

A trade-off between two resource-demanding functions: post-nuptial moult and immunity during reproduction in male pied flycatchers

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Summary

1. Costs of reproduction might be mediated by a resource-allocation trade-off between immunity and reproductive effort. Recent studies have suggested that the moult–breeding overlap observed mainly in males may affect this trade-off. In order to test whether increased investment in immunity may affect the onset of moult or reproductive output in a species with moult–breeding overlap, we immunized breeding male pied flycatchers *Ficedula hypoleuca* (Pallas) with a non-pathogenic antigen (sheep red blood cells) in the first week post-hatching and compared their moult score, parental effort and reproductive success shortly before fledging with that of control males injected with sterile phosphate-buffered saline.

2. Immunized males provisioning nestlings delayed the onset of post-nuptial primary moult compared to control males. The experimental activation of the immune defence of males affected their stress levels (heterophile/lymphocyte ratio, heat-shock proteins) on day 13 after chicks hatched. An activated immune response tended to depress reproductive output, although the experimental design was not efficient enough to find a significant effect because the manipulation was not performed early enough in the nestling period.

3. The results suggest that experimental activation of the males' immune system decreased the resources necessary for initiating post-nuptial moult. The trade-off between immunity and moult onset may imply costs of delayed moult in cases of infection and may interact with reproductive immunosuppression in cases of moult–breeding overlap. Moult, immunity and reproduction are locked in a three-way interaction which may markedly affect avian life histories.

Key-words: H/L ratio, HSP, immunity, moult–breeding overlap, moult, pied flycatcher.

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Introduction

One physiological mechanism underlying the cost of reproduction is the trade-off between reproductive effort and immunity (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Schmid-Hempel 2002). Several studies on birds have shown that some components of the immune system (antibody production, cell-mediated immunity) may be affected adversely by increased reproductive effort (Deerenberg *et al.* 1997; Moreno,

Sanz & Arriero 1999; Cichón, Dubiec & Chadzińska 2001; Moreno *et al.* 2001; Saino *et al.* 2002; Bonneaud *et al.* 2003; but see Ilmonen *et al.* 2003). The reciprocal experiment has met with contrasting results (Williams *et al.* 1999; Ilmonen, Taarna & Hasselquist 2000). Thus, there is at present some empirical ground to sustain a claim for the ecological relevance of reproductive effort–immunity trade-offs in birds. Recently, Moreno *et al.* (2001) have shown that for female pied flycatchers *Ficedula hypoleuca*, but not for males, the trade-off between parental effort and immunity could underlie the cost of reproduction in this migratory species. However, they have suggested that for male pied flycatchers their frequent moult–breeding overlap may offset this trade-off (Moreno *et al.* 2001).

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An energetically costly activity which may coincide with breeding is avian moult, as many birds already initiate the post-nuptial moult while raising young (Jenni & Winkler 1994). This moult–breeding overlap represents a conflict in resource allocation between two energy-demanding processes: reproduction and post-nuptial moult (Murphy 1996; Hemborg & Lundberg 1998). There is also a tendency for males of several species to initiate moult earlier than females, and thus increase the period of moult–breeding overlap (Hemborg 1999; Sanz 1999; Hemborg, Sanz & Lundberg 2001). This fact suggests that the two sexes have different control mechanisms for the allocation of resources to reproduction and to moult (Hemborg 1998; Sanz 1999). A trade-off between investments in reproduction and moult has been proposed as another possible mechanism underlying the cost of reproduction (Nilsson & Svensson 1996; Dawson *et al.* 2000).

In at least some bird species, moult processes have been associated with increases in organs of the immune system, such as the thymus or spleen (Ward & D’Cruz 1968; Silverin *et al.* 1999). This hypertrophy may result from a need for increased lymphocyte production associated with expansion of circulation (Ward & D’Cruz 1968) or to the need to avoid infection of wounds resulting from the breakage of growing feather follicles (Silverin *et al.* 1999). In an aviary study, Nava, Veiga & Puerta (2001) have shown that moult induced an increase in basophils and monocytes in juvenile and adult house sparrows (*Passer domesticus* L.), suggesting that moult imposes immunological challenges for this species. From poultry studies, we know that moulting mature hens infected experimentally with cestodes are more resistant to primary and secondary infection than non-moulting hens (Gray 1982), and that proglottis production by several cestodes ceases when hens started to moult (Sawada 1960). From this evidence we may conclude that large components of the energy budget devoted to moult could therefore be associated with a higher, not lower immune response. In general, this increase in immune response associated with post-nuptial moult would make it difficult to detect trade-offs between reproductive effort and immunity particularly in males with moult–breeding overlap (Moreno *et al.* 2001). If moult activates the immune system, any challenge by pathogens may result in a fast and strong immune response which may detract resources from moult itself, and from reproduction in cases of moult–breeding overlap.

The aim of the present study was to examine whether an experimentally activated immune response of male pied flycatchers while feeding nestlings would result in a delay in the onset of post-nuptial moult. The experiment also aimed secondarily at exploring possible fitness costs in terms of reduced reproductive success due to depressed male parental effort. We investigated this physiological trade-off by challenging breeding males with a non-pathogenic antigen (sheep red blood cells; SRBC) to stimulate a specific immune response.

Materials and methods

The study was conducted in 2001 using a pied flycatcher population breeding in nestboxes in central Spain (for details about the study species and long-term study see Sanz *et al.* 2003). Pied flycatchers perform a complete moult on the breeding areas before autumn migration (Lundberg & Alatalo 1992). In several populations, moult has been found to overlap with the feeding of nestlings more often in males as compared to females (Lundberg & Alatalo 1992; Hemborg *et al.* 2001).

On the day after eggs hatched, pairs of nests with the same hatching date were created for the experiment. For each pair of nests the experimental treatment (control, immunized) was assigned randomly. On day 6 after hatching of the chicks, males were captured for the first time, banded with numbered rings (ringing permit by regional authorities), weighed to the nearest 0.1 g, and sampled for blood (see below). Males were aged as yearling or older. In the present study, only monogamous males with a minimum age of 2 years that were not moulting on their first capture were included in the experiment. Thus, the actual sample of males included did not allow pairwise tests based on initial assignments. Hatching date and brood size on day 6 after hatching did not differ between experimental treatments (hatching date: $t_{28} = 0.23$, $P = 0.82$; brood size on day 6: $t_{28} = 0.91$, $P = 0.37$). Moult–breeding overlap differs between yearling and older males (Hemborg *et al.* 2001), and the onset of moult could not be manipulated for males already moulting on first capture. Immunized males were injected intraperitoneally with 0.1 mL of a 10% suspension of washed sheep red blood cells (SRBC; Sigma R-3378) in sterile phosphate-buffered saline (PBS). SRBC is a non-pathogenic T lymphocyte-dependent complex antigen used commonly in immunological studies (Hay & Hudson 1989). Control males received a placebo injection (0.1 mL PBS). Males were captured for the second time on day 13 after hatching of the chicks, weighed to the nearest 0.1 g, and resampled for blood (see below). Although we have not performed haemagglutination tests on bird sera to verify directly that the SRBC treatment elicited increased investment in immunity, some recent studies have shown that this procedure induced a measurable humoral response in 96.4% (Williams *et al.* 1999), 87% (Cichón 2000) and 68% (Ardia, Schat & Winkler 2003) of the free-living breeding individuals sampled. Obviously, the undetected response by the rest of individuals sampled in these studies does not mean that these birds did not activate their immune system after injection of large volumes of a foreign organic substance, but that the haemagglutination assay may not be sensitive enough to measure low humoral responses. Thus, the assumption of the study is that the SRBC treatment elicited a response similar as in other studies of free-living birds. To determine if there was a physiological response to the experimental treatment we have used indices of stress (see below).

All chicks were weighed on days 6 and 13 with a Pesola spring balance to the nearest 0.1 g. Nestling growth between days 6 and 13 and fledging success (percentage of hatchlings that resulted in fledged young) were considered as partial measures of reproductive success. On days 6 and 13, all captured males were inspected for wing moult to determine a moult score (see Ginn & Melville 1983). The moult status of males on those days was presented as either moulting (moult score > 0), or non-moulting (moult score = 0).

PARENTAL HEALTH AND STRESS

Lymphocytes and heterophils are the most abundant types of leucocytes in avian blood (Campbell 1995). Davison, Rowell & Rea (1983) and Gross & Siegel (1983) described a ratio calculated from the proportions of heterophils and lymphocytes present in the circulation of domestic fowl as a measure of stress. The heterophils/lymphocyte (H/L) ratio has now become accepted widely as a reliable and accurate physiological indicator of the stress response (Maxwell & Robertson 1998). To estimate H/L, a drop of blood obtained from the brachial vein of males on days 6 and 13 after their chicks hatched was smeared on individually marked microscope slides. A detailed description of the method and protocol followed to estimate H/L ratios is given elsewhere (Moreno *et al.* 2002a). The H/L ratio has shown a low measurement error in field situations (Ots, Murumägi & Horak 1998). Given the presence of parasites (*Trypanosoma* spp., *Haemoproteus balmorali* Peirce) in peripheral blood of male pied flycatchers (Sanz *et al.* 2002) and the effect of haematzoa on heat shock protein (HSP) levels (Merino *et al.* 2002), smears were screened in search of haematzoa following methods described in Merino, Potti & Fargallo (1997). Males were classified as being either infected or non-infected.

On day 13 after chicks hatched, blood (80–100 µl) was sampled from the brachial vein of males into heparinized capillary tubes to use for centrifugation for HSP analyses. HSPs, also called heat stress proteins, are evolutionary highly conserved molecules that help cells in recovering from stress situations by correcting misconfigurations in protein structures (Morimoto 1991). A detailed description of the HSP analysis and protocol followed is given elsewhere (Merino *et al.* 1998; Moreno *et al.* 2002a). HSP (HSP70 and HSP60) levels were expressed as arbitrary units of optical density per area. All samples were processed and analysed at the same time in two separate blots. Because there are differences between blots (see below), we have included the blot as a factor in all the analyses presented.

PROVISIONING RATES

Provisioning rates by both parents were recorded during 1-h periods between 10 : 00 h and 16 : 00 h on day 12 after hatching of their broods ($n = 23$ pairs). This day represents the plateau in provisioning rates of

almost-fledged young (Lundberg & Alatalo 1992). Nests were filmed with a video camera in order to count the number of feeding visits performed by both mates. The video camera was placed 5–10 m away from the nestbox. There were no significant differences among experimental groups in the time of day that the nests were filmed ($t_{21} = 1.48$, $P = 0.15$).

DATA ANALYSES

To test the changes in some variables (male body mass, nestling body mass, H/L ratio) between experimental treatments, repeated-measures ANOVAs were performed. To allow the use of parametric tests, the H/L ratios were logarithmically transformed and fledging success was arcsine square root-transformed. The sample sizes differ between analyses because in some cases the quality of the blood smears did not allow their examination.

Results

MALE MOULT–BREEDING OVERLAP, HEALTH AND STRESS PARAMETERS

The probability of starting the moult while feeding nestling between days 6 and 13 after chicks hatched differed significantly between experimental treatments (Fisher's exact test, $P = 0.042$). While 33.3% of control males ($n = 15$) presented a moult–breeding overlap on day 13 after chicks hatched, none of the immunized males ($n = 15$) presented this overlap.

Male body mass did not differ between experimental treatments (repeated-measures ANOVA; $F_{1,27} = 0.98$, $P = 0.33$), and did not differ between days 6 and 13 ($F_{1,27} = 2.99$, $P = 0.10$). Moreover, there was no significant experimental treatment by brood age interaction effect ($F_{1,27} = 0.79$, $P = 0.38$). The percentage of infected males by blood parasites did not differ between experimental treatments on days 6 or 13 (all Fisher's exact tests, $P > 0.10$). The H/L ratio of males did not differ between experimental treatments (repeated-measures ANOVA; $F_{1,24} = 3.60$, $P = 0.07$) and differed significantly between days 6 and 13 ($F_{1,24} = 13.42$, $P = 0.001$), with lower values on day 6 (Fig. 1). There was, however, a significant two-way interaction between experimental treatment and brood age ($F_{1,24} = 4.65$, $P = 0.041$), owing to a significant increase in the levels between days 6 and 13 in immunized males (Fig. 1).

The level of HSP60 of males on day 13 after chicks hatched did not differ between experimental treatments when the effects of blot were controlled for (two-way ANOVA; factor experimental treatment, $F_{1,27} = 0.05$, $P = 0.83$; factor blot, $F_{1,27} = 14.65$, $P < 0.001$). However, the level of HSP70 of males tending 13-day-old nestlings differed significantly between experimental treatments when the effects of blot were controlled for (two-way ANOVA; factor experimental treatment, $F_{1,27} = 5.22$, $P = 0.030$; factor blot, $F_{1,27} = 29.64$, $P < 0.001$). Levels of HSP70 on day 13 after chicks hatched were

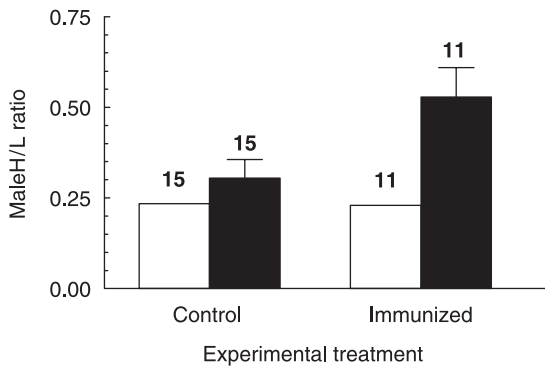


Fig. 1. Mean H/L ratios of males on days 6 (open bars) and 13 (black bars) after hatching according to experimental treatment. Sample sizes are given above of the bars. The error bars represent the SE of the mean.

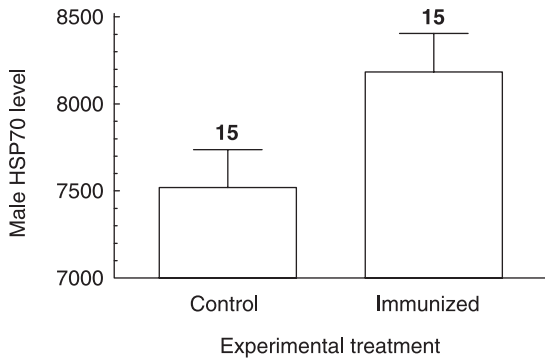


Fig. 2. Mean HSP70 levels (optical density per area) of males on day 13 after hatching according to experimental treatments. Sample sizes are given above of the bars. The error bars represent the SE of the mean.

significantly higher in immunized than in control males (Fig. 2). The level of HSP70 of males on day 13 after hatching was correlated positively with their H/L ratio ($r_{24} = 0.41$, $P = 0.036$).

BREEDING PERFORMANCE AND PROVISIONING RATE

Fledging success did not differ between experimental treatments ($t_{28} = 0.73$, $P = 0.47$). Nestling body mass did not differ between experimental treatments (repeated-measures ANOVA; $F_{1,28} = 2.06$, $P = 0.16$), and differed significantly between days 6 and 13 ($F_{1,28} = 1400.14$, $P < 0.001$). Moreover, there was no significant experimental treatment by brood age interaction effect, although the interaction was close to significant ($F_{1,28} = 3.76$, $P = 0.06$). Nestling mass growth between days 6 and 13 tended to be smaller in the immunized treatment compared to the control treatment.

Provisioning rates on day 12 after hatching did not differ between parental sexes or between experimental treatments, when the effect of brood size on that day was controlled for (ANCOVA; factor parental sex, $F_{1,44} = 2.65$, $P = 0.11$; factor experimental treatment, $F_{1,44} = 1.30$, $P = 0.26$; covariate brood size, $F_{1,44} = 12.33$, $P = 0.001$).

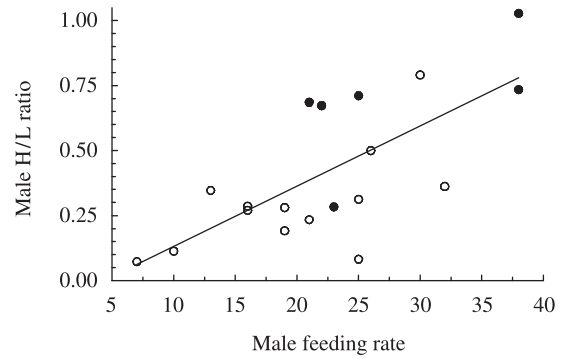


Fig. 3. Relationship between male H/L ratios on day 13 after their chicks hatched and male feeding rates per hour on day 12 after hatching of their brood (open circles: control treatment; black circle: immunized treatment).

Male and female feeding rates were correlated positively ($r_{21} = 0.64$, $P = 0.001$). Finally, male feeding rate on day 12 after hatching was related positively to their H/L ratio ($r_{17} = 0.70$, $P = 0.001$; Fig. 3).

Discussion

In the present experimental study, we investigate the phenotypic costs for males of mounting an immune response while feeding nestlings by examining trade-offs between immunity and the onset of post-nuptial moult or breeding performance, respectively. Given that for male pied flycatchers parental effort interacts with immunity depending on moult status (Moreno *et al.* 2001), we explored whether for males an experimentally activated immune response interacts with post-nuptial moult, as trade-offs can be revealed only through experimental manipulation. For female pied flycatchers, but not for males, a trade-off between effort expended in provisioning young and immunity has been previously shown in the same population (Moreno *et al.* 2001). In this population, males moult regularly during nestling feeding and initiate their moult earlier in the breeding cycle when compared to females (Sanz 1997; Hemborg *et al.* 2001). The ultimate factors explaining this sexual difference in moult remain unclear (Hemborg 1998; Hemborg & Merilä 1998). The somatic investment in a new plumage can be seen as an annual repair mechanism, and in some situations it may be more important for one of the sexes to allocate resources to moult than to the current brood. These sexual differences in timing of moult should be taken into account when determining the physiological mechanism of the cost of reproduction in passerine birds.

The results indicate that breeding pied flycatcher males (more than 2 years old), which were challenged with a non-pathogenic antigen some days after their chicks hatched, presented a delayed onset of post-nuptial moult compared to control males. A similar experiment, although not dealing with natural moult processes, was presented previously by Ilmonen *et al.* (2000). During the incubation period they removed one of the outermost

tail feathers of control and immunized females, showing that immunized females were less likely to regrow removed tail feathers than control ones (Ilmonen *et al.* 2000). Their and our results suggest the existence of a trade-off between two self-maintenance processes such as immunity and feather renewal. However, the present study is the first to show this trade-off explicitly for natural post-nuptial moult in males, which is the sex that presents a moult–breeding overlap more frequently.

The most straightforward explanation for our result is that experimental activation of the males' immune system decreased the resources necessary for the onset of post-nuptial moult. Therefore, we present the first experimental evidence for a link between allocation of resources to the immune system and to the natural onset of avian post-nuptial moult. If our experimental challenge simulates a pathogen attack, this study suggests that in some situations it may be more important for male passerines to allocate energetic or nutritional resources to the immune response than to the onset of post-nuptial moult. This competition for resources between moult and immunity would add a further trade-off to the life-history implications of immunity (Sheldon & Verhulst 1996; Schmid-Hempel 2002). Alternatively, males would need to activate their immune system before moulting (Moreno *et al.* 2001), and in cases in which the immune system has been challenged previously by pathogens, no sufficient resources of the immune system would be available to devote to the activation necessary for post-nuptial moult. It has been shown that post-nuptial moult in wild passerines is associated sometimes with an increase in the size of immune organs or in abundance of immune cells (Ward & D'Cruz 1968; Silverin *et al.* 1999; Nava *et al.* 2001). This interpretation is based on a potential trade-off within the immune system between defence against pathogens and body repair.

Neither fledgling body mass nor fledging success differed significantly between experimental treatments. This fact indicates that control and immunized males, or their mates, were able to adjust their effort to brood demand. This suggestion is confirmed in the present study by the fact that provisioning rates did not differ between experimental treatments. However, the number of nests recorded was small and may not provide sufficient statistical power to detect subtle effects. An alternative explanation could be that the methods and the experimental design in this study were not efficient enough to find such effects. Males were immunized on day 6 after their chicks hatched, and this may be a late timing for treatment if the intention is to explore effects of immunization on reproductive performance. Because this late timing ignores the early nestling phase when the provisioning of nestlings is mainly the responsibility of males, the net effect of the treatment on reproductive output is likely to be small. In fact, immunized males had the same fledging success but nestling growth tended to be smaller in the immunized treatment. Therefore, the results partially support the prediction that an activated immune response may depress

reproductive output (Ilmonen *et al.* 2000; Råberg *et al.* 2000; but see Williams *et al.* 1999).

Immunized and control males did not differ in condition (body mass), stress (H/L ratio) or in the prevalence of blood parasites on day 6 after chicks hatched, so we have no evidence that health was different between experimental treatments. On day 13, these males did not differ in condition or in the prevalence of blood parasites. However, immunized males tending 13-day-old nestlings presented higher levels of some stress indicators (H/L ratio, HSP70). The H/L ratio has been shown to be a reliable stress indicator in field situations (Ots *et al.* 1998; Moreno *et al.* 2002a). On the other hand, laboratory studies have detected that immunological challenges increase the concentration of specific stress proteins (McComb & Spurlock 1997), indicating that there is a link between the immune response and stress protein synthesis. In the present study, more than 2-year-old males that were challenged with SRBC after their chicks hatched presented higher levels of HSP70, but not of HSP60. The results suggest that the experimental procedure involved an increased stress due possibly to an activation of the immune system of the immunized males. On the other hand, we have shown that parental stress was associated positively with provisioning rates at the nest. This confirms our previous results obtained with breeding female pied flycatchers in the same population (Moreno *et al.* 2002b). Ilmonen *et al.* (2003) have recently found a similar association between experimentally manipulated parental effort and stress hormone levels in male pied flycatchers breeding in south-west Finland.

Given the widespread occurrence of moult–breeding overlap in birds (Jenni & Winkler 1994), the main consequence of the trade-off between moult and immune defence against pathogens is that infections may delay the onset of annual moult. The costs of delayed moult may be related to the time constraints faced by migrants and to the problems of overlapping premigratory fat deposition with moult (Lindström, Daan & Visser 1994). This may lead to compressed moult, which in turn may affect plumage quality (Nilsson & Svensson 1996; Dawson *et al.* 2000). The costs of arresting moult to avoid overlap (Hyytiä & Vikberg 1973) in terms of flight efficiency may also affect survival prospects. More studies are needed to determine which factors are affecting the onset of post-nuptial moult in breeding males, given its potential effects on fitness (Hemborg & Merilä 1998). The immunity–moult trade-off would affect tropical birds especially, which show a high rate of moult–breeding overlap (Foster 1974) and are subjected to stronger parasite pressures (Møller 1998). The trade-off between immunity and post-nuptial moult should also be considered when trying to determine the physiological mechanisms underlying the cost of reproduction (Moreno *et al.* 2001). If reproduction leads to immunosuppression and moult to activation of the immune system, pathogens could affect adult survival prospects by benefiting from the first effect and by delaying moult

through the second effect. Moulting, immunity and reproduction appear to be locked in a complex three-way interaction which may profoundly affect avian life histories and whose mechanisms remain to be elucidated.

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