

Disentangling the evolution of weak warning signals: high detection risk and low production costs of chemical defences in gregarious pine sawfly larvae

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Abstract Evolution of costly secondary defences for a cryptic prey is puzzling, if the prey is already well protected by camouflage. However, if the chemical defence is not sufficient to deter all predators, selection can favour low signal intensity in defended prey. Alternatively, if the costs of chemical defence are low or cost-free, chemical defences can be expected to evolve also for non-signalling prey, particularly if conspicuous signalling is costly. We tested these assumptions with pine sawfly larvae (*Neodiprion sertifer* and *Diprion pini*) that are cryptically coloured and chemically defended with resin acids sequestered from their host plant (*Pinus* sp.). Larvae feed in large aggregations, which we hypothesise could function as a signal of unprofitability. Our results show that even though the birds found *N. sertifer* larvae unprofitable in the controlled laboratory assays, they continued attacking and consuming them in the wild. When we tested the signal value of aggregation we found that a large group size did not offer protection for a defended larva: the survival was higher in groups of 10 individuals compared to groups of 50, suggesting increased detectability costs for individuals in larger groups. Finally, we tested how costly the production and maintenance of a chemical defence is for *D. pini* larvae by manipulating the resin acid content of the diet. We did not find any life history or immunological costs of the chemical defence for the larvae. In contrast, pupal weights were higher on the high resin diet than on the low resin diet. Also, larvae were able to produce higher amounts of defence fluids on the high diet than on the low diet. Thus, our result suggests high detectability costs and low production costs of defences could explain why some unprofitable species have not evolved conspicuous signals.

Keywords Aggregation · Chemical defence · Costs of defence · Warning signal evolution

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Introduction

Animals exhibit a diversity of colours and patterns to reduce the risk of predation (Cott 1940; Ruxton et al. 2004). The most common strategy is to hide from predators' eyes and rely on camouflage that makes prey more difficult to detect (Endler 1991; Ruxton et al. 2004; Stevens and Merilaita 2009). An alternative strategy is aposematism where a prey species informs predators about its defences, such as toxicity or unprofitability, with conspicuous warning signals (Poulton 1890; Ruxton et al. 2004). Signals are often bright colour patterns (typically red, orange, yellow and white patterns combined with black markings), that has been shown to accelerate predator learning (Sillén-Tullberg 1985; Mappes and Alatalo 1997; Gamberale-Stille and Tullberg 1999; Exnerová et al. 2006). Also, the size of the stimulus, such as large pattern elements (Forsman and Merilaita 1999; Lindström et al. 1999; Lindstedt et al. 2008), the large body size of the aposematic prey (Gamberale and Tullberg 1996; Hagman and Forsman 2003; Nilsson and Forsman 2003) and prey aggregations (e.g. Sillén-Tullberg and Leimar 1988; Gagliardo and Guilford 1993; Alatalo and Mappes 1996; Mappes and Alatalo 1997; Gamberale and Sillén-Tullberg 1998; Tullberg et al. 2000; Gamberale-Stille 2000; Riipi et al. 2001; Beatty et al. 2005; but see also e.g. Gamberale and Tullberg 1996; Lindstedt et al. 2006; Skelhorn and Ruxton 2006) can increase the survival of prey due to enhanced avoidance learning of predators. In addition to elements making prey more conspicuous, greater toxicity of conspicuous prey increases the learning efficiency of the predator (Leimar et al. 1986; Skelhorn and Rowe 2006a; Rowland et al. 2007). Thus, selection should favour conspicuous signals in well defended individuals.

In spite of the benefits of being brightly coloured and defended, there are several examples of species that possess secondary defences, but do not advertise their defences with typical aposematic colour patterns (reviewed in Endler and Mappes 2004). Instead, their coloration can be rather dull (green, grey and brown patterns with black markings) which makes them camouflaged against their natural backgrounds. For example, *Utetheisa galapagensis* -moth (Lepidoptera, Arctiidae) is chemically defended (Roque-Albelo et al. 2002) without any bright colour markings. Pine sawfly species *Neodiprion sertifer* and *Diprion pini* larvae are chemically defended against avian (Sillén-Tullberg 1990) and arthropod (Eisner et al. 1974; Codella and Raffa 1995a; Björkman et al. 1997; Lindstedt et al. 2006) predators; when disturbed they actively regurgitate resinous droplets of defence fluid from their mouth (e.g. Eisner et al. 1974; Sillén-Tullberg 1990). They are also gregarious, but their colouration makes them difficult to detect from their background (green larvae with darker patterns living among the pine needles).

This kind of weakly conspicuous coloration (referred here as colour patterns that do not include any typical aposematic colours) can evolve for a defended prey if the predators vary in their tendency to attack defended prey (Endler and Mappes 2004). In addition, it is possible that conspicuousness may not be as important as long as the signal is distinguishable from the cryptic prey (Holloway et al. 2001; Sherratt and Beatty 2003; Rowe et al. 2004; Merilaita and Ruxton 2007). Also, if the species is gregarious, the high density itself could act as a signal (e.g. Sillén-Tullberg and Leimar 1988; Alatalo and Mappes 1996; Gamberale and Tullberg 1996; Riipi et al. 2001; Endler and Mappes 2004; Beatty et al. 2005). Large aggregations could also both increase the size of the stimulus (Gamberale and Tullberg 1996; Hagman and Forsman 2003; Nilsson and Forsman 2003) and increase the learning efficiency of predators, since predators learn to avoid defended non-signalling prey more quickly if they are clumped (Sillén-Tullberg 1990; Alatalo and

Mappes 1996; Riipi et al. 2001; but see Tullberg et al. 2000; Beatty et al. 2005). In addition, a large group size can be beneficial via the dilution effect; if the predator can not eat the whole group, an individual's risk of being eaten is lower in larger than in smaller groups (Lawrence 1990; Codella and Raffa 1995b; Hunter 2000; Riipi et al. 2001 but see Lindstedt et al. 2006).

Alternatively, animals with restricted resources may allocate less to conspicuous colouration (Grill and Moore 1998; Speed and Ruxton 2007; Blount et al. 2009; Ruxton et al. 2009). For example, the production and maintenance of conspicuous signals may trade off with toxicity (e.g., Leimar et al. 1986; Darst et al. 2006; Speed and Ruxton 2007; Blount et al. 2009) or with other fitness traits such as development time (Longson and Joss 2006; Ojala et al. 2007), immunology (Friman et al. 2009), effective thermoregulation (Lindstedt et al. 2009a, b) and/or detoxification costs of the host plant's defence chemicals. In addition, if toxicity and conspicuousness are expensive to produce and maintain, but an increase in either component offers equally good protection against predators (Darst et al. 2006), it could relax the selection for conspicuous signalling. Finally, chemical defences could evolve for non-signalling prey if defences are inexpensive to produce but they further increase prey survival (Leimar et al. 1986; Longson and Joss 2006).

In this article, we evaluated the costs and benefits of chemical defence in gregarious pine sawfly larvae. We assessed (1) the relative unprofitability of pine sawfly larvae (*N. sertifer*) for birds (great tits) and (2) how group size affects the survival of *N. sertifer* larvae in the conditions where the risk of bird predation is high. We assumed that large group size would increase the survival of larvae if predators found them unprofitable (e.g. Sillén-Tullberg 1990; Riipi et al. 2001). If predators do not find larvae unprofitable, a large group size can increase mortality due to the increased detectability (e.g. Sillén-Tullberg 1990; Lindstedt et al. 2006; Skelhorn and Ruxton 2006).

In the second part of the study, we tested how investment in chemical defences affects the fitness of the prey individual. Sequestration of resin acid compounds in pine sawfly larvae has been shown to be positively associated with the resin acid content of its diet (Björkman et al. 1991; Codella and Raffa 1995a; Lindstedt et al. 2006) as has the efficacy of these defences (Codella and Raffa 1995b; Björkman et al. 1997; Lindstedt et al. 2006). For example, the high resin acid content of the diet has been shown to increase defence efficacy against ant predation (Codella and Raffa 1995a; Lindstedt et al. 2006) and it enables production of larger and stickier defence droplets (Björkman and Larsson 1991). Despite of the benefits of a high resin acid diet, it has been shown to be costly for *Neodiprion* –family of pine sawflies by decreasing the growth rate of the individuals (Björkman and Larsson 1991; Björkman et al. 1997). If these kinds of trade-offs do occur it could constrain the evolution of conspicuous signals. On the other hand, many specialist herbivores are well adapted to chemical compounds of host plants and they often benefit from the high concentrations of plant's defence chemicals in their diet (e.g. Holloway et al. 1991, 1993; Camara 1997; Del Campo et al. 2005; Harvey et al. 2005; Saastamoinen et al. 2007). If the defence chemicals are inexpensive to acquire and maintain, other costs such as variation in the probability of attack by visual predators could explain the selection for weak warning signals. To investigate these assumptions, we conducted a factorial rearing experiment, where we reared *Diprion pini* larvae on low and high resin acid diets. We tested the effect of diet quality on life history traits, antipredatory defence (size of the defence droplet) and immunological defence (measured as encapsulation rate).

Materials and methods

Pine sawflies

The studies were carried out using two pine sawfly species, *Neodiprion sertifer* and *Diprion pini* (Hymenoptera, Diprionidae). These species are univoltine in northern European coniferous forests and ecologically very similar; they are specialist on pines (*Pinus* spp.) and use pine needles as their diet. Eggs are laid in clusters in the pine needles and larvae feed gregariously. They have same kind of antipredatory strategy; larvae move their front end and produce oral effluents when approached by predators (e.g. Prop 1960). Both species are common in Finland, *D. pini* being more common in southern and eastern parts of the country.

Pine branches supporting *N. sertifer* eggs were collected from Kerimäki and Puumala in eastern Finland and brought to the laboratory. Larvae were fed with fresh pine branches ad libitum before they were transferred on to experimental branches in the field.

Pupae of *D. pini* were collected from one field site in eastern Finland (Ilomantsi) and reared to adulthood in the laboratory. Freshly emerged and randomly chosen *D. pini* adult pairs were introduced onto pine (*Pinus sylvestris*) branches which were then enclosed within mesh bags. Only a small proportion of the eclosed adults were males (14 individuals). Therefore, a proportion of the females (12 individuals) did not mate and produced only unfertilized eggs. Altogether we had 14 sexually produced families (part of the eggs fertilized) and 12 asexually produced families (all the eggs unfertilized). Pine sawflies (Hymenoptera) are haplodiploids, thus unfertilized eggs developed to males (Cook 1997; Heimpel and de Boer 2008) that are also smaller in size (pupal weight <90 mg) (Mopper et al. 1990). From our experimental individuals only 12 (6.3%) were diploid females (5 non-implanted and 3 implanted on the high diet treatment and 2 non-implanted and 2 implanted on the low diet treatment). Due to low sample size for females and sexual dimorphism in size, we only included males into the analyses in order to be able to run the full statistical model.

Branches supporting eggs were kept in the laboratory where eggs were allowed to hatch. After hatching larvae were fed twice a week with fresh pine branches supporting current and one-year-old needles until the beginning of the experiment. The temperature in the laboratory reflected the temperature outdoors.

Profitability of *Neodiprion sertifer* pine sawfly larvae

Great tits ($N = 9$) used in the prey preference assays were trapped from feeding sites around Konnevesi Research Station (Central Finland), and were subsequently ringed for identification. As a prey species in all predation experiment we used *Neodiprion sertifer* larvae as they were easily available during the experiments. Each bird was kept individually in an illuminated and air-conditioned plywood cage (65 cm × 65 cm × 80 cm, w × d × h) with a daily light period of 11.5 h and at 15°C. Each cage contained three perches offering roosting and prey-handling sites for the birds. Sunflower seeds, tallow and fresh water were available ad libitum for great tits. The experiment with great tits was performed in October to avoid disturbing the birds' breeding. The birds were released at their capture sites after the experiments. All the birds remained in good health throughout their captivity. Permission numbers for the experiment were KSU-2006-L249 and 18/22.5.2006.

Profitability assays were conducted in experimental cages (plywood, 50–70 cm) in Konnevesi research station (see e.g. Ham et al. 2006). Birds were first familiarized to

forage on sunflower seeds and nuts from a white dish in the experimental cage for 2 h. The cages were lit with energy-saving bulbs (Osram Dulux el longlife 7 W) that do not reflect UV-light. The behaviour of the birds was observed through a small mesh-covered window in one side of the box and experiments took place in a dark room so that the birds were unaware of the presence of the observer. The cages contained a perch and a water bowl. Prey items were offered through a hatch against white plate behind a visual barrier during both training and experiments. The visual barrier enabled us to measure the exact time of prey detection as the bird had to come from behind the barrier or fly to the top of it to observe the prey.

We offered three different prey types for each bird: a dead palatable mealworm larva, unpalatable aposematically coloured prey (yellow-black painted mealworm larva filled with Bitrex-solution) and a dead final instars of *N. sertifer* pine sawfly larva; respectively. All offered prey types were similar in size. By comparing the bird's eagerness to attack and kill pine sawfly in comparison to profitable mealworm (positive control) and unprofitable aposematic prey (negative control), we were able to determine the relative profitability of pine sawflies. All the prey items were matched to be approximately the same size. Pine sawflies and mealworms were killed by freezing. In order to make mealworms unpalatable and aposematic (aposematic prey type), we injected similar amounts of Stop and Grow liquid (Bitrex) into the larva (the Mentholatum co., Scotland, UK) that is unpalatable for humans. It is possible that artificial unpalatable taste is not as effective as natural compounds (Skelhorn et al. 2008), but the bitter taste of the Stop and Grow made mealworms clearly unpalatable (see results). Larvae were also painted with non-toxic water-soluble yellow (Perinnemaali, Farnia Oy (colour 5515), Finland) and black (Perinnemaali, Farnia Oy (colour 5511), Finland) acrylic colours to make them look aposematic (black and yellow stripes).

Before the experiment birds were food deprived for 1 h in order to increase their motivation to forage. During the experiment, prey types were offered to birds one by one. All the prey types were offered to birds 4 times. We measured the number of attacked (bird pecked the larvae), killed (bird ate part of the prey) and consumed (bird ate the whole prey) prey. Before the birds were released to their capture sites, they were fed with sunflower seeds, nuts and mealworms.

We also made additional profitability tests in the field in the vicinity of nest boxes in June 2006. Great tits on four territories were first familiarized to feed on a 1 m high feeding tray (30 cm × 20 cm) by offering them dead mealworms (killed by freezing) ad libitum on petri dish. The feeding tray was placed approximately 2 m from the nest box. We spent as little time as possible in vicinity of the nestboxes in order to not to disturb birds. 7 of 8 birds learned to use the feeding tray. We next offered one living pine sawfly larva and one mealworm simultaneously on the petri dish on the white background and observed with a telescope whether the birds attacked and killed pine sawfly larvae. Mealworms were offered to control feeding motivation of birds. Finally, we also offered pine sawflies in groups of 30 on pine branches for the birds. Branches supporting larvae were attached standing on the feeding tray with the help of Styrofoam. Again we observed whether the birds were willing to attack and/or eat pine sawfly larvae.

Efficacy of group size of *Neodiprion sertifer* larvae against avian predation

Predation experiments in the field were conducted in Konnevesi in Central Finland (62.62°N, 26.32°E) in June and July 2006. In early spring, we distributed nest boxes for great tits in habitats which had pines nearby. After the nesting period started, we chose 22

territories of great tits ($N = 22$) for the experiment. During the experiment, we disturbed birds as little as possible. Birds were not handled during the experiment. As a prey species we used *N. sertifer* larvae.

In order to control the predator community, the experiment was conducted in the great tit territories. The experiment was started after nestlings hatched. In the 22 great tit territories we attached large pine branches (50 cm long) with wire on to the tree trunks of any species of tree which was within 3–5 m of the nest box. This was done to equalize the likelihood for birds to notice pine sawfly larvae among feeding territories. Branches were in 50 ml tubes to keep them in water. Water was changed and added if needed during the experiment. In order to ensure that the mortality of pine sawfly larvae was caused by the avian predation and not by arthropod predation (e.g. ants) we blocked the admission of insect predators on experimental branches with a square shaped barrier made from transparencies that was covered with liquid silicon (Lindstedt et al. 2006).

We also tested whether the mortality of pine sawfly larvae correlated with the resin acid content of the host plant. In *P. sylvestris*, more and larger resin ducts in needles reflects the higher resin acid concentrations of the needles (Björkman et al. 1991). As the chemical concentration of the host plants affects chemical defence of sawfly larvae (Codella and Raffa 1995a; Lindstedt et al. 2006), we used the resin duct number of the needles on the host plant as indirect measure of the resin acid concentration of larvae (Björkman et al. 1991). We took random needle samples (20 needles/tree) from trees where experimental branches originated. The number of resin ducts was counted from the thin slices of each needle under a microscope (Björkman et al. 1991; Lindstedt et al. 2006). Branches from experimental trees with different resin acid concentrations were divided equally for both group size treatments in a pair-wise manner. Thus, the variation in resin acid concentration and other properties of the diet was similar in both treatments.

Twenty four hours before transferring the branches with larvae to the field, third instars larval groups of 10 and 50 were moved onto the pine branches so that they had enough time to re-aggregate. During the experiment we observed the mortality of the larvae once a day during the first 9 days and after that every second day for 5 days. Pine branches were changed once a week and they were replaced with branches from the same trees. This was done to ensure a sufficient amount of fresh needles for larval growth.

Effect of diet quality on fitness of *Diprion pini* pine sawfly larvae

We tested the effect of diet resin acid content on larval performance, chemical defence efficiency and immunological traits of *Diprion pini*. We conducted a factorial rearing experiment during May–July 2005 at the University of Jyväskylä in Central Finland. We had two diet treatments: high and low. The resin acid content of the wild pine trees were determined as in Björkman et al. (1991) and Lindstedt et al. (2006) (see above). Trees in which the resin acid duct number of the needles was ≤ 4 and ≥ 10 represented a low and high resin acid diet respectively. Larvae were fed with a mixture of branches within the diet treatments. Before the larvae were divided to low and high diet treatments, they were fed with branches with average levels of resin ducts (6–7). Pine branches (approx. 20–30 cm long) were kept in the water in the glass jar.

At 9 days of age, larvae from each family were randomly divided between low and high resin pine branches. We had 5 larvae per pine branch and the maximum number of individuals per family per diet was 10. Water was added and fresh branches provided for larvae twice a week. Larvae normally moved on to the fresh branches within 24 h, if not, they were carefully moved to the fresh branch on the following day.

Diet and life history traits

Larvae were weighed before they were divided into the diet treatments in order to minimize differences in weights between the treatment groups. Larvae were also weighed at 15 days when their encapsulation reaction was measured. After the second weighing larvae were moved on to petri dishes and reared individually until they pupated. Larvae were checked daily and fresh needles were added *ad libitum* while removing old ones. Individuals were also weighed on the day of their pupation. Growth rate was calculated as \ln pupal mass (mg)/larval development time (days) to pupal stage in days.

Diet and defence traits

The encapsulation reaction is an immunological response to foreign intrusions in insects (Rantala et al. 2000; Ryder and Siva-Jothy 2000; Koskimäki et al. 2004; Ojala et al. 2005). The encapsulation response of all larvae was measured in their final instars (age of 15 days). We measured 5 larvae per treatment per family. Before the encapsulation reaction measurements larvae were weighed. Larvae were anaesthetized with CO₂, after which a small nylon implant (4 mm long, 0.11 mm thick) was inserted inside the larvae on the end of dorsal part of the body in a way that 3 mm of the implant was inside the larvae and 1 mm was left out (enabled the removal of implant). The immune system of the larvae was allowed to react for 48 h. The reaction time was determined in preliminary experiments. Shorter times would have given very low encapsulation and longer times would have given too strong a reaction. Subsequently, the implant was removed, dried and photographed under a microscope with 10× magnification with a Panasonic wv-CL702 video recorder. The mean grey value of the implant was measured with ImagePro Plus 4.0 (Media Cybernetics) on 1 mm of the implant, measured from the end implanted inside the larva. The grey value of the background was subtracted from the grey value of the implant to correct for any variation in lighting during photography. Higher grey values (darker implant) indicated a stronger encapsulation response (see Ojala et al. 2005).

When threatened by predators, larvae regurgitate sticky and resinous droplet from their foregut diverticulae (e.g. Björkman and Larsson 1991). Resinous fluid repels both vertebrate and invertebrate predators (e.g. Prop 1960; Codella and Raffa 1995b). Anesthetization triggers this same regurgitation behaviour and therefore we collected defence droplets from larvae by putting them on blotting paper while anesthetized.

In order to test whether the defence against parasites trades off with defence droplet production, we measured the size of the defence droplet from implanted larvae when the implant was removed. Non-implanted larvae were treated at the same age and in the same way except for implantation. We next photographed the blotting papers with Canon EOS D60-digital camera (50 mm macro object) and measured the area (mm²) of absorbed droplets from the photographs with the help of Image Pro Plus 4-programme.

Statistical analyses

Defence of Neodiprion sertifer larvae against visual predators

Due to the non-parametric structure of the data, the total number of attacked and killed prey in prey profitability tests (study 1) were analysed with non-parametric Kruskal–Wallis tests and more specific pairwise comparisons were made with Wilcoxon tests.

The effects of group size ($N = 11$) and mean resin acid content (mean number of resin ducts on experimental branches) of a host plant on larval survival in the beginning (during first 9 days) and in the end (during last 8 days) of the experiment were analyzed with a Cox-regression. This was done because the group sizes were rather similar from the 9th day onwards (Fig. 2). Due to non-significance (all P values > 0.393), interaction between mean resin acid content and group size was omitted from the final models. Effect sizes are described as odds ratios (OR), where a value of 1.00 indicates that two treatments have identical survival probabilities.

Correlations between the mean survival time of individuals during first 9 days and the mean resin acid content (mean number of resin ducts on experimental branches) of a host plant were analysed separately for both group size treatments.

Effect of diet quality on the fitness of Diprion pini larvae

Even though growth rate and pupal weight differed among sexually and asexually produced larvae (Lindstedt, Huttunen, Mappes unpublished), there were no interactions between diet and asexually or sexually produced offspring (all P values > 0.426). Moreover, sexual and asexual individuals were divided evenly among all the treatments. We therefore pooled asexually and sexually produced haploid males together in order to increase the power of the test and keep results and analyses on the focus of our questions.

We used One Way ANOVA to analyse the effect of diet (factor) on encapsulation rate (dependent variable). The effects of diet and implantation treatment (fixed factors) on development time of larvae to pupa, growth rate, pupal weight and defence droplet size (dependent variables) were analyzed with two-way ANOVAs. Altogether on a low diet we had 50 implanted individuals and 56 non-implanted individuals. On a high diet we had 48 implanted and 57 non-implanted individuals.

Results

Profitability of *Neodiprion sertifer* pine sawfly larvae for avian predators

Birds attacked all the mealworms offered (Table 1). Attack risks differed among the prey items ($\chi^2 = 18.7$, $n = 9$, $df = 2$, $P < 0.001$). Attack risk of the *N. sertifer* pine sawfly larvae was significantly lower compared to mealworms ($Z = -2.598$, $n = 9$, $P = 0.009$) but did not differ from the aposematic prey ($Z = -1.134$, $n = 9$, $P = 0.257$) (Fig. 1). Attack risk of aposematic prey was significantly lower compared to the mealworms ($Z = -2.810$, $n = 9$, $P = 0.005$). Birds consumed all the attacked mealworms but only 24% (mean number of attacked 0.22 ± 0.15 SE) of aposematic prey and 30% (mean number of attacked 0.78 ± 0.43 SE) of *N. sertifer* pine sawfly larvae (Table 1). This experiment confirms that pine sawfly larvae are unprofitable prey for birds.

Table 1 Percentage of prey items attacked, killed and consumed by bird predators

	Mealworm (%)	Aposematic prey (%)	<i>N. sertifer</i> larva (%)
Attacked	100	52.5	57.5
Killed	100	40	45
Consumed	100	25	30.4

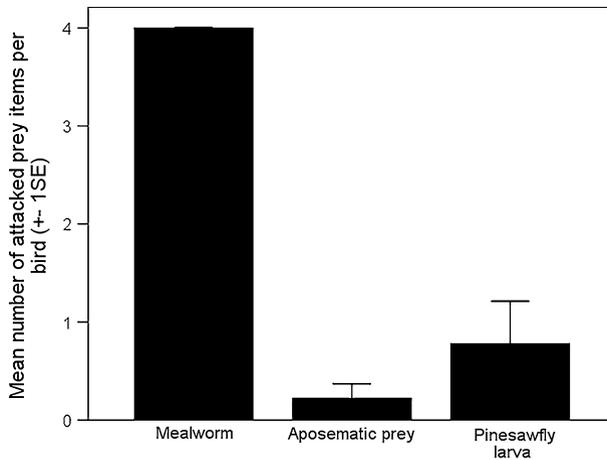


Fig. 1 Mean number of prey items (mealworm, aposematic, unprofitable prey and *Neodiprion sertifer* larva) attacked by the bird predators

When pine sawfly larvae were offered to birds in the vicinity of their nest boxes from the feeding trays, three out of seven birds attacked and killed pine sawfly larvae. Two birds attacked once, but avoided sawflies after the first encounter. Two other birds did not attack at all for pine sawflies. All the birds attacked and ate mealworms. Interestingly, we observed that none of the birds consumed the sawfly larvae themselves but they fed them to their offspring.

Efficacy of group size of *Neodiprion sertifer* larvae against avian predation

Survival rate of larvae in the groups of 50 was lower than for larvae in the groups of 10 ($W = 8.819$, $df = 1$, $P = 0.003$) during the first 9 days of the experiment (Fig. 2) suggesting that on a short term, predation risk is higher in larger than smaller groups. Also, the resin acid content of the diet affected significantly the survival rate ($W = 16.189$, $df = 1$, $P < 0.001$). However, survival was not significantly correlated with the resin acid content of the diet in the groups of 10 ($r = -0.196$, $n = 11$, $P = 0.564$) or 50 ($r = -0.431$, $n = 11$, $P = 0.186$) larvae.

After the first 9 days, the group sizes reached approximately the same level due to relatively higher mortality in the groups of 50 larvae (Fig. 2). After this point (during the last 8 days of the experiment) the survival did not differ significantly among the group size treatments ($W = 0.025$, $df = 1$, $P = 0.875$) or according to resin acid content of the diet ($W = 0.001$, $df = 1$, $P = 0.940$).

Effect of diet quality on the fitness of *Diprion pini* pine sawfly larvae

The resin acid content of the diet did not have any significant effect on larval growth rate or development time (Table 2), but larvae that were parasitized (implanted) grew more slowly than non-implanted larvae (Table 2). However, the development time did not differ significantly between the implanted or non-implanted larvae (Table 2). There were no interactive effects between the diet quality and implant treatment on the growth rate or development time (Table 2).

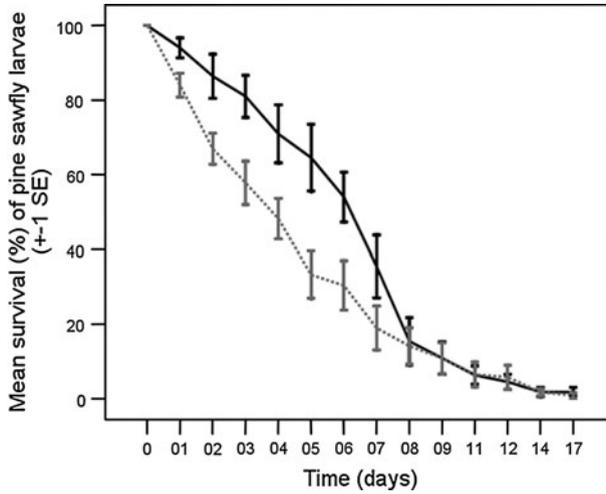


Fig. 2 Mean survival (%) of *Neodiprion sertifer* larvae in groups of 10 (solid black line) and 50 (dashed grey line). Error bars show ± 1 SE

Table 2 Effect of diet chemistry and artificial parasitism on the life history and defence traits of *Diprion pini* pine sawfly larvae

Source of variation	df	MS	F	P
Growth rate				
Diet	1	<0.001	0.001	0.982
Parasitization	1	0.003	4.499	0.035
Diet \times Parasitization	1	<0.001	0.215	0.643
Error	170	0.001		
Development time				
Diet	1	26.292	1.131	0.289
Parasitization	1	3.982	0.171	0.680
Diet \times Parasitization	1	4.189	0.180	0.672
Error	170			
Pupal weight				
Diet	1	1535.218	20.825	<0.001
Parasitization	1	1529.786	20.752	<0.001
Diet \times Parasitization	1	37.401	0.507	0.477
Error	170	73.719		
Defence droplet size				
Diet	1	8.738E + 07	5.637	0.019
Parasitization	1	1.475E + 08	9.516	0.002
Diet \times Parasitization	1	1.055E + 07	0.681	0.411
Error	128	1.550E + 07		
Encapsulation rate				
Diet	1	85.964	1.011	0.318
Error	77	85.036		

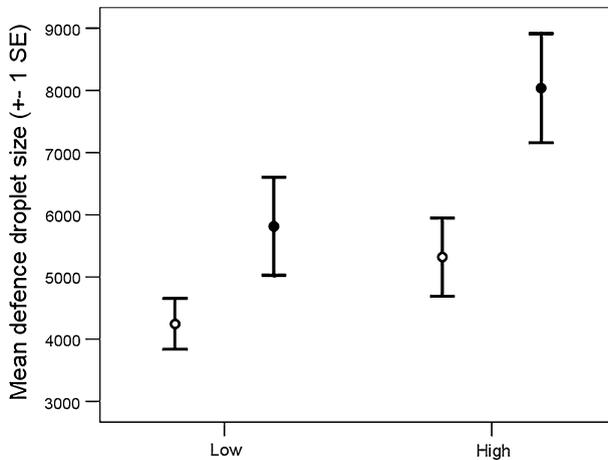


Fig. 3 Mean size of defence droplet (area, mm^2) that parasitized (*open circles*) and non-parasitized (*closed circles*) *Diprion pini* larvae produced on low and high resin acid diet

Resin acid content had positive effect on pupal weights since pupae were heavier on the high than on the low resin diet (low resin = mean $59.0 \text{ g} \pm 0.99 \text{ SE}$, high resin = mean $65.1 \text{ g} \pm 0.94 \text{ SE}$; Table 2). Pupae of the non-implanted individuals were heavier than implanted individuals, which suggest that parasite treatment (implantation and encapsulation reaction) was costly for larvae (parasitized = mean $58.1 \text{ g} \pm 1.1 \text{ SE}$, unparasitized = mean $64.5 \text{ g} \pm 0.88 \text{ SE}$; Table 2). There was no significant interaction between the diet quality and implant treatment (Table 2).

Diet quality did not significantly affect the encapsulation rate, but both diet and implantation had effects on the size of the defence droplet (Table 2). Parasitized (implanted) larvae produced smaller droplets than non-parasitized larvae (Fig. 3). Interestingly, droplets were larger on high than on low diet (Table 2, Fig. 3). There was no diet \times implant treatment interaction for defence droplet size (Table 2).

Discussion

Efficacy of group size of *Neodiprion sertifer* larvae against avian predation

Grouping has shown to be beneficial for defended prey (Sillén-Tullberg 1990; Gagliardo and Guilford 1993; Alatalo and Mappes 1996; Gamberale and Tullberg 1996; Mappes and Alatalo 1997; Hunter 2000; Riipi et al. 2001) even if the prey is cryptic, as predators learn to avoid noxious prey faster when they are clumped (Sillén-Tullberg 1990; Alatalo and Mappes 1996; Riipi et al. 2001; Beatty et al. 2005). Large group size also benefits prey via diluted mortality risk (Sillén-Tullberg and Leimar 1988; Lindström et al. 2001; Riipi et al. 2001). It has also been suggested that the benefit of gregarious lifestyle of chemically defended prey may give a cumulative effect of noxious substances for a predator (Aldrich and Blum 1978). Here we show that large aggregations did not increase the survival of chemically defended *Neodiprion sertifer* pine sawfly larvae. Even though great tits found *N. sertifer* larvae clearly unprofitable as food, the *per capita* mortality was higher in larger groups, giving benefit for individuals in smaller groups with lower *per capita* attack rate.

After the group sizes were approximately on the same level (Fig. 2), the survival decreased on the same rate in both group sizes. Also, the resin acid content affected significantly on larval survival during the first 9 days of the experiment. However, we did not find a significant correlation between the resin acid content of the diet and mean survival time. This suggest that in addition to variation in resin acid content, other features of the diet (e.g. variation in water or nutrition content) could also affect the mortality risk of larvae (see also Ojala et al. 2005, Klemola et al. 2007).

Our results suggest that if the predator finds prey edible, aggregation can be an unnecessarily risky strategy for a prey that is already well protected by crypsis as large aggregation increases its detectability for predators (Ruxton et al. 2004; Lindstedt et al. 2006). This was opposite to results by Sillén-Tullberg (1990) (see also Alatalo and Mappes 1996; Riipi et al. 2001), who found that groups of *N. sertifer* sawflies were attacked less than solitary individuals by birds. However, also in her experiment the sawfly larvae were attacked more than aposematic swallowtail (*Papilio machaon*) larvae suggesting that pine sawfly larvae were not extremely unprofitable as a prey item. Feeding groups of pine sawfly larvae are normally approximately 5–20 larvae (Codella and Raffa 1995b), thus groups of 50 larvae used in our experiment should be high enough to observe the benefits of grouping. It is also important to notice that all studies that have shown benefits of aggregation for defended prey have been conducted in the laboratory conditions (Sillén-Tullberg 1990; Alatalo and Mappes 1996; Riipi et al. 2001). Thus, predators have had a choice (sequential or simultaneous) between preys of different group sizes. Moreover, those experiments typically use one predator at the time whereas in the field prey individuals most likely have to face several predator species and individuals.

The benefits of aggregation found for pine sawfly larvae in previous studies (Codella and Raffa 1995b, Codella and Raffa 1996) may inflate on low quality territories with low amounts of alternative prey (Merilaita and Kaitala 2002; Lindström et al. 2004; Barnett et al. 2007) or against hungry (Barnett et al. 2007; Sandre et al. 2010) or otherwise less susceptible predators (Lindstedt et al. 2006). When the damaging effects of prey unprofitability are relatively low for a predator, a high abundance of unprofitable prey may offset the possible costs, making prey more desirable to consume (Beatty et al. 2005; Lindstedt et al. 2006; Ruxton and Sherratt 2006). Even though pine sawfly larvae were clearly unprofitable for birds in our experiments, we did not observe any highly harmful effects of their defences for the birds; birds did not vomit and/or had no other serious symptoms of poisoning after eating the pine sawfly larvae (personal observation). In addition, during the predation experiments in the field, the weather conditions were rather harsh (cold and rainy) possibly making conditions especially challenging for the nesting birds that need to feed their nestlings. Thus, it is possible that nutritional needs and the risk of starvation are so high that it pays off to feed unpalatable prey as long as it contain sufficient amounts of nutrients (i.e. energy-toxin trade-off) (Barnett et al. 2007) or the tolerance for those particular toxic compounds is relatively high (Turner and Speed 1999). Pine sawfly larvae were also easily available as the branches with larvae were located in the proximity of the nest boxes. Furthermore, if the parents do not have previous experience of the pine sawfly larvae (and they remain naïve as they do not eat them by themselves), it may make them more willing to feed their nestlings with the unprofitable prey items. However, in this case the defence movements of larvae combined to regurgitation of resin fluid would not have influence on bird's decision to attack.

Feeding in groups can offer also other benefits for herbivorous insects than protection from natural enemies (reviewed in Hunter 2000) such as enhanced thermoregulation (Seymour 1979; Casey and Knapp 1987) and exploitation of the host plant (e.g. Kalin and

Knerer 1977; Hunter 2000; Reader and Hochuli 2003; Aukema and Raffa 2004), or improved immune responses (e.g. Wilson and Reeson 1998; Cotter et al. 2004). These other benefits could balance the costs of increased detectability in larger groups. Other benefits of grouping can also act indirectly, increasing prey survival against predators by diminishing exposure time of larvae to natural enemies via increased growth rate (Kalin and Knerer 1977; Hunter 2000; Reader and Hochuli 2003; Aukema and Raffa 2004).

Pine sawflies are one of the most common forest pests of pine trees which cause significant economic harm for the forestry industry during their massive outbreaks. During these outbreaks, larvae are highly clustered both in space and time. It is possible that our experimental setup underestimates the effect of aggregation during the outbreak peaks when efficacy of chemical defences and grouping may be enforced. Nevertheless, our experiment describes the efficacy of the pine sawfly larvae's defence strategy at low- and intermediate population densities when avian predation could have a significant effect on population dynamics of pine sawflies at least on a local scale (see also Lindstedt et al. 2006 for ant predation). As risk of the outbreak has been suggested to be highest when needle resin acid concentration or larval predation pressure is low (Larsson et al. 2000), it could be beneficial to add nest boxes in potential outbreak areas in order to increase predator density that could prevent pine sawfly populations to reach the outbreak levels.

Costs of defences in *Diprion pini* pine sawfly larvae

Evolution of costly secondary defences for a cryptic prey is puzzling, if the prey is already well protected by camouflage (Ruxton et al. 2004). However, if the defences are inexpensive to produce and maintain, but they offer further protection against predators, secondary defences may evolve (Leimar et al. 1986). Results from our rearing experiment suggest that chemical defences did not appear to incur costs in chemically defended but cryptic *D. pini* larvae. In contrast, larvae seem to actually benefit from the resin acids as it increased the size of their defence droplets and pupal weights. Low production and maintenance costs of chemical defences for *D. pini* larvae combined with increased survival against predators (see also Prop 1960; Björkman and Larsson 1991; Björkman et al. 1997) can explain the evolution of chemical defences in this cryptically coloured species.

Our results are in contrast to the studies done with *Neodiprion sertifer* pine sawfly larvae that have shown to suffer from high resin acid concentration of their host plant especially in their first larval instars (Larsson et al. 1986; Björkman and Larsson 1991). However, while *Diprion pini* are known to be able to use also new pine foliage for food in addition to old foliage, *N. sertifer* larvae mainly use old foliage for food. Old foliage is normally lower in resin acids and other allelochemicals than young foliage (Larsson et al. 1992). Thus, it is possible that *D. pini* larvae are able to handle higher resin acid concentrations with lower costs than *N. sertifer* larvae. It is also possible that there are differences between the sexes in pine sawfly larvae. In our experiment we only included male larvae into our analyses to be able to compare both sexually and asexually reproduced individuals (see methods). However, studies done with *N. sertifer* larvae have shown that costs of defences could be lower for males than for females (see Codella and Raffa 1995a) since they are smaller in size. Despite the difference in pupal weights, we did not find any differences in development times or growth rates between the diet treatments. One possibility is that pine sawfly larvae are known to retain the resin filled cuticular sacs during pupation and discard them filled with resin into the pupa (Eisner et al. 1974). Thus, if the larvae on the high diet have larger amounts of resins in their cuticular sacs (see also

Björkman and Larsson 1991) that could cause the difference in pupal weights between the treatments but not in growth rate or development time.

We found that allocation to defence against parasites decreased the pine sawfly larvae's defence capacity against predators. Manipulated larvae (i.e. implantation and encapsulation reaction) produced smaller defence droplets than non-manipulated larvae. However, the defence droplet sizes were always higher on high resin acid diet compared to low resin acid diet even though larvae were implanted. This further suggests that high resin acid diet is beneficial for *D. pini* larvae under selection by predators and parasites (Larsson et al. 2000). According to a previous study (Björkman and Larsson 1991), pine sawfly larvae are able to compensate for low resin acid availability in their host plant by changing their feeding preferences to the plant parts that are high in resin acids (e.g. bark). However, in our experiment the parasitized larvae and non-parasitized larvae were not able to do this, since we fed them individually in petri dishes with needles.

Even though olfactory cues can be important in locating prey over large distances (Hedlund et al. 1996; Powell et al. 1998; Mattiacci et al. 2000), many parasitoid wasps and flies are shown to use visual cues (Powell et al. 1998; Fischer et al. 2001) to locate their hosts, at least over short distances. In *D. pini* larvae, the overall defence capacity against parasitoids was low; pine sawfly larvae need a long time to produce a clearly visible encapsulation reaction compared to other species (e.g. Rantala et al. 2000; Ojala et al. 2005). In the wild, the parasitisation rate of Diprionid larvae can be rather high parasitoids been one of the main sources of mortality (Herz and Heitland 1999). Also, according to our own observation the prevalence of parasitoids in pupae collected from the field can be as high as 44% during an outbreak. Thus, increased detectability to parasites could favour lower detectability.

Evolution of weak signals in chemically defended species

It is possible that conspicuousness can only be maintained in well defended species (Sherratt and Beatty 2003; Endler and Mappes 2004). Thus, if predators vary in their susceptibility to a prey's defences, it may only cause weak selection toward conspicuous signalling (Endler and Mappes 2004; Mappes et al. 2005; Ruxton et al. 2009). Similarly, chemical defences that are effective against birds or ants may be ineffective against parasitoids (Gentry and Dyer 2002). When the efficacy of defences varies between different enemies, conspicuousness may be useful as a signal to some predators, but invite attacks from other predators and parasites. We showed here that increased detectability (i.e. large group size) was costly for pine sawfly larvae as it increased their predation risk. Even though high resin acid concentration increased the amount of defensive secretion in pine sawfly larvae, it did not have effects on their defence capacity against parasitoids. Therefore, high detectability costs and a low defence capacity against parasites could offer one explanation why low signal intensity may be selected for instead of overtly conspicuous signals in pine sawfly larvae. Also, as pine sawfly larvae do not need to travel long distances for food, opportunity costs of crypsis are probably not very high for them, in which case optimal conspicuousness may be low (Speed et al. 2010).

It is also important to note that crypsis and aposematism are not necessary mutually exclusive strategies (Marshall 2000; Tullberg et al. 2005). Even though cryptic from a long distance, at short distances pine sawfly larvae can appear rather conspicuous to predators when they do their defence movements. Pine sawfly larvae also perform some kind of self-advertisement of their secondary defences as they regurgitate the resinous content from their mouth. Display of defensive secretion can act as a startle behaviour (Eisner et al.

1974; Ruxton et al. 2004) aiming to scare the predator away or purely as a defence making pine sawfly difficult for predators to handle (sticky) and unpalatable. However, it may also have a value as a signal of defensive potential (Ruxton et al. 2004, Skelhorn and Rowe 2006b). Efficacy of this behaviour may be further enhanced when performed in larger groups. Thus, even though hiding from predators' eyes would be most optimal defence strategy for pine sawfly larvae in the first place, after detection by predators, conspicuous behavioural displays and advertisement of secondary defences could ensure accurate recognition from a closer distance. However, the signal value of these different components (defence behaviour, defence droplet and aggregation size) for visual predators needs further studies.

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