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Experimentally activated immune defence in female pied flycatchers results in reduced breeding success

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Traditional explanations for the negative fitness consequences of parasitism have focused on the direct pathogenic effects of infectious agents. However, because of the high selection pressure by the parasites, immune defences are likely to be costly and trade off with other fitness-related traits, such as reproductive effort. In a field experiment, we immunized breeding female flycatchers with non-pathogenic antigens (diphtheria–tetanus vaccine), which excluded the direct negative effects of parasites, in order to test the consequences of activated immune defence on hosts' investment in reproduction and self-maintenance. Immunized females decreased their feeding effort and investment in self-maintenance (rectrix regrowth) and had lower reproductive output (fledgling quality and number) than control females injected with saline. Our results reveal the phenotypic cost of immune defence by showing that an activated immune system *per se* can lower the host's breeding success. This may be caused by an energetic or nutritional trade-off between immune function and physical workload when feeding young or be an adaptive response to 'infection' to avoid physiological disorders such as oxidative stress and immunopathology.

Keywords: cost of immune defence; ecological immunology; *Ficedula hypoleuca*; host–parasite interactions; life-history trade-offs; parental effort

1. INTRODUCTION

Life-history theory assumes that there is a trade-off between an organism's current reproductive effort and its future survival and reproductive success (Roff 1992; Stearns 1992). This concept of the costs of reproduction is based on physiological trade-offs between resource-demanding functions within an individual. Recently, the costs of immune defences, traits essential for an organism's survival in pathogenic environments, have been emphasized (Sheldon & Verhulst 1996). Allocation decisions between reproductive effort and immune defence are suggested to be targets for optimizing selection, favouring individuals which allocate their resources in a way which maximizes their lifetime reproductive success.

Parasites are often known to reduce the reproductive success of their avian hosts (Loye & Zuk 1991; Møller 1997). The decreased reproductive output of hosts can either be a direct pathogenic effect of the parasite or a consequence of the adaptive adjustment of reproductive effort to reduce the indirect negative impact of the parasite on the host's physiology (Forbes 1993; Møller 1997). Traditional explanations for how parasites affect hosts are that parasites cause dysfunction of somatic systems and reduce metabolic efficiency (e.g. Schall *et al.* 1982; Thompson 1990; Chapman & George 1991) or that parasites deplete resources essential for host reproduction and self-maintenance (Connors & Nickol 1991). However, as parasites evidently exert high selection on hosts, it is probable that immune defences have evolved to such an extent that they become costly (Keymer & Read 1991; Sheldon & Verhulst 1996). However, direct evidence for costly immune defences is still lacking.

In this study, we experimentally investigated the costs of mounting an immune response on the parental effort and breeding success of the hosts. We challenged breeding female pied flycatchers (*Ficedula hypoleuca*) with novel antigens and measured their investment in reproduction and somatic functions (immune response and feather regrowth). By using killed pathogens (human diphtheria–tetanus vaccine) we excluded the direct negative effects of parasites and, thus, tested only for the effects of activating the host immune defence.

We also wanted to study whether the costs of activating immune defence depend on environmental stress, as any trade-off is more likely to be uncovered in stressful conditions (Tuomi *et al.* 1983; Bell & Koufopanou 1986). For this, we carried out the experiment within two subgroups of a population of flycatchers, one breeding close to and the other breeding far from a copper smelt, i.e. in high- and low-pollution stress environments, respectively.

2. MATERIAL AND METHODS

(a) *Study species*

The pied flycatcher is a small (*ca.* 12–13 g) insectivorous passerine bird which ranges over most of northern and eastern Europe. Males arrive at our study area from wintering grounds in West Africa in May, approximately one week earlier than females. In our study population egg laying starts at the end of May and females lay a clutch of three to nine eggs (mean of six eggs). The female incubates alone for approximately two weeks. Both sexes feed the young, which fledge at the age of 14–16 days. Females rear only one brood per breeding season.

(b) *Study area*

The study was conducted in the surroundings of the city of Harjavalta (61°20′ N, 22°10′ E), south-west Finland in 1998. The study was carried out in two areas; at six sites within 3 km of a copper smelter complex (centre) and at another six sites more

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than 5 km away from the smelter (background). Each site contained 30–50 nest-boxes, in total 490 nest-boxes. Elevated heavy metal concentrations occur at the sites in the centre area due to the current and long-term effects of atmospheric deposition from the copper smelter. The metal contents decrease exponentially with increasing distance from the factory complex and approach normal background levels at sites greater than 5 km from the centre (e.g. Jussila & Jormalainen 1991; Koricheva & Haukioja 1995). Pied flycatchers breeding in the centre suffer from low breeding success (Eeva & Lehikoinen 1996; Eeva *et al.* 1997) and they have reduced survival rates (Eeva & Lehikoinen 1998) compared to birds breeding in the background area. The two-area design, together with the fact that the breeding season in 1998 was exceptionally rainy, with the highest monthly rainfall in June during the last ten years (unpublished data from Peipohja Meteorological station), provided a good opportunity of investigating possible allocation trade-offs, because organisms are more likely to face such trade-offs under environmental stress (Tuomi *et al.* 1983; Bell & Koufopanou 1986).

(c) *General methods*

Nest-boxes were visited at least once a week to determine the clutch initiation date, clutch size, hatching date and the number of hatched and fledged young. Females were captured a first time five days before their clutch hatched. To investigate the ability of the females to allocate resources for self-maintenance, we removed one of their outermost tail feathers. Females were captured a second time when their chicks were seven days old and a third time when their chicks were 13–14 days old (close to fledging). Parents and 13–14-day-old chicks were ringed, weighed to the nearest 0.1 g with a Pesola spring balance and their tarsus length measured to the nearest 0.1 mm with sliding callipers. As a measure of female body condition, we used the ratio of body mass on tarsus length. Female subcutaneous fat was scored into five classes (0–4) following Busse & Kania (1970). We used the mean body mass of 13–14-day-old chicks and fledging success (the number of fledglings per hatched young) as a measure of female reproductive success.

(d) *Immunization protocol*

Females with clutch sizes of five to seven eggs were immunized five days before the expected hatching day of their clutch by intramuscular injection in the pectoral muscle with 100 µl of diphtheria–tetanus vaccine (Finnish National Public Health Institute; diphtheria 38 Lf (limit of flocculation) and tetanus 10 Lf, mixed with the adjuvant aluminium phosphate at 1.0 mg ml^{-1}). Control females with similar laying dates (mean ± s.e. for females in the centre area, vaccine group 29.20 ± 1.64 and control group 31.21 ± 1.64 and mean ± s.e. for females in the background area, vaccine group 32.20 ± 1.58 and control group 31.64 ± 1.64; 1 = 1 May) and clutch sizes (mean ± s.e. for females in the centre area, vaccine group 6.33 ± 0.19 and control group 6.29 ± 0.20 and mean s.e. for females in the background area, vaccine group 6.00 ± 0.19 and control group 5.93 ± 0.19; in both cases the effects for the main factors and their interaction in two-way ANOVAs were non-significant) were injected with 100 µl of saline. Females were blood sampled (120–150 µl in heparinized capillary tubes by brachial venipuncture) prior to injection and 12–13 days after the injection (when their chicks were approximately seven days old) to measure activation of the humoral immune system. The blood was transferred into Eppendorf tubes containing 3 µl of heparin. The tubes were immediately stored in an icebox and centrifuged within 3 h at 3000 rpm for 8 min. Plasma was extracted and stored at −20 °C until later enzyme-linked immunosorbent assay (ELISA) analysis.

(e) *ELISA assay*

We measured humoral immune system activation as the antigen-specific antibody levels in the females' sera using an ELISA previously developed for red-winged blackbirds (for details of the methods, see Hasselquist *et al.* (1999)), and this assay has also been proved to work with high accuracy for other passerines (D. Hasselquist, unpublished data). This ELISA method provides sensitive measures of the amount of passerine antibodies which specifically bind to a certain antigen (here diphtheria or tetanus toxoid).

Ninety-six-well ELISA plates were first coated with antigen (diphtheria or tetanus toxoid). Diluted pre- and post-injection serum samples from female pied flycatchers were added to the wells and the plates were then incubated overnight at 4 °C. After washing the plates, a secondary rabbit anti-red-winged blackbird immunoglobulin (Ig) antiserum was added. After a second incubation (1 h at 37 °C) and wash, a commercial peroxidase-labelled goat anti-rabbit-Ig antiserum diluted 1:2000 (cat. A6154, SIGMA; Sigma-Aldrich Sweden AB, Lund, Sweden) was added to the plates. Following incubation (30 min at 37 °C) and wash, peroxidase substrate (2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, ABTS; cat. A1888, SIGMA) and peroxide were added and the plates were immediately transferred to a V_{max} (molecular dynamics) kinetics ELISA reader. The plates were read at 30 s intervals for 14 min using a 405 nm wavelength filter. All antibody concentrations are given as the slope of the substrate conversion (in 10^{-3} × optical densities (OD); m_{OD}) over time (m_{OD} min^{-1}), with a higher slope indicating a higher concentration of antigen-specific antibodies in a sample.

We used a diluent of 1% powdered milk in 0.01 phosphate-buffered saline (pH 7.2) to produce 1:1600 dilutions of each pre- and post-injection serum sample. To avoid between-plate variation, we ran serum samples from all studied females on three 96-well ELISA plates for each of the two antigens and analysed all plates on the same day. Pre-injection serum samples from each female were run in order to investigate each individual's background level of antigen-specific antibodies. For each individual, post-injection serum samples were added to the plate in duplicate and the average of these was our measure of antibody titre for each dilution. We ran at least two wells with blank samples on each plate (these wells were treated in the same way as the test sample wells except for not adding any bird serum). As our measure of pre- and post-injection antibody titres of individual females, we subtracted the mean value of these blanks from the measured antibody concentration. We ran three standard samples covering the range of antibody titres for the injected females on each plate. We used the differences between the standard curves to adjust pre- and post-injection antibody titres to control for between-plate variation.

The antibody production against diphtheria and tetanus in the post-injection samples was at least two times higher than the pre-injection samples in all but one case among immunized females. By investigating the plots of substrate conversion over time, we confirmed that the antibody titres of all individuals were within the linear range of the ELISA reader.

(f) *Monitoring feeding rates*

The parental feeding rates (feedings per hour) were documented with video cameras when the nestlings were ten days old.

Table 1. *Body condition (body mass/tarsus length) and subcutaneous fat at the end of the nestling period and percentage of individuals regrowing their removed rectrix for female pied flycatchers injected with saline and diphtheria–tetanus vaccine close to the centre and in the background area in 1998*

	close to the centre		background area	
	control	vaccine	control	vaccine
sample size	9	11	13	14
body condition	7.2 ± 0.09	7.1 ± 0.09	7.3 ± 0.07	7.2 ± 0.08
subcutaneous fat	0.8 ± 0.31	1.2 ± 0.29	0.8 ± 0.26	1.2 ± 0.24
rectrix regrowth (%)	44.4	9.1	15.4	7.1

Cameras were placed *ca.* 10 m from the nest-boxes between 8.00 and 18.00 h. Each nest was monitored for 80 min. Monitoring was done only during rainless periods. The first 15 min and last 5 min of the video recordings were excluded from the analyses to prevent possible effects of disturbance.

(g) *Statistical analyses*

To meet the requirements of parametric tests, we $\log_{10}$ transformed the female and male feeding rates and the values of the antibody titres against diphtheria and tetanus toxoids. Treatment (vaccination or saline injection) and area (centre or background) and their interactions were entered into ANCOVA models. Brood size at the time of the video recordings was used as a covariate in the analyses of parental feeding rates and laying date as a covariate in the analysis of fledgling body mass. The logistic regression procedure in SPSS (Norušis 1993) was used to estimate the likelihood of the rectrix regrowth probabilities of females relative to treatment and area. In logistic regressions, treatment and area were treated as categorical (dummy) variables. The model with the best fit was chosen using backward (stepwise) model selection. The GENMOD procedure in SAS statistical software (SAS Institute, Inc. 1989) was used to estimate the likelihood of fledging success (number of fledged young/number of hatched chicks). We used a scale parameter to control for the effects of over-dispersion on the binomial variance (McCullagh & Nelder 1989). Polygynous males and their nests were excluded from the analyses. All results are reported with two-tailed probability values.

3. RESULTS

Females immunized with diphtheria–tetanus vaccine showed a clear increase in both their diphtheria- and tetanus-specific antibody titres, whereas among the saline-injected control females their antibody levels sustained at low, close to initial levels (ANCOVA, initial level as covariate $F_{1,42} = 4.42$ and $p = 0.04$, effect of treatment on diphtheria $F_{1,42} = 44.1$ and $p < 0.001$ and least square mean (±s.e.) of $\log_{10}$-transformed antibody titres, saline group 0.04 ± 0.09 and vaccine group 0.84 ± 0.07; ANCOVA, initial level as covariate $F_{1,42} = 3.19$ and $p = 0.08$, effect of treatment on tetanus $F_{1,42} = 125.1$ and $p < 0.001$ and saline group 0.31 ± 0.08 and vaccine group 1.40 ± 0.06). Neither the area nor the area–treatment interaction had a significant effect on the diphtheria- or tetanus-specific antibody titres (all p-values > 0.1). The antibody responses for diphtheria and tetanus were highly correlated within immunized birds ($r = 0.62$, $p < 0.001$ and $n = 28$).

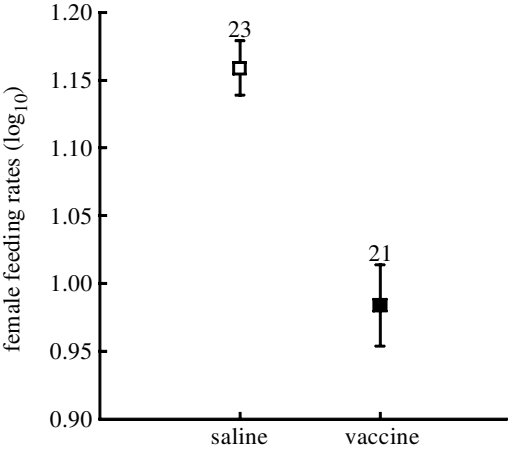

Figure 1. Feeding rates (feedings per hour, $\log_{10}$-transformed least-square means ± s.e.) of female pied flycatchers injected with saline and diphtheria–tetanus vaccine. The figures denote the sample sizes.

(a) *Effects of immunization on female body condition, fat stores and rectrix regrowth*

The treatment, area or their interaction did not have any significant effect on female body condition at the end of the nestling period (ANCOVA, incubation body condition as covariate $F_{1,42} = 5.13$ and $p = 0.03$, treatment $F_{1,42} = 0.18$ and $p = 0.68$, area $F_{1,42} = 0.79$ and $p = 0.38$, and treatment × area $F_{1,42} = 0.13$ and $p = 0.72$) (table 1).

The treatment, area or their interaction did not have any significant effect on female subcutaneous fat at the end of the nestling period (ANCOVA, incubation fat as covariate $F_{1,42} = 13.76$ and $p = 0.001$, treatment $F_{1,42} = 2.16$ and $p = 0.15$, area $F_{1,42} = 0.00$ and $p = 0.94$, and treatment × area $F_{1,42} = 0.01$ and $p = 0.93$) (table 1).

Only 17.0% of females ($n = 47$) started to regrow removed tail feathers during the nestling period (table 1). Control females near the centre had a higher rectrix regrowth probability compared to immunized females breeding in the same area (treatment × area $\chi^2 = 4.95$, d.f. = 1 and $p = 0.03$) (table 1).

(b) *Effects of immunization on parental effort and breeding success*

Immunized females tended to feed their young less intensively than control females (ANCOVA, brood size during recordings as covariate $F_{1,39} = 3.35$ and $p = 0.08$, and treatment $F_{1,39} = 3.77$ and $p = 0.06$) (figure 1), but area ($F_{1,39} = 1.30$ and $p = 0.26$) or treatment × area ($F_{1,39} = 1.33$

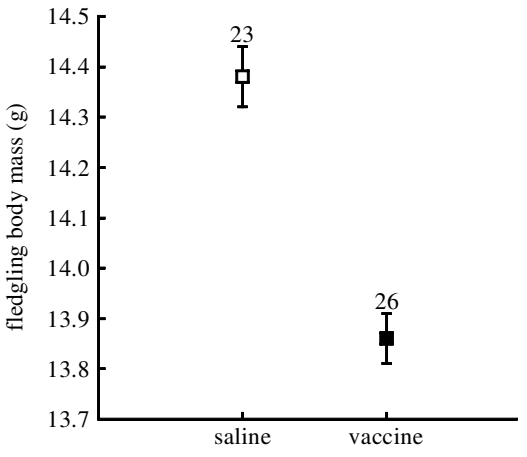

Figure 2. Body mass of 13–14-day-old fledglings (least-squares means ± s.e.) of female pied flycatchers injected with saline and diphtheria–tetanus vaccine. The figures denote the sample sizes.

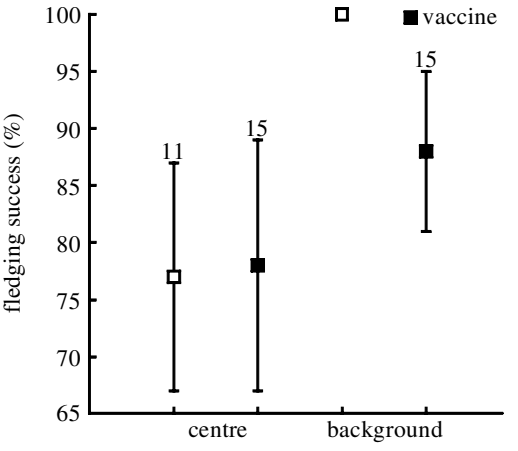

Figure 3. Fledgling success (mean percentage of fledglings/hatchlings ± s.e.) of female pied flycatchers injected with saline and diphtheria–tetanus vaccine. The figures denote the sample sizes.

and $p = 0.26$) were not significant. Male feeding rates were not significantly affected by treatment (ANCOVA, brood size $F_{1,39} = 2.80$ and $p = 0.10$, and treatment $F_{1,39} = 2.62$ and $p = 0.11$) or area ($F_{1,39} = 0.08$ and $p = 0.78$), but males of the vaccinated females in the background area tended to feed their chicks less intensively than males of control females (area × treatment $F_{1,39} = 3.59$ and $p = 0.07$).

Vaccinated females produced fledglings in poor condition (body mass) compared to control females (ANCOVA, laying date as covariate $F_{1,45} = 5.19$ and $p = 0.03$, and treatment $F_{1,45} = 4.10$ and $p = 0.05$) (figure 2), whereas area ($F_{1,45} = 0.03$ and $p = 0.86$) or treatment × area ($F_{1,45} = 0.72$ and $p = 0.40$) had no significant effect on fledgling body mass.

The fledgling success of females (fledglings/hatched chicks) was affected both by treatment, area and their interaction (GENMOD, treatment $\chi^2 = 12.71$, d.f. = 1 and $p = 0.0004$, area $\chi^2 = 25.82$, d.f. = 1 and $p = 0.0001$ and treatment × area $\chi^2 = 11.27$, d.f. = 1 and $p = 0.0008$) (figure 3). Pairwise comparisons within areas revealed that, in the background area, vaccinated females produced significantly less fledglings than control females (treatment $\chi^2 = 4.88$, d.f. = 1 and $p = 0.0001$), whereas in the centre area, treatment had no effect on fledgling success (treatment $\chi^2 = 0.01$, d.f. = 1 and $p = 0.92$).

4. DISCUSSION

We investigated the phenotypic costs of mounting an immune defence by examining trade-offs between immune system activation and reproductive effort. Breeding pied flycatcher females which were challenged with non-pathogenic diphtheria–tetanus vaccine showed reduced feeding rates compared to control birds injected with saline. As a result of the reduced female parental effort, immunized females produced fledglings in poorer condition compared to control females, manifesting the cost. Among the birds breeding in the background area unaffected by deposition from a copper smelter, vaccinated females produced fewer fledglings per hatchling than control females. The lowered reproductive success of vaccinated females in the background area may also have been affected by a reduction in feeding rates by the males as a response to the vaccination of their females. Among the birds breeding in the centre area close to the copper smelter, vaccinated females were less likely to regrow removed tail feathers than control ones, but there was no difference in fledging success between female treatment groups. Although any trade-off between immune defence and reproduction can be expected to be more pronounced under stressful rather than benign conditions (Wiehn & Korpimäki 1998; Ilmonen *et al*. 1999; Wiehn *et al*. 1999), the exceptionally rainy breeding season in 1998 (see § 2) may have made the conditions bad enough for birds breeding in the background area. However, near the smelter the effects of our immune challenge were apparently overridden by stronger effects of heavy metal pollution on nestling mortality.

The most straightforward explanation for the observed reduction in parental effort and rectrix regrowth of vaccinated females is that experimental activation of the humoral immune defence decreased the females' resources available for other functions. As we excluded the direct negative effects of parasitism on host resources by using non-pathogenic antigens, our study shows that activation of the immune defence can be costly *per se*. This is in agreement with the general belief among evolutionary ecologists that immunological defences against pathogens compete for the host's resources (energy and nutrients) needed for other resource-demanding processes, such as reproduction and self-maintenance (Keymer & Read 1991; Sheldon & Verhulst 1996). Recently, there has been some direct and indirect evidence for the physiological costs of immune function (for a review, see Lochmiller & Deerenberg (2000), but also see Klasing (1998)) and it has been shown that increases in a birds' parental effort can result in immunosuppression (Deerenberg *et al*. 1997; Nordling *et al*. 1998; Moreno *et al*. 1999). All this evidence supports the idea that both immune defence and reproductive effort may not be maximized simultaneously within an individual.

However, the strongest evidence for energetic costs of immune defence so far comes from the biomedical literature and is mainly based on studies of human or

laboratory mammal models (see the review in Lochmiller & Deerenberg (2000)) and, thus, may not be extended to field situations on wild birds. In the only study where energetic costs of mounting an immune response has been measured in wild birds, Svensson *et al*. (1998) found no increase in the basal metabolic rates (BMR) of blue tits (*Parus caeruleus*) immunized with diphtheria–tetanus vaccine as compared with saline controls. This and other results have made several authors suggest that mechanisms other than energy limitation should be examined as possible explanations for the trade-off between immune defence and reproduction effort (Klasing 1998; Råberg *et al*. 1998; Svensson *et al*. 1998; Von Schantz *et al*. 1999). Alternative or additive to the energetic cost scenario is the fact that immunized pied flycatcher females reduced their reproductive effort in order to avoid oxidative stress caused by deleterious free oxygen radicals (see Von Schantz *et al*. 1999) or the risk of immunopathology (Råberg *et al*. 1998; Svensson *et al*. 1998). Hence, by reducing parental workload individuals could avoid immunopathological effects when mounting an immune response against the vaccine or during an infection they could decrease their net production of free radicals (see Coyle & Puttfarken 1993; Liebler 1993; Packer *et al*. 1994) and, thus, keep oxidative stress within critical limits for the body to function without disorders.

Whatever the physiological mechanisms for the observed reduction in parental effort of immunized female pied flycatchers, hosts are expected to alter their current reproductive effort adaptively in order to minimize the negative effects of parasitism on their lifetime reproductive success (Forbes 1993). If so, the benefits in terms of lifetime reproductive success have to offset the short-term costs. On the one hand, immunized females paid the cost of mounting an immune response by producing fewer offspring of poorer quality. However, the benefits of reduced parental workload may, during a real pathogen challenge, accrue if it allows an increased parasite resistance. The contracted infection will be kept under control, allowing parent birds to produce at least a few offspring during the current reproductive event and at the same time increase the prospects for survival and successful future reproduction. Furthermore, due to the development of long-term immunological memory after initial exposure, subsequent re-exposure to the same pathogen results in enhanced efficiency in defence response (e.g. Wakelin & Apanius 1997), most likely with positive effects on hosts' lifetime expectations.

Our results suggest that birds, as a response to exposure to parasitism, can reduce their investment in self-maintenance and parental effort, in the latter case even at the expense of current reproductive success. More specifically, we were able to show that an activated immune system *per se* can lead to reduced investment in parental effort and other somatic functions and, hence, the phenotypic costs of immune defence. No doubt, future studies will show the proximate mechanisms behind the observed trade-offs and the possible genotypic basis for the costs of immune defence.

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