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Impact of starvation on immune defense and other life-history traits of an outbreaking geometrid, *Epirrita autumnata*: a possible causal trigger for the crash phase of population cycle

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Outbreaking populations of the autumnal moth, *Epirrita autumnata*, are regularly facing a shortage of food and starvation. We found that starvation led to a prolonged larval development and a marked reduction in pupal weights. A decrease in female pupal weight caused a 50% decrease in the realized fecundity. Due to starvation the melanotic encapsulation rate decreased but the activity of PO — a key enzyme in the melanotic pathway that may indicate scarcity of PO substrates due to starvation — increased. Our results suggested that the shortage of food during high-density years reduce markedly the fecundity of *E. autumnata*, and may also reduce the ability of the autumnal moth to resist parasitoids and pathogens. Altogether, starvation may potentially destabilize the pathogen dynamics of insects and may be a proximate reason for moth population crashes.

Introduction

Food scarcity often plays a key role in the regulation of population dynamics in animals (Bonsall & Eber 2001). Starvation is a problem especially for outbreaking herbivore populations. Autumnal moth, *Epirrita autumnata* (Lepidoptera: Geometridae), is the most severe pest species of mountain birch forests in Fennoscandia. *Epirrita autumnata* outbreaks are often characterized by a total destruction of the leaves of its main host, the mountain birch. As a result, the starving larvae migrate to alternative host species (Klemola *et al*. 2004). During the most severe outbreaks, polyphagous moth larvae defoliate all available deciduous trees and shrubs. Both the body weight and the fecundity of *E. autumnata* decrease during the peak years of population cycle prior to population crash (Haukioja *et al*. 1988a, Ruohomäki *et al*. 2000). The reasons are suggested to be extrinsic (food limitation, use of alternative host plants), intrinsic (genetic or maternal) or the interaction of these factors (*see* Haukioja *et al*. 1988b, Ruohomäki *et al*. 2000, Klemola *et al*. 2004).

Individuals in good condition are often able to generate an immune response better than the ones in poor condition (Møller *et al*. 1998, West-

neat & Birkhead 1998, Rantala *et al*. 2003b). Lochmiller (1996) proposed that reduced immunocompetence resulting from limited food availability is the main process that leads to the population fluctuation in small mammals. However, in *E. autumnata* a mild stress such as slight pollution (van Ooik *et al*. 2007, *see* also Sorvari *et al*. 2007), overcrowding (J. Kilpimaa unpubl. data) or the induced defence of plants (Kapari *et al*. 2006, S. Sillanpää unpubl. data) may also enhance immunity.

Parasitism has been shown to be a key determinant in the regulation of the *E. autumnata* population cycle (Tanhuanpää *et al*. 1999, Ruohomäki *et al*. 2000, Klemola *et al*. 2004), although the rate of parasitism may be connected to food quality i.e. the induced resistance of birches increasing the melanotic encapsulation rate of autumnal moths (Kapari *et al*. 2006). In another study, we found that the shift of *E. autumnata* larvae to alternative host species decreased their body weight but did not affect immune defence (S. Yang unpubl. data). In this study, we tested the hypothesis whether starvation might act as a causal factor for the *E. autumnata* population crash in the last phase of its cycle. We gradually decreased food availability for the final 5th instar of *E. autumnata* and measured the effects of starvation on pupal weights, development times and immune defense.

Material and methods

Study species

The autumnal moth, *Epirrita autumnata* (Borkhausen) is a holarctic geometrid with a univoltine life cycle (Haukioja *et al*. 1988a). Females lay eggs into the lichen cover of trees in autumn, and larvae hatch in the beginning of spring in synchrony with the bud break of its main host, the mountain birch (*Betula pubescens* ssp. *czerepanovii*).

The autumnal moth is a major pest in mountain birch forests in NW Europe. Population cycle of *E. autumnata* is characterized by regular regional outbreaks that occur with a period of 9 to 10 years and usually last for 2–3 years (Tenow 1972, Ruohomäki & Haukioja 1992a, Hogstad 1997). During an outbreak, larvae are able to defoliate vast areas of mountain birch forests (Tenow 1972, Haukioja *et al*. 1988a, Ruohomäki *et al*. 2000).

The experimental procedure

We conducted the experiment at the Kevo Subarctic Research Station of Turku University, in northernmost Finland (69°45´N, 27°00´E) in summer 2004. We used two separate strains of *E. autumnata* larvae from two populations, Kevo (northern Finland) and Abisko (northern Sweden). We collected both populations from the wild two years in advance and reared them in laboratory conditions for one progeny prior to the experiment. The Abisko population was collected as larvae from an outbreak area, whereas Kevo population represents a population which does not reach outbreak densities due to climatic reasons, but shows a similar cyclic density fluctuation. We reared the larvae individually in 48-ml jars in natural light and temperature regime and fed them *ad libitum* with fresh mountain birch leaves until they reached the final 5th instar. The newly molted 5th instars were kept without food in a refrigerator at +1 °C to stop the development until all larvae had molted to the 5th instar. Thus, all larvae in the starvation experiment were of the same age.

We selected 100 healthy larvae (5th instar) from each population (Abisko and Kevo) and allocated these larvae randomly to the control and starvation treatments. The starvation treatment mimicked the gradual reduction in food availability during the peak years of outbreaks in nature. We gradually reduced the amount of food for the treatment larvae (*see* also Tammaru 1998) before total starvation, while the control larvae were fed *ab libitum* with fresh leaves throughout the experiment (*see* Table 1). By the end of the 5th day, most of the larvae had shortened and we transferred them into moss where they pupated. Those that had not shortened before the end of the 5th day were kept in starvation every other day for 24 h until also they pupated. Seven days after the pupation, we weighed and sexed the pupae and measured the encapsulation rate (*see* below). We froze the pupae in liquid nitrogen

and afterwards stored them in –80 °C until they were analyzed.

Encapsulation rate assay

One of the easiest and most informative ways to evaluate the strength of immune defense in arthropods is to challenge the immune system with an artificial object (e.g. a piece of monofilament) and to analyze the degree of melanization in the attached cell mass (Rantala *et al*. 2002, Ahtiainen *et al*. 2004, 2005, 2006, Koskimäki *et al*. 2004, Vainio *et al*. 2004). We inserted a piece of nylon monofilament (2 mm long, 0.1 mm diameter, rubbed with sandpaper and knotted at one end) in the abdomen of *E. autumnata* pupae (as described in Kapari *et al*. 2006). The immune system of insects was allowed to react to this object for an hour at +24 °C. The implanted pupae were then frozen in liquid nitrogen and stored at –80 °C until they were analyzed. The implants were removed and photographed from three different angles with a microscope camera. The melanization degree was analysed using an image analysis software (Image J-Program). The melanotic encapsulation rate of each pupa was calculated as an average of three pictures. The scale was calibrated with the grey value of a blank implant that had only been rubbed with sandpaper (for details of the method, *see* e.g. Rantala *et al*. 2000, 2003a, 2003b).

Phenoloxidase activity assay

The pupae were thawed and crashed with tweezers in a microcentrifuge tube, and the haemolymph was isolated by a short centrifugation for 30 s at 15 000 rpm at +4 °C. The clear haemolymph (1.5 μl) was diluted with 28.5 μl of potassium phosphate buffer (pH 7.5, 0.1 M), which contained 1 mM PMSF (phenylmethanesulfonyl fluoride, Sigma-Aldrich), vortexed and stored at –80° C prior to enzymatic assays. PO activities were examined in duplicates by using 10 mM L-DOPA as a substrate and following the changes in absorbance at 490 nm for 30 minutes (*see* Rantala *et al*. 2002, 2003b) with a Multilabel counter (1420 Victor, Wallac Oy, Turku, Finland). PO activities were expressed both as the absolute activity (dAbs min^{-1} ml^{-1}) and the specific activity (U/mg protein). One unit was the amount of enzyme required to increase the absorbance by 0.001 min^{-1}. The protein content of the haemolymph was measured using the BioRad protein assay kit (*see* Ruuhola & Yang 2006) based on the Bradford method (Bradford 1976).

Statistical analysis

The hypothesis that starvation will decrease both the fecundity and immunocompetence of autumnal moth was tested with the univariate analysis of variance (UNIANOVA). Pupal weight, protein content of the haemolymph, melanotic encapsulation rate, and both the absolute and specific PO activities were set as dependent variables. Pupal weight, both the absolute and specific PO activities were square-transformed to meet assumptions of parametric testing. Treatment, location, sex and their interactions were fixed factors in the analysis. In the analysis of protein contents and PO activities, the plate was also set as a random factor. The realized fecundity of females was calculated according to Tammaru (1998) with the following equation: realized fecundity = (2 × pupal weight) – 60. The realized fecundity was tested with the univariate analysis of variance, where treatment and location were fixed factors. The normal distribution and the equality of variances were tested with Kolmogorov-Smirnov's and Levene's tests, respectively.

Table 1. Starvation experiment. The experiment was conducted with the final 5th instar of *E. autumnata*, which is the most voracious instar often facing starvation in years when its population reaches high densities.

Date	Starvation		Control	
	Starving (h)	Feeding (h)	Starving (h)	Feeding (h)
26 June	6	18	0	24
27 June	8	16	0	24
28 June	12	12	0	24
29 June	16	8	0	24
30 June	24	0	0	24

To test which effects of the treatment were mediated by pupal weights and in which cases there were treatment effects that could not be wholly ascribed to the induced change in body size, we used the pupal weight as a co-variate in the model (ANCOVA). The effects of the treatment, location and sex on the duration of the 5th instar were analyzed with the Kruskall-Wallis non-parametric test. All tests were performed with the SPSS 11.5.1 software (SPSS, Chicago, IL, USA).

Results

Starvation treatment during the final phase of larval development resulted in a remarkable decrease in pupal weights. The decrease was larger in females than in males (Table 2 and Fig. 1a). The population had a marginally significant effect on pupal weights and there was a significant treatment × location interaction (Table 2). Pupal weighs of control animals of the Abisko population were lower as compared with those of the Kevo population, whereas the decrease in pupal weights caused by starvation was smaller in the Abisko population than in the Kevo population (Fig. 1a).

Treatment, sex and population all had a significant effect on the duration of the 5th instar (Table 3 and Fig. 1b). Starvation treatment prolonged the development time of both males and females in the Kevo population, whereas in the Abisko population, only the development time of females increased (Fig. 1b).

The starvation treatment, sex and population all affected significantly the protein content of the haemolymph (Table 2 and Fig. 1c), although there were no significant interactions between these factors. Starvation decreased the protein content of the haemolymph and in the Kevo population the decrease was more pronounced in males than in females, whereas in the Abisko population the decrease was more pronounced in females. The starvation treatment increased both the absolute (dAbs min^{-1} ml^{-1}) and the specific (U mg $protein^{-1}$) PO activities, whereas neither sexes nor populations differed in their PO activities (Table 2, Fig. 1d and e). In addition, there were no significant interactions. Removing non-significant items from the model did not affect the results.

Table 2. UNIANOVA and ANCOVA of the effects of treatment, sex, population and their interaction on the life history traits of *Epirrita autumnata*. Statistically significant cases are set in black-boldface ($P \leq 0.05$) and nearly significant cases in grey-boldface ($P < 0.1$). df = 1 in all tests.

Dependent variable		Treatment	Sex	Population	Treatment × sex	Treatment × population	Pupal weight
Pupal weight	*F*	**172.949**	**9.887**	3.203	**12.319**	**4.908**	
	P	**< 0.001**	**0.002**	0.077	**0.001**	**0.028**	
Protein content of haemolymph	*F*	**5.423**	3.828	**6.507**	0.206	0.191	
	P	**0.021**	0.052	**0.012**	0.651	0.663	
Protein content of haemolymph ANCOVA	*F*	0.022	1.717	**7.942**	0.000	0.678	**5.042**
	P	0.883	0.192	**0.005**	0.991	0.412	**0.026**
Absolute PO-activity	*F*	**5.079**	0.108	0.012	1.058	1.805	
	P	**0.026**	0.743	0.913	0.305	0.181	
Absolute PO-activity ANCOVA	*F*	**5.818**	0.000	0.130	0.600	1.275	1.287
	P	**0.017**	0.989	0.719	0.440	0.261	0.258
Specific PO-activity	*F*	**8.452**	0.000	0.107	0.841	1.378	
	P	**0.004**	0.993	0.744	0.360	0.242	
Specific PO-activity ANCOVA	*F*	**6.408**	0.028	0.058	0.579	1.088	0.392
	P	**0.012**	0.868	0.810	0.448	0.299	0.532
Encapsulation rate	*F*	3.172	2.105	0.033	0.087	0.212	
	P	0.077	0.149	0.855	0.769	0.645	
Encapsulation rate ANCOVA	*F*	**5.081**	3.031	0.137	0.006	0.474	1.964
	P	**0.025**	0.083	0.712	0.940	0.492	0.163

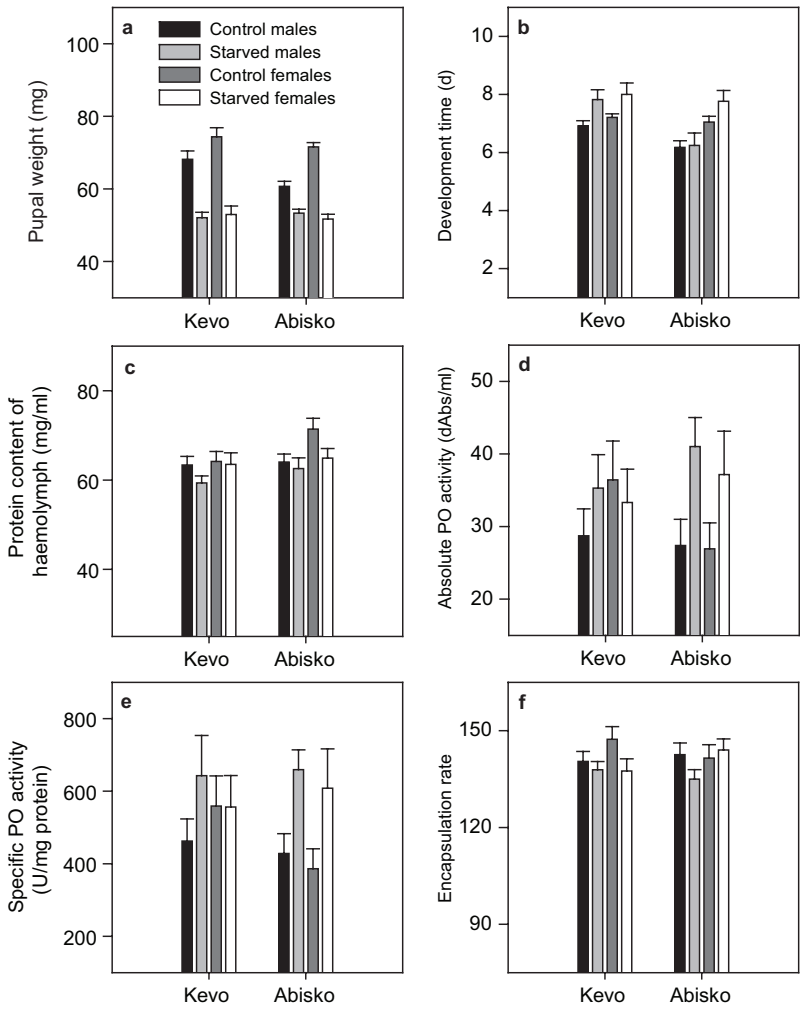

Fig. 1. The effect of starvation on (**a**) the pupal weight, (**b**) the development time, (**c**) the protein content of haemolymph, (**d**) the absolute PO, (**e**) the specific PO activities, and (**f**) the rate of melanotic encapsulation of autumnal moth. The bars represent SE.

Protein content of the haemolymph was condition-dependent, i.e. pupal weight as a covariate having a significant effect on the protein content, and the effect of starvation and sex disappeared, although the differences between populations remained significant (Table 2). On the contrary, pupal weight as a covariate had no significant effect on the PO activities; neither was the treatment effect altered by this statistical adjustment. The starvation treatment had a marginally significant effect on the melanotic encapsulation rate, and when pupal weight was set as a covariate, the differences were statistically significant and also the sexes tended to differ in their encapsulation rates (Table 2 and Fig. 1f).

Starvation resulted in a significant decrease of the realized fecundity of females ($F = 116.571$, $P < 0.001$) (Fig. 2). Populations did not differ significantly in their response to the starvation treatment ($F = 1.117$, $P = 0.282$).

Discussion

Populations with fluctuating densities are regu-

Table 3. Kruskal-Wallis non-parametric test of the effects of treatment, sex and population on the duration of the 5th instar of *Epirrita autumnata*.

		Treatment	Sex	Population
Duration of the 5th instar	df	1	1	1
	χ^2	3.718	13.310	12.573
	P	0.054	< 0.001	< 0.001

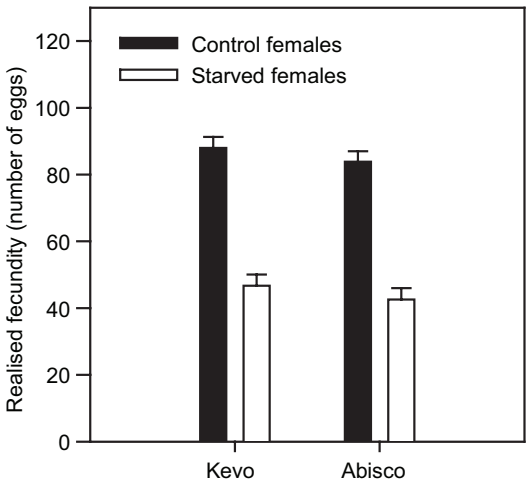

Fig. 2. The effects of starvation on the realized fecundity of autumnal moth females. Error bars represent SE.

larly facing food deprivation during the high-density years. Larvae of *Epirrita autumnata* suffer from the lack of food during the most intensive outbreaks. However, parasitism is generally considered as the major factor regulating the density of northern *E. autumnata* populations (Tanhuanpää *et al*. 1999, Ruohomäki *et al*. 2000, Klemola *et al*. 2004). In our study, we found that starvation during the ultimate 5th instar phase of *E. autumnata* significantly reduced the pupal weights, and prolonged the duration of this final instar. The effects of starvation were more pronounced in females than in males, which is a general phenomenon among insects (*see* Teder & Tammaru 2005). The pupal weights of females decreased 20 mg, whereas the pupal weighs of males decreased 10 mg. As a result of starvation, the realized fecundity of females collapsed markedly (ca. 50%; Fig. 2).

Parasites are suggested to benefit from the large size of hosts (Teder & Tammaru 2002). However, we found that the melanotic encapsulation rates of starved animals decreased due to the limited food availability, which suggests a lowered defence against parasites in small, starved animals. The melanotic encapsulation response against a nylon monofilament in *E. autumnata* resembles that against an entomopathogenic fungus (Rantala & Roff 2007). Thus, a failure to respond to a nylon filament would indicate an impaired immunity against real pathogens and parasites. Our results are consistent with previous studies on several insect species, where the food limitation has been shown to reduce the immune defence (Schmid-Hempel & Schmid-Hempel 1998, Suwanchaichinda & Paskewitz 1998, Moret and Schmid-Hempel 2000, Siva-Jothy & Thompson 2002). However, in noctuid larvae (Lepidoptera: Noctuidae), the increased nutrient intake decreased the disease resistance against baculoviruses (Hoover 1998).

Haemolymphal phenoloxidases (POs) are key enzymes that regulate the melanization of capsules (Gillespie *et al*. 1997, Söderhäll & Cerenius, 1998, Shiao *et al*. 2001). POs take also part in other important physiological processes such as the hardening of cuticle in insects and wound-healing processes (Ashida & Brey 1995, Gillespie *et al*. 1997). In the present study, we found that food stress significantly increased PO activities of the haemolymph of *E. autumnata*, indicating enhanced resistance to pathogen infection. However, our finding is contrary to Rantala *et al*. (2003b) and Siva-Jothy and Thompson (2002), who reported that a short-term nutrient deprivation (only *ad libitum* water) reduced the haemolymphal PO activities of the mealworm beetles (*Tenebrio molitor L)*. In those studies, the beetles have been starved for 3–7 days, whereas in our experiment, moth larvae experienced gradually food deprivation. Tyrosine, the limiting factor in the melanization process (Johnson *et al*. 2003), is ultimately acquired from food. Thus, starvation probably leads to the exhaustion of this and other melanin precursors. The increase in the PO activities might indicate that starved animals attempt to compensate for the reduced encapsulation capacity by increasing the PO activity of the haemolymph, but due to the limited amount of PO substrates, this compensation failed.

The larvae of the Kevo population, whose grandparents were collected from a non-outbreaking population, managed better when food was abundant but suffered more when food was scarce as compared with the larvae of the Abisko population, which represents an outbreaking population. Since we reared the experimental larvae as well as their parents in identical lab conditions, thus these differences were due, most probably, to phenotypic difference and/or local adaptation rather than to maternal or genetic dif-

ference (*see* also Ruohomäki & Haukioja 1992a, 1992b).

We found no significant differences either in the encapsulation rate or PO activities between the two *E. autumnata* populations, although the protein content of the haemolymph differed between subpopulations. It is important to note that the subpopulations were not isolated ones. Instead, they were parts of a huge continuous transholarctic population of this species. Thus, gene exchange may occur frequently, even though females of *E. autumnata* are weak fliers (*see* Snäll *et al*. 2004). This explains why there were no significant differences in immunocompetence between the two studied populations.

Previously, Kapari *et al*. (2006) showed that during the increasing phase of the cycle, a delayed induced resistance of the host plant increased the immune defence of the autumnal moth, which in turn might have prevented parasitoids and diseases from controlling its population size of the autumnal moth (Kapari *et al*. 2006). In the present study, during the last phase of the population cycle, when the larvae experience the shortage of food, the fecundity of females collapsed and the ability to resist parasitoids and pathogens was apparently also reduced. This might, in turn, allow parasitoids (or diseases) to catch up with the growth of the host population and subsequently, cause it to crash.

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