 4th instar, and thus not able to produce a costly, large orange signal.

Second, there was a significant positive correlation calculated from family means between growth rate and signal size in the last instar, which suggests that a genetic correlation between these traits is likely. Linkage between these traits would mean that selection on either one of these traits would also affect the other. If for example, in some years or environments slower growth would be favoured, the frequency of small signals would increase in the population although small signal *per se* would not be favoured.

Third, it seems likely that the predation pressure on *P. plantaginis* larval warning signal changes as larvae develop. Young larvae are greenish and cryptic and gain their orange warning signal in the third instar. It is likely that in a very small larva a warning signal would not be sufficiently effective to counter the detectability costs of conspicuous colouration (Gamberale & Tullberg 1996, Forsman & Merilaita 1999, Hagman & Forsman 2003, but see Nilsson & Forsman 2003). Also, in a small larva the absolute size of a warning signal can obviously not be very large and therefore aposematic colouration might not be a successful anti-predatory strategy.

Fourth, there is also the possibility that the selection pressure against variation in warning signal is not as intensive as it is usually assumed (III). Perhaps learning and generalization by predators allows for variation in warning signal pattern as long as a signal is separable from cryptic prey (Wallace 1867, Sherratt & Beatty 2003). Recent experiments by Rowe et al. (2004), Lindström et al. (2006) and Ihalainen & Suutari (2003) indeed show that predators are able to learn and remember rather variable signals suggesting that the “paradoxical” variability might not be so paradoxical. This is also supported by the study by Holloway et al. (2001), which showed that there is more variation in the defended *Vespula* wasps than in their non-defended (Batesian) hoverfly mimics; suggesting more relaxed selection on the colour pattern of a defended species, i.e. in Müllerian co-mimics than in Batesian mimics. However, more studies are needed to clarify warning signal architecture and its fitness correlations before we can make straightforward conclusions about this phenomenon.

The hypotheses presented here are not exclusionary: it is likely that many or all of them play a part in explaining the variation in warning signal of *P. plantaginis*. I suggest that variable growing conditions and genetic correlation between signal size and growth rate explain variation in signal size. It is also likely that the relative fitness benefit of large signal varies in space and time, causing variable selection pressure on the colouring of the aposematic species. The variable selection can be caused e.g. by differences in the predation pressure or by variation in the demands for thermoregulatory ability of the larva. Thus, although the variation in warning signals might seem paradoxical in the light of the theory of warning signals (e.g. Joron & Mallet 1998), it is likely a result of varying selection pressures on the colouration of warningly coloured animals (Mappes et al. 2005).

The warning signal of *P. plantaginis* was assigned to category 2 (moderate warning signal) and the warning signal of *D. sannio* was assigned to category 1 (some features of warning signals) (Fig 1). Thus, these species do not have a very strong warning colouration, which seems to be in accordance with the result that they are not always avoided by predators (IV, Lindstedt et al. unpublished manuscript).

3.2.2 Predator perspective

My results from the predation experiment with great tits and artificial prey items in a novel world setup show that both signal and taste are important for the survival of aposematic Müllerian co-mimics (II). The signal denoting unpalatability is not irrelevant; an irregular star was a much more effective signal producing avoidance by birds than a square, even though there was no initial bias against either of the signals. Surprisingly, when both star- and square-signalling aposematic prey were presented to the birds, the predation pressure was not increased compared to perfect mimicry, which is suggested by the classical Müllerian mimicry theory (Müller 1879, Joron & Mallet 1998, but see Beatty et al. 2004, Rowe et al. 2004). Birds learned to avoid highly unpalatable prey faster than mildly unpalatable prey. However, variation in palatability levels did not unequivocally increase the total mortality of strongly unpalatable models as suggested by quasi-Batesian theory (Speed 1993), but the effects were dependent on the signal of the prey: the squares survived poorly when they were paired with highly unpalatable stars. It seemed that predation pressure even tended to decrease for the mildly unpalatable weaker signal (square) when it was paired with the strong signal (irregular star), indicating that for prey with a weak warning signal even imperfect Müllerian mimicry to a strong signal would be a more beneficial strategy than perfect mimicry to another weak signal. These results suggest that signals and unpalatability levels can produce different effects depending on the configuration of the whole mimicry complex. Also, quasi-Batesian effects may be more dependent on the signal than on the taste of the unpalatable prey.

In study III, I tested the predation pressure on artificial prey types with either a conspicuous warning signal, an intermediate sized warning signal or without a warning signal in a natural environment. The efficacy of warning colours as a defence strategy against predation was found to vary between seasons. Warningly coloured prey were at an advantage compared to more cryptic prey at spring and late summer, but in the middle of the summer the cryptic artificial larvae were at an advantage and suffered less predation. This pattern likely exists because of changes in the predator community structure, which changes over time in a seasonal environment. When there are mostly experienced predators present that have already learnt to avoid prey with warning signals, a conspicuous warning signal is beneficial to its bearer. However, when there are many young, naïve predators present, having a conspicuous color signal also increases the likelihood of being preyed upon by naïve predators, and cryptic individuals are at an advantage. Also, it is possible that warning colouration is more beneficial at the end of the summer when there is plenty of food for the predators, since then the predators can find alternative prey easily and the cost of possibly rejecting edible prey is likely relatively low.

In this study, the predation pressure on warningly coloured artificial larvae was likely somewhat similar for larvae with either a large or a small warning colour patch. Therefore, it seems that a large, strong colour signal is not necessarily needed for the signal to function efficiently, and a smaller, less conspicuous warning signal can be enough to deter predators. On the other hand, it is possible that a small signal combines the benefits of two anti-predator strategies: a small signal is not extremely conspicuous especially from a distance and so predators are not likely to detect the larva, but from a close distance even a small signal can be deterrent (see also Tullberg et al. 2005).

Since I found that even a small warning signal at least can be nearly as beneficial as a large one in generating predator avoidance, it is possible that warningly coloured species do not have a trade-off between the effectiveness of their warning signal and other demands on their colouration, such as thermoregulation and UV-protection. However, it is important to note that in the experiment with artificial larvae we studied only the effect of a warning signal that the predators likely had not encountered previously, and not how signal size affects the learning and memory of the predators. It is possible that signal size affects learning and not the initial reactions of the birds towards prey. However, since many macrolepidopteran species are not abundant in Finland (Mikkola & Jalas 1977, 1979, 1983, 1989, Marttila et al 1991, 1996) and aposematism is surprisingly scarce strategy among Lepidoptera (III), bird predators are not likely to encounter many of the species often enough to facilitate learning, the initial reaction of the birds towards the warning signal is expected to be extremely important to the survival of the larva. It seems likely that previous experiments based on laboratory trials have overestimated the significance of learning in evolution of aposematism.

I also tested the occurrence of warning colouration at different times of the year with a comprehensive dataset of pictures of macrolepidopteran larvae (III). The proportion of macrolepidopteran species in Southern Finland with warningly coloured larvae was lower in summer than in spring and autumn. I conclude that the benefits of warning colours as an anti-predator strategy change, as was demonstrated in the study with artificial larvae, and this change is reflected in the proportion of warningly coloured species at different times of the year.

Predation experiments both in the novel world setup (II) as well as in nature with wild bird predators (III) did not provide evidence that predation pressure causes close similarity in aposematic animals. Actually, variation is exactly what we find in nature: despite some well-known and well-studied examples of striking similarity among species in Müllerian mimicry rings (e.g. burnet moths: Turner 1971, bumblebees: Plowright & Owen 1980, heliconid butterflies: Mallet & Barton 1989 and poison arrow frogs: Symula et al. 2001), the majority of insect species with warning signals are not very much alike even when they share predators (at least in Northern Europe) (Carter & Hargreaves 1986).

4 CONCLUSIONS

Even though the mimicry theories were among the first evidences for the theory of evolution (Blaisdell 1992), and even today they serve as schoolbook examples of natural selection (Ridley 2004), there is still great need for experimental research on aposematism and Müllerian mimicry to understand their dynamics. Studies that take into account more selection pressures than just predation e.g. towards warning colouration are especially needed. My aim was to study the interactions between host plants, aposematic herbivores and their natural enemies. In particular, I have been interested in how variation at all of these levels affects the evolutionary dynamics of aposematism.

The first part of this study deals with a main question of how variation in diet affects the defence of aposematic animals against their natural enemies. Especially polyphagous aposematic animals face a challenge, since eating several host plants can affect their immunocompetence (V), chemical defence (IV) and warning signal (I), and they can face trade-offs e.g. among growth and immunocompetence (V) when choosing their diet. It is possible that aposematism is a more demanding anti-predator strategy for a polyphagous herbivore than a specialist on a particular host plant species from which they can sequester defensive chemicals. Indeed, it has been found that aposematic insect species are more often specialised on one host plant than cryptic species (Bernays & Cornelius 1989).

This study has demonstrated that a generalist herbivore *Diacrisia sannio* is unpreferred prey to great tits (IV). An additional study by Lindstedt et al. (unpublished manuscript) has shown that *Parasemia plantaginis* is not eaten by ants, blue tits or pied flycatchers; however the species is eaten, but not preferred by great tits. Thus, one can conclude that the aposematic defence of these arctiid moths does not totally protect them from predation. However, these larvae can still be classified as aposematic because most predators will likely avoid eating them and they both have colouration which is typically used for a warning signal, although the signal on these species is not extremely conspicuous against the natural background. The behaviour of these larvae is then understandble: they can move relatively fast and also live in the midst of vegetation so that finding them in nature is difficult (K. Ojala personal observation). Strongly aposematic species are often found in exposed positions (Ruxton et al 2004). Such behaviour indicates strong secondary defence, something not possible for these two species because they are only moderately unpalatable. Since some predators can cope with their defence, it is not beneficial for them to advertise themselves too conspicuously (Endler & Mappes 2004)

In this study, I found considerable variation in the warning colour pattern of *P. plantaginis*; and both genetic and environmental factors generated variation in the warning signal (I). This observed within-species and within-localities variation in the warning signal is usually not expected according to the theory of aposematism. Based on e.g. the study with artificial prey items and great tits as predators (II), the efficacy of a warning signal can profoundly affect the learning of predators and therefore effect the survival of the insect. Thus, if different signals differ in their efficacy in deterring predators, the signal in expected to evolve towards uniformity. However, because the *P. plantaginis* larvae spend much of their time hiding in the vegetation, because there is an abundance of alternative insect prey to bird predators in Northern European summers, and because predator generalisation seems to be wide, small differences in warning signal efficacy do not necessarily affect the outcome of predation pressure on fitness.

The efficacy of warning colouration as a defence against predation was found to vary temporally in a seasonal environment, and this variation was likely due to variation in the predator community experience level. As many environments are seasonal, this effect could widespread in nature. This observation could at least partly explain why warningly coloured species seem to be more common in stable environments such as a Bolivian rainforest (K. Ojala personal observation) than in seasonal Northern Europe (Chinery 1986, IV). In Finland, the best period for insect growth occurs at the same time when numerous insectivorous bird fledglings and in the environment, and thus warning colours are not necessarily beneficial for the bearer. In a more stable environment, the predators are likely to be more local, the hunger level of the predators is likely to be more stable and also the breeding period of predators much more widespread, and consequently the benefits from warning colouration will be higher. However, until properly tested, this reasoning remains speculation only (but see Pinheiro 2003).

The effect of variation in unpalatability of aposematic animals and Müllerian mimics in particular has been studied theoretically (Huheey 1976, 1984, Owen & Owen 1984, Speed 1993, 1999, Speed and Turner 1999) but rarely with real predators (Ihalainen & Suutari 2003, II). The level of unpalatability profoundly affects the efficacy of aposematism, but the effect of variation in the level of unpalatability still remains unclear. Other research highlighted that some signals are more efficient in facilitating predator learning than others, and that predators have innate preferences as well as aversions for some colours and colour patterns (Roper 1990, Mastrota 1995). However, in models of the dynamics of Müllerian mimicry, the only qualities of aposematic co-mimics affecting the evolution of mimicry have been differences in density and variation in palatability level. As this study has shown, the efficacy of the warning signal is not irrelevant for the fitness of the bearer of a signal, and the effect of the signal strength is dependent on the unpalatability level of the bearer. Thus, the implications from theoretical models including only density and palatability level can change profoundly if variation in signal efficacy is added. I found that one of the artificial signals was somewhat surprisingly more efficient in producing predator avoidance, although it was not larger, more conspicuous or initially preferred or avoided by the birds. Therefore, assessing the efficacy of the warning signal of real-life aposematic organisms can be very difficult. Also, predators' initial reactions towards preys' signals (Roper 1990, Mastrota 1995), their reactions after being exposed to aversive chemicals (Rowe & Skelhorn 2004), and their learning and remembering are likely to be different. The same rules probably do not apply when determining which signals are the most beneficial from the preys' perspective in an environment with many kinds of predators. Some predators can overcome the defences and prey on the species, some are naïve and could attack, some are experienced and not likely to attack, and yet others are experienced, but hungry and could also attack. Therefore, the benefits of aposematism as a strategy against predation depend heavily on the structure of the predator community. To make things even more complicated, also other selection pressures can affect the colouration and possibilities for chemical defence of an organism (Endler & Mappes 2004, Mappes et al. 2005 Ruxton et al. 2004).

My study has demonstrated that there is variation in the defence against predators in aposematic animals. Although this variation can appear problematic if we only consider one selection pressure at a time, when we consider tri-trophic interactions, variation in aposematic defence becomes understandable. Also, the benefit of aposematism is not absolute, but depends e.g. on time, place and the enemy community.

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YHTEENVETO (RESUME IN FINNISH)

Puolustuskyvyn vaihtelu ja sen merkitys aposemaattisten eläinten kelpoisuuteen: ravinnon, loisten ja saalistajien vuorovaikutus

Puolustautuminen saalistajia ja loisia vastaan on erittäin tärkeää kaikille eläinlajeille, sillä onnistuneesta pedon tai loisen hyökkäyksestä voi olla seurauksena eläimen haavoittuminen tai kuolema. Toisaalta pedot ja loiset ovat evoluutiohistorian aikana sopeutuneet saalislajien puolustukseen. Tämä saalistajien ja petojen välisen kilpavarustelun tutkiminen on yksi evoluutioekologian keskeisimpiä tutkimuskohteita. Puolustautumiskyvystä voi kuitenkin olla myös kustannuksia: Esimerkiksi piikkien, karvojen, myrkyllisyyden tai immuunijärjestelmän ylläpito voi viedä resursseja kasvulta ja lisääntymiseltä, tai saalistajilta piilottelu voi vähentää ruuanhankintaan käytettävissä olevaa aikaa. Lähes aina kutakin lajia saalistaa useita petoja ja loisia, jotka voivat käyttää hyvin erilaisia saalistusstrategioita. Koska yksilöillä on vain rajallinen määrä resursseja käytettävissään, saaliseläimet pystyvät harvoin varustautumaan kaikkia petoja ja loisia vastaan yhtä tehokkaasti. Saattaa olla myös niin, että hyvä puolustusstrategia yhdenlaista saalistajaa vastaan altistaakin saaliseläimen toisentyyppiselle saalistajalle. Yksilön puolustusstrategia onkin kompromissi monien erilaisten valintapaineiden välillä. Väitöskirjatutkimukseni kohteena olivat erilaiset puolustautumistavat saalistajia vastaan.

Aposematismiksi kutsutaan puolustusstrategiaa, jossa syömäkelvoton eläin mainostaa puolustuskykyään potentiaalisille saalistajille (esim. myrkyllisyyttä tai pahanmakuisuutta) varoitussignaalilla (esim. näkyvä väritys). Esimerkkejä aposemaattisista eläimistä ovat mm. kimalaiset, joiden varoitussignaalina toimii musta yhdistettynä valkoiseen, keltaiseen tai oranssiin väritykseen ja joiden naaraat voivat puolustautua saalistajia vastaan pistämällä. Värikkäillä leppäkertuilla puolestaan on ruumiinnesteessä pahanmakuista pyratsiinia. Saalistajat oppivat, tunnistavat ja muistavat varoitussignaalin helpommin silloin, kun varoitussignaalit ovat hyvin näkyviä eli ne esimerkiksi koostuvat selkeistä oransseista ja mustista alueista. Toisaalta varoitussignaalin näkyvyydestä voi olla myös haittaa, jos saalistajat eivät ole vielä oppineet välttämään sitä. Aposematismiteoria ennustaakin vähäistä vaihtelua varoitussignaaleissa ja puolustuskyvyssä sillä saalistajien on todennäköisesti vaikeampi oppia välttämään useita kuin vain yhtä varoitussignaalia.

Väitöskirjassani olen tutkinut puolustuskyvyssä saalistajia ja loisia vastaan ilmenevän vaihtelun syitä ja seurauksia. Tärkeimmät tutkimuslajini olivat aposemaattiset, siilikkäisiin kuuluvat perhoset karhusiilikäs (*Diacrisia sannio*) ja täpläsiilikäs (*Parasemia plantaginis*), joiden toukien kasvua, varoitussignaalin kokoa (*P. plantaginis*) ja puolustautumiskykyä loisia vastaan (*D. sannio*) tutkin. Tutkiakseni, onko puolustautumisesta kustannuksia näille lajeille, kasvatin toukkia erilaatuisilla ravintokasveilla (I, IV, V). Tutkiakseni kasvien kvalitatiivisen ja kvantitatiivisen puolustusaineiden merkitystä karhusiilikkäiden puolustautumiseen, lisäsin perhosten keinoruokaan joko klorogeenihappoa, joka on yleinen kasvien kvantitatiivinen puolustusaine kasvinsyöjiä vastaan tai monokrotaliinia, joka on kvalitatiivinen puolustusaine. Vertasin toukkien kasvua ja niiden kykyä puolustautua petoja vastaan toukkiin, jotka olivat kasvaneet kontrolliravinnolla (keinoruoka, johon ei ollut lisätty kasvien puolustusaineita) tai toukkien luontaisella ravintokasvilla (voikukka) (IV). Osoitin, että täpläsiilikkään toukkien varoitussignaalissa on suhteellisen paljon vaihtelua yksilöiden välillä, toisin kuin teoreettisesti olisi ollut odotettavissa (I). Varoitussignaalin kokoon vaikuttavat sekä geneettiset tekijät että yksilön ravinto, ja toukat, joilla on suuri varoitussignaali, kasvavat nopeammin ja selviytyvät todennäköisemmin aikuiseksi kuin toukat joilla on pieni signaali (I). Lisäksi ravinnon kemiallinen koostumus ja varsinkin sen antioksidanttipitoisuus vaikuttaa toukkien puolustautumiskykyyn loisia vastaan (V). Toisaalta ravinnon kemiallinen koostumus vaikutti hämmästyttävän vähän karhusiilikkään toukkien kykyyn puolustautua lintupetoja vastaan, vaikka se vaikutti huomattavasti yksilöiden kasvuun ja elossasäilymiseen (IV).

Laboratoriokokeet, joissa talitiaiset saalistivat keinosaaliita, osoittivat, että vaihtelu varoitussignaalissa ja kemiallisen puolustuksen voimakkuudessa vaikuttaa yllättävän vähän saalistajien välttämisoppimiseen, ja siten vaihtelulla varoitussignaalissa ei ehkä olisikaan niin suurta merkitystä kuin aiemmin on ajateltu (II). Toinen luonnossa tehty osakoe, jossa varoitussignaalin tehokkuutta manipuloitiin keinotoukilla, kuitenkin osoittaa, että varoitusvärien tehokkuus vaihtelee vuodenaikojen mukaan: varoitusvärit ovat tehokkaampia saalistuksen estäjiä kuin kryptinen (ympäristöön sulautuva) väritys keväällä ja syksyllä, mutta keskellä kesää varoitusvärisiä yksilöitä saalistetaan enemmän kuin kryptisiä (III). Tämän tuloksen selittää vuodenaikainen vaihtelu saalistajayhteisön rakenteessa: kun ympäristössä on paljon nuoria ja siten naiiveja saalistajia, varoitussignaalien näkyvyys lisää niihin kohdistunutta saalistusta, kun taas aikoina jolloin suurin osa saalistajista on vanhempia ja kokeneita, varoitussignaalit suojelevat yksilöä. Tämä ajallinen vaihtelu varoitusväristen ja kryptisten yksilöiden suhteellisessa kelpoisuudessa on vaikuttanut myös eri perhoslajien toukkien varoitusvärityukseen sillä samassa tutkimuksessa totesin, että keskikesällä toukkina elävissä perhosentoukissa on suhteellisesti vähemmän varoitusvärisiä lajeja kuin keväällä ja syksyllä toukkana elävillä (III).

Väitöskirjani tulokset osoittavat, että sekä geneettiset tekijät (I) että ravinto (I, IV, V) voivat rajoittaa puolustuksen tehokkuutta saalistajia ja loisia vastaan. Lisäksi vuodenaikaiset muutokset petoyhteisössä saavat aikaan erilaisia valintapaineita, jolloin erilaisten puolustusstrategioiden (esim. kryptinen vs. aposemaattinen) suhteellinen tehokkuus vaihtelee ajallisesti ja paikallisesti (III). Nämä tekijät yhdessä ylläpitävät muuntelua aposemaattisten eläinten varoitussignaaleissa ja puolustuskyvyssä ja osaltaan selittävät sitä suurenmoista värivaihtelua, jota luonnossa näemme.

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Considerable phenotypic and genetic variation in the warning signal expression of *Parasemia plantaginis* (Arctiidae)

by

Katja Ojala, Leena Lindström & Johanna Mappes

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II

Relative importance of taste and visual appearance for predator education in Müllerian mimicry

by

Leena Lindström, Anne Lyytinen, Johanna Mappes & Katja Ojala

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III

Changes in predator community generate seasonally varying selection for warning signals

by

Katja Ojala, Hanna Kokko, Leena Lindström & Johanna Mappes

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IV

The effects of qualitative versus quantitative plant secondary metabolite on growth and defence against predators and parasites in an arctiid moth *Diacrisia sannio.*

by

Katja Ojala, Leena Lindström & Johanna Mappes

Manuscript

THE EFFECTS OF QUALITATIVE VERSUS QUANTITATIVE PLANT SECONDARY METABOLITES ON GROWTH AND DEFENCE AGAINST PREDATORS AND PARASITES IN AN ARCTIID MOTH *DIACRISIA SANNIO*

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Abstract

Plant secondary metabolites can have dual effects on herbivores. On one hand they can inhibit growth and decrease survival, but at the same time they can provide precursors for the defence against predators and parasitoids. We reared the polyphagous moth *Diacrisia sannio* (Arctiidae) on artificial diets that contained chlorogenic acid (a quantitative plant secondary metabolite), monocrotaline (a qualitative plant secondary metabolite), on a control (no plant secondary metabolites) and on their natural host plant dandelion (*Taraxacum* sp.), and measured if these substances have negative effects on their growth and survival. We also assessed whether any of the diets benefited the encapsulation ability, a reliable indicator insects ability to fight parasites. Finally we studied whether plant secondary metabolites benefit larvae against bird predation. We did not find any evidence that even the qualitative secondary metabolite was harmful or beneficial for *D. sannio*. Larval growth rate and survival on diets containing secondary metabolites was as good as larval performance on their natural host plant. Surprisingly, larvae performed worst on the control diet which did not contain secondary metabolites. When larvae were reared in a sterile environment, differences in performance disappeared, which indicated together with an additional contamination experiment that secondary metabolites act as preservatives benefiting larvae indirectly. Secondary metabolites did not benefit larvae in their defence against enemies. Encapsulation ability was not affected by the diet of larvae although the larvae that had the highest encapsulation ability survived best. Although larvae were not preferred prey for great tits, the unprofitability was not due to their diet. Our results suggest that polyphagous insects can be surprisingly tolerant to plant secondary metabolites and their effects can be indirect and complex.

Introduction

Quality of a diet has a profound importance to the fitness of an herbivore, and the quality of a plant-based diet is largely affected by its chemical composition. Plant species differ in the amount and quality of plant secondary metabolites they contain. Often plant secondary chemicals are a part of the plants' defence against herbivory (Frankel 1959; Rosenthal & Janzen 1979; Strauss & Zangerl 2002), and it has been well established that plant secondary chemicals can have negative effects on the growth and survival of an herbivore (e.g. Gordon 1961; Erickson & Feeny 1974; Cates 1980; Price et al. 1980, Berenbaum & Zangerl 1999). Plant secondary metabolites either are a definitive feeding barrier to most herbivore species (qualitative defence; e.g. Hartmann 1991) or reduce the digestibility of the plant material and thus slow down the development of most herbivore species (quantitative defence; e.g. Swain 1979; Seigler 1998). Qualitative defence chemicals can be well tolerated by adapted specialists but not by generalist feeders, whereas quantitative defence chemicals are often found in a wide variety of plant species and reduce the quality of the food for both specialists and generalists (Rosenthal & Janzen 1979).

However, plant secondary compound can also be beneficial to an herbivore by aiding them to gain a chemical defence against their predators. Chemically defended herbivore species often sequester plant secondary metabolites from their host plants and use the chemicals in their own defence (Rothschild 1973, Dobler and Rowell-Rahier 1994, Camara 1997, Stamp 2001). Many of these species also have warning signal advertising to potential predators that the individual is not profitable prey, and this combination of a warning signal and a secondary defence is called aposematism (after Poulton 1890). It has been shown that the stronger the defence (unprofitability) is in an aposematic species, the more they are avoided by their potential predators (Duncan & Sheppard 1965; Lindström et al. 2006). Therefore, the capability to sequester noxious chemicals effectively is critical to the survival of an aposematic herbivore.

Another mechanism in which plant secondary metabolites can benefit herbivores is via the ability to defend against parasitoids (Nieminen et al. 2003; Singer & Stireman 2003; Ojala et al. 2006), baculoviral disease (Hoover et al. 1998) and to reduce the parasitism rate of individuals (Barbosa et al. 1991). Also, antioxidants such as carotenoids and flavonoids reduce the harmful effects of stress caused by, for example, UV-radiation or infection (Demming-Adams & Adams 1996; Ouchane et al. 1997). Therefore, in addition to the chemical defence against predators, plant secondary compounds can also reduce the parasitism rate of individuals. It has been shown that plant toxins present in herbivore bodies can be barriers to successful attack by generalist parasitoids. For example, iridoid glycosides, nicotine and tomatine have been found to reduce parasitism in insects (Campbell & Duffey 1979; Barbosa et al. 1991, Nieminen et al. 2003). However, this topic has not received much study (but see Ojala et al. 2005).

Thus, plant secondary metabolise can have dual effects on herbivore. Evidence for the costs of acquiring the chemical defence in aposematic species has been studied mostly with monophagous species. For example, the well studied monarch butterflies (see review in Malcolm & Zalucki 1993) do not seem to bear any costs in eating their milkweed host plant and thus are considered to be well co-adapted to their host plants (but see Zalucki et al. 2001). However, many aposematic species are polyphagous (Bernays & Cornelius 1989), and may incorporate different plant secondary metabolites into their defence, thus increasing their defence arsenal; and maintaining two or more different detoxification mechanisms simultaneously might be costly (e.g. Hatle &

Spring 1998). Given these conclusions we can assume that possible underlying costs of chemical defence might be easier to detect in a generalist herbivore that has not co-adapted to use a specific host plant, but has to maintain a broad detoxification mechanism. We can also assume that generalist herbivores would be more adapted to diets containing common plant secondary metabolites and more sensitive to defensive compounds that only occur in some plant genera (e.g. Levins & McArthur 1966).

Here we examined whether the fitness of an aposematic insect herbivore is affected by the secondary metabolite contents of their larval diet. We studied the growth and survival of an arctiid moth *Diacrisia sannio* in laboratory conditions, as well as their defence ability against parasitoids and bird predators, which are likely to be important causes of mortality for lepidopteran larvae in nature, and thus extremely important to the fitness of the moths. We chose plant secondary metabolites that we expected to differ in their effect to a generalist herbivore: the phenolic chlorogenic acid, which is a common, quantitative plant chemical in the diet of this species; and the pyrrolizidine alkaloid monocrotaline, which is a qualitative plant defence chemical. These chemicals were added to an artificial diet to ensure the diets did not differ in other qualities such as nitrogen content, and as a control, we used an artificial diet without plant secondary metabolites. We were particularly interested to see if consuming plant secondary metabolites, which can potentially be used in chemical protection of the larvae, can also have negative effects on e.g. the growth of the larvae, predicting costs that in turn, could affect the evolutionary dynamics of chemical defence. Because in our first rearing experiment the survival of the larvae was the lowest on the diet without plant secondary metabolites, we repeated the experiment in a sterile environment to examine if the effect was because of a higher bacterial content of the diet without secondary chemicals. We also studied which diet the larvae preferred to feed on and if this is the most beneficial diet for their growth and survival.

Material and methods

Study species

We used *D. sannio* as our study species, since it feeds on numerous herbaceous plant species (Marttila et al. 1996) and the larvae are likely to be aposematic, i.e. have warning colouration to warn potential predators that they are not beneficial prey. In northern Europe this species usually has only one generation per year. *D. sannio* overwinters as larvae, but in laboratory conditions, larvae keep growing if kept in warm and light conditions and they can be several generations per year. Arctiid moths are capital breeders, i.e. the adults do not feed. Thus the larvae have to collect all the nutrients and other substances the pupae and adult moths need to survive and reproduce, making the larval diet critical to the fitness of the individual.

The effect of qualitative and quantitative plant secondary metabolites on growth

To study the effects of the qualitative and quantitative plant secondary metabolites on the growth, survival and defence against predators and parasites, we reared larvae of the moth *D. sannio* on four different diets: 1) Plain artificial diet, which is based on semolina and wheat germ (modified from Poitout & Bues 1974 by leaving out benzoic acid and formaldehyde and adding 0.5 % Vanderzant vitamin mixture for insects). This diet contains the nutrients needed by the larvae but only traces of plant secondary metabolites (Ojala et al. 2005). 2) Artificial diet with 8% chlorogenic acid of the dry weight. This corresponds to a natural, albeit high concentration in some plant species (Seigler 1998). 3) Artificial diet with 0.06% of monocrotaline. This corresponds to a natural concentration in the genus *Crotalaria* (Hartman 1991). 4) *Taraxacum sp.*, which is a natural, common host plant of *D. sannio*, was used as a control diet to assess the development of the larvae on a natural diet as opposed to an artificial diet.

We chose the two plant secondary metabolites because they are known to protect the plant against herbivory (Matsuda & Senbo 1985; Hartmann 1991) and also because they can potentially be used in chemical defence against predators. Monocrotaline can be considered qualitative defence: it is a pyrrolizidine alkaloid and is the highly toxic product of the tropical plant genus *Crotalaria* (Fabaceae, Mattocks 1986). Many aposematic species are known to sequester pyrrolizidine alkaloids and use them in their defence (Wink & van Nickisch-Rosenegk 1997; Weller et al. 1999), and the phylogeny of arctiid moths suggests that *D. sannio* has the ability to sequester pyrrolizidine alkaloids (Weller et al. 1999). However, monocrotaline-containing plant species do not occur sympatracally with *D. sannio*, and thus the substance can potentially be harmful to this species.

Chlorogenic acid, on the other hand, is a very common phenolic substance present in many plant species. It has been reported to depress development rate in a generalist herbivore (Stamp & Yang 1996) and to decrease digestibility of leaves to a specialist feeder (Johnson & Felton 2001), and can therefore be considered a quantitative defence of the plant. We expected *D. sannio* to be relatively well adapted to the presence of chlorogenic acid in this diet, but possibly suffer from reduced growth rate when feeding on it. Also, chlorogenic acid has been reported to act as chemical protection of an herbivore against predators (Traugott & Stamp 1997).

Fifteen adult *D. sannio* females were caught with a butterfly net in late June and early July 2002 from three populations: one individual from Jyväskylä area in central Finland, six from Jomala in the Åland Islands in south-western Finland and eight from

Tartu area in Estonia. All the wild-caught females had already mated in the wild and laid eggs in plastic containers in the laboratory.

We conducted this study as a full-sib common garden design. Eighteen-day-old larvae which were in 3rd -4th instar (total n = 547) of the 15 *D. sannio* families were weighed and allocated to diet treatments. Before the beginning of the experiment, all the larvae had been fed with *Taraxacum sp*. The larvae were reared in individual containers in a greenhouse, where the temperature and lighting followed outside conditions. Larvae were checked every day and fresh food was provided *ad lib* every second day or more often if needed. The day after pupation, pupae were weighed and kept in warm and light conditions. Adults normally hatched within 6-12 days from pupation. If the moth had not hatched a month after pupation, the pupa was sexed when possible.

The effect of novel and common plant secondary metabolite on encapsulation

Encapsulation reaction can be quantitatively measured by using a novel and standardized “parasite” such as nylon monofilament implants (König & Schmid-Hempel 1995; Ryder & Siva-Jothy 2000) that mimic a parasitoid inside the insect. The advantage of the implant method is that the “parasite” is neutral and does not have means to overcome the defence of the host. Thus the outcome of encapsulation is solely dependent on the host, giving a relevant quantitative measure of the insect’s ability to fight parasitoids. However, this technique does not take into account the effect that plant secondary metabolites *per se* can sometimes prevent parasitism (Nieminen et al. 2003).

One hundred ninety-five pupae from the 15 families were implanted 1-3 days after pupation, 1-19 larvae per family (mean = 13 larvae). We used nylon implants that were 0.11 mm in diameter and 4 mm long. The larvae were anesthetized with CO_2 and a small cut was made into the sixth abdominal segment with a sterilized needle. Two thirds of the implant was inserted inside the pupa and the rest of the implant was left outside. The immune system of the pupa was allowed to react to the implant for five hours.

After removing the implant, it was dried and photographed under a microscope with 57 x magnification with a Panasonic wv-CL702 video recorder. Three black and white pictures from different angles were taken of each implant to ensure they were seen from all sides (Rantala et al. 2000). From each picture, the mean grey value of the implant was measured with ImagePro Plus 4.0 (Media Cybernetics) from the 1 mm of the end of the implant that had been inside the larva. Since there was variation in lighting during the photographing we subtracted the grey value of the background from the grey value of the implant. The absolute value of the mean of the three grey values was used in all analyses.

To increase the number of families and individuals when testing the correlation between survival and encapsulation ability, we also included five families in which a total of 30 individuals had been implanted. These families originated from the Tartu area in Estonia and had been reared simultaneously and similarly to the rearing experiment on artificial diets with either monocrotaline or chlorogenic acid or on *Taraxacum* (but not on the control diet of plain artificial diet).

The effect of qualitative and quantitative plant secondary metabolite on defence against predators

We used wild adult great tits (*Parus major*) to test if the larvae are unpalatable to their potential predators. Birds were trapped from feeding sites at Konnevesi Research

Station in central Finland and subsequently ringed for identification. Each bird was kept individually in a plywood cage indoors with a daily light period of 12 hours. Sunflower seeds, tallow and fresh water were available *ad libitum* except prior to the experimental trials when the birds were food deprived to ensure motivation to eat the larvae. The experiment was run in October 2002 by the permissions from Central Finland Regional Environment Centre (permission number KSU-2002-L-382/254) and Experimental Animal Committee of the University of Jyväskylä (permission number 29/4.6.2002). Unfortunately, we were not able to use *D. sannio* larvae which had been feeding on the artificial diet with no plant secondary metabolites (control), since the larvae on this treatment were growing more slowly than the other ones and thus size differences would have made comparing the effects of treatment to the chemical defence of a larva impossible.

The experiment was conducted in an aviary (3.4 x 3.9 m^2 (height 2.5 m)) to which the birds had been habituated prior to the experiment. We placed two poles (height 103 cm, diameter 10 cm) in an aviary. Poles had black paper as a blind facing the birds. The birds were first trained to find food from the trays that were placed behind the blinds, and thus we could conclude that a bird was motivated to eat when it approached the trays. The poles with trays were placed 1 m apart, and a petri dish with experimental larvae was placed on each tray. One of the petri dishes had three living *D. sannio* larvae (either from chlorogenic acid, monocrotaline or *Taraxacum* treatment) and one petri dish always contained three mealworms matched to the size of *D. sannio* larvae. Therefore, we were able to compare which larvae the birds preyed on first, mealworms or *D. sannio* larvae, and if they ate less of either species. As a control, we had experiments in which both of the petri dishes contained three mealworms, and thus we were able to study if the birds ate more mealworms than *D. sannio* larvae. The order in which the bird ate or killed the larvae was recorded. The first choice was scored 1, the second 2 etc. until score of 6, which was the last choice. If the bird did not touch the larva during the 30 min trial, it was scored 7. The scores were then added together for each species of larvae and this total score was used in statistical analyses.

Larval diet preference test

We studied which artificial diet the larvae preferred to feed on and if this is the most beneficial diet for their growth and survival. This experiment was performed in July-September of 2002, and the individuals were the offspring of the individuals used in the rearing experiment. On the day of hatching from eggs, 44 individual larvae from 17 families were tested for their preference of the three artificial diets. Cubes ($1cm^3$) of each of the diets were placed on a petri dish (Ø 9cm), with equal distance to the centre of the dish and from other diet squares. The larva, which had never fed before, was placed in the middle of a petri dish and observed until it reached one of the diets. When the larva reached a diet and started feeding, it was placed in the middle of the dish again and the procedure was repeated for a second and a third time. After the initial preference test on the first day after hatching, 16 of the larvae were left on petri dishes. The diet was changed every other day and the location of the larva on the petri dish was checked every day, and we recorded which diet the larvae were touching or feeding on or if they were not on any of the diets. This experiment was continued for 26 days.

Sterile rearing

Surprisingly, in the rearing experiment, the survival of the larvae was the lowest on the diet without plant secondary metabolites (control). We observed that the control diet became contaminated much faster than diets with either monocrotaline or chlorogenic acid. Therefore we reared the moths in a sterile environment to determine if the low survival and slower growth rate on control diet was due to a higher microbial content of the diet rather than low secondary metabolite content on the diet. In other words, it is possible that plant secondary metabolites acted as a preservative in the artificial diets. Thirty-three females were caught from the Jyväskylä area between the first and seventh of July 2003, and three larvae per female were allocated to each of the artificial diets at the age of 18 days (N = 99). The same diets were used as in the rearing experiment in the previous year: 1) a control, plain artificial diet, 2) artificial diet with 8% chlorogenic acid or 3) artificial diet with 0.06% monocrotaline. As in the main experiment, prior to the experiment these larvae had been feeding on *Taraxacum*. The larvae were reared individually in sterile petri dishes in a laminar flow chamber with natural light from a window and a constant temperature of 22° C. The artificial diets were made, handled and kept as sterile as possible. The larvae were placed every day in new sterile petri dishes with fresh food. The larvae were handled only with hands and forceps sterilized with alcohol. The pupae were weighed on the day of pupation and the moth was sexed after the adult emerged.

Bacterial contamination of the diets

We tested whether there were differences in contamination rate among the artificial diets. Cubes (1cm^3) of artificial diets were kept in petri dishes for two days in the greenhouse similarly as in the rearing experiment but without larvae. Thus, we tested how different diets were naturally contaminated with microbes. After two days, one gram of each of the three types of artificial diet were ground fine and mixed with 1 ml of distilled water, and further diluted to 1/10 000. Then the dilution was spread on agar plates. Agar plates contained 10 g of Difco™ nutrient broth, 2.5 g of Bacto™ yeast extract and 15 g of Bacto™ agar (Becton, Dickinson and Co., Franklin Lakes, NJ) in one litre of distilled H_2O. Before use, agar plates were autoclaved (121°C for 25 minutes). After allowing for bacterial growth for two days at 22° C, plaques on the plates were counted and the ln of the count was used in ANOVA.

Statistical analysis

In this study, larval growth rate, which combines the effect of development time and adult mass, was used as a measure of the ability of larvae to grow on different diets, and it was calculated using the formula ln (pupal mass – larval mass at the beginning of the experiment)/larval development time in days. We used analysis of variance, in which larval growth rate and encapsulation score were dependent variables and diet, family and sex were used as factors. Family was treated as a random factor in all analyses. Other factors (sex and diet) were fixed. All data were checked for normality and homogeneity of variances. Data for growth rate and encapsulation score were heteroscedastic and thus we used ranked data for ANOVA. All statistical analyses were performed with SPSS 11.0.1 (SPSS Inc. 2003).

Results

The effect of qualitative and quantitative plant secondary metabolites on growth and survival

The larval growth rate did not differ between females and males ($F_{1,342} = 2.509$, $p = 0.114$) so we pooled the sexes and used growth rate as the measure of growth. Also, population of origin did not have an effect on growth rate ($F_{2,54.167} = 0.351$, $p = 0.706$) and it was omitted from further analyses. Both larval diet and family had a significant effect on the growth rate (ANOVA for treatment: $F_{3,78.686} = 3.340$, $p = 0.023$; for family $F_{14,39.091} = 4.298$, $p < 0.001$), and the interaction between family and diet was marginally significant ($F_{37,464} = 1.419$, $p = 0.056$). Growth rate was the lowest on plain artificial diet, but did not differ among artificial diet with chlorogenic acid, artificial diet with monocrotaline or *Taraxacum* (Paired T-test, plain artificial diet vs. chlorogenic acid $t_{224} = -2.299$, $p = 0.022$, plain artificial diet vs. monocrotaline $t_{188} = -3.394$, $p = 0.001$, plain artificial diet vs. *Taraxacum* $t_{265} = -4.249$, $p < 0.001$, *Taraxacum* vs. chlorogenic acid $t_{327} = 1.838$, $p = 0.067$, chlorogenic acid vs. monocrotaline $t_{250} = -0.918$, $p = 0.360$; Fig. 1). Survival from 18-day-old larva to pupa differed significantly among diets ($N = 547$, $\chi^2 = 10.346$, $p = 0.016$), and was the lowest on plain artificial diet (44.4%), and higher on artificial diet containing monocrotaline (54.9%), *Taraxacum* (61.3%) and artificial diet containing chlorogenic acid (64%).

The effect of qualitative and quantitative plant secondary metabolites on encapsulation

The pupae's ability to encapsulate a foreign object was higher in females than in males ($F_{1,94} = 4.372$, $p = 0.039$). However, because many of the pupae died as a result of implantation, we were able to sex only about half of the implanted individuals (95 out of 195). Therefore, in further analyses, we used the pupal mass as a covariate, since it correlated positively with encapsulation ability ($r_s = 0.180$, $N = 195$, $p = 0.012$) and differs between males and females ($F_{1,343} = 19.271$, $p < 0.001$). Within sexes, there was no significant correlation among pupal mass and encapsulation ability (p-values > 0.505). There were no differences in the encapsulation ability among the diets ($F_{3,61.898} = 1.088$, $p = 0.361$), but the families differed significantly ($F_{14,58.525} = 2.289$, $p = 0.014$) indicating a genetic basis for the defence against parasites. We found a positive correlation between the families mean encapsulation ability and their mean likelihood to pupate ($N = 20$, $r_s = 0.492$, $p = 0.028$). Therefore, although we did not find evidence that plant secondary metabolites influence encapsulation ability, encapsulation is a reliable measure of immunocompetence and a correlate of fitness.

The effect of qualitative and quantitative plant secondary metabolites on defence against predators

The great tits preferred to eat mealworms over *D. sannio* larvae (Kruskal-Wallis $\chi^2 = 9.998$, df = 3, $p = 0.019$; Fig 2). On average, great tits ate 0.818 (± 0.38) (out of three) *D. sannio* larvae fed on *Taraxacum*, 1.333 (± 0.37) fed on diet with monocrotaline, 1.571 (± 0.48) fed on diet with chlorogenic acid and 2.313 (± 0.24) controls (mealworms). Also, birds ate the mealworms before the *D. sannio* larvae (Kruskal-Wallis $\chi^2 = 9.084$, df = 3, $p = 0.028$). Thus, *D. sannio* is not a totally ignored prey item for birds but not preferred either. There are some indications that larvae that were reared on *Taraxacum* might have been more unpalatable than larvae reared on artificial diet containing monocrotaline or chlorogenic acid. For example, attack probability against larvae fed on *Taraxacum* was only 0.27 (± 0.13) whereas it was 0.44 (± 0.12) and 0.52 (± 0.16) against larvae fed on monocrotaline or chlorogenic acid, respectively (Fig. 2).

Larval diet preference among artificial diets

On the day of hatching, the neonate larvae did not have any preferences among the artificial diets ($N = 44$, $\chi^2 = 0.614$, $p = 0.736$). However, later in their lives (days 2-26), the larvae did not feed on the three diets equally ($\chi^2 = 21.877$, $N = 17$, $p < 0.001$), but spent more of their time feeding on the chlorogenic acid containing diet than on the other two diets (Wilcoxon signed ranks test, chlorogenic acid vs. plain: $z = -3.437$, $p = 0.001$, chlorogenic acid vs. monocrotaline: $z = -3.393$, $p = 0.001$); however the time spent on the plain artificial diet and monocrotaline containing diet did not differ (Wilcoxon $z = -1.338$, $p = 0.181$) (Figure 3).

Growth and survival of *D. sannio* in a sterile environment

When the larvae were reared on three different artificial diets in a sterile environment, the growth and survival did not vary among diets (growth rate: $F_{2,76} = 0.099$, $p = 0.906$; survival: $N = 98$, $\chi^2 = 0.818$, $p = 0.664$; Fig. 3). This suggests that the better growth on diets that contained plant secondary metabolites compared to control diet without the secondary metabolites in our main rearing experiment arose because the plant secondary metabolites may act as preservatives in the diet. Thus, secondary metabolites do not necessarily improve larval growth *per se* but may keep food clean of microbes and indirectly improve larval growth and survival.

Bacterial contamination of the diets

The number of bacterial plaques differed significantly among diets ($F_{2,20} = 5.549$, $p = 0.013$). The plain artificial diet had the highest amount of plaques, and the amount of plaques on chlorogenic acid and monocrotaline containing diets were significantly lower ($LSD = 1.017$, $p = 0.011$ and $LSD = 1.044$, $p = 0.009$, respectively). The number of plaques on chlorogenic acid and monocrotaline containing diets did not differ ($LSD = 0.026$, $p = 0.943$). This result suggests that plant secondary metabolites can act as preservatives in artificial diets.

Discussion

Surprisingly, neither the qualitative nor the quantitative plant secondary metabolite in the diet had negative effects on the growth or survival of *D. sannio*. This was against the expectation that the plant secondary metabolites would have adverse effects on a generalist herbivore (Rosenthal & Janzen 1979). On the contrary, when fed with artificial diets containing either monocrotaline (qualitative secondary metabolite) or chlorogenic acid (quantitative secondary metabolite) the larvae grew and survived as well as on their natural diet *Taraxacum*, and significantly better than on the artificial diet without plant secondary metabolites (Figure 1.). This could suggest that plant secondary metabolites could be even beneficial for the survival and growth of this species similarly as has been found in some monophagous aposematic species that are co-adapted to the secondary metabolites of their host plants (Ollf et al. 1999; Macel et al. 2002). Secondary metabolites did not affect the encapsulation ability of this species either. Also somewhat surprisingly, the larvae that ate diets containing plant secondary metabolites were not avoided by birds although birds preferred to eat the alternative prey (mealworms). Thus, we did not find clear negative effects of plant secondary metabolites on larval growth and survival or positive effects on defence against parasites or predation.

However, the benefits for the growth and survival of *D. sannio* on diets containing secondary metabolites may be explained by the preservative function of these metabolites in the artificial diets. When the larvae were reared on artificial diets in sterile environment, the growth rate and survival of the larvae on plain artificial diet and the larvae on artificial diet with plant secondary metabolites did not differ. Furthermore, the agar plates spread with artificial diets containing either chlorogenic acid or monocrotaline had lower microbial content compared to the control diet when all diets were naturally contaminate with microbes in the greenhouse. Therefore, the better growth and survival of the individuals in the main rearing experiment on artificial diets with plant secondary metabolites could arise from the differences in the bacterial contents of the diets rather than the plant secondary metabolites *per se*. It seems that the larvae are well adapted to eating chlorogenic acid, and thus do not suffer from any adverse effects from it. Also, it is possible that even though *D. sannio* does not naturally feed on monocrotaline-containing plants, it has an ability to detoxify pyrrolizidine alkaloids as do some other species of arctiid moths (Wink & van Nickisch-Rosenegk 1997; Weller et al. 1999). Thus, combining results from both rearing experiments, these two plant secondary metabolites seemed to have very similar effects on the growth and survival of *D. sannio*.

It is possible that we did not find negative effects of plant secondary metabolites because we used artificial diets in the study and not living plant material with differing chemical concentrations. For example, Ali et al. (1999) found in their experiment with tobacco budworm that the effect of chlorogenic acid in the diet was different when the chemical was found in plant material and when it was added to an artificial diet. It could be possible that their results, similarly as our results with artificial diets, can be explained by differential concentration of bacteria in the diets and not by the effect of chlorogenic acid *per se*. However, it is also possible that this result can be generalized to herbivores eating living plants containing secondary chemicals, since these chemicals inhibit bacterial growth and the secondary metabolite-containing food thus contains less harmful bacteria or prevents bacterial growth in the intestine of the herbivore. This could explain e.g. the result that eating lettuce (*Lactuca sativa*), which has very low levels of plant secondary metabolites, causes high mortality in *Parasemia plantaginis* (Arctiidae) larvae although eating lettuce is beneficial for their growth (Ojala et al 2005). It should be noticed however that the plant secondary metabolites did not have any negative effects on larval survival nor growth in the sterile environment.

As expected, great tits preferred eating mealworms over *D. sannio* larvae. However, they did not totally reject *D. sannio* larvae, and, on average ate half of the experimental larvae presented to them. However, the maximum number of *D. sannio* larvae the birds were able to eat was three, and it is possible that this was not enough for them to learn to avoid this species (see also Pinheiro 2003). It seems that *D. sannio* is not strongly defended against bird predators, but not preferred prey either. This slight aversion could still potentially protect the larvae of this species in nature, since there is usually plenty of alternative prey available for insectivorous birds in Northern Europe and no need to eat somewhat unwanted prey (Ojala et al. unpubl.). There was no difference in the palatability of the *D. sannio* larvae among the diet treatments, which was surprising, since the result that the larvae did not suffer adverse effects from monocrotaline would suggest that they can sequester this substance from the diet and use it for chemical defence (Weller et al. 1999). Unfortunately, in this experiment, we were not able to use larvae which had been feeding on the plain artificial diet and which should have been most palatable to birds. Thus, we can not rule out the possibility that the larvae are able to use both chlorogenic acid (also present in *Taraxacum*, Ojala et al. 2005) and monocrotaline for their chemical defence, and that these two substances are equal in deterring predators.

In this study, the larval diet did not affect the encapsulation ability of the pupae. This was not expected, since the diet treatment affected the growth and survival of the larvae, and therefore it was been expected that individuals on a beneficial diet would be in better condition (Salt 1964; Benrey & Denno 1997) and have had better immunocompetence (see Ojala et al. 2005) However, mean encapsulation ability per family correlated positively with survival, suggesting that the families in better condition also had better immunocompetence.

The neonate larvae did not prefer any of the artificial diets but randomly fed on any of them. This indicates that for the neonate larvae, being able to feed quickly is more important than selection of the most beneficial diet. However, as the larvae grew, they clearly preferred eating artificial diet containing chlorogenic acid over the plain artificial diet and monocrotaline-containing artificial diet. Therefore, the larvae were able to choose a diet which was beneficial for their survival and growth. It could be argued that larvae preferred chlorogenic acid-containing diet because it is a common secondary metabolite and they may be well adapted to ingesting it. However, it is also possible that they selected chlorogenic acid containing diet because of its lower bacterial content or because chlorogenic acid can act as a feeding cue (see also discussion by Singer et al. 2002)

Taking all these results together, our study suggests that polyphagous insects can be surprisingly tolerant to plant secondary metabolites. Also, the effects can depend on the co-evolutionary history of the species and the specific chemicals involved, and so the effects can be indirect and complex.

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Legends to the figures

Figure 1. The mean growth rate on the diet treatments. Bars show standard errors of the mean.

Figure 2. The mean attack probability by birds on *D. sannio* larvae compared to control (mealworm). Larvae were reared on artificial diet with different plant secondary metabolites and on their natural diet (*Taraxacum*). Bars show standard errors of the mean.

Fig. 3. Growth rate of the larvae on different artificial diets in a sterile environment. Bars show standard errors of the mean. Note that temperature in this sterile rearing was lower than in the main rearing experiment.

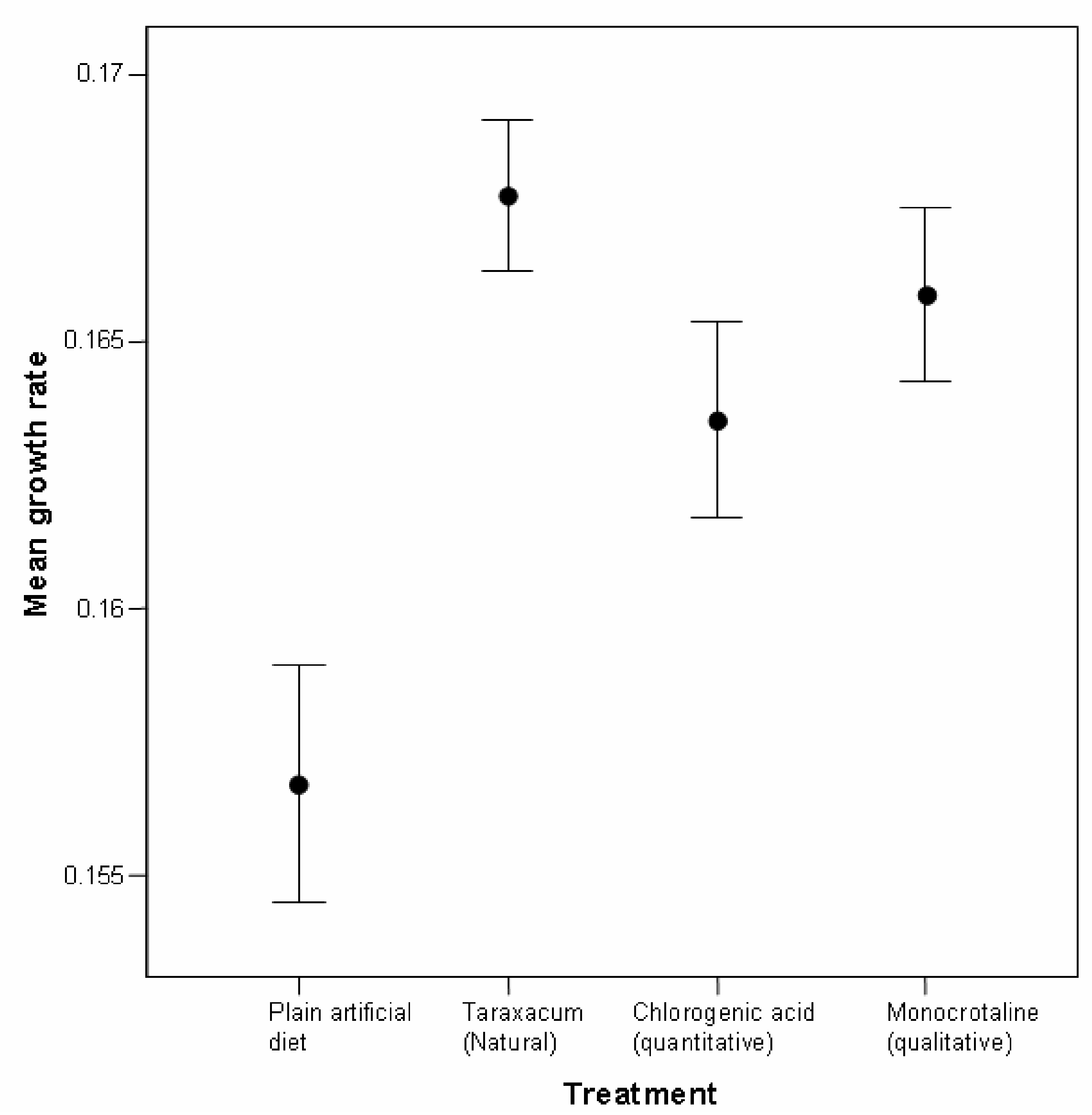

Fig. 1. Ojala et al.

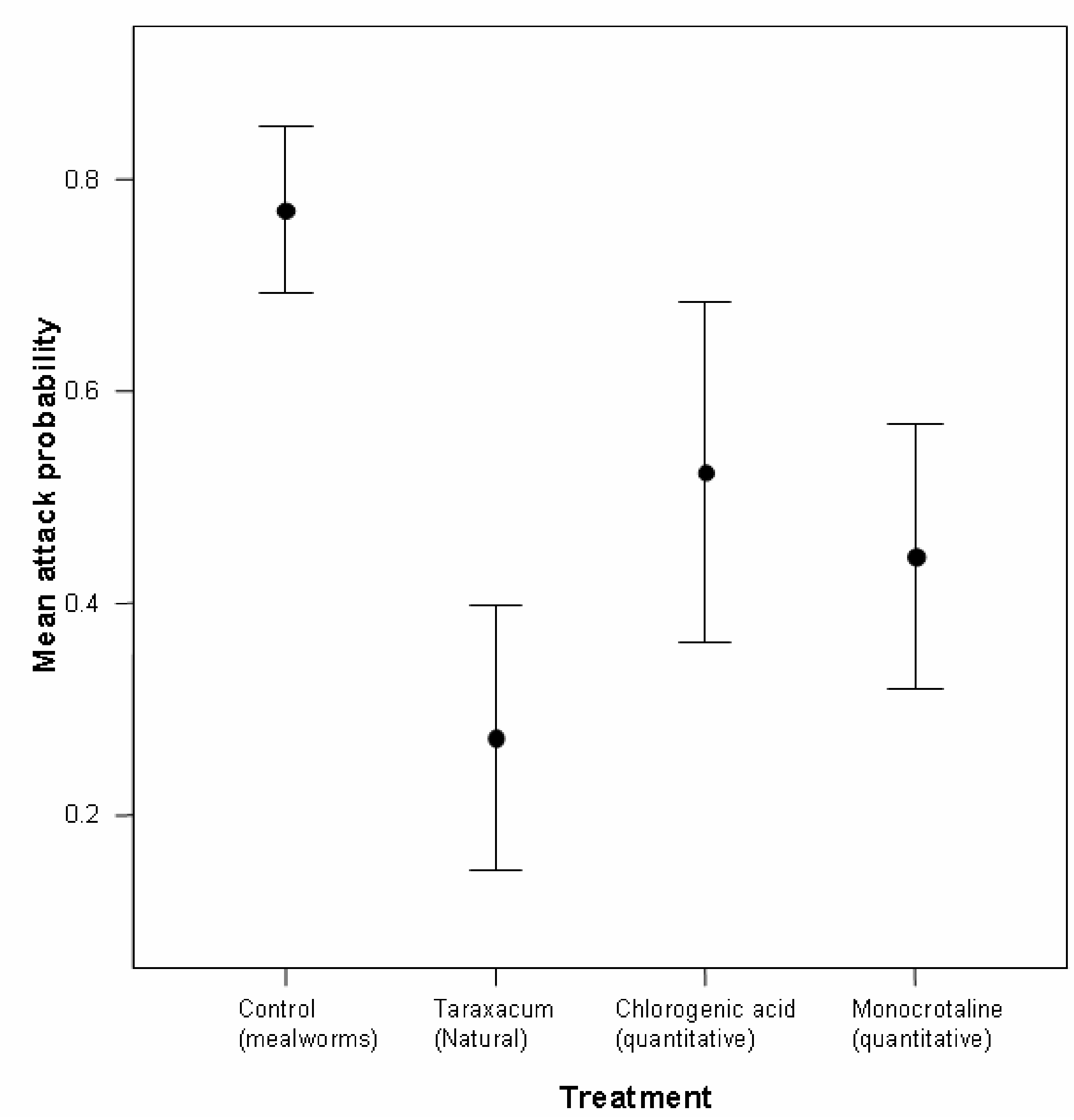

Fig. 2. Ojala et. al.

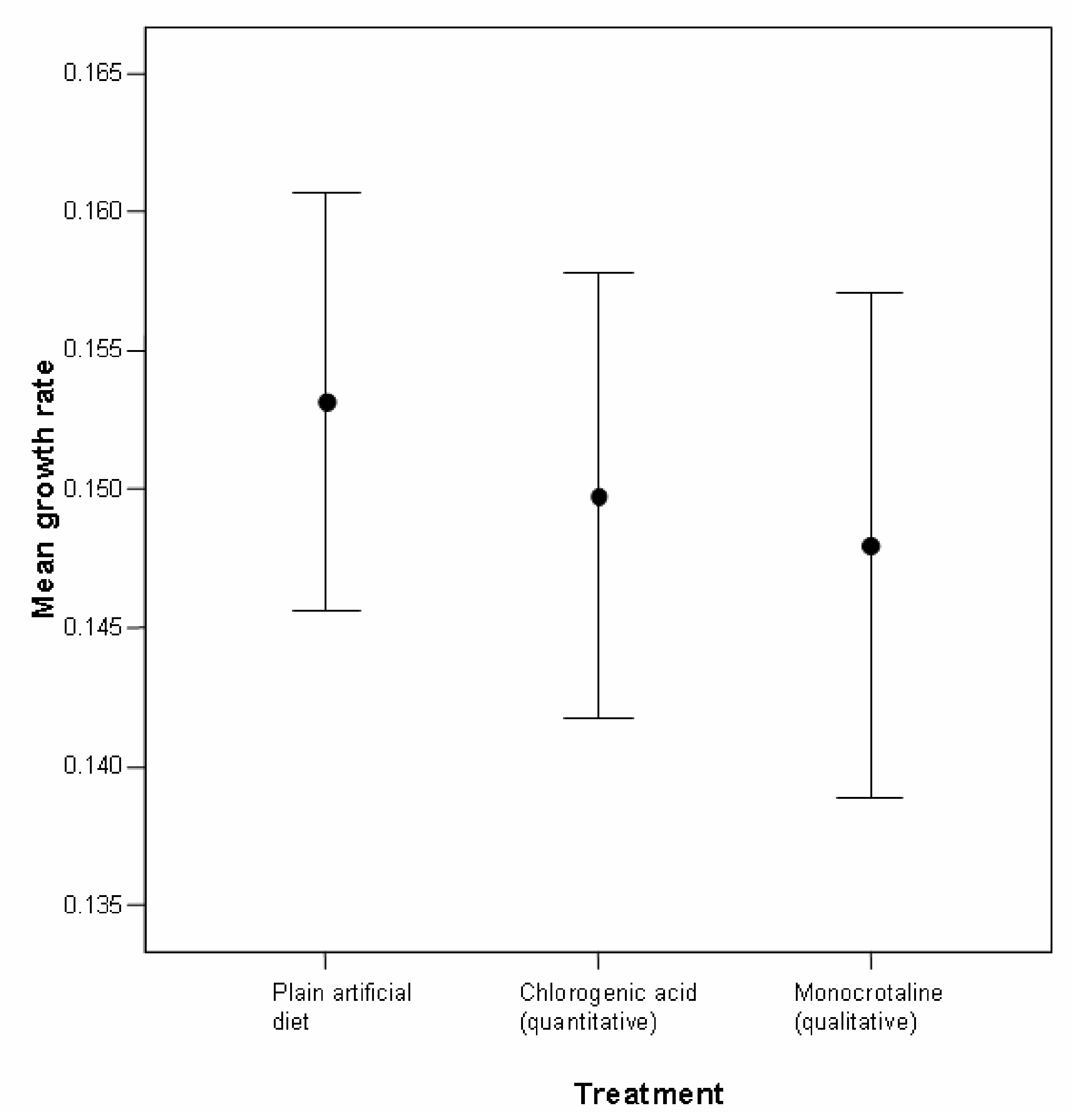

Fig 3. Ojala et al.

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Diet affects the immune defence and life-history traits of an Arctiid moth *Parasemia plantaginis*

by

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