

Cost of reproduction, T-lymphocyte mediated immunocompetence and health status in female and nestling barn swallows *Hirundo rustica*

Péter L. Pap and Róbert Márkus

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We investigate the trade-off between reproductive effort, health status and T-lymphocyte acquired immunity in female and nestling barn swallows *Hirundo rustica* using a brood size manipulation experiment. Maternal and total feeding effort increased with experimental brood size. Parents did not fully compensate for the increased food demand of the enlarged broods and as a consequence the per capita feeding rate of nestlings decreased with increasing experimental brood size. Body mass and a measure of T-cell mediated immunity in 12 days old nestlings also decreased with increasing experimental brood size. Different leucocyte concentrations and the heterophile/lymphocyte ratio – an index of stress – of nestlings did not change in relation to experimental brood size, suggesting that within brood competition did not affect stress to nestlings. The brood size manipulation had a significant effect on maternal T-cell mediated immunity, measured by the phytohemagglutinin skin test, but not on maternal body mass, haematocrit or differential or total white blood cell counts. Our results seem to support the prediction that under mild work stress females respond first by reducing the energetically expensive acquired immunity. Different leucocyte types and the heterophile/lymphocyte ratio appear less sensitive to parental workload.

P. L. Pap (correspondence), Behavioural Ecology Research Group, Department of Evolutionary Zoology, University of Debrecen, H-4010 Debrecen, Hungary, and Department of Zoology, Babes-Bolyai University, R-3400 Cluj Napoca, Clinicilor street no. 5-7, Romania. E-mail: peterpl@hasdeu.ubbcluj.ro. R. Márkus, Biological Research Center of the Hungarian Academy of Sciences, Institute of Genetics, Lab. of Molecular Immunology, H-6726 Szeged, Temesvári street no. 62, Hungary.

An increase in parental activity has been shown to increase parasite infestation, probably due to a reduction in immune function and health status of breeding individuals (e.g. Richner et al. 1995, Nordling et al. 1998). The immune system consists of two parts, the innate and the acquired immune system. Innate immunity acts as a primary defense mechanism during the initial stages of infestation. Heterophils, the most numerous granulocytes in peripheral blood, and monocytes are known as a primary means of controlling extracellular bacterial infestations and macro-parasites (Gergely and Erdei 1998). Natural killer cells act primarily during intracellular bacterial and viral infections. The more specific acquired immunity acts more

effectively against various pathogens, including viruses, bacteria and ectoparasites (Wakelin 1996, Gergely and Erdei 1998), but with a high metabolic cost due to rapid cell proliferation (Apanius 1998).

Previous studies of the effects of parental effort on parental health status have been of two kinds. First, a study of the great tit *Parus major* showed an increase in the number of heterophils (derived from the innate immune system) and a decrease in lymphocyte numbers (derived from both the innate and the acquired immune system) in peripheral blood of the great tits, when the broods were experimentally increased (Ots et al. 1998). The elevated heterophile concentration and a reduction of the lymphocyte number in the blood due to altered

parental effort could indicate either elevated stress, or it may express a depression of innate immunity. Second, other studies, based on responses to challenges of the acquired immune system by a novel antigen, have also investigated the effects of altered parental effort on the health status of reproducing birds. These studies show that cell mediated and humoral immunity can be suppressed by increased parental activity (Deerenberg et al. 1997, Nordling et al. 1998, Moreno et al. 1999). However, our knowledge of the effects of experimentally altered reproductive efforts on the immunocompetence of free living birds is limited (e.g. Nordling et al. 1998, Moreno et al. 1999).

The physiological background of the trade-off between parental effort and immunocompetence is two-fold. First, due to the cost of the immunological defence, both traits compete for the same resources, and as a consequence an increase in reproductive effort should result in suppression of the immune system. Second, an increased locomotory activity, like parental effort, results in increased work stress, which through neurological and endocrine linkage inhibits the immune system (Besedovsky and del Rey 1996, Wingfield et al. 1997, Apanius 1998).

In this study we used several health-state and immunological tests to investigate the physiological background responsible for the trade-off between reproductive effort and immunocompetence in female barn swallows *Hirundo rustica*. We did this by manipulating parental effort by increasing or decreasing the brood size with one nestling. To assess the stress level of females we determined the differential leucocyte number and the heterophile/lymphocyte (H:L) ratio in the peripheral blood based on blood smears. Furthermore, a change in differential leucocyte types could indicate the effect of our brood size manipulation on the innate immunity of the birds. The activity of the acquired immune system (henceforth immunocompetence) of the birds was assessed based on the activity of the T-cells towards to an antigen injected in the left wing web. Based on studies on poultry and wild birds (reviewed in Goto et al. 1978, Alonso-Alvarez and Tella 2001), we assumed that a strong response (inflammation) of the bird to an antigen reflects the activity of the T-cells and according to this, the stronger the response, the more immunocompetent the individual.

Just as in the adults, the health status and immunocompetence of the nestlings in increased broods can be suppressed by food shortage (Lochmiller et al. 1993, Saino et al. 1997a). Furthermore, in experimentally enlarged broods, due to within-brood competition, stress can render nestlings even more susceptible to parasites via the negative effect of the stress hormones on the immune system. Accordingly, we investigated the effect of our brood-size manipulation on the condition, health state and immunocompetence of the nestlings (see review by Alonso-Alvarez and Tella 2001).

Study area and methods

General

The study was conducted in 2000 and 2001 in three colonies near Balmazújváros (47°37'N, 21°21'E), eastern Hungary. The barn swallow breeds in colonies of up to 15 pairs, inside buildings. Nests were checked systematically from 20 April, and date of clutch initiation, clutch size and number of fledged young was noted. Nestlings were weighed 12 days after hatching (hatching day = 0), and their tarsus and wing length were measured. Adult birds were captured with mist-nets during the pre-laying period and while feeding 12-day old nestlings.

Manipulation of reproductive effort

We performed a brood size manipulation experiment by transferring nestlings between broods that hatched on the same day and with no more than a two nestlings difference in brood size among pairs of broods. We exchanged the nestlings between broods on the day after hatching (for a detailed description of the method, see Saino et al. 1997a). By this manipulation we created two experimental groups, where the nests were either randomly increased or decreased by one nestling. In the third group (control), half of the nestlings were taken out the day after the nestlings hatched for a period of 10 minutes (the average time needed for the exchange of nestlings between manipulated broods), in order to control for the effect of the manipulation. There was no difference in original clutch size (two-way ANOVA with year and groups as factors, $F_{2,75} = 0.42$, $P = 0.66$) or laying date ($F_{2,75} = 0.16$, $P = 0.85$) among groups after controlling for the effect of year. To control for the effect of year, we included year as a factor in the subsequent analyses. There was no significant difference among experimental groups in body mass, tarsus length, wing length and tail length of females ($P > 0.1$).

We recorded provisioning rates of males and females on days 10–12 after hatching for 1 h between 7.00 and 12.00, the time of day that feeding rate is highest. We recorded feeding rate only on sunny days and whenever possible on the same day for the pairs in a group of three manipulated nests.

Health status and immunocompetence

We collected blood samples (80 µl) from the brachial vein of breeding female barn swallows, 12 days after the hatching of nestlings, in heparinized capillary tubes in order to assess haematocrit. After collection, the blood samples were stored in a cooling box until centrifugation for 10 min at 10,000 r.p.m. Haematocrit is a serological variable that can be regarded as an indicator

of metabolic activity for the period preceding the date of blood sampling, due to the effect of dehydration and increased erythropoiesis on haematocrit (Carpenter 1975). Birds raise their individual haematocrit levels in response to intense locomotory activity (Palomeque and Planas 1978).

For estimating the number and proportion of different leucocyte types, a drop of blood was smeared on a microscope slide, air-dried, fixed in methanol and stained with May-Grünwald and Giemsa. All smears were examined by the same person (RM) at $\times 1000$ magnification and the proportion of different types of leucocytes was assessed on the basis of the examination of 100 leucocytes. The number of different white blood cells was expressed per approximately 10,000 erythrocytes. The repeatability of leucocyte counts estimated by rescanning 16 smears was significantly high ($r = 0.86$, $F = 13.5$; $r = 0.74$, $F = 6.8$ and $r = 0.86$, $F = 11.7$ for heterophile, lymphocyte and total white blood cell (WBC) counts, respectively, $df = 1,15$, $P < 0.001$) or intermediate ($r = 0.51$, $F = 3.1$ and $r = 0.54$, $F = 3.3$ for monocytes and H:L ratio, respectively, $df = 1,15$, $P < 0.05$), while eosinophils and basophils did not show significant repeatability. In the subsequent analyses we excluded eosinophils, basophils and monocytes due to their low repeatability and low proportion in the blood. Total leucocyte counts and the number and level of different leucocyte types are considered to be indicators of health status, parasitic infestation and stress (Davis 1981, Ots et al. 1998). Leukocytosis (increase in the number of leucocytes) is most commonly due to infectious diseases (Davis 1981, Fudge 1989). Heterophils are non-specific phagocytic immune cells which are important components of the innate immune system together with monocytes, basophils, eosinophile granulocytes and natural killer cells. They act in the primary defense against parasites, during stress and various inflammations. The H:L ratio is widely used as an indicator of stress in poultry (Maxwell 1993), and it is known to increase under various stressful conditions. The adaptive immune system is comprised of the lymphocytes, highly specific immune cells that can be classified into two main types: T-lymphocytes and B-lymphocytes. The T-lymphocytes are considered to be the main part of cell-mediated immunity and comprise around 70% of the lymphocytes. Lymphocyte concentration in peripheral blood can be an indirect indicator of adaptive immune system activity, while the T-lymphocyte response to an antigen measure the cell mediated immune response of an individual (see below).

We assessed the cell-mediated immunity of breeding female barn swallows in response to brood size manipulation by T-lymphocyte activity to an antigen (phytohemagglutinin, Sigma, L-8754) injected intradermally in the wing web on day 12 after hatching (Saino et al. 1997a). The phytohemagglutinin skin test is considered a useful method for the evaluation of the thymus-de-

pendent function (Goto et al. 1978) and has been used as an estimate of T-lymphocyte cell mediated immune activity (Goto et al. 1978, Cheng and Lamont 1988, Alonso-Alvarez and Tella 2001). The immunization protocol was as follows: female birds were captured immediately before sunset at the nest, and after the measurements and blood samples had been taken, one of us (PLP) injected 0.2 mg PHA diluted in 0.04 ml of phosphate buffered saline (PBS) in the right wing web. The activity of T-lymphocytes was estimated by measuring the increase in inflammation of the wing web between injection and 12 hours after immunization with a pressure sensitive spessimeter (accuracy 0.01 mm). After the immunization protocol, the birds were put in a cloth bag until the next morning when the birds were re-measured for the immune response. None of the birds deserted their nests due to the manipulation. The nestlings were injected and measured too, but the immunization protocol differed from that of the adults by measuring the increase of the wing web 24 hours after immunization. We consider the 12 h immunization protocol on adult birds reliable in measuring the immunocompetence of the birds, since in a study on domestic chickens *Gallus gallus* the T-cell immunocompetence of birds was highly repeatable within individual birds, between measurements at 12 and 24 hours after immunization (Goto et al. 1978). We used the simplified protocol proposed by Smits et al. (1999) which avoids the injection of PBS in the opposite wing-web as a control. The advantage of this method is a decrease in handling time and a reduction in the probability of errors, by decreasing the coefficient of variation due to the difficulties in measuring accurately the thin wing web and the correct injection of the antigen. Because previous knowledge of the manipulation can influence the wing web measurements, we tried to avoid this bias as follows. First, on days when more than one experimental pair of birds was measured (31% of females), we measured the birds blindly without any previous knowledge about the group to which they belonged. Second, in order to reduce measurement errors as much as possible, we measured the wing web of each bird three times. The repeatability of wing web measurements was high ($r = 0.86$, $F = 13.28$, $df = 2,27$, $P < 0.0001$). Third, an indirect way which might indicate the reliability of our measurements is the positive correlation found between wing web index and body mass (see Results), a relationship generally found in other studies as well (Alonso-Alvarez and Tella 2001). Although we cannot exclude completely that our results on immunocompetence were influenced by our knowledge on the experimental status of the birds, we consider this effect to be minor. Because the PHA response was stronger in 2001 than in 2000 ($t = 4.72$, $df = 93$, $P < 0.0001$), we included year as a factor in multivariate analyses (two-way ANOVA).

Table 1. Effect of brood size manipulation on parental provisioning rate. The confounding effects of year was controlled for in a two-way ANOVA, where year was entered as a factor. Values are means $\pm$ SD (n).

	Treatment			F	P
	Increased	Control	Reduced		
Female feeding rate	22.14 $\pm$ 6.07 (22)	19.43 $\pm$ 5.52 (30)	17.62 $\pm$ 6.53 (26)	3.26	0.0442
Total feeding rate	42.41 $\pm$ 11.73 (22)	37.33 $\pm$ 10.99 (30)	32.35 $\pm$ 9.82 (26)	4.82	0.0108
Per capita feeding rate	7.94 $\pm$ 2.37 (22)	8.35 $\pm$ 2.36 (30)	10.11 $\pm$ 3.66 (26)	4.26	0.0178

Table 2. The mean brood size at 12 days old nestlings, and the effect of brood size manipulation on offspring body mass, T-cell response and health status. The confounding effects of year was controlled for in a two-way ANOVA, where year was entered as a factor. Values are means $\pm$ SD (n).

	Treatment			F	P
	Increased	Control	Reduced		
Brood size at day 12	5.46 $\pm$ 0.86 (22)	4.55 $\pm$ 1.03 (33)	3.42 $\pm$ 0.95 (26)	25.93	<0.0001
Body mass (g)	20.99 $\pm$ 1.19 (22)	21.77 $\pm$ 1.95 (33)	22.4 $\pm$ 1.47 (26)	4.16	0.0194
PHA response	83.92 $\pm$ 23.21 (22)	98.08 $\pm$ 32.45 (31)	111.46 $\pm$ 24.32 (26)	9.67	0.0002
Heterophils	20.21 $\pm$ 7.12 (21)	24.64 $\pm$ 16.34 (33)	19.39 $\pm$ 9.58 (26)	1.86	0.16
Lymphocytes	40.96 $\pm$ 9.93 (21)	37.96 $\pm$ 17.36 (33)	41.15 $\pm$ 18.25 (26)	0.87	0.43
Total White Blood Cell Counts	67.43 $\pm$ 14.52 (21)	66.81 $\pm$ 30.03 (33)	66.19 $\pm$ 25.53 (26)	0.01	0.99
H:L	0.52 $\pm$ 0.25 (21)	0.67 $\pm$ 0.35 (33)	0.52 $\pm$ 0.27 (26)	2.90	0.06

All variables were normally distributed except for the H:L ratio for females and lymphocyte count for nestlings, which were log-transformed to obtain a normal distribution. Because some individuals were sampled and assessed for immunocompetence in both years, we randomly excluded one item from the data in order to avoid pseudo-replication. All tests are two-tailed and the values reported are means $\pm$ SD.

Results

The brood size manipulation resulted in a significant difference in provisioning rate when nestlings were 12 days old (Table 1). Female barn swallows responded to the brood size manipulation experiment by increasing their feeding rate in enlarged broods, and significantly reducing the feeding effort in reduced broods. Total feeding rate also differed significantly among experimental groups, since parents fed their nestlings more frequently in increased than in decreased broods. Nevertheless, the per capita feeding rate of nestlings was significantly higher in reduced than in the control and the enlarged groups (Table 1, Tukey's post hoc test, $P < 0.05$). The difference among treatments in nestling body mass and T-cell mediated immune response was significant (Table 2), while differential and total leucocyte counts were similar between experimental groups.

The T-cell immune response of female barn swallows captured for the first time in 2001 did not differ from that of birds with a previous immunization in 2000 (immunized in 2000 vs. unmanipulated: 27.2 ± 12.5 , $n = 15$ vs. 25.3 ± 8.5 , $n = 26$, $t = 0.58$, $P = 0.57$), even

after controlling for the potentially confounding effects of age, laying date and manipulation. Thus the difference in the cell mediated immune response of females in the two years could not be accounted for by differences in previous contact with the antigen. T-cell immune response of female barn swallows when nestlings were 12 days old was significantly and positively correlated with their body mass in 2000 (Fig. 1). Brood size manipulation significantly affected T-cell mediated immune response after controlling for differences among years (Table 3, Fig. 2). The effect of brood size manipulation on the T-cell response of females remained significant after controlling for body mass on day 12 of the nestling period (two-way ANCOVA, $F_{2,68} = 5.67$, $P = 0.005$). The effect of manipulation did not change

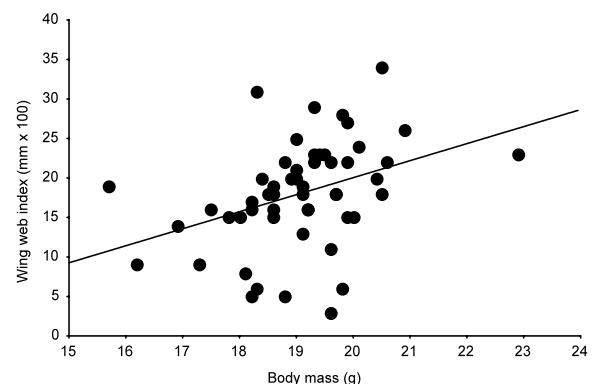


Fig. 1. T-cell response of breeding female barn swallows at 12 day old nestlings in relation to the body mass (g). The data are from 2000, $r = 0.38$, $n = 53$, $P = 0.005$, wing web index = $2.16 \times$ body mass $- 23.06$.

Table 3. Effect of brood size manipulation on T-cell response, body mass, haematocrit and health status of adult female barn swallows when nestlings were 12 days old. The confounding effect of year was controlled for in a two-way ANOVA, where year was entered as a factor. Values are mean $\pm$ SD (n).

	Treatment			F	P
	Increased	Control	Reduced		
PHA response	18.55 $\pm$ 8.39 (22)	19.87 $\pm$ 7.40 (30)	24.42 $\pm$ 8.00 (24)	5.60	0.0039
Female body mass (g)	18.8 $\pm$ 1.11 (22)	18.97 $\pm$ 1.23 (32)	19.13 $\pm$ 0.87 (26)	0.57	0.57
Haematocrit	51.56 $\pm$ 1.95 (22)	51.42 $\pm$ 3.22 (33)	51.61 $\pm$ 3.19 (26)	0.06	0.95
Heterophils	10.73 $\pm$ 8.34 (19)	12.97 $\pm$ 8.41 (32)	11.09 $\pm$ 6.95 (26)	0.24	0.79
Lymphocytes	29.09 $\pm$ 22.97 (19)	28.04 $\pm$ 14.77 (32)	25.53 $\pm$ 18.44 (26)	0.10	0.91
Total White Blood Cell Counts	45.02 $\pm$ 33.75 (19)	45.10 $\pm$ 19.64 (32)	39.83 $\pm$ 25.47 (26)	0.20	0.82
H:L	0.45 $\pm$ 0.35 (19)	0.68 $\pm$ 0.83 (32)	0.52 $\pm$ 0.40 (26)	0.19	0.83

after controlling for other variables such as tarsus, wing and tail length of the females ($P < 0.05$). Finally, we did not find any significant effects of brood size manipulation on adult body mass, haematocrit level, absolute lymphocyte count, heterophile count, total white blood cell count and H:L ratio, respectively.

Discussion

Our brood size manipulation had a significant effect on the feeding activity of female barn swallows, and adult females caring for enlarged broods provisioned their nestlings more frequently than females provisioning control or reduced broods. As a consequence, females that decreased parental activity had a stronger T-cell immune response than birds in the control and increased groups, while none of the condition indices, like body mass, haematocrit and differential and total leucocyte counts differed between experimental groups. This was also the case for the H:L ratio, an index of stress in birds.

In recent years several studies have indicated that the health status and the immunocompetence of breeding birds may play a role in mediating trade-offs between reproductive effort, parasitism and survival prospects,

and hence in the evolution of optimal parental decisions (Deerenberg et al. 1997, Saino et al. 1997b, 1999, Christe et al. 1998, Ots et al. 1998, Tella et al. 2000). Mounting an effective immune response against parasites is crucial due to the deleterious effects of parasites on mate choice, breeding success and survival of hosts (Møller 1997). An increase in parasite infestation during the breeding season can be linked directly to the depression of the immune system, due to the negative effects of sex hormones on the immunocompetence of breeding birds and the immunosuppressive effect of stress hormones on the immune system (see Besedovsky and del Rey 1996, Wingfield et al. 1997 and Apanius 1998 for reviews). Alternatively, the suppression of the immune system during intense exercise can be explained by the "nutrition limitation" hypothesis (Råberg et al. 1998). This hypothesis states that the down-regulation of the immune system during increased activity is a result of the resource limitation for the immune system and other fundamental processes, like parental effort. In this case individuals should suppress their immune system in order to save nutrients for life history traits which promote fitness more directly. Our brood size manipulation resulted in a significant reduction in the acquired immunity, supporting the previous findings on wild birds, namely a trade-off between acquired immunity and reproductive effort (Nordling et al. 1998, Moreno et al. 1999). The absence of a difference among experimental groups in H:L ratio of the female barn swallows indicates that birds were not strongly affected by stress under our experimental design, leading us to suppose that the principal mechanistic base of the immunosuppression of females is nutrition limitation, while stress probably had a minor effect in the down-regulation of T-cell activities. However, the use of H:L ratio cannot be accepted in all situations as an accurate measure of stress, since it depends much on the duration and form of stress (Maxwell 1993). Until now, we are aware of only one study series on wild birds where the H:L ratio was tested in response to parental activity (Ots et al. 1998). Thus, in order to elucidate the mechanistic background of the trade-off between the immune system and reproductive effort, further research is

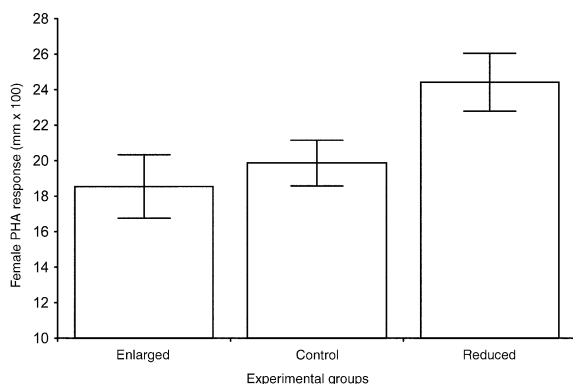


Fig. 2. T-cell mediated immune response of female barn swallows in enlarged, control and reduced broods at 12 day old nestlings (mean $\pm$ SE (n)).

needed, with more exact stress measurements and with more than one component of the immune system measured simultaneously and under different experimental designs.

Studies on poultry have shown that during mild stress only the energetically costly acquired immunity is down-regulated, while phagocytes, the principal components of innate immunity in the blood, respond only during acute stress or during infestation with ectoparasites (review in Apanius 1998). We manipulated the brood size of the barn swallow by only a single nestling, which caused a significant, but not a dramatic increase in the provisioning rate of females. The lack of effect of the experimental treatment on body mass and haematocrit-level of adult females also indicates that the brood size manipulation experiment only had weak effects on parental condition. We suggest that this mild increase in stress due to increased reproductive effort could explain why the experiment only caused an effect on the energetically costly T-cell immunity. Another explanation of the moderate effect of the manipulation on females could be that we manipulated parental effort only during the nestling period, and not during the entire breeding cycle. However, when analyzing the cost of reproduction in birds, we should also take into consideration the energetic costs of egg laying for adults and the cost of incubation for both adults and nestlings (Monaghan and Nager 1997). Thus, we consider that the effects recorded in our study will be further increased if these additional components of parental effort would also be manipulated.

Components of immunocompetence in birds are strongly related to nutritional and physiological status, as indicated by a relationship between body mass and T-cell immune response in adult and nestling birds (review in Alonso-Alvarez and Tella 2001). Furthermore, T-cell mediated immune response can predict survival probability of breeding birds (González et al. 1999, Soler et al. 1999, Tella et al. 2000). Moreover, adult barn swallows trade their survival against the T-cell mediated immune response of their offspring (Saino et al. 1999). Saino et al. (1999) in their study of the barn swallow showed that brood size manipulation affected the survival probability of adults, reducing it in enlarged broods and increasing it in reduced broods. The survival prospects of parent barn swallows were negatively correlated with nestling T-cell response and body size. However, the proximate mechanisms affecting the survival of breeding barn swallows were not determined. In the present study, we used the same manipulation protocol as used by Saino et al. (1999), and we showed that female birds caring for enlarged broods had a lower T-cell mediated immune response than females in the control and reduced groups. Thus, we can infer that the T-cell mediated immune response of adult birds is mediating the trade-off between self-maintenance and reproduction. The crucial assumption

that a stronger immune response of adults is beneficial in terms of adult survival but detrimental in terms of reproduction has yet to be tested in a single experiment.

Parents did not fully compensate for the increased food demand of the nestlings. Thus per capita provisioning rate decreased significantly with experimental brood size. Mounting an effective immune response (e.g. T-lymphocyte response) to an antigen is limited by nutrition (Lochmiller et al. 1993, Saino et al. 1997a). Further, the reduced cell mediated immune response of nestlings from enlarged broods with a lower per capita provisioning rate than nestlings from reduced broods is in line with previous findings in the barn swallow (Saino et al. 1997a). The absence of a difference in differential and total leucocyte counts of nestlings between experimental groups could indicate – as in the case of adult females – an absence of stress caused by within-brood competition.

In conclusion, we have shown that T-cell mediated immunity is a good measure of the condition of birds as indicated by a strong positive correlation with body mass. At the same time T-cell mediated immunity is a sensitive indicator of one of the components of the immunocompetence as demonstrated by the effect of brood size manipulation on T-lymphocyte activity of adult females and nestlings. Our brood size manipulation had no significant effect on haematocrit-level, differential and total leucocyte numbers of females and nestlings, nor on the body mass of female barn swallows. These results support previous findings in wild birds, showing that mounting an effective immune response against an antigen is costly, and that it can result in a trade-off between parental effort and parental immunity. We consider these findings important in understanding the physiological background of the trade-off between the immune system and reproductive effort in birds.

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