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Stress protein levels and blood parasite infection in blue tits (*Parus caeruleus*): a medication field experiment

Gustavo Tomás[1]*, Santiago Merino[1], Javier Martínez[1,2], Juan Moreno[1] & Juan J. Sanz[1]

[1)] *Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), C/ José Gutiérrez Abascal 2, ES-28006 Madrid, Spain (*e-mail: gtomas@mncn.csic.es)*
[2)] *Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Alcalá, ES-28871 Alcalá de Henares, Spain*

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It has been suggested that blood parasites are a source of physiological stress for avian hosts in the wild. We report the first experimental evidence relating blood parasite infection to the physiological stress response in a wild avian population. We reduced through medication the intensity of infection by *Haemoproteus majoris* and the prevalence of infection by *Leucocytozoon majoris* in half of a sample of female blue tits (*Parus caeruleus*). Results show that (i) control females had a higher final level of the stress protein HSP60 than medicated ones, (ii) the initial immunoglobulin level was negatively correlated with final HSP60 level, (iii) control females had a lower final body mass than medicated ones, body mass being negatively correlated with the number of blowfly pupae in the nest only for control females, and (iv) final female body mass was positively correlated with nestling tarsus length only for control females, which produced nestlings with shorter tarsi than medicated females. Responses of HSPs to parasitism, and associations with aspects of immune function and condition, suggest that the stress response may allow blue tits to maintain blood parasites under control during reproductive stress.

Introduction

Effects of parasites on their hosts have been frequently reported and a vast literature exists on host–parasite interactions in birds (Loye & Zuk 1991, Clayton & Moore 1997). However, it is still not well established what kind of costs and trade-offs are induced in hosts which maintain chronic infections. In any case, parasites impose undoubtedly a drain of resources upon their hosts, thereby forcing them to evolve abilities to reduce these costs by mounting effective immune and/or stress responses. Blood parasites could cause physiological stress to the host by means of cell destruction and haemoglobin consumption (Kirkpatrick & Suthers 1988, Atkinson & Van Riper 1991). More recently, it has been suggested that parasites may induce heat shock protein expression in the host (Merino *et al.* 1998, 2002).

Heat shock proteins (HSPs) represent one of the main molecular barriers against cellular homeostasis alteration, and are involved in many different functions like protein synthesis, folding and transport, and degradation of misfolded, non-functional proteins (Morimoto 1991). Ecological and evolutionary concerns about the HSP-mediated stress response have received attention only in recent years (Krebs & Loeschcke 1994a, Merino *et al*. 1998, Feder 1999, Feder & Hofmann 1999, Buchanan 2000, Sørensen *et al*. 2003). In addition, measures of the stress response, while relatively routine in areas such as poultry or veterinary sciences, have not been normally employed in field studies on wild species (Feder 1996). The need to sacrifice animals to perform protein analyses (e.g. Eeva *et al*. 2000, Zatsepina *et al*. 2000) may be one cause for low interest in the role played by the so-called stress or heat shock proteins. Both ethical and research reasons in field studies make it desirable to avoid animal sacrifice if at all possible (ASAB Ethical committee & ABS Animal Care committee 2003). Therefore, the non-destructive assessment of the HSP-mediated stress response from blood cells has been shown to be an accurate and reliable method for evaluating physiological or environmental stress of birds *in vivo* (Merino *et al*. 1998, Moreno *et al*. 2002, Tomás *et al*. 2004). It has been found that HSPs respond to a wide array of stressors, including heat (Gehring & Wehner 1995), toxins (Mariño *et al*. 1999), oxidant compounds (Martínez *et al*. 1999a), cold (Martínez *et al*. 2001) and parasites (Martínez *et al*. 1999b). Furthermore, each one of the several types of existing HSPs could respond in a different way to each particular stressor, including absence of response (Garbe 1992). For example, Merino *et al*. (2002) found a parasite-induced HSP60 response, but no effect was detected on HSP70 levels.

Evolutionary ecologists started to pay more attention to immunology since the idea became widespread that parasitism may drive selection on most aspects of animal ecology (Folstad & Karter 1992, Sheldon & Verhulst 1996, Zuk *et al*. 1996, Schmid-Hempel 2003, Schmid-Hempel & Ebert 2003). Given the great ubiquity and diversity of parasites, a similar diversity of anti-parasite defences should have evolved in hosts. Such defences may comprise behavioural, physiological and immunological mechanisms. Immunological responses against parasite infection, changes in white blood cell profiles (e.g. Graczyk *et al*. 1994, Massey *et al*. 1996, Ots & Horak 1998), total serum proteins (e.g. de Lope *et al*. 1998, Ots & Horak 1998), and antibody titres in serum (e.g. Isobe & Suzuki 1987, Ots & Horak 1998, Atkinson *et al*. 2001a) have been emphasised in studies of diverse host–parasite systems. Measures of humoral immune response are becoming widely used in many ecological studies dealing with host–parasite interactions (Norris & Evans 2000), and the development of immunological procedures to evaluate immune responses against specific antigens (Isobe & Suzuki 1987, Graczyk *et al*. 1993) have been crucial in the approach called ecological immunology. Immunoglobulins play an important role in the specific humoral immune response of vertebrates, being responsible for antigen recognition (Roitt *et al*. 1996). Isobe and Suzuki (1987) documented for the first time an IgM and IgG antibody response in chickens experimentally infected with *Leucocytozoon caulleryi*. Specific antibody responses against malarial infections were also reported by Atkinson *et al*. (2001a, 2001b). Thus, haemoparasite infection may exert a cost on hosts by increasing their immune response. To date, few studies have explored relationships between parasite infections and immunoglobulin levels in wild birds, and to our knowledge, no antibody-mediated response in avian hosts infected with *Haemoproteus* or *Leucocytozoon* species in the wild has been reported. Recent data indicate that certain HSPs play key roles in the modulation of the immune system (Feder & Hofmann 1999). The evaluation of humoral immunity in conjunction with the stress response has the potential to shed light on the physiological mechanisms underlying host defences against parasite infections.

Our aim in this study was to determine experimentally whether there exists a relationship between blood parasitism and HSP60, HSP70 and immunoglobulin levels in host peripheral blood, using as a model system a wild population of blue tits (*Parus caeruleus* L.) infected by several blood parasite species. We medicated half of a sample of females with Primaquine, an antima-

larial drug, and derived three predictions regarding effects of the experimental manipulation:

1. Medication with Primaquine should reduce the intensity of infection by *Haemoproteus majoris* and the prevalence of infection by *Leucocytozoon majoris* (*see* Merino *et al*. 2000).
2. The HSP-mediated stress response against parasites should differ between experimental groups. In particular, we predicted an increase in HSP60 levels in the course of the brood rearing period for control females (*see* Merino *et al*. 1998, 2002).
3. Fitness costs should be higher in the control group as compared with the medicated group due to the higher parasitaemias and/or the induced HSP60 response. Thus, we predicted that control females should lose more weight during the brood rearing period, and/or produce fewer nestlings of poorer quality than medicated females.

Material and methods

Study population

The study was carried out in a Pyrenean oak (*Quercus pyrenaica*) deciduous forest located in Valsaín (Segovia, central Spain, 40°54´N, 4°01´W, 1200 m a.s.l.). A population of blue tits breeding in nest boxes in this area has been studied since 1991 (Sanz 1995). Every year, nest boxes were periodically inspected in order to determine reproductive parameters. The high prevalence of infection by blood parasites in this population (Fargallo & Merino 1999) allowed us to test for the effect of reduction of parasitemia on host stress and immune responses.

Experimental treatment

During the breeding season of 2001, nests were paired according to clutch size and laying date (day 1 = 1 April), and randomly assigned to one of the following experimental treatments. Females were trapped at nest boxes when their nestlings were 3 days old (hatching date = age 0), and injected subcutaneously with either 0.1 mg of Primaquine (Sigma, St Louis, MO, USA) diluted in 0.1 ml of saline solution (medicated females; approx. 9.25 mg kg^{-1}), or alternatively, the same volume of saline solution (control females). Primaquine is an antimalarial chemical compound which has been successfully employed to reduce blood parasitization in the species under study (Merino *et al*. 2000). Immediately after capture and before the injection, we obtained blood (80–100 μl) from the brachial vein with the aid of a capillary tube (initial sample). One drop of this blood was smeared on a slide for detection of blood parasites and the rest of the blood was centrifuged (2000 × *g*, 5 min) with a portable centrifuge (Labnet, Mini Centrifuge, Cat. N° 1201-220V, Woodbridge, NJ, USA). Serum and cell fractions were separated and maintained in a cool box below 15 °C before being frozen for later analysis. Time elapsed from collection until freezing did not exceed eight hours. It has been previously shown that this storing procedure leads to no alteration in HSP levels (Tomás *et al*. 2004). At this capture, females were weighed to the nearest 0.05 g with a Pesola (Baar, Switzerland) spring balance and banded individually with numbered metal rings when necessary.

When nestlings were 13 days old, we attempted to recapture as many of the females as possible to obtain a second blood sample (final sample). This allowed us to determine post-treatment HSP levels, immunoglobulin levels and parasitaemia. Females were reweighed and female tarsus length was measured with a digital calliper (accuracy 0.01 mm) at this time. Tarsus length of all nestlings was measured at 13 days of age, wing length was measured with a rule to the nearest 0.5 mm and nestling mass recorded to the nearest 0.05 g.

Parasite quantification

Blood smears were immediately air-dried and later fixed with ethanol (96%) and stained with Giemsa (1/10 v/v) for 45 min. Half of the symmetrical smear was scanned at 200× magnification in search of large blood parasites such as *Trypanosoma* or *Leucocytozoon*, whereas small intra-erythrocytic parasites such as *Haemopro-*

teus were detected using 1000× magnification (Merino & Potti 1995a, Merino *et al*. 1997). Intensity of infection by *Haemoproteus* parasites was estimated as the number of infected cells per 2000 erythrocytes (Godfrey *et al*. 1987). We use presence/absence indexes for *Trypanosoma* and *Leucocytozoon* parasites due to their low intensities of infection. Larvae of the fly *Protocalliphora azurea* (Diptera: Calliphoridae) are common blood sucking ectoparasites of Holarctic birds (Bennett & Whitworth 1992). In our study population, *Protocalliphora* are probably the most harmful ectoparasites for nestlings, as in other blue tit populations at similar latitudes (Hurtrez-Boussès *et al*. 1997). After the nestlings had fledged, nests were removed and nest material was carefully dismantled over a white piece of paper in order to count *P. azurea* pupae (Merino & Potti 1995b).

HSP determination

We determined HSP levels from the blood cellular fraction by means of the widely employed Western blot technique (e.g. Lyashko *et al*. 1994, Gehring & Wehner 1995, Merino *et al*. 1999, Tomás *et al*. 2004). In brief, blood cells were homogenised in approximately 0.2 ml of distilled water by a sonicator. The homogenate was then centrifuged (14 000 × *g*, 20 min) at 4 °C and the supernatant collected. The total protein concentration was determined using the Bio-Rad Protein Assay. Samples of soluble proteins (70 μg/well) were separated by SDS-PAGE. The total protein value has been found to be in the linear range of the antibody–antigen response for the species and antibodies studied. Stacking gels containing 4% and separating gels with 10% acrylamide were used. Electrophoresis was carried out at 200 V. Electroblot transfer from the polyacrylamide gels was performed as described by Towbin *et al*. (1979). The polyvinylidene fluoride (PVDF) blots were washed in phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tw), and incubated with 5% non-fat powdered milk in PBS-Tw for 1 h. After incubation, blots were tested with antiserum. The primary monoclonal antibodies (Sigma) were anti-HSP70 (clone BRM22) and anti-HSP60 (clone LK2) diluted 1/5000 and 1/1000 in PBS-Tw. These antibodies react specifically with HSP70 and HSP60 respectively, as shown by the immunoreactive bands of appropriate molecular weights.

A peroxidase-conjugated secondary antibody (Sigma) was used at 1/6000 dilution. This dilution was chosen because it allows clear detection of HSPs without unspecific binding. Positive bands were detected using 50 mM Tris buffer containing 0.06% diaminobenzidine and 0.1% hydrogen peroxide. Primary and secondary antibodies were incubated overnight at 4 °C and for 2 h at room temperature respectively. Three washes with PBS-Tw were performed after each step. Finally, protein bands were quantified using image analysis software for Windows (Scion Corporation™, Frederick, MD, USA). HSP levels were expressed in arbitrary units as area per mean density of the bands. In previous blotting experiments the same monoclonal antibodies used here were found to produce linear response curves to HSP70 and HSP60 ($r^2 > 0.95$).

Immunoglobulin determination

We determined immunoglobulin levels from the blood serum fraction by means of a direct ELISA (enzyme-linked immunosorbent assay) technique. The antibody used to detect immunoglobulins was rabbit anti-chicken IgG conjugated with peroxidase (Sigma). A previous study has shown that this commercial antibody recognizes by cross-reactivity immunoglobulins from blue tits (Martínez *et al*. 2003). The linear range of the sigmoidal curve for this antibody–antigen response, as well as the optimal dilution have been determined previously and we have shown that this procedure is reliable and accurate for determining total immunoglobulins of blood serum fractions from blue tits (Martínez *et al*. 2003). ELISA plates (Maxi-sorp, Nunc) were coated with serums (100 μl) diluted at 1/4000 in carbonate–bicarbonate buffer (0.1 M, pH = 9.6, overnight at 4 °C). Later, the plates were blocked with 5% defatted milk diluted in PBS-Tw buffer for 1 h at 37 °C (200 μl). Anti-chicken conjugate was added at 1/250 dilution in PBS-Tw and incubated for 2 h at 37 °C (100 μl). After incubation

with a substrate comprising ABTS (2,2´-azinobis (3-ethylbenzthiazoline-6-sulphonic acid)) and concentrated hydrogen peroxide diluted to 1/1000 for 1 h at 37 °C, absorbance was measured using a plate spectrophotometer at $\lambda = 405$ nm. In these conditions, we achieved the maximum values of absorbance.

There were no significant plate-to-plate differences in immunoglobulin levels. Given the important role immunoglobulins may play in mediating HSP responses to parasitism, we have included in the present study only those females for which both initial and final serum and cellular fraction of the blood could be analysed. Serum samples were initially processed by means of electrophoretic techniques, which were afterwards found to be unreliable. As a consequence, several samples were not available for ELISA analyses and experimental groups have unequal sample sizes.

Statistical analysis

Parametric statistics were used when variables fitted normal distributions. Otherwise, nonparametric tests were employed. To examine differences in changes of *Trypanosoma* and *Leucocytozoon* infection status in medicated and control females across the experiment (birds changing infection status and birds maintaining infection status), we used McNemar χ^2 tests. Fledging success was subjected to arcsine square-root transformation before using parametric statistical tests. *Haemoproteus* infection intensities were logarithmically transformed to fit normal distributions. GRM models (StatSoft 2001) were built by introducing final value of the variable (when nestlings were 13 days old) as the dependent variable, and treatment as the categorical predictor. Initial value of the variable (when analysing variables with pre-treatment value), initial and final immunoglobulin, HSP70 and HSP60 levels, and number of *Protocalliphora* pupae in the nest were introduced as continuous predictors. Non significant variables were backward stepwise removed. Differences between blots were tested only when comparing HSP levels between groups, as initial and final samples of each individual were always processed in the same blot. To explore relationships between parasite infection and HSP and immunoglobulin levels, we have analysed individual changes in HSP60, HSP70 and immunoglobulin levels (i.e. value at day 13 of nestling age – value at day 3 of nestling age) against individual changes in *Haemoproteus* infection intensity and classes of change of infection status by *Leucocytozoon*. All tests are two-tailed.

Results

Treatment and infection

A total of 36 experimental individuals were included in the analyses (21 control and 15 medicated). Thirty four out of 36 females were infected by blood parasites at the beginning of the nestling period. The most common blood parasite detected was *Haemoproteus majoris*, infecting 72.2% of the birds, followed by *Leucocytozoon majoris* (63.9%) and *Trypanosoma avium* (33.3%). Before treatment, there were no differences in intensity of *Haemoproteus* infection between experimental groups ($P > 0.05$). The same was true in the cases of *Leucocytozoon* and *Trypanosoma* parasites (χ^2_1: $P > 0.05$ in both cases).

In accordance with prediction 1, we found that medicated females had lower final *Haemoproteus* infection intensity than control females (Table 1; ANCOVA: treatment: $F_{1,33} = 5.85$, $P = 0.021$; covariate: initial *Haemoproteus* intensity: $F_{1,33} = 196.65$, $P < 0.001$). Moreover, final prevalence of infection by *L. majoris* was significantly different between treatments ($\chi^2_1 = 6.22$, $P = 0.013$; 20.0% for medicated females, 61.9% for controls), and there was a tendency towards more birds recovering from infection by *Leucocytozoon* than acquiring it in the medicated group (McNemar $\chi^2_1 = 3.13$, $P = 0.077$). No such tendency appeared in the control group (McNemar $\chi^2_1 = 0.00$, $P = 1.000$). There were no significant differences when comparing the number of birds maintaining the uninfected status with the number of birds maintaining the infected status by *Leucocytozoon* in any group (McNemar χ^2_1: $P > 0.10$ in both cases). Nevertheless, we did not find any apparent effect of medication with

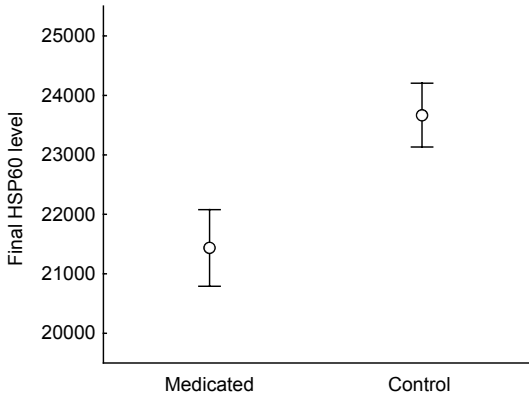

Fig. 1. Least square means (± SE) of final HSP60 level in medicated and control female blue tits.

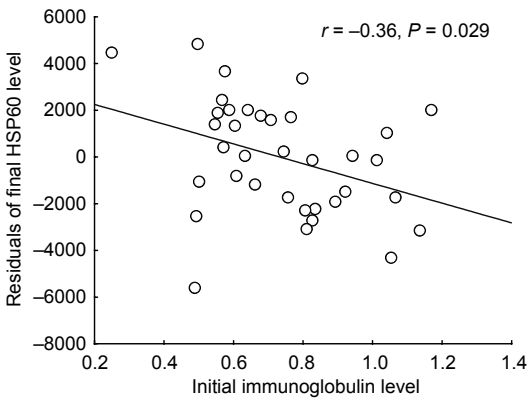

Fig. 2. Relationship between initial immunoglobulin level and final HSP60 level in female blue tits. HSP60 values are residuals after controlling for treatment and initial HSP60 level.

Primaquine on the prevalence of infection by *T. avium* (McNemar χ^2_1: $P > 0.18$ for birds changing infection status in both groups and $P > 0.72$ for birds maintaining infection status in both groups). Thus, the experimental treatment had the expected effect on infection by the two most common blood parasites.

Blood parasites and physiological host responses

Before treatment, there were no significant differences either in HSP70, HSP60 or immunoglobulin levels between females subsequently injected with Primaquine and those injected with the placebo ($P > 0.05$ in all cases). In accordance with prediction 2, there was a significant difference in final HSP60 level between treatments, due to its increase in control females (Table 1), when both initial HSP60 and immunoglobulin levels were controlled for (ANCOVA: $F_{1,32} = 6.58$, $P = 0.015$; covariates: initial HSP60 level: $F_{1,32} = 56.20$, $P < 0.001$; initial immunoglobulin level: $F_{1,32} = 5.95$, $P = 0.020$; Fig. 1). When treatment and initial HSP60 level were controlled for, it appeared that initial immunoglobulin level was negatively and significantly associated with final HSP60 level ($r = –0.36$, $P = 0.029$; Fig. 2). There were no significant differences in final HSP70 levels between treatments, after controlling for initial HSP70 level (ANCOVA: $F_{1,33} = 1.19$, $P = 0.284$; covariate: initial HSP70 level: $F_{1,33} = 166.79$, $P < 0.001$; Table 1). Final immunoglobulin level did not significantly differ between medicated and control females, after controlling for initial immunoglobulin level (ANCOVA: $F_{1,33} = 1.34$, $P = 0.255$; covariate: initial immunoglobulin level: $F_{1,33} = 20.87$, $P < 0.001$; Table 1).

Table 1. Average intensity of infection by *Haemoproteus majoris*, HSP60, HSP70 and immunoglobulin levels, and female body mass at 3 (initial) and 13 (final) days of nestling age for medicated ($n = 15$) and control ($n = 21$) female blue tits. Numbers in parenthesis are standard errors.

	Medicated		Control	
	Initial	Final	Initial	Final
Haemoproteus	12.60 (3.57)	7.21 (2.22)	12.14 (4.26)	11.05 (2.96)
HSP60	22488.42 (800.15)	22422.68 (865.67)	21313.99 (882.32)	22963.43 (900.89)
HSP70	29780.19 (1661.98)	30182.05 (1925.49)	29713.63 (1446.78)	29054.61 (1451.64)
immunoglobulins	0.66 (0.05)	0.64 (0.06)	0.80 (0.05)	0.80 (0.05)
female mass (g)	10.88 (0.12)	10.39 (0.13)	10.66 (0.11)	10.01 (0.12)

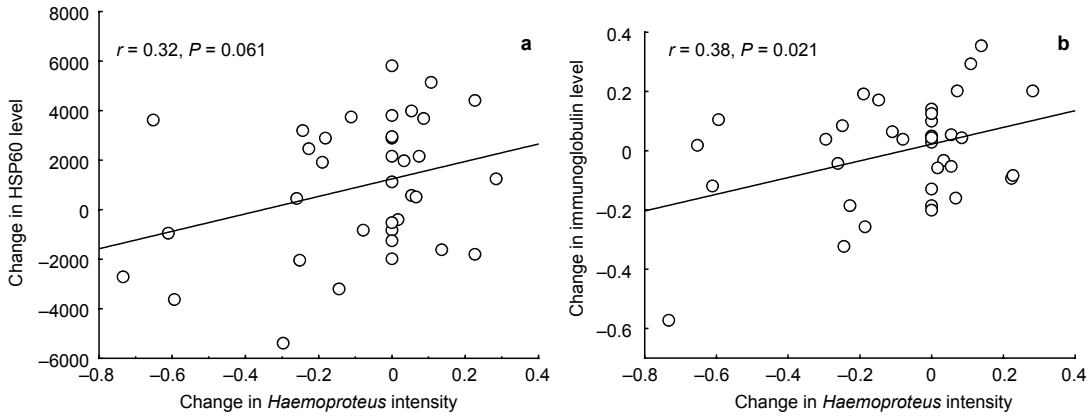

Fig. 3. Relationship between individual change in *Haemoproteus* infection intensity (logarithmically transformed) from day 3 to day 13 of nestling age (final – initial values) and individual change in the same period in (**a**) HSP60 level, and (**b**) immunoglobulin level, in female blue tits.

Individual change in *Haemoproteus* intensity (final intensity – initial intensity) showed a marginally significant positive correlation with individual change in HSP60 levels ($r = 0.32$, $P = 0.061$: Fig. 3a), and a significant and positive correlation with individual change in immunoglobulin levels ($r = 0.38$, $P = 0.021$; Fig. 3b). Individual change in *Haemoproteus* intensity was not correlated with individual change in HSP70 level ($r = –0.26$, $P = 0.123$). There were no significant differences in the change in HSP60, HSP70 or immunoglobulin levels between birds that either remained uninfected by *Leucocytozoon*, remained infected, acquired the infection or recovered from it (ANOVA: all $P > 0.19$).

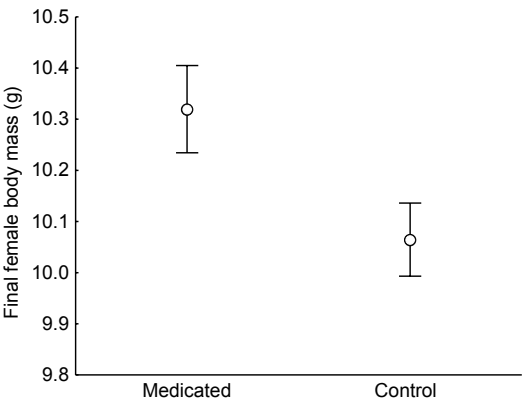

Fig. 4. Least square means (± SE) of final body mass in medicated and control female blue tits.

Effects on parental and offspring body measurements

There were no significant differences between groups in laying date, clutch size, brood size or parental tarsus length and mass before the experiment (one-way ANOVA: $P > 0.05$ in all cases). In accordance with prediction 3, control females had lower final body mass than medicated ones (Table 1 and Fig. 4), when correcting for initial body mass and the negative effect of number of blow fly pupae in the nest (ANCOVA: $F_{1,32} = 5.01$, $P = 0.032$; covariates: initial body mass: $F_{1,32} = 42.88$, $P < 0.001$; blow fly pupae: $F_{1,32} = 6.85$, $P = 0.013$). To confirm whether or not the relationship between female mass and blow fly infestation differed between treatment groups, we included in the analysis the interaction between treatment and number of blow fly pupae, which proved significant ($F_{1,32} = 5.44$, $P = 0.026$). Final body mass of control females was negatively and significantly correlated with number of *Protocalliphora* pupae, while this was not the case for medicated females (Fig. 5). By including this interaction term, the treatment factor was no longer significant. Moreover, there was a tendency for female body mass change (i.e. female mass loss = final body mass – initial body mass) to correlate positively with number

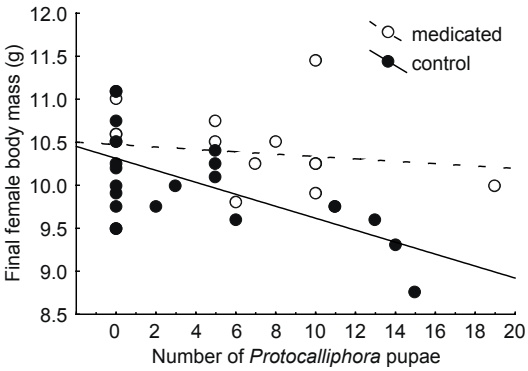

Fig. 5. Relationship between female body mass and number of *Protocalliphora* pupae in blue tit nests (r = –0.16, P = 0.582 for medicated females; r = –0.66, P = 0.001 for control females) at the time nestlings were 13 days old.

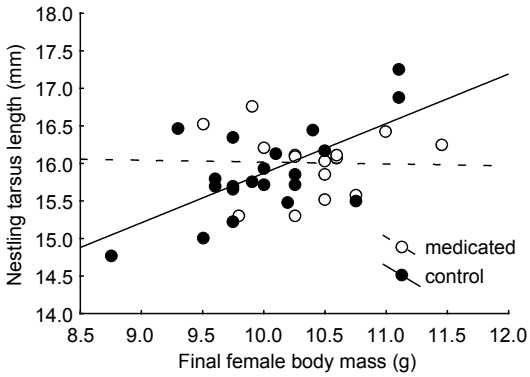

Fig. 6. Relationship between female body mass and mean nestling tarsus length when the nestlings were 13 days old (r = –0.03, P = 0.920 for medicated females; r = 0.64, P = 0.002 for control females).

of blow fly pupae in the nest (r = 0.29, P = 0.084). This tendency turns significant when excluding nests lacking blow flies (r = 0.43, P < 0.05, N = 21).

Contrary to our expectations, there were no significant differences between groups in fledging success ($F_{1,34}$ = 0.17, P = 0.681), or nestling measurements on day 13 (nestling mass: $F_{1,34}$ = 0.10, P = 0.760; nestling tarsus length: $F_{1,34}$ = 0.53, P = 0.473; nestling wing length: $F_{1,34}$ = 0.20, P = 0.654). Nevertheless, nestling tarsus length and nestling mass were significantly and positively correlated with female final body mass (nestling tarsus length: r = 0.45, P = 0.006; nestling mass: r = 0.45, P = 0.006). To confirm whether the relationships between nestling size and final female body mass differed between treatments, we carried out ANCOVAs with treatment and final female body mass as factors and nestling tarsus length or nestling mass as the dependent variable. Both treatment (nestlings of control females having shorter tarsi), and final female body mass (nestlings with shorter tarsi were attended by females with lower mass), had a significant effect on nestling tarsus length (treatment: $F_{1,32}$ = 4.96, P = 0.033; final female mass: $F_{1,32}$ = 4.31, P = 0.046). In addition, we found a significant interaction between treatment and final female body mass ($F_{1,32}$ = 5.02, P = 0.032). Only tarsus lengths of nestlings reared by control females were positively and significantly correlated with final female body mass, while this was not the case for nestlings reared by medicated females (Fig. 6). Nestling body mass was significantly affected by female final body mass ($F_{1,32}$ = 6.83, P = 0.014), but not by treatment or the interaction between treatment and final female body mass (both P > 0.5).

Discussion

The role of stress responses in the life histories of wild animals is receiving increasing attention by ecologists. Study of stress responses is stimulating as integrated knowledge of a wide range of behavioural, ecological and evolutionary processes are still not fully understood (Feder 1999, Feder & Hofmann 1999, Buchanan 2000, Sørensen *et al.* 2003). The function of HSPs in response to stress in almost all organisms so far studied could turn this group of proteins into one of the best candidates for acting as bioindicators in wild animal populations (Buchanan 2000). Despite the vast literature on host–parasite interactions, the relation between parasitism and HSP levels in hosts has not been examined in wild populations until recent years (Merino *et al.* 1998, 2002, Feder & Hofmann 1999).

Our experimental manipulation produced a reduction in intensity of infection by *H. majoris* and in prevalence of infection by *L. majoris* in female blue tits as expected (Merino *et al.* 2000). We also found experimental evidence for

a parasite-induced stress response in the host: final HSP60 levels were significantly higher in control females, whose parasite infection intensity was not experimentally reduced through medication (prediction 2, Fig. 1). Our data show that when parasitism is experimentally reduced, HSP60 levels show little variation, but in control females which maintain similar infection intensities throughout the nestling stage, a physiological response mediated by HSP60 levels is triggered. The tendency for individual change in *Haemoproteus* infection intensity to correlate positively with individual change in HSP60 level (Fig. 3a) adds support to the hypothesis that parasites are the stressors that cause the increase in HSP60 values in blue tits.

Some previous studies on birds, all dealing with hirundines, suggest a role for HSP60 in the response to parasitism. Merino *et al*. (1998) found higher levels of this protein in nestling house martins (*Delichon urbica*) infected by *Trypanosoma* sp. In another study with adult barn swallows (*Hirundo rustica*), Merino *et al*. (2002) linked parasite infection with higher levels of HSP60 by comparing uninfected birds with those infected by different species of blood parasites. However, to our knowledge, this is the first study reporting experimental evidence of a positive association between parasite infection and HSP expression in a wild avian population. The mechanistic basis underlying this process will require further research. Parasites may cause increases in HSP60 by at least three different, non mutually exclusive mechanisms: (i) it could be a response to the fever occasioned by the infection, i.e. HSP60 could be increased in response to a heat shock (Garbe 1992); (ii) the increase in HSP60 could be due to a pathogen-induced necrosis (Matzinger & Fuchs 1996, Moseley 2000); and (iii) secretion/excretion products from parasites could induce protein expression (Martínez *et al*. 1999b).

In stressful conditions such as those produced by parasite infections, an increase in HSP60 expression by the host could be adaptive in order to maintain appropriate levels of erythrocyte functionality. Intraerythrocytic parasites could probably cause physiological stress to their hosts by means of cell destruction and haemoglobin consumption (Kirkpatrick & Suthers 1988, Atkinson & Van Riper 1991). In addition, parasites may obtain some benefit from the maintenance and correct functioning of cell machinery. It has been demonstrated that certain HSPs, such as HSP70 and HSP65, play key roles in inhibiting apoptotic death of cells infected by intracellular parasites (Heussler *et al*. 2001, Kroemer 2001). This could be beneficial both for the parasite and for the host, because if HSP synthesis is induced in infected cells, the apoptotic machinery would be inhibited, resulting in mild disease. However, if HSP expression is suppressed, infected cells would be destroyed rapidly, and severe disease will follow (Hisaeda *et al*. 1997). Whether one mechanism or the other is favoured could depend on the virulence of the parasite strain (Hisaeda *et al*. 1997).

As previously found (Merino *et al*. 2002), blood HSP70 is not apparently associated with a host response against infection by blood parasites in birds. The exact function of this protein in the blood cells of birds remains unknown, and it is likely that HSP70 responds in a different manner, or in response to different stressors, than HSP60 does (Krebs & Loeschcke 1994b).

Trade-offs between physiological antiparasite defences and reproductive performance have been frequently suggested in birds (Svensson *et al*. 1998, Ilmonen *et al*. 1999, 2000, Hasselquist *et al*. 2001). Merino *et al*. (1998) showed that HSP60 could be costly to produce in growing birds, since they found a negative relationship between HSP and wing length that may be due to a trade-off between energy allocations. In addition, it is important to indicate that the induction of HSPs has been shown to be deleterious in the absence of stress (Feder 1996). Further evidence that HSP expression is costly in ecological terms has been reported by Silbermann and Tatar (2000). In the present study, some of the costs imposed directly by parasites and/or indirectly through HSP60 expression were paid for by female blue tits in terms of post-breeding condition (in accordance with prediction 3; Fig. 4). In a similar experiment, Merino *et al*. (2000) showed that final body mass in non-medicated female blue tits was negatively related to final infection intensity by *Haemoproteus*.

Medicated females seemed to obtain some advantage from the reduction in blood parasitaemia, as they did not lose mass differentially in response to the degree of blow fly infestation suffered by their brood. We should expect condition-dependent survival prospects in female blue tits, especially in control females (Brown *et al*. 1995, Richner & Tripet 1999). In contrast to Merino *et al*. (2000), who found that broods attended by control females had lower fledging success than those of medicated females, we found no short-term detrimental effects of parasites on fledging success, maybe because *Haemoproteus* infection intensities — as well as sample sizes — were lower in this study than in Merino *et al*. (2000). Nevertheless, we found significant effects of treatment, final female mass and their interaction on nestling tarsus length, in addition to a significant positive relationship between final female body mass and nestling tarsus length observed only for control females (Fig. 6). These results are in accordance with prediction 3, showing that control females produced nestlings of poorer quality than medicated females.

On the other hand, the fact that the initial immunoglobulin level is significantly related to final HSP60 level (Fig. 2), suggests that the initial health condition of birds plays an important role and must be taken into account to properly reveal relationships underlying host stress responses to blood parasites. The positive association between intensity of infection by *Haemoproteus* and immunoglobulin levels (Fig. 3b) also points out the role of immunoglobulins in managing parasite infections in wild birds. These results offer a new insight into how parasitism, stress response and immune function may be connected in a three-way interaction, and thus merit further research.

To conclude, we have experimentally shown that infection by two of the most common avian blood parasites induces stress responses in wild birds. Fitness costs of either parasites and/or responses against them were detected with respect to current reproduction. The evaluation of the long-term effects on host fecundity and survival could help to better interpret the role played by HSPs in stress responses of wild birds facing parasite infections.

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