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# Pollution related effects on immune function and stress in a free-living population of pied flycatcher *Ficedula hypoleuca*

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We investigated whether exposure to heavy metal pollution affected the immune function of individuals in a free living population of a small insectivorous passerine bird, the pied flycatcher *Ficedula hypoleuca*. We measured humoral immune responses in two study areas: a polluted area in the vicinity of a copper smelter and a control area far from the smelter. Plasma corticosterone level and blood heterophil/lymphocyte ratio (H/L) were used as more general physiological measures of stress. The immune response of *F. hypoleuca* was not suppressed by pollution stress. In contrast, we found that *F. hypoleuca* males showed stronger humoral immune responses to a novel antigen (tetanus toxoid) in the polluted environment than in the unpolluted one. After the immunization of males, numbers of lymphocytes rose significantly more in the polluted area, leading to a smaller H/L ratio than in males from the control area. Females showed no pollution related effects on their immune responses. Corticosterone levels of males and nestlings were not related to pollution levels. Nestlings showed somewhat higher H/L ratios and lower fledging success in the polluted area, both factors indicating increased stress levels in a polluted area. Our results suggest that humoral immune response of male *F. hypoleuca* may be enhanced under moderate levels of heavy metal pollution. Enhanced immune function may, however, also be costly for birds and the higher humoral immune responses in polluted areas may thus have negative effects on the birds' breeding performance and survival.

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Various chemicals can affect the functional activities of the vertebrate immune system (Wong et al. 1992, Trust et al. 1994, Krzystyniak et al. 1995). Many environmental contaminants, like heavy metals and organochlorine insecticides are known to change immune functions (Melancon 2003). For example, some heavy metals may have detrimental effects on the immune system at doses where other toxicological changes are not evident (Wong et al. 1992, Liu et al. 1999). Moreover, chemically induced immunosuppression may increase the susceptibility of an individual to viral, bacterial and parasitic infections (e.g. Morahan et al. 1984). Environmental pollution could thus have harmful effects on animals even at relatively low doses in the target population (e.g. Smits et al. 1999).

Information on pollution induced changes in immune functions mainly derives from laboratory studies, whereas relatively little is known about its significance in free-living animal populations. In addition to possible toxic effects, wild bird populations are susceptible to various indirect effects of pollution, such as reduced quality or quantity of food, or habitat changes (Eeva et al. 1997). The complex combination of direct and indirect effects of air pollution can only be measured in free-living populations. Therefore, we compared the immune function between two populations of a small insectivorous passerine bird, the pied flycatcher *Ficedula hypoleuca*: one population inhabiting an area polluted with heavy metals (near a copper smelter) and another population living in an unpolluted area.

To evaluate the effects of pollution on humoral immune responses of *F. hypoleuca* males and females, we measured their primary antibody response to an immunization with human diphtheria-tetanus vaccine (Ilmonen et al. 2000). To control for the possible effects of immunization procedure itself on stress and breeding performance, an additional group of females was given saline. We also recorded birds' stress hormone (corticosterone) levels and leucocyte profiles to estimate their physiological stress. Various stress situations (e.g. food deprivation and environmental pollutants) are known to elevate plasma glucocorticoid levels (Assenmacher 1973, Brown 1993, Kishikawa et al. 1997). Because corticosterone levels may show short-term variation due to acute stressors, we used heterophil/lymphocyte (H/L) ratio as an indicator of longer-term physiological stress. High values of H/L ratio are found to indicate stress in birds (Gross and Siegel 1983, Maxwell 1993, Hõrak et al. 2002).

## Methods

### Study area and experimental design

The study was conducted in the surroundings of the town Harjavalta (61°20′N, 22°10′E), SW Finland in 1998 and 1999. The main source of local air pollutants is a factory complex producing copper, nickel and fertilizers in the centre of the town. Sulphuric oxides and heavy metals (especially Cu, Zn, Ni, Pb and As) are common pollutants in the area (Kubin 1990, Jussila and Jormalainen 1991). Thirteen study sites, each with 40–50 nest-boxes, were established along the air pollution gradient in three main directions (SW, SE and NW) away from the copper smelter complex. Special attention was paid in selecting study plots so that they would represent a similar forest type, i.e. relatively barren pine dominated forests typical of the study area. Elevated heavy metal concentrations occur in soil, vegetation, insects and birds in the polluted area due to current and historical deposition, and metal contents decrease exponentially with increasing distance from the smelter (e.g. Jussila and Jormalainen 1991, Koricheva and Haukioja 1995, Eeva and Lehikoinen 1996, Derome and Nieminen 1998). For example, increased concentrations of Pb have been measured in faeces and bone tissue of *F. hypoleuca* nestlings (Eeva and Lehikoinen 1996, 2000). *F. hypoleuca* breeding in the vicinity ($<2$ km) of the smelter exhibit low breeding success (Eeva et al. 1997), and they have reduced survival rates (Eeva and Lehikoinen 1998) compared to birds breeding in the non-polluted area. The five sites within 2 km of the copper smelter were thus considered polluted (hereafter 'polluted area'), and eight sites more than 2 km from the smelter were considered to be unpolluted (hereafter 'control area').

The experimental nests were chosen so that treatments (pollution vs. control) were randomly assigned to parents with the same laying date and clutch-size. To ensure that the quality of birds and situation at the start of the experiments would be as similar as possible in all groups, we selected nests (n = 68 in 1998, n = 35 in 1999) with similar clutch-sizes (5–7 in 1998 and 6–7 in 1999) for our experiment. Nests were then visited to determine the hatching date, and number of hatched and fledged young. Because it was not always possible to measure all parameters in every nest and some nests were depredated during the experiment (7 in 1998, 1 in 1999) sample sizes vary among the experimental groups and analyses. At the ages of 7–8 days (in 1998) and 11–12 days (in 1999), the nestlings were weighed to the nearest 0.1 g with a spring balance.

### Immunization, saline treatment and blood sampling

We performed three separate experiments: 1. female immunization, 2. male immunization, and 3. female saline injection. Females (1998, $n = 15_{pollution} + 15_{control}$) were captured and immunized 5 days before the hatching day and males (1999, $n = 17_{pollution} + 18_{control}$) were immunized when their chicks were 2 days old. The birds were immunized by intramuscular injection of 100:l of diphtheria-tetanus vaccine (Finnish National Public Health Institute; diphtheria 38 Lf and tetanus 10 Lf, mixed with the adjuvant aluminiumphosphate 1.0 mg/ml) in the pectoral muscle. To control for the possible effects of immunization procedure itself we injected females (1998, $n = 15_{pollution} + 15_{control}$) with similar laying dates as in immunized birds with 100:l of saline.

Blood samples were taken prior to injection (120–150 μl in heparinized capillary tubes by brachial venipuncture). Females were sampled again 12–13 days after the injection (when chicks were ca. 7 days old) and males 9–10 days after the injection (when chicks were ca. 11 days old), for measuring the activation of their humoral immune system. Blood was transferred into Eppendorf tubes containing 3 μl heparin. Tubes were immediately stored in an ice-box and centrifuged at 3000 rpm for 8 min within 3 hours of sampling. The plasma of males was further divided into separate samples for antibody and corticosterone analyses. Blood from females was not analysed for corticosterone. Males were sampled $10.2 \pm 0.97$ minutes after capture and sampling time did not differ among the experimental groups (ANOVA, $F_{1,24}=0.35$, $P=0.56$). Plasma was extracted and stored at $-20°C$ until used for ELISA-analysis (females) or at $-195°C$ until used for ELISA and corticosterone analysis (males). For both sexes, a drop of blood was further used to make smear samples for leukocyte profiles.

When nestlings were 11–12 days old (in 1999) we chose randomly two nestlings from each experimental nest ($n=34$). Blood samples (80–100 µl by brachial venipuncture) were taken from two nestlings for assessment of leukocyte profiles and circulating corticosterone levels. The first handled nestling was bled on average $3.6 \pm 0.28$ min and the second nestling $6.0 \pm 0.38$ min after removal from the nest. Blood sampling times did not differ among the experimental groups (ANOVA; $F_{1,33}=3.2$, $P=0.09$ and $F_{1,33}=1.69$, $P=0.20$, respectively). A drop of blood was used to make smear samples for leukocyte profiles.

## ELISA assay

We measured humoral immune response as the antigen-specific antibody levels in plasma using an enzyme-linked immunosorbent assay (ELISA) previously developed for red-winged blackbirds (for details of methods, see Hasselquist et al. 1999, 2001, Ilmonen et al. 2000). This assay has proved to work well also for all other passerines in which it has been tried (e.g. blue tits *Parus caeruleus*, Råberg et al. 2003; red-winged blackbirds *Agelaius phoeniceus*, Westneat et al. 2003; house sparrows *Passer domesticus*, starlings *Sturnus vulgaris*, Hasselquist unpubl. data). This ELISA method provides a sensitive measure of the amount of passerine antibodies (in millioptical densities [mOD]/min) that specifically bind to a certain antigen (here diphtheria or tetanus toxoid). The interassay variation was 10.7%. As a measure of antibody responses to tetanus toxoid and diphtheria toxoid we used the difference between pre- and post-immunization antibody titres. The mean pre-immunization titres in the experimental groups were as follows: female immunization, $n=28$, $\bar{x}_{tetanus}=2.64 \pm 0.21$, $\bar{x}_{diphtheria}=1.14 \pm 0.21$; male immunization: $n=25$, $\bar{x}_{tetanus}=1.35 \pm 0.18$, $\bar{x}_{diphtheria}=1.16 \pm 0.13$; female saline injection: $n=18$, $\bar{x}_{tetanus}=2.17 \pm 0.39$, $\bar{x}_{diphtheria}=0.73 \pm 0.39$.

## Measurement of leukocyte profiles

Smear samples were air-dried, fixed in absolute methanol and stained with azure-eosin. The proportion of different types of leukocytes was assessed by examining a total of 100 leukocytes under $\times 1000$ magnification. Total white blood cell counts (WBC) were estimated by counting the number of leukocytes per approximately 10 000 erythrocytes (see Ots et al. 1998 for the details of the method). Differential leucocyte counts for heterophils and lymphocytes were obtained by multiplying their proportions with WBC. Heterophil/lymphocyte ratio (H/L) was used as a measure of longer-term physiological stress (i.e. lasting from several days to weeks) levels of males and nestlings. Hõrak et al. (2002) found a significant individual consistency in H/L ratio within 4-month period. Leukocyte counts obtained by using the same method are also found to be highly repeatable (Ots et al. 1998). The investigator examining blood samples was unaware of the phenotypic values and treatments of the birds.

## Measurement of corticosterone levels

The male and nestling plasma levels of corticosterone were measured using a radioimmunoassay (RIA) kit (Biotrak rat corticosterone [$^{125}I$], Amersham, England). Since corticosterone levels tended to increase with increasing handling time (see also Silverin and Wingfield 1998) all values were corrected to correspond to the value of an average handling time (males: $n=24$, $\bar{x}=9.6 \pm 0.79$ min; nestlings: $n=68$, $\bar{x}=4.1 \pm 0.28$ min) by adding residuals from linear regression to the predicted values for average handling times.

## Statistics

Differences in humoral immune responses, H/L ratios and corticosterone levels between study areas were compared with one-way ANOVAs. Because nestlings were slightly heavier in a polluted area in 1999, we controlled for the effect of body mass on H/L ratio by using mean body mass as covariate. Fledging success (a probability of a hatchling to fledge) was analysed by using generalized linear models (GENMOD procedure of SAS, type 3 analysis with binomial probability distribution and logit link function). As a binomial response variable we used the proportion: fledglings/hatchlings. The area (polluted vs. unpolluted) was used as the explanatory variable. Depredated and destroyed nests were omitted from the analyses of fledging success. Normality of residuals was tested for each parametric test with Kolmogorov-Smirnov test (UNIVARIATE procedure in SAS).

# Results

Female humoral immune responsiveness was not significantly affected by pollution stress (Table 1). Instead, the mean male humoral immune responsiveness against tetanus toxoid was 3.5 times higher in the polluted than in the control area (Fig. 1), whereas the difference was not significant for diphtheria (Table 1). Note, however,

Table 1. Humoral immune response of *F. hypoleuca* females and males (primary antibody titres, mOD/min) for tetanus and diphtheria in polluted and control areas. ANOVAs for differences between areas.'I' denotes birds immunized with diphteria-tetanus vaccine and 'S' denotes control group with saline injection.

| | Polluted area | | | Control area | | | | |
|---|---|---|---|---|---|---|---|---|
| | n | x̄ | SE | n | x̄ | SE | F | P |
| Females 1998[1] | | | | | | | | |
| Anti-tetanus, I | 13 | 34.8 | 6.40 | 15 | 27.2 | 5.70 | 0.69 | 0.41 |
| Anti-diphtheria, I | 13 | 15.0 | 4.25 | 15 | 8.6 | 2.52 | 1.0 | 0.33 |
| Anti-tetanus, S | 9 | 0.45 | 0.21 | 9 | 0.09 | 0.22 | 0.02 | 0.89 |
| Anti-diphtheria, S | 9 | 0.26 | 0.13 | 9 | 0.16 | 0.07 | 0.22 | 0.64 |
| Males 1999[1] | | | | | | | | |
| Anti-tetanus, I | 12 | 20.6 | 6.66 | 13 | 5.9 | 1.83 | 5.99 | 0.022 |
| Anti-diphtheria, I | 12 | 5.7 | 3.20 | 13 | 1.3 | 0.64 | 1.4 | 0.25 |

[1]Values were log10-transformed before analyses to conform more to a normal distribution.

that the means were higher in the polluted area for all measures of humoral immune responses but since variation was relatively high in our samples the power of these analyses is low. Immunized females showed a clear increase in their diphtheria and tetanus antibody levels, whereas among the saline-injected control females antibody levels remained at low, close to initial levels (ANOVA, treatment vs. control; tetanus: $F_{1,44}=134.7$, $P<0.0001$; diphtheria: $F_{1,44}=57.5$, $P<0.0001$; Table 1). The number of nestlings at the time of blood sampling did not correlate with antibody levels either in males (tetanus: $n=25$, $r=0.069$, $P=0.74$; diphtheria: $n=25$, $r=-0.096$, $P=0.65$) or in females (tetanus: $n=28$, $r=0.14$, $P=0.49$; diphtheria: $n=28$, $r=0.083$, $P=0.67$).

H/L ratios of nestlings were higher in the polluted area than in the unpolluted area (Table 2). In both areas their H/L ratio was significantly and negatively related to the mean body mass (Table 2). No difference was found in female H/L ratios between polluted and unpolluted areas whereas immunized males showed smaller H/L ratio in the polluted area (Table 2). In both sexes, H/L ratios increased significantly after the immunization

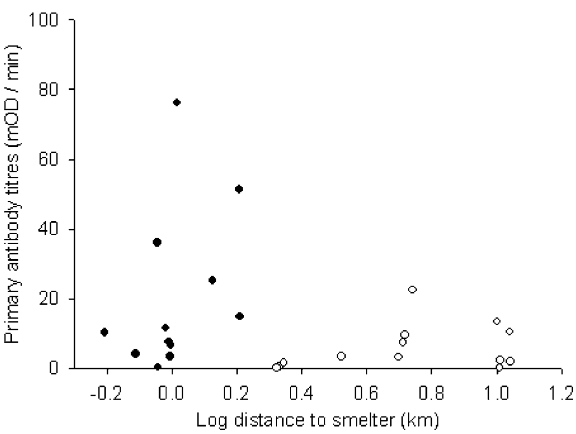


Fig. 1. Humoral immune response (primary antibody titres, mOD/min) of *F. hypoleuca* males against tetanus toxoid along the pollution gradient (experimental groups: ● = polluted area, ○ = unpolluted area).

(female: $F_{1,47}=6.87$, $P=0.012$; male: $F_{1,37}=22.8$, $P<0.0001$), but not after injection with saline (female: $F_{1,50}=1.02$, $P=0.32$). The increase in H/L ratio of males in the polluted area was smaller than that of males in the control area (area × time interaction: $F_{1,37}=6.45$, $P=0.016$), because the increase in heterophil count was similar in both groups ($F_{1,19}=0.13$, $P=0.72$) but lymphocyte numbers increased more in birds from polluted than from control area ($F_{1,19}=6.26$, $P=0.022$). No significant pollution related temporal change was observed in females (area × time interaction: $F_{1,47}=3.07$, $P=0.087$).

Neither males nor nestlings showed any differences in their corticosterone levels between the two areas (Table 2). The two measures of stress, corticosterone level and H/L ratio, were not significantly correlated (males: $n=21$, $r=-0.22$, $P=0.35$; nestlings: $n=27$, $r=-0.059$, $P=0.77$).

Five days before hatch, females were, on average, 0.7 g heavier in the polluted than in control area, but there were no significant differences between areas in female body mass during the nestling period (Table 3). At the age of 11 days, nestlings were 0.7 g heavier in the polluted area in 1999, but not in 1998 (Table 3). Furthermore, nestlings were 0.5 g heavier in saline-treated nests than in those nests where females were immunized with diphteria-tetanus vaccine (ANCOVA, laying date as covariate: treatment $F_{1,44}=4.62$, $P=0.037$). There were no differences in male body mass between the polluted and control area (Table 3).

In both years, fledging success (a probability of a hatchling to fledge) in untreated nests was lower in the polluted than in unpolluted area (Table 4). This was also the case for the saline and immunization treatments, although in 1999 the difference was only marginally significant (Table 4). There was a strong negative association between H/L ratio of nestlings and fledging success ($n=24$, $\chi^2=10.5$, $P=0.001$), i.e. nestlings in the unsuccessful nests had higher H/L ratios than those in successful nests. In contrast, no association was found between mean corticosterone levels of nestlings

Table 2. Blood heterophil/lymphocyte ratios (H/L) and serum corticosterone levels (ng/ml) of *F. hypoleuca* in polluted and control areas. Time of sampling is shown in parenthesis as days in relation to the hatching date. ANOVAs for differences between areas. The days of sampling in relation to hatching dates are given in brackets. 'O' denotes situation before immunization, 'I' denotes situation after injection of females (1998) or males (1999) with diphteria-tetanus vaccine and 'S' denotes situation after saline injection.

| | Polluted area | | | Control area | | | | |
|---|---|---|---|---|---|---|---|---|
| | n | $\bar{x}$ | SE | n | $\bar{x}$ | SE | F | P |
| H/L ratio[1] | | | | | | | | |
| 1998 | | | | | | | | |
| Females (−5 days), O | 24 | 0.13 | 0.011 | 28 | 0.14 | 0.014 | 0.49 | 0.49 |
| Females (7 days), I | 13 | 0.22 | 0.019 | 13 | 0.19 | 0.036 | 1.14 | 0.30 |
| Females (7 days), S | 13 | 0.14 | 0.025 | 14 | 0.15 | 0.029 | 0.09 | 0.76 |
| 1999 | | | | | | | | |
| Males (2 days), O | 10 | 0.16 | 0.020 | 9 | 0.12 | 0.022 | 2.39 | 0.14 |
| Males (11 days), I | 11 | 0.23 | 0.021 | 11 | 0.34 | 0.050 | 4.41 | 0.049 |
| Nestlings (11 days), I | 15 | 0.19 | 0.018 | 12 | 0.16 | 0.010 | 9.02 | <0.01 |
| Corticosterone[2] | | | | | | | | |
| Males (11 days), I | 11 | 24.7 | 2.28 | 12 | 20.1 | 2.00 | 2.23 | 0.15 |
| Nestlings (11 days), I | 17 | 16.1 | 1.77 | 17 | 17.7 | 1.82 | 0.41 | 0.53 |

[1]An arcsin square root -transformation was performed for all H/L values before ANOVA to normalize distributions. For nestlings, mean body mass was used as a covariate ($F_{1,24}$ = 12.0, P = 0.0020).
[2]Values were corrected for the effect of variation in blood sampling time by linear regressions.

(two per nest were sampled) and the fledging success of the brood (n = 33, $\chi^2$ = 0.95, P = 0.33).

## Discussion

Our data suggest that humoral immune response of *F. hypoleuca* was not suppressed by pollution stress in our study area. In contrast, *F. hypoleuca* males showed stronger humoral immune responses to a novel antigen in the polluted environment when compared to the unpolluted one. Moreover, this was also reflected in a greater increase in male lymphocyte numbers (and smaller H/L ratio) after immunization in the polluted area, which is in agreement with general immunological principles, as many specific immune responses are primarily based on lymphocyte activation and proliferation. Because of the relationship between immune response and lymphocyte numbers in the immunized males, it is difficult to estimate how the H/L ratio reflects stress after immunization.

There were no significant differences in stress hormone (corticosterone) levels of *F. hypoleuca* males or nestlings between polluted and unpolluted sites. Similarly, another study in the same area showed that corticosterone levels of great tit *Parus major* females and nestlings were not increased in the polluted environment (Eeva et al. 2003). In agreement with our results, Wayland et al. (2003) found no relationship between corticosterone levels and Hg or Cd levels in common eider *Somateria mollissima* females, even though their body mass was affected. It is possible that long-term stress results in the adjustment of the corticosterone production pathway such that any possible initial differences disappear. Thus, either corticosterone cannot be used to assess stress caused by long-term metal

Table 3. The mean body mass (g) of birds in the experimental groups. ANOVA for the differences between areas. The days of sampling in relation to hatching dates are given in brackets. 'O' denotes situation before immunization, 'I' denotes situation after injection of females (1998) or males (1999) with diphteria-tetanus vaccine and 'S' denotes situation after saline injection.

| | Polluted area | | | Control area | | | | |
|---|---|---|---|---|---|---|---|---|
| | n | $\bar{x}$ | SE | n | $\bar{x}$ | SE | F | P |
| 1998 | | | | | | | | |
| Females (−5 days), O | 26 | 15.3 | 1.23 | 29 | 14.6 | 1.75 | 9.26 | <0.01 |
| Females (7 days), I | 13 | 12.7 | 2.12 | 15 | 12.8 | 2.20 | 0.23 | 0.63 |
| Females (7 days), S | 10 | 12.8 | 1.89 | 14 | 12.9 | 1.30 | 0.31 | 0.58 |
| Males (7 days), I | 13 | 12.4 | 1.35 | 13 | 12.4 | 1.99 | 0.04 | 0.85 |
| Nestlings (11 days), I[1] | 12 | 14.0 | 0.30 | 14 | 13.7 | 0.27 | 0.52 | 0.48 |
| Nestlings (11 days), S[1] | 10 | 14.3 | 0.23 | 13 | 14.5 | 0.19 | 0.61 | 0.44 |
| 1999 | | | | | | | | |
| Males (2 days), O | 15 | 12.5 | 0.18 | 15 | 12.5 | 0.14 | 0.04 | 0.84 |
| Males (11 days), I | 12 | 12.7 | 0.25 | 13 | 12.4 | 0.12 | 1.28 | 0.27 |
| Nestlings (11 days), I[1] | 17 | 14.0 | 0.26 | 17 | 13.3 | 0.22 | 4.32 | 0.046 |

[1]Calculated from brood means.

Table 4. The probabilities (95% confidence intervals) of *F. hypoleuca* hatchlings to fledge in untreated nests and the experimental groups at the polluted and unpolluted area. Generalized linear model[1] results for the differences between the polluted and unpolluted area.

| | Polluted area | | | Control area | | | | |
|---|---|---|---|---|---|---|---|---|
| Experimental group[2] | n | Prob. | C.I. | n | Prob. | C.I. | $\chi^2$ | P |
| Untreated nests 1998 | 32 | 0.87 | 0.81–0.92 | 24 | 0.95 | 0.89–0.97 | 4.7 | 0.030 |
| Immunization 1998 | 11 | 0.76 | 0.65–0.86 | 15 | 0.96 | 0.88–0.99 | 11.0 | <0.001 |
| Saline injection 1998 | 12 | 0.79 | 0.68–0.87 | 15 | 0.98 | 0.91–0.99 | 13.8 | <0.001 |
| Untreated nests 1999 | 44 | 0.91 | 0.87–0.94 | 51 | 0.97 | 0.94–0.98 | 6.6 | 0.010 |
| Immunization 1999 | 17 | 0.91 | 0.84–0.95 | 16 | 0.97 | 0.91–0.99 | 3.7 | 0.053 |

[1]GENMOD, type 3 analysis with bionmial probability distribution and logit link function.
[2]Nests with clutch sizes of 5–7 were included in 1998 and clutch sizes of 6–7 were included in 1999.

exposure or the exposure has been too low to cause hormonal stress responses. On the contrary, the H/L ratio, a more general measure of physiological stress was significantly higher in nestlings from the polluted area. In agreement with earlier studies (Gross and Siegel 1983, Maxwell 1993, Hõrak et al. 1998, Moreno et al. 2002) H/L ratio was increased in lighter nestlings and in broods with low fledging success. An earlier study in the same study area showed lowered invertebrate food availability at polluted sites (Eeva et al. 1997) and it is possible that differences in H/L ratio are caused by differences in food abundance or food quality. Alternatively, activated detoxication of nonessential metals might lead to increased stress response. Blanco et al. (2004) found that H/L ratio increased in Cd-exposed black kite *Milvus migrans* nestlings. They suggested that participation of metallothioneins in detoxification and metal regulation may have indirectly enhanced the stress response to contaminants. In our study area the activity of one of the detoxication enzymes, ethoxyresorufin-O-deethylase (EROD) has been found to be increased in *F. hypoleuca* nestlings (Eeva et al. 2000).

Why did *F. hypoleuca* males living in a polluted area show a stronger humoral immune response, although heavy metals are generally considered to be immunosuppressive (Wong et al. 1992, Bernier and Brousseau 1995)? It is known that immune responsiveness can be traded off against brood-rearing effort (e.g. Deerenberg et al. 1997, Cichon et al. 2001, Ardia et al. 2003, Lozano and Lank 2003, Pap and Márkus 2003, Soler et al. 2003). One possibility is that males in the polluted area put less effort in brood-rearing than their counterparts in the unpolluted area, thus allowing them to invest more in immune defence. However, this hypothesis is not supported by our data because brood size did not correlate with male antibody levels and in 1999 nestlings actually were slightly heavier in the polluted area. Furthermore, male feeding rates seem not to differ between the study areas (T. Eeva unpubl. data from years 2000 and 2002). Alternatively, moderate heavy metal exposure may have increased antibody levels in males. In Pb-treated mice, low doses enhanced the immune response, medium doses did not have any effect and high doses resulted in immunosuppression (Lawrence 1981). Also in Japanese quail *Coturnix coturnix* Pb was observed to suppress antibody-mediated immunity only at dosages that also caused clinical Pb poisoning (Grasman and Scanlon 1995). Since Pb is one of the main pollutants in the study area, Pb concentrations were measured in 1996 from the femurs of *F. hypoleuca* nestlings (Eeva et al. 2000). The average Pb concentrations were 7.3 μg/g, d.w. (moderate) and 0.6:g/g, d.w. (low) in nestlings from the polluted and unpolluted areas, respectively. Enhanced immune response of *F. hypoleuca* males might thus be explained by moderate levels of exposure in our study. This explanation is also supported by the observation that males breeding very close ( <1 km) to the smelter, and being most exposed to heavy metals, did not seem to show higher responses than those breeding slightly farther (1–2 km) away (Fig. 1).

Female responses to a novel antigen did not differ significantly between the polluted and the unpolluted area, although the means were generally higher in the polluted area. Since the variation in our data was high making the power of tests low we cannot finally rule out the possibility of pollution related effects also in females. On the other hand, gender differences in immunotoxicity of heavy metals and PCBs have been found in some studies. After early exposure to lead, male chickens produced more antibodies while such exposure did not markedly alter female antibody levels (Bunn et al. 2000). PCB-exposed female American kestrels *Falco sparverius* showed increased antibody response while exposed males showed decreased production (Smits and Bortolotti 2001). It should also be recalled that our data sets for females and males were collected in different years. So, it is possible that different conditions (e.g. in weather or food availability) during breeding in two years would produce different natural stress levels and different outcomes in responses.

Clearly, the immunization itself (i.e. the effect of antigens, adjuvant or both) was stressful for females, since their H/L ratio increased by about 47% after the treatment whereas no change was observed in saline-treated birds. Furthermore, nestlings in saline-treated nests were heavier than those in nests where females were immunized. This is in agreement with previous studies on *F. hypoleuca* (Ilmonen et al. 2000) and blue tits (Råberg et al. 2000), where females injected with diphtheria-tetanus vaccine decreased their feeding rates and had nestlings of lower body mass than control females injected with saline.

Our study shows that *F. hypoleuca* males living in a polluted area have activated, rather than suppressed, humoral immune response. High immune responsiveness may, however, be costly for birds in the long run. Keeping up enhanced immune response may cause trade-offs in some other life-history traits (Lochmiller and Deerenberg 2000, Ilmonen 2001). It is possible that the higher humoral immune response in some males in the polluted areas reflects a hyper-activated immune system (Råberg et al. 1998), which, in turn, may affect negatively on birds' breeding performance (Ilmonen et al. 2000) or survival (Hanssen et al. 2004). Furthermore, activated immune responsiveness may potentially increase the susceptibility of birds to other environmental stressors. The relationships between heavy metal detoxification and immune response clearly call for further studies.

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