Associations between immune parameters, parasitism, and stress in breeding pied flycatcher (*Ficedula hypoleuca*) females

Judith Morales, Juan Moreno, Santiago Merino, Gustavo Tomás, Javier Martínez, and Laszlo Zsolt Garamszegi

Abstract: There are two major interpretations of serum IgY concentration in wild birds. On the one hand, it has been considered an indication of susceptibility to stress and parasite infection. Therefore, immunoglobulin concentration is expected to change in response to variation in these factors owing to reproductive activities. On the other hand, it has been considered a measure of immune capacity. We measured the IgY level and the lymphocyte proportion at the beginning of incubation and at the end of the nestling period in female pied flycatchers, *Ficedula hypoleuca* (Pallas, 1764). We assessed the immune response to phytohaemagglutinin (PHA) at the latter stage. We found that the total IgY level remained constant throughout the season. Initially, it was positively associated with the PHA response, lymphocyte proportion, intensity of infection by *Haemoproteus* spp., and concentration of stress protein HSP70 in peripheral blood. These variables explained nearly 80% of the variation in IgY concentration. In the final phase, only the PHA response was correlated with the IgY level. We discuss the hypothetical mechanisms underlying these associations and the need to control for parasite infection and physiological stress in ecological studies including measurements of immunoglobulin concentration.

Résumé : Il y a deux façons principales d'interpréter la concentration d'IgY dans le sérum des oiseaux sauvages. D'abord, elle peut être considérée comme un indice de la susceptibilité au stress at à l'infection par les parasites. Ainsi, la concentration d'immunoglobuline doit changer, croit-on, en réaction à ces facteurs au cours des activités de reproduction. En second lieu, on a suggéré qu'elle pouvait être une mesure de la capacité immunitaire. Nous avons mesuré les concentrations d'IgY, ainsi que les proportions des lymphocytes, chez des gobe-mouches noirs, *Ficedula hypoleuca* (Pallas, 1764) femelles au début de l'incubation et à la fin de la nidification. À cette dernière période, nous avons évalué leur réaction immunitaire à la phytohémagglutinine (PHA). Les concentrations d'IgY restent constantes au cours de la saison. Dans la phase initiale, elles sont en corrélation positive avec la réaction à la PHA, les proportions de lymphocytes, l'intensité de l'infection à *Haemoproteus* spp. et à la concentration de la protéine du stress HSP70 dans le sang périphérique. Ces variables expliquent 80 % des variations de concentration d'IgY. Dans la phase finale, seule la réaction à la PHA est en corrélation avec la concentration d'IgY. Nous discutons des mécanismes présumés qui expliquent ces associations, ainsi que de l'importance de tenir compte des infections parasitaires et du stress physiologique dans les études écologiques, en particulier des dosages des concentrations d'immunoglobuline.

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Introduction

The immune system has evolved to protect organisms from pathogens (Roitt et al. 2001) and may play an important role in the mechanisms underlying life-history trade-offs (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Norris and Evans 2000). Understanding the complex role of immune function has recently become the main focus of many studies on evolutionary ecology (Zuk and Stoehr 2002; Schmid-Hempel 2003). Variation in immune defenses is expected to be maintained in natural populations through trade-offs with other fitness components, through host–parasite interactions, and through the existence of immunopathological costs (Sheldon and Verhulst 1996; Westneat and Birkhead 1998; Schmid-Hempel 2003). The cellular and humoral components of the acquired immune response have been quantified mainly by exposing organisms to a novel antigen and then measuring the subsequent response (Norris

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and Evans 2000). The cell-mediated component of the immune system has routinely been measured by the phytohaemagglutinin (PHA) challenge injection (Merino et al. 1999; Moreno et al. 1999; Smits et al. 1999; Martin et al. 2001; Tella et al. 2002). Likewise, the humoral component can be quantified by measuring the increase in the production of specific antibodies against an antigen such as diphtheria-tetanus (Ilmonen et al. 2000; Råberg et al. 2000) or sheep red blood cells (Deerenberg et al. 1997; Saino et al. 1997). It has been suggested that the humoral component may also be quantified by estimating the circulating level of total non-specific gamma globulins (IgY in birds) (Ots and Hõrak 1998; Johnsen and Zuk 1999; Szép and Møller 1999). These are the most important serum proteins involved in humoral immune responses (Roitt et al. 2001) and may provide information about the health status of organisms (Gustafsson et al. 1994). Nevertheless, the interpretation of immunoglobulin levels remains controversial because they can be an indication of both prior exposure to infection (Gustafsson et al. 1994; Saino et al. 1999) and immune capacity (Johnsen and Zuk 1999).

Parasite infection and stress may have significant effects on IgY levels (Gustafsson et al. 1994). Parasites presumably provoke an activation of the immune system of their host, which has been detected as an elevation of IgY levels in peripheral blood (Wakelin and Apanius 1997; Roitt et al. 2001). Thus, high IgY levels may be interpreted as a sign of bad health. Linkages between physiological stress and immunocompetence have been reviewed in detail (Apanius 1998; Pruett 2003). Stress responses mediated by parasitism and reproductive activities may directly alter immune function, adding more complexity to the interactions that affect general measures of the immune response. Physiological stress promotes the synthesis of heat-shock proteins (HSPs), a set of highly evolutionarily conserved molecules that facilitate protein folding and assembly (Lindquist 1986; Feder and Hofmann 1999; Sørensen et al. 2003). Under a wide variety of environmental stressors, HSP levels are increased to maintain cellular homeostasis. Members of the HSP60 and HSP70 families have been measured in a few studies of wild avian populations (Merino et al. 1998*a*, 2002; Eeva et al. 2000; Moreno et al. 2002). The intensity of infection by certain parasites (Weatherhead and Bennett 1991) and the stress effected by reproductive activities may change in the course of the breeding season (Atkinson and Van Riper 1991; Apanius 1998; Sanz et al. 2002). Thus, we could expect changes in the total IgY level in response to changes in these factors.

However, total IgY level has also been used as a measure of immunocompetence (Johnsen and Zuk 1999; Saino et al. 2001*a*) and has been found to predict postfledging survival of house-martins, *Delichon urbica* (L., 1758) (Christe et al. 2001). When characterizing the immune system, several immune parameters should be measured rather than a single component (Norris and Evans 2000; Tella et al. 2002; Blount et al. 2003). One of the most widely used measures of the cell-mediated component of the immune response is the PHA assay (Norris and Evans 2000). An indirect measure of acquired immune function is the number of circulating lymphocytes as a percentage of total leucocytes (lymphocyte proportion) (Zuk and Johnsen 1998; Blount et al. 2003). Lymphocytes are wholly responsible for the specific immune recognition of pathogens, initiating acquired immune responses (Roitt et al. 2001). Given the complexity of the organization of the immune system, we cannot raise a directional prediction concerning the association between the immune parameters measured. The assumption that an estimate of antiparasite defense reflects the universal importance of parasites would require a positive association between immunological traits supposed to reflect general health status. However, recent studies have provided contradictory results regarding the correlation between different measures of immune function (Gonzalez et al. 1999; Johnsen and Zuk 1999; Westneat et al. 2003). These contradictory patterns may indicate that different immune responses may at least partly involve different and independent mechanisms.

Therefore, the purpose of this study was to explore the possible relationships between total IgY levels and other measures of the immune system such as the PHA response and the lymphocyte proportion in female pied flycatchers, *Ficedula hypoleuca* (Pallas, 1764), at two stages of the breeding cycle. The initial sample was taken as close as possible in time to the costly metabolic processes involved in egg laying, while the second sample was taken during the period of most intense effort associated with nestling provisioning. In addition, we characterized the effects of various physiological factors potentially mediating the relationships between the immune parameters, such as intensity of haemoparasite infection (Apanius 1991; Gustafsson et al. 1994; Ots and Hõrak 1998) and physiological stress (Sapolsky 1992; Apanius 1998; Dhabhar 2003). The potential connections between the traits measured and their change throughout the reproductive period may contribute to our understanding of the complexity of adaptive immune responses and may provide insight into the physiological problems that animals confront in the wild under different circumstances.

Methods

Study species and study area

This study was conducted during the 2002 breeding season in a deciduous forest of Pyrennean oak (*Quercus pyrenaica* Willd.) at an elevation of 1200 m in the vicinity of La Granja, Segovia province, central Spain (40°48′N, 4°01′W). A study of nest-box-breeding birds has been conducted in this area since 1991 (Sanz 1995; Sanz and Moreno 2000). The pied flycatcher is a small (12–13 g) hole-nesting passerine of European woodlands. For details about its biology see Lundberg and Alatalo (1992). Egg laying in the population under study typically begins in late May, and clutch sizes in our population range from 4 to 7 eggs with a mode of 6 eggs (mean = 5.73 eggs).

Females were captured at the beginning of incubation (on the 8th day after the first egg was laid) with nest box traps. Mass was recorded with a Pesola® spring balance (precision of 0.05 g). A blood sample was collected from the brachial vein. After a blood smear was obtained, the blood sample was centrifuged at 2000*g* for 5 min (Mini Centrifuge, Catalog No. 1201-220V, Labnet, Woodbridge, New Jersey). Cellular and plasma components were separated and maintained in a cool box below 15 °C until being frozen on the same day for later analyses.

On day 12 of the nestling period (i.e., on the 12th day after at least half of the brood had hatched), feeding adults were captured at the nest. A second blood sample was obtained from females to obtain a smear and to estimate IgY and HSP levels. Parents and nestlings were weighed. Tarsus length was measured with a digital calliper to the nearest 0.01 mm and wing length was measured with a rule (precision of 0.5 mm), following Svensson (1984). All chicks were ringed with numbered aluminum bands (Dirección General de Conservación de la Natureza bands; ringing permitted by regional authorities). Males and females were assigned a moult score following Ginn and Melville (1983). Unringed adults were aged as yearlings or older according to Svensson (1984). The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Provisioning rates

When nestlings were 10 days old, parental provisioning rates were determined by monitoring the number of feeding trips performed by both parents. The entrance of each nest box was videotaped for 1 h between 0900 and 1800 using a video camera placed 5–10 m from the nest box. The female feeding rate was not related to the time of day ($P > 0.1$).

Immunization

The T-cell response was assessed by the dermal reaction to PHA in the wing web. This assay has long been used in poultry science (Goto et al. 1978) and has been proved not to alter HSP levels or other haematological values (Merino et al. 1999). This method has been used to measure the T-cell immune response in studies of poultry and in field studies (Sorci et al. 1997; Martin et al. 2001; Moreno et al. 2001; Tella et al. 2002). We used the simplified protocol proposed by Smits et al. (1999), which avoids the injection of saline solution into the opposite wing web as a control. Smits et al. (1999) showed that their protocol reduces inaccuracies inherent in the technique.

On day 12 of the nestling period, after the second blood sample was obtained, 0.2 mg of PHA in 0.04 mL of saline solution was injected into the left wing web of females after measuring the thickness of the wing web at the point of injection. Three consecutive measurements of the thickness, to the nearest 0.01 mm, were taken with a digital spessimeter (Mitutoyo 7/547, Tokyo, Japan) at constant pressure to calculate the repeatability of wing web measurements. Upon recapture the following day, three new measurements of the thickness at the point of injection were taken. The immune response was estimated as the difference between the average initial and average final measurements. The repeatability of this value was calculated from three randomly selected differences between postinjection and preinjection measurements ($r = 0.74$, $F_{[26,54]} = 9.73$, $P < 0.001$).

Nest mites

All ectoparasites introduce saliva into the wound made by their mouthparts. The proteins present in saliva are potent immunogens that elicit strong immune responses, frequently hypersensitive in nature (Wakelin and Apanius 1997). Therefore, inferring that nest mites could affect the female entering the nest box, we recorded the presence/absence of mites and estimated mite abundance following Merino et al. (1998*b*).

Leucocyte count and haemoparasite quantification

A drop of blood was smeared on an individually marked microscope slide, air-dried, fixed in absolute ethanol, and stained with Giemsa stain (1/10 *v*/*v*) for 45 min. To prevent the possibility that the symmetry of the blood smear might lead to a nonrandom distribution of haemoparasites, one half of each smear was scanned at × 200 magnification in search of large, extraerythrocytic parasites such as *Trypanosoma* spp. Small intraerythrocytic parasites, such as *Haemoproteus* spp., were detected using × 1000 magnification (Merino et al. 1997). Intensity of infection by *Haemoproteus* spp. was estimated as the number of infected cells per 2000 erythrocytes (Godfrey et al. 1987).

Leucocytes form the basis of the immune system, and their main function is protection against pathogens. Lymphocytes are central to all acquired immune responses because they specifically recognize individual pathogens (Roitt et al. 2001). The other leucocytes are phagocytes, which mediate innate immune responses but also facilitate acquired immune function (Campbell 1995; Roitt et al. 2001). Slides were examined under × 1000 magnification with oil immersion to assess lymphocytes as a percentage of total leucocytes (lymphocyte proportion). We differentiated and counted different types of leucocytes according to Hawkey and Dennett (1989) and Campbell (1995). Fields with similar densities of erythrocytes were scanned for all cells. Examination was arrested when the first 100 leucocytes had been found, excluding thrombocytes, which normally present an irregular, aggregated distribution. The counts of leucocytes and parasites were highly repeatable (10 blood smears were each scanned twice; $r = 0.91$, 0.90, 0.97, 0.90, 0.91, and 0.84 for lymphocytes, heterophils, basophils, eosinophils, monocytes, and thrombocytes, respectively; $r = 0.96$ and 0.86 for *Haemoproteus* and *Trypanosoma* spp. counts, respectively; all $P < 0.001$).

HSP determination

We determined HSP levels from the blood cellular fraction by means of Western blot. Samples of soluble proteins (70 µg/well) were separated by SDS-PAGE; this amount of total protein is in the linear range of the antibody–antigen response for the species and antibodies studied. The primary monoclonal antibodies used were anti-HSP70 (clone BRM22, Sigma H-5147) diluted 1/5000 and anti-HSP60 (clone LK2, Sigma H-3524) diluted 1/1000. The peroxidase-conjugated secondary antibody was goat anti-mouse specific for the Fc region (Sigma A-0168) at 1/6000 dilution. Protein bands were quantified using 1D image analysis software (Scion Image for Windows, Scion Corp., Frederick, Maryland). Immunoreactivity of the bands was measured in arbitrary units using the following formula: mean optical density × area. For details see Moreno et al. (2002).

Immunoglobulin Y determination

To measure circulating levels of total IgY, the blood serum fraction was analyzed by means of a direct ELISA using peroxidase-conjugated anti-chicken IgY antibodies (Sigma A-9046). The linear range of the sigmoidal curve for this

Table 1. Traits (mean ± SD) of female pied flycatchers, *Ficedula hypoleuca*, at the beginning of the incubation period (8th day after first egg was laid; initial stage) and at day 12 of the nestling period (final stage).

Trait	Initial stage	Final stage	*N*	*t*	*P*
Intensity of *Haemoproteus* spp. infection[a]	1.35±2.15	0.55±1.13	42	3.41	0.002
HSP70[b]	160.64±81.09	143.39±65.73	34	3.87	<0.001
HSP60[b]	97.71±40.06	77.90±29.72	34	5.93	<0.001
Lymphocyte proportion	0.65±0.15	0.56±0.12	42	3.50	0.001
Lymphocyte number[c]	15.47±9.48	8.85±6.79	42	3.45	0.001
Heterophile number[c]	3.17±2.6	3.10±2.54	42	0.12	0.91
IgY[d]	0.79±0.27	0.76±0.16	34	0.78	0.44

[a]Number of infected cells per 2000 erythrocytes.
[b]Mean optical density × area.
[c]Absolute lymphocyte and heterophile numbers (number of cells per 10.000 erythrocytes).
[d]Absorbance (λ = 405 nm).

antibody–antigen response, as well as the optimal serum dilution (1/8000), had been previously determined. Absorbances were measured using a plate spectrophotometer at λ = 405 nm. For details see Martínez et al. (2003).

Sample size and statistical analyses

We included in the sample only females older than 1 year, as the cellular and humoral components of the immune system can vary significantly with age (Ots and Hõrak 1998). We could not obtain enough blood for HSP and IgY analyses from all birds. Therefore, we took only a blood smear from some individuals. In addition, we could not trap all females on the day following PHA injection. This explains the different sample sizes in the correlations between the predictor variables, as we have entered the maximum amount of data available.

All variables were normally distributed (Kolmogorov–Smirnov test for continuous variables, $P > 0.05$) except for intensity of blood parasite infection, which was square-root transformed. Replacing intensity with presence/absence of blood parasites led to similar results and we therefore used the variable intensity, because it more precisely represents the degree of infection. Blots made on different days showed variation due to slight temperature fluctuations or other subtle conditions affecting the Western blot technique. To remove this effect we obtained the residuals of the HSP–blot association in an ANOVA. We used them in all analyses except paired *t* tests for detecting possible seasonal changes between initial and final values in the same individuals, as the initial and final samples for the same individual were analysed on the same blot.

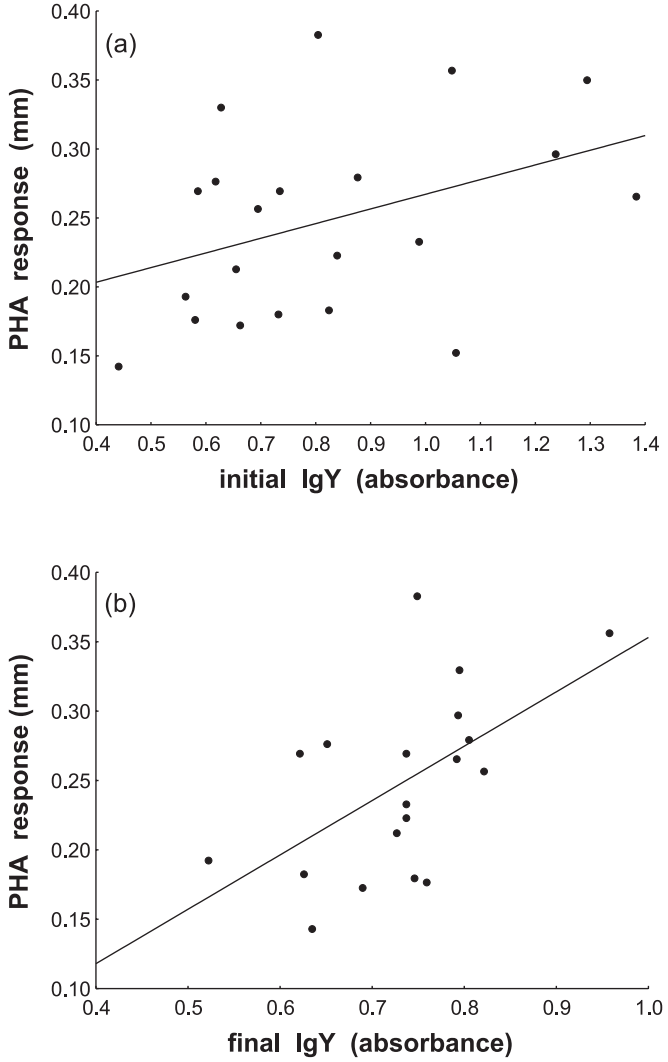
The female IgY level was related to different continuous or discrete parameters with the STATISTICA® GLM module (StatSoft Inc. 2001) to account for all the effects of the independent variables simultaneously. We performed two GLM tests separately to detect possible variations between initial (i.e., beginning of incubation; incubation model) and final (i.e., day 12 of the nestling period; nestling model) associations of traits. In both models, we entered IgY level as the dependent variable and the following indices as predictor variables: intensity of infection by *Haemoproteus* spp., residuals of HSP70, lymphocyte proportion, and PHA response. Although the PHA response was measured on day 12 of the nestling period, we also included it in the incubation model to examine its possible connection with the initial IgY level. HSP60 and HSP70 levels were strongly positively correlated (initial levels, $r = 0.93$, $n = 46$, $P < 0.001$; final levels, $r = 0.87$, $n = 37$, $P < 0.001$). Therefore, HSP60 was left out of the definitive models, as it did not explain as much variance as HSP70 and the use of both stress indicators might have introduced redundancy.

Results

For each individual, the initial and final measurements of the traits were compared using paired *t* tests. As expected, the intensity of *Haemoproteus* spp. infection declined from incubation to nestling feeding (Table 1). Likewise, HSPs and the lymphocyte proportion decreased over the season (Table 1). This decline in the lymphocyte proportion was due to a decrease in the absolute number of lymphocytes (number of lymphocytes per 10.000 erythrocytes) and was not accompanied by an increase in the absolute number of the other common type of leucocyte in birds, namely heterophiles (Table 1). Finally, the circulating levels of IgY did not change over the course of the breeding cycle (Table 1), being individually repeatable ($r = 0.54$, $F_{[33,34]} = 3.304$, $P < 0.001$).

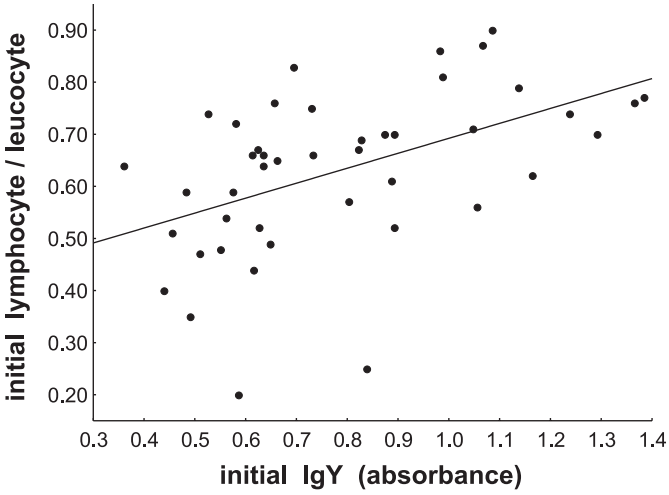
The initial *Haemoproteus* spp. infection intensity was not associated with the other predictor variables (HSP70 residuals, $r = –0.28$, $n = 44$, $P = 0.06$; HSP60 residuals, $r = –0.006$, $n = 44$, $P = 0.97$; lymphocyte proportion, $r = 0.03$, $n = 50$, $P = 0.84$; PHA response, $r = –0.29$, $n = 21$, $P = 0.20$). The initial lymphocyte proportion was neither related to stress protein levels (HSP70 residuals, $r = 0.12$, $n = 44$, $P = 0.44$; HSP60 residuals, $r = 0.24$, $n = 44$, $P = 0.11$) nor associated with PHA response ($r = 0.09$, $n = 21$, $P = 0.71$). PHA response was not related to HSP levels (HSP70 residuals, $r = –0.04$, $n = 21$, $P = 0.86$; HSP60 residuals, $r = 0.03$, $n = 21$, $P = 0.91$). In the incubation model, the IgY level of the female was positively related to the other measures of immune function: PHA response during the nestling stage (Fig. 1*a*), initial lymphocyte proportion (Fig. 2), initial intensity of infection by *Haemoproteus* spp., and HSP70 level (Table 2). The whole model explained 76% of variation (adjusted r^2) in the IgY values and was highly significant ($P < 0.001$). These results show that the initial IgY level was strongly positively related to the other measures of acquired immune function after controlling for the effects of stress and parasites.

Fig. 1. Regression of the PHA response (increase in thickness of wing web at point of injection; mm) on (*a*) initial (i.e., beginning of incubation period) and (*b*) final (i.e., day 12 of nestling period) IgY absorbance (λ = 405 nm) for female pied flycatchers (*Ficedula hypoleuca*).



The final *Haemoproteus* spp. infection intensity was not significantly associated with the other explanatory variables (HSP70 residuals, $r = 0.28$, $n = 37$, $P = 0.09$; HSP60 residuals, $r = 0.15$, $n = 37$, $P = 0.39$; lymphocyte proportion, $r = –0.22$, $n = 66$, $P = 0.07$; PHA response, $r = –0.35$, $n = 22$, $P = 0.11$). The final lymphocyte proportion was not significantly related to stress protein levels (HSP70 residuals, $r = –0.04$, $n = 37$, $P = 0.82$; HSP60 residuals, $r = –0.12$, $n = 37$, $P = 0.47$) or PHA response ($r = 0.11$, $n = 22$, $P = 0.62$). PHA response was not significantly related to HSP levels (HSP70 residuals, $r = 0.10$, $n = 19$, $P = 0.69$; HSP60 residuals, $r = 0.20$, $n = 19$, $P = 0.41$). The pattern of association between IgY level and the explanatory variables included in the models showed a clear change during the breeding cycle, as IgY level was no longer associated with *Haemoproteus* spp. infection intensity, stress indicators, or lymphocyte proportion (Table 3) during the nestling feeding stage. The whole model for this stage was not significant ($P = 0.15$) and explained only 19% of the variance (adjusted r^2). However, female IgY level on its own was significantly positively related to the PHA response (Fig. 1*b*), even when controlling for the effect of other explanatory variables.

Fig. 2. Regression of lymphocyte number as a proportion of total leucocytes on IgY absorbance (λ = 405 nm) at the beginning of the incubation period for female pied flycatchers ($r = 0.49$, $n = 44$, $P < 0.001$).



We found no effect of the other two parasites, mites and *Trypanosoma* spp., on IgY level, PHA response, or lymphocyte proportion (all $P > 0.1$). Breeding variables were also incorporated, but none of them contributed significantly to improving the models (incubation model: laying date and clutch size; nestling stage model: hatching date, brood size on day 12, mean nestling mass on day 12, final female mass, female provisioning rate, and female moult score).

Discussion

As predicted, the intensity of *Haemoproteus* spp. infection decreased over the course of the breeding season. This trend has been detected previously in this population (Sanz et al. 2002). This phenomenon is well known in birds, as haemoparasite infections relapse in spring, probably owing to the reproductive activities of the host, and subsequently enter a chronic phase in which the immune system of the host controls the parasite (Atkinson and Van Riper 1991; Apanius 1998). We also predicted that the initial IgY level and intensity of infection would be positively associated, as previously reported in other studies (Gustafsson et al. 1994; Ots and Hõrak 1998). The absence of association between IgY level and intensity of *Haemoproteus* spp. infection on day 12 of the nestling period could be due to the drop in intensity of infection, as the end of the nestling period coincided with the latent phase of the parasite. Therefore, our results are in accordance with the hypothesis that serum IgY concentration might increase as a consequence of haemoparasite infection. However, although in very general terms the humoral response is the most important defense against extracellular parasites (Roitt et al. 2001), the prevalence and abundance of *Trypanosoma* spp. were, for unknown reasons, not related significantly to IgY levels.

Table 2. Results of the GLM test for the incubation model with IgY as the dependent variable and intensity of *Haemoproteus* spp. infection, HSP70 residuals, lymphocyte proportion, and PHA response as continuous explanatory variables.

Effect	β	df	MS	*F*	*P*
Intercept		1	0.04	2.18	0.16
Intensity of *Haemoproteus* spp. infection[a]	0.69	1	0.56	32.88	<0.001
HSP70[b] residuals	0.38	1	0.18	10.70	0.0052
Lymphocyte proportion	0.46	1	0.27	16.10	0.0011
PHA response[c]	0.59	1	0.41	24.32	<0.001
Error		15	0.02		

[a]Number of infected cells per 2000 erythrocytes.
[b]Mean optical density × area.
[c]Difference in thickness of wing web (mm) before and after phytohaemagglutinin injection.

Table 3. Results of the GLM test for the nestling stage model with IgY as the dependent variable and intensity of *Haemoproteus* spp. infection, HSP70 residuals, lymphocyte proportion, and PHA response as continuous explanatory variables.

Effect	β	df	MS	*F*	*P*
Intercept		1	0.103	14.03	0.002
Intensity of *Haemoproteus* spp. infection[a]	0.23	1	0.007	0.95	0.35
HSP70[b] residuals	0.02	1	0.00005	0.007	0.94
Lymphocyte proportion	0.17	1	0.004	0.50	0.50
PHA response[c]	0.57	1	0.05	6.84	0.02
Error		14	0.007		

[a]Number of infected cells per 2000 erythrocytes.
[b]Mean optical density × area.
[c]Difference in thickness of wing web (mm) before and after phytohaemagglutinin injection.

Similarly, levels of both stress proteins, HSP60 and HSP70, were higher at the beginning of incubation than at the end of the nestling period. Egg formation is an energy-demanding activity for female birds (Ward 1996; Monaghan and Nager 1997; Nilsson and Råberg 2001) owing to the investment of additional energy in the synthesis and mobilization of reproductive materials such as IgYs, carotenoids, hormones, antibacterial factors, and other substances (Carey 1996; Deeming 2002). Therefore, HSP60 and HSP70 might have been produced in response to the stress resulting from oviposition and then declined after this period, being unaffected by further stressors. We expected stress proteins to be produced in response to parasitism (Merino et al. 1998*a*, 2002), but we found no evidence of a relationship with haemoparasites. We found a strong positive relationship between IgY and HSP70 levels in the incubation model. The association between IgY and HSP70 levels could be explained by the key role of HSP70 in antigen processing and presentation and the function of the HSP70 family in immunoglobulin assemblage (Kaufmann 1990; Srivastava 2002). Alternatively, although numerous studies have shown that stress can be immunosuppressive, it has also been reported that acute stressors may enhance immune function and that the subsequent stress response could be an evolutionarily adaptive survival mechanism that may have health-promoting consequences (Cocke et al. 1993; Wood et al. 1993; see Dhabhar 2003 for a review). According to this prediction, an unknown source of stress at the beginning of the reproductive period could have contributed to triggering the high initial level of circulating immunoglobulins. However, it is difficult to speculate which stressor can be considered sufficiently acute to enhance immune function rather than depress it. Our capacity to predict the effects of stressors on IgY levels is marred by our ignorance about the relative importance of infection and stress in activating the humoral component of the immune system.

Lymphocytes as a proportion of total leucocytes declined throughout the season, and this decrease was due to a drop in the absolute lymphocyte number and not to an increase in heterophile concentration. This suggests that lymphocytes may have played a pivotal role against pathogens at the beginning of the breeding period. Immune function may be activated at the onset of egg laying because of daily secretion of IgY into egg yolk (Klasing and Leshchinsky 1999; Saino et al. 2001*b*). This could explain the initial positive relationship between lymphocyte proportion and IgY level. This association may be expected, as B lymphocytes and, indirectly, a significant proportion of T lymphocytes are responsible for the production of IgY. However, we could not distinguish different types of lymphocytes in the blood smears and therefore we have not estimated the relative amounts of each type implicated in the production of IgY.

We found that total IgY levels remained constant throughout the breeding season. Similarly, Saino et al. (2001*b*) found that the postlaying concentration of circulating IgY in barn swallow (*Hirundo rustica* L., 1758) females was similar to the concentration well before laying; the concentration peaked just prior to laying of the first egg. Our study included the nestling stage and also showed that IgY concentration remained very stable, even though parasite infection intensity and physiological stress indicators changed significantly during this period. Hõrak et al. (1998) reported that

IgY level was higher prior to egg laying than in the middle of the nestling period and decreased in the middle of the nestling period in great tits, *Parus major* L., 1758. This also corroborates the idea that there is a peak before oviposition owing to the transfer of maternal IgY to the egg (Saino et al. 2001*b*) and that after this phase the IgY level drops to its previous level. Apanius and Nisbet (2003) observed no clear trend in IgY level from hatching to fledging in common terns, *Sterna hirundo* L., 1758, which is also consistent with our results. However, Hollmén et al. (2001) found in one year a decline in the IgY level during the incubation period in common eiders, *Somateria mollisima* (L., 1758), but they also found that the level remained stable in the following season. The fact that the associations of IgY levels with infection and stress were not detected at the end of the nestling period indicates that IgY levels may reflect physiological processes operating after laying and may not be responsive to seasonal variation in stress and health.

The most striking result was that the cellular response to PHA was positively correlated with the final IgY level. Owing to the correlative nature of this study, the exact mechanisms that generated this relationship remain undetermined. However, we can propose two hypothetical mechanisms that could result in such covariation. First, it is plausible that IgY levels and the PHA response are affected by the same physiological factors, resulting in their indirect association. For example, the immune system is known to be condition-dependent, with responsiveness being directly associated with body condition and ingestion of protein-rich food and essential nutrients (Lochmiller et al. 1993; Møller et al. 1998; Alonso-Alvarez and Tella 2001). Hence, the condition dependence of functionally different components of the immune system may cause them to respond in a similar fashion. Another possibility would be a common trade-off of different immune parameters with other organismic functions such as growth (Fair et al. 1999; Soler et al. 2003). For example, Saino et al. (2001*a*) found that the total immunoglobulin concentration and the PHA response were larger in late-hatched barn swallow nestlings. Second, the theory of general immune defense predicts that high-quality individuals maintaining an efficient T-cell-mediated immune response also sustain superior humoral immune function (Schmid-Hempel and Ebert 2003). Some studies seem to support this idea. For example, Heller et al. (1992) selected chickens that exhibited simultaneously enhanced cellular and humoral responses against unrelated antigens. Our results are partially in accordance with the general immune defense hypothesis. That the PHA response was also positively correlated with the initial IgY level was probably due to the seasonal inertia of immunoglobulin levels, which are known to remain high for weeks after infection (Ots and Hõrak 1998). However, Johnsen and Zuk (1999) showed the opposite trend in captive jungle fowl, *Gallus gallus* L., 1758, suggesting a trade-off between both arms of the acquired immune system. The fact that the jungle fowl were previously infected with an intestinal parasite that normally attacks this species and causes harmful effects could explain in part the discrepancies with this study, as the birds were induced to fight against a natural enemy, presumably being unable to mount efficient cellular and humoral immune responses simultaneously. It is also feasible that conditions of captivity might have produced a different pattern of association between the immune traits than would be observed in a wild population. We are aware of no previous study of adult wild birds documenting positive correlations between total immunoglobulin levels and the cell-mediated response. Nevertheless, other authors have measured the humoral response by means of a challenge technique and have reported contradictory results regarding these correlations. For instance, Møller et al. (2001) showed that the PHA response and the humoral immune response to sheep red blood cells were positively correlated across closely related species of hirundine birds. In contrast, Parmentier et al. (1993) and Gonzalez et al. (1999) reported the opposite pattern in captive birds. Therefore, we concur that a single measure of an individual component may not reflect an overall immune strategy (Norris and Evans 2000; Blount et al. 2003) and that the complex actions of the immune system are context-dependent, making generalizations difficult unless other variables involved, such as intensity of parasitization and stress, are controlled (Zuk and Johnsen 1998).

In conclusion, this is the first study, to our knowledge, reporting that the level of total circulating IgY is positively associated with the PHA response in adult wild birds and that this relationship remains stable in different stages of the breeding cycle. Other traits involved, such as intensity of haemoparasite infection and physiological stress, exerted a strong effect on IgY level at the beginning of the incubation period. The hypothetical mechanisms mediating these correlations have been discussed. Our results add to the growing literature on the connections between immune traits, parasites, and stress, and may provide new insight into the intricate adaptive immune responses in natural populations.

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