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Increase in a heat-shock protein from blood cells in response of nestling house martins (*Delichon urbica*) to parasitism: an experimental approach

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Abstract Heat-shock proteins (HSPs) are synthesized by animals and plants in response to various stressors. The level of the HSP60 stress protein was measured from the cell fraction of peripheral blood obtained from nestling house martins (*Delichon urbica*) to test whether ectoparasitism increased the concentration of stress protein. We assessed HSP from nestlings raised in nests previously treated with an insecticide or infested with 50 martin bugs (*Oeciacus hirundinis*). In addition, haematozoa infections were checked in blood smears. Nestlings from parasite-infested nests, or nestlings infected with trypanosomes, had increased levels of HSP in their blood cells. Nestling growth as determined from wing length was negatively related to HSP60 levels and within-brood variation in wing length increased with increasing levels of the stress protein independently of treatment and infection by trypanosomes. These results suggest HSPs may play a role in host-parasite interactions, and that they can be used reliably for measuring physiological responses to parasites.

Key words Disease · Heat-shock protein · Martin bug · Sedimentation rate · Trypanosomes

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Introduction

Parasitism is a common environmental factor faced by all animals in the wild, and almost all living organisms are attacked by parasites during their lifetime (Price 1980; Clayton and Moore 1997). Parasites not only drain their hosts of energy reserves, they also deplete resources indirectly in hypersensitivity reactions, irritation, behavioural responses and immune defences. Thus, parasites are undoubtedly a source of stress for hosts. A plethora of research shows that stress induced by parasitism has important consequences for the evolution of life history traits (see Møller 1997 for a recent review). In addition, recent work has emphasized the role of immunity as a mechanism mediating life history decisions to parasitism (Gustafsson et al. 1994; Saino et al. 1997).

In the early 1960s, the observation that some areas of chromosomes from the salivary glands of *Drosophila* were altered under adverse conditions initiated studies of stress proteins (Macario 1995). These changes appeared in *Drosophila* chromosomes after the flies had been subjected to heat shock. Thus, the proteins encoded by genes in puffing areas were termed heat-shock proteins (HSPs). These proteins have now been found in all kinds of organisms from bacteria to higher eucaryotes (Morimoto 1991). HSPs have been classified in different families by molecular weight, and although their functions continue to be determined, some have been shown to act as molecular chaperones, that is, molecules helping other proteins to fold correctly during their production or to refold after partial denaturation (Morimoto 1991). In the last few years, many stressors have been demonstrated to induce HSPs, and have therefore been termed stress proteins. However, the terms HSP, stress protein and chaperone are currently used interchangeably.

Most ecological work on stress proteins centres around their relationship to thermotolerance (Coleman et al. 1995; Krebs and Feder 1997). However, in vitro studies have shown that HSPs may be induced by other

stressors such as disease (Garbe 1992). Cell stressors causing induction of genes producing these proteins include infections and other responses to disease such as fever and inflammation (Macario 1995).

Here we experimentally test whether a stress protein is induced in the presence of ectoparasitism. To our knowledge, this is the first study reporting a relationship between parasitism and stress protein induction.

Materials and methods

The study was carried out during April–June 1998 in a colony of house martins located in the surroundings of Badajoz (38°50′N, 6°59′W), Spain. Nests were inspected every 2 days with the aid of a dental mirror to determine laying date. In some cases where laying had begun at the first visit, we calculated the laying date assuming that house martins lay one egg per day (Cramp 1988). Only nests with incomplete clutches when we started inspecting nests were included in the experiment. Fifteen days after completion of the clutch, the nest was inspected to determine hatching date.

At an age of 13 days, nestling tarsus length was measured as the distance between the bending points with a dial caliper to the nearest 0.01 mm. Nestling mass was measured with a spring balance to the nearest gram and wing length, as the distance from the carpal joint to the longest primary feather, was measured with a ruler to the nearest millimetre. Subsequently, we obtained a drop of blood from the brachial vein, which was used to produce a blood smear that was air dried. Another sample of blood was collected in a heparinized capillary tube to obtain the haematocrit value and the sedimentation rate (see below).

Experimental treatment

All nests were fumigated with a solution containing pyrethrin (1.85 gl^{-1}) and piperonil butoxid (2.4 gl^{-1}) before completion of the clutch. In previous studies (Møller et al. 1994), this insecticide had no apparent detrimental effects on eggs or nestlings of this species. On the day of hatching, nests were randomly either fumigated (F-nest, $n = 16$) again or infested with 50 individuals of the martin bug *Oeciacus hirundinis* (I-Nest, $n = 18$). The martin bug is a common ectoparasite of house martins, and is easily collected from old nests in the colony prior to experimental infestation. F-nests were refumigated every 2 days to prevent immigration of ectoparasites from other nests. This treatment has been shown to effectively create differences in ectoparasite load using nests in the same colony (Møller et al. 1994; de Lope et al. 1998).

Blood analysis

Blood smears were fixed in absolute ethanol and later stained with Giemsa. Half of the symmetrical smear was checked for blood parasites at ×500 magnification (Merino and Potti 1995). This allowed us to obtain a good measure of infection status. However, due to difficulties ascertaining trypanosome infection in blood smears, especially for weak infections, we assumed that most chicks in the same nest were infected by trypanosomes (see Merino et al. 1996). In any case, unidentified infections should make our results conservative.

The capillary tube containing blood was positioned vertically for 3 h at 4°C. The length of plasma and blood cells was measured with a dial caliper to the nearest 0.01 mm. The sedimentation rate was calculated as the distance occupied by plasma divided by the total length of the blood column (plasma + cells). Capillaries were centrifuged at 12 000 rpm for 10 min immediately afterwards to calculate the haematocrit value (packed cell volume).

Finally, the capillaries were cut to separate plasma from cells. The latter part was frozen for later analyses. The concentration of HSP was determined as follows. Blood cells were homogenized in 0.2 ml distilled water by a sonicator. This homogenate was centrifuged at 12 000 rpm. for 30 min at 4°C. The supernatant was collected and the protein concentration determined (Bradford, Bio-Rad). Samples (60 μg/well) were separated by SDS–PAGE. Stacking gels containing 4% and separating gels 12% acrylamide were routinely used. Electrophoresis was carried out at a constant current of 40 mA. Electroblot transfer from the polyacrylamide gels was performed as described by Towbin et al. (1979). The polyvinylidene fluoride blots were washed in PBS containing 0.05% Tween-20, and incubated with 5% non-fat powdered milk for 1 h. After incubation, blots were tested with antisera. Primary monoclonal antibodies (Sigma) were anti-HSP60 (LK1) diluted (1/1500) in PBS. The peroxidase-conjugated secondary antibody (Sigma) was used at 1/6000 dilution. This dilution was chosen because it allows a clear detection of HSPs using the lowest quantity of secondary antibody. The peroxidase reaction was carried out in 0.05 M Tris buffer containing 0.03% diaminobenzidine, and 0.001% hydrogen peroxide. For densitometric quantification of blot immunoreactivity, a digital analysis image system (Microm, Spain) was used.

Statistical analysis

Blots made on different days may show variation due to small differences in temperature or other subtle conditions affecting some or all the steps in the Western blot technique. To minimize this effect, we attempted to simultaneously process as many samples as possible to maintain similar conditions during the process. Thus, all samples were processed in seven different blots. However, there was a clear blot effect on HSP values. We controlled for this effect using the residuals of the relationship HSP-blot to calculate the within-brood average.

As the haematocrit greatly influences the sedimentation rate, we used the residuals of the regression of sedimentation rate on haematocrit as a corrected sedimentation rate.

To avoid pseudoreplication due to the shared environment for chicks in a nest, we used brood means and measures of variation in all analyses. All test are two-tailed.

Results

Only four nests had chicks infected by *Trypanosoma* spp. (probably *Trypanosoma everetti* based on their small size).

To test the possible effect of parasitism on HSP induction, we carried out an ANCOVA with treatment and infection by trypanosomes as factors and HSP levels as the dependent variable (Table 1). Both trypanosome infection and ectoparasite treatment had a significant effect on HSP induction. The mean HSP60 levels for fumigated and infested nestlings were −113.98 and 1833.24 units, respectively (Table 2). The mean values for nestlings infected and uninfected by trypanosomes were −123.08 and 1842.33, respectively (Table 2).

We found a significant interaction between ectoparasite treatment and trypanosome infection, implying that nests fumigated and uninfected by trypanosomes had the lowest values of HSP. Either infection with trypanosomes or ectoparasite infestation gave a similar higher level of HSP induction. Finally, infection by both kinds of parasites produced the highest level of HSP

Table 1 Results of an ANCOVA with HSP60 levels as a dependent variable and experimental treatment and infection by trypanosomes as factors

Source of variation	Sum of Squares	*df*	Mean square	*F*-ratio	*P*
Trypanosome infection (A)	10 517 678	1	10 517 678	10.701	0.0027
Experimental treatment (B)	10 323 976	1	10 323 976	10.504	0.0029
A × B	5 386 608.7	1	5 386 608.7	5.481	0.0261
Residual	29 484 995	30	982 833.17		
Total (corrected)	44 274 782	33			

induction (Fig. 1). However, this interaction should be interpreted cautiously: only one nest suffered from multiple infections, although it did show the highest HSP value in the sample.

Low growth rate, high within-brood variability in size and body mass (nutritional stress), and a high sedimentation rate (general infection) reflect stressful conditions for nestling birds (Sharma et al. 1984; Møller 1997). We tested whether these factors were related to HSP induction by introducing these variables as covariates in the previous ANCOVA. We found a negative relationship between wing length and HSP induction ($F = 5.55$, $df = 1,33$, $P = 0.03$; slope = −62.97, SE = 26.93; Fig. 2). Furthermore, the within-nest coefficient of variation in wing length was positively related to stress protein levels ($F = 4.86$, $df = 1,33$, $P = 0.04$; slope = 61.76, SE = 28.65; Fig. 3), while controlling for treatment and infection effects. Body mass and tarsus length and their coefficients of variation were not significantly related to HSP levels ($F = 2.15$, $df = 1,33$, $P > 0.15$ in all cases). Haematocrit and corrected sedimentation rate were not significantly related to stress protein level ($F = 3.58$, $df = 1,33$, $P > 0.07$).

Brood size was not significantly correlated with HSP levels ($F = 0.42$, $df = 1,33$, $P = 0.53$). The relationship between size and body mass of nestlings was unaffected by brood size.

Table 2 HSP60 level (SE) in relation to experimental parasite treatment and infection by trypanosomes

	n	HSP60 level
Treatment		
Fumigated	16	−113.99 (317.50)
Infested	18	1833.24 (510.06)
Trypanosome infection		
Uninfected	30	−123.08 (182.63)
Infected	4	1842.33 (572.37)

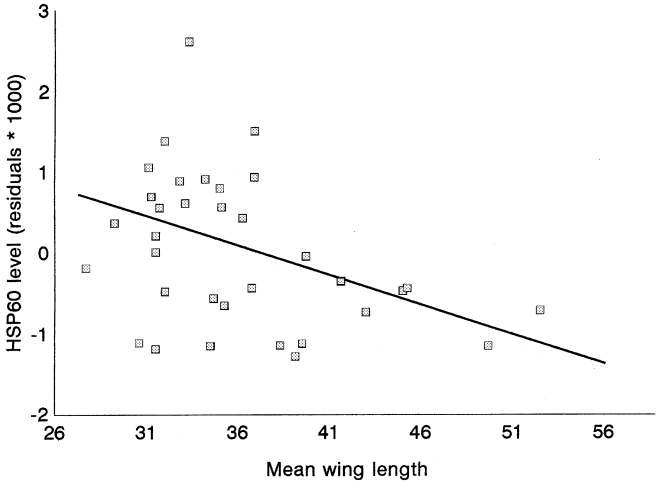

Fig. 2 Relationship between HSP60 level and mean wing length after controlling for experimental treatment and trypanosome infection

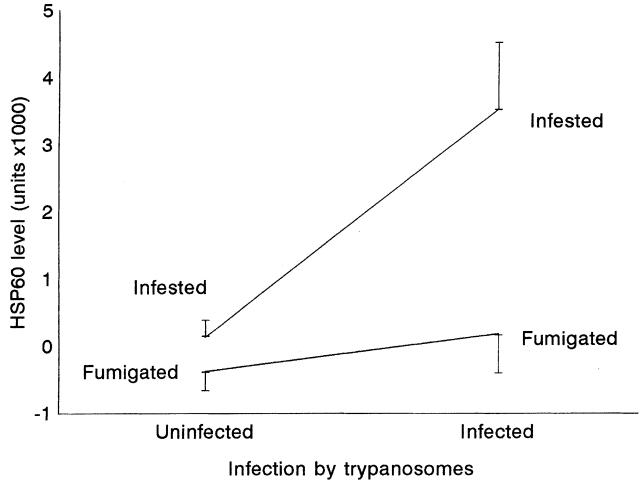

Fig. 1 Stress protein level in relation to the interaction between experimental treatment and infection by trypanosomes in nestling house martins. *Bars* show 1 SE

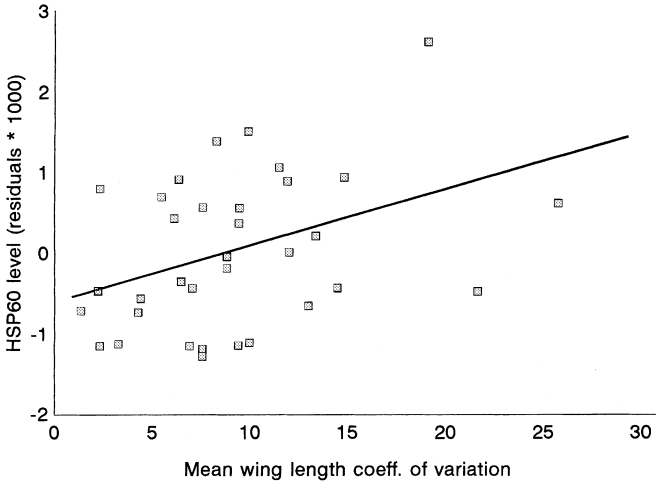

Fig. 3 Relationship between HSP60 level and within-nest coefficient of variation in wing length after controlling for experimental treatment and trypanosome infection

Discussion

The positive association between parasitism as determined by the bug infestation treatment and HSP60 levels clearly shows that the levels of this protein in blood cells increase in the presence of ectoparasites. To our knowledge, this is the first report of increased HSP production by hosts in response to parasitism. Martin bugs are blood feeders and their impact may consequently be directly noted in blood cells. Thus, the increase in production of HSP in cells from the peripheral blood may be directly related to a response to substances secreted by bugs during feeding preventing coagulation. In addition, biochemical substances transferred by parasites are the target of an inflammatory process following a bite. Most physiological processes implicated in the response to parasite biochemical substances may affect blood cells which then increase the production of HSP60. For example, the high levels of stress protein shown by parasitized nestlings may be a simple consequence of fever. Thus, a rise in temperature due to infection may activate the production of HSP as a response to heat shock (Garbe 1992). This response may be adaptive for hosts to maintain blood functions while preventing blood consumption by parasites (inflammation, fever). The role of stress proteins in preventing infections is unclear, although some of these proteins are related to the folding and secretion of important proteins related to an immune response (i.e. immunoglobulins) under stressful conditions (Haas 1994). In addition, stress proteins may be considered analogous to immunoglobulins due to their role in molecular surveillance (Morimoto 1991), helping to maintain homeostasis.

The relationship between infection by trypanosomes and stress protein levels may also be a direct consequence of the presence of parasites in the blood. It is possible that the detected increase in HSP was partly due to the synthesis of a similar HSP60 by parasites. However, as we used a monoclonal antibody specific for vertebrate HSP60, which therefore provides a low signal for the presence of a similar stress protein from trypanosomes, most of the response can be assigned to host cells. In addition, the infections would have to be very high to show a significant effect on parasite HSP levels.

As shown in Fig. 1 and Table 2, the increase in HSP level due to trypanosome infection is mainly due to the only nest where nestlings suffered from both parasite addition and infection by trypanosomes. Chicks from this nest presented the highest level of HSP60 in the sample, while the remaining nestlings infected by trypanosomes only showed a slight increase in HSP60 compared with nestlings from uninfected nests. Therefore, multiple infections appear to induce the production of high concentrations of HSP60 in house martin blood.

HSP60 from microbial parasites induces antibody production by host cells as well as a cellular immune response (Kaufmann 1992; Mollenhauer and Schulmeister 1992). In fact, HSP is so conserved across species, that an antibody response against epitopes cross-reactive between microparasites and host species can give rise to an auto-immune response. Thus, HSPs produced by infectious parasites, including trypanosomes, may increase the immune response and as a consequence induce HSP in host cells. Interestingly, a recent study reported an increase in the concentration of stress protein in some tissues when the individual was suffering an immunological challenge (McComb and Spurlock 1997).

The negative relationship between HSP and wing length, and the positive association between HSP and within-nest variation in wing length, may be caused by the same process. Individual hosts have a limited amount of energy to allocate to different functions. Therefore, not all activities can be maximized simultaneously and a trade-off emerges (Roff 1992; Stearns 1992). The responses against infections may drain energy resources that could otherwise be allocated to processes such as growth. It is plausible that an increase in HSP synthesis may require energy partially reallocated from growth.

In a stressful state, the energy available for growth is reduced compared with maintenance requirements (Parsons 1991). Thus, HSP60 may be highly adaptive by helping to maintain erythrocyte function in house martin nestlings. HSP induction has been shown to be costly in populations of *Drosophila melanogaster* (Krebs and Loeschcke 1994), with the production of HSPs being adaptive in populations suffering from stress but maladaptive in their absence (Krebs and Feder 1997).

The present study suggests that HSPs may be a measure of infection in wild populations of birds, with important adaptive implications. The small amount of blood required for quantifying these proteins is easily obtained without impairing the hosts, thus facilitating the study of the importance of HSPs in wild populations of birds. Furthermore, due to the specificity of the reaction, as shown by the Western blot technique, it is possible to use a cheaper and easier technique such as ELISA to measure HSP60 levels in peripheral blood. Thus, stress proteins may represent a reliable tool for the study of covariation between disease and life history traits in wild populations.

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