Reproductive effort and T-lymphocyte cell-mediated immunocompetence in female pied flycatchers *Ficedula hypoleuca*

Juan Moreno*, Juan J. Sanz and Elena Arriero

Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales-CSIC, J. Gutierrez Abascal 2, E-28006 Madrid, Spain

The physiological mechanism underlying the cost of reproduction may consist of immunodepression caused by increased parental effort. Here, we report effects of experimental manipulation of clutch size on T-lymphocyte cell-mediated immune response in female pied flycatchers, *Ficedula hypoleuca*. Parents with reduced broods provisioned at lower rates than those caring for control and enlarged broods three days after hatching. Parents caring for enlarged broods provisioned nests at higher rates 13 days after chick hatching than those feeding control and reduced broods. Females with enlarged broods weighed less than females with control or reduced broods. No effect of experimental treatment on nestling mass and size was found. The response to the injection of phytohaemagglutinin in the wing-web of females decreased with increasing brood size and with increasing provisioning rate when the chicks were three days old, when controlling for the negative effect of female mass on response. The T-lymphocyte cell-mediated response decreased from the reduced to the control, and from this to the enlarged group, when controlling for female mass. This effect of experimental manipulation of clutch size was significant and consistent with a trade-off between maternal effort and immunocompetence.

Keywords: cost of reproduction; immunocompetence; parental effort; pied flycatcher; clutch-size manipulation; body mass

1. INTRODUCTION

Life-history theory predicts that reproduction should entail costs in terms of parental survival or future reproduction (Williams 1966; Roff 1992). The trade-off between current reproductive effort and future reproduction has been repeatedly documented in birds (Lindén & Møller 1989), with scant evidence apart from survival costs of brood manipulations (Daan *et al.* 1996). This last fact may be due to the inherent difficulty in detecting significant effects on survival with the sample sizes usually found in field studies (Roff 1992). Even if it is not easy to detect survival costs in a manipulative situation, their existence is still the most plausible explanation for the widespread limitation of reproductive rates in avian populations (Drent & Daan 1980). However, our understanding of the physiological basis of such costs is still very limited. It is important to unravel the mechanism behind physiological costs of reproduction to be able to predict their importance and distribution in nature (Roff 1992).

The search for physiological costs of reproduction (Moreno 1993) has led in recent years to a focus on the immune system as the crucial link between energetic exertion and subsequent survival probabilities (Sheldon & Verhulst 1996). Several lines of evidence point in the direction of the immune system as a possible source of life-history trade-offs. Several studies of altricial birds

have detected effects of an experimentally increased parental effort on parasitism rate by haematophagous parasites (Møller 1996; Allander 1997). The mechanism underlying this association could be an increased exposure to vectors or an increased susceptibility to infection due to reduced immunocompetence. Also, it is clear from brood manipulation experiments that effects of reproductive effort on future fecundity may be paid one year after the experimental manipulation (Gustafsson & Sutherland 1988). The immune system offers the best explanation of delayed long-term effects of exertion given the importance of its so-called memory. It is still contentious if the effects of workload on immunocompetence are due to the differential allocation of energetic resources or to alternative physiological mechanisms (Svensson *et al.* 1998; Råberg *et al.* 1998). However, the consensus is growing that intense work may depress the immune system (Hoffman-Goetz & Pedersen 1994), offering a mechanism for crucial life-history trade-offs (Sheldon & Verhulst 1996). The crucial, as yet untested, assumption is that a stronger immune response is beneficial in terms of survival or future reproduction. Manipulation of the immune response itself is needed for testing this assumption (Sheldon & Verhulst 1996).

Although understanding the specific physiological link between work rate and immunocompetence is of the utmost interest in ecological immunology (Svensson *et al.* 1998), there is still need for evidence that immunodepression follows increased reproductive effort in natural

* Author for correspondence: (jmoreno@mncn.csic.es).

populations. Deerenberg *et al.* (1997) have shown a significant effect on the production of antibodies to sheep red blood cells in captive zebra finches, *Taeniopygia guttata*. Additionally, they have been able to relate this depressed response to increased work rate independently of brood demand. Nordling *et al.* (1998) have shown a depressed humoral response to the injection of a pathogen in parent collared flycatchers, *Ficedula albicollis*, caring for enlarged broods in a field study. To our knowledge, these two studies are the only evidence yet that increased reproductive effort actually depresses the immune response, and only one has been performed in the field. It is thus necessary to widen the spectrum of species and populations tested in field situations before drawing strong conclusions about the generality of the reproduction–immunity link.

The two earlier studies have focused on the humoral immune response using methods to detect the production of antibodies. These methods require repeated captures in the field and the drawing of relatively large amounts of blood. A possible caveat is that these manipulations may affect the reproductive behaviours studied and be harmful to the specimens handled, especially in small animals. They may affect the results in unknown ways depending on the sensitivity to capture and handling of the birds studied. The skin test provides a measure of the proliferative response potential of circulating T lymphocytes to an injected mitogen (Lochmiller *et al.* 1993). This method requires two captures on subsequent days with brief handling and no sampling of blood, and is probably less stressful and harmful to the individuals studied. In the present study we use the phytohaemagglutinin (PHA) skin testing technique (Smits *et al.* 1999) to determine the immunocompetence of parent pied flycatchers, *Ficedula hypoleuca*, feeding manipulated broods resulting from manipulated clutches. Thus we have included the potentially costly incubation phase in the experimental protocol (Thomson *et al.* 1998), something which is frequently disregarded in experimental manipulations of reproductive effort (e.g. Nordling *et al.* 1998).

2. METHODS

(a) Study area and species

The study was conducted in 1998 in a deciduous forest of Pyrenean oak, *Quercus pyrenaica*, at 1200 m above sea level in the vicinity of La Granja, Segovia province, central Spain (40° 48' N, 4° 01' W). A study of a population breeding in nest-boxes in this area has been conducted since 1991. Nest-boxes (125 mm × 117 mm bottom area) are cleaned every year after the breeding season. Every year the nest-boxes are checked for occupation by pied flycatchers, and the dates of clutch initiation, clutch sizes and number of fledged young are determined. Nestlings are weighed 13 days after hatching (hatching day = day 0), to the nearest 0.1 g, with a spring balance, and their tarsi are measured with a digital caliper to the nearest 0.01 mm.

The pied flycatcher is a small (12–13 g), hole-nesting passerine of European woodlands (Lundberg & Alatalo 1992). It is a summer visitor, which adapts readily to breeding in artificial nest-boxes. Egg laying in the population being studied typically begins in late May, and clutch sizes range from two to eight eggs with a mode of six eggs (mean 5.73). The female incubates alone and receives part of her food from her mate (Lundberg &

Alatalo 1992). Young are brooded by the female only up to an age of seven days (Winkel & Berndt 1972). Both sexes feed the young. Young fledge within 14–16 days of hatching (Lundberg & Alatalo 1992). This occurs in the second half of June in our study area. There are no second clutches in this species.

(b) Manipulation of reproductive effort

On the day after clutch completion, two randomly selected eggs from nests included in the reduced treatment were transported to nests included in the enlarged treatment. Two eggs of nests in the control treatment were briefly removed and put back in their nests. Trios of nests with the same clutch size and laying date were thus created (23 trios of six-egg clutches and three trios of seven-egg clutches were used for the experiment). There were no differences in original clutch size ($F_{2,71}=0.12$, $p=0.90$) or laying date ($F_{2,71}=0.25$, $p=0.78$) among treatments. Nests were randomly assigned to treatments. Nests where the male was not observed on either day 3 or day 13 after hatching (see provisioning rates) and was not captured feeding nestlings, have not been included in analyses of the effects of experimental treatment.

(c) Immunization

The skin test provides a measure of the proliferative response of circulating T lymphocytes to an injected mitogen. Phytohaemagglutinin (PHA) has long been known for its mitogenic properties (Hungerford *et al.* 1959). The PHA-injection test has been routinely used in poultry science (Goto *et al.* 1978). The main cellular response observed 6–12 h after injection consists of a prominent perivascular accumulation of T lymphocytes followed by macrophage infiltration (Goto *et al.* 1978). The PHA-stimulated hypercellularity disappears normally 48 h post-injection. The PHA skin test is considered to be a useful method to evaluate thymus-dependent function (Goto *et al.* 1978) and has been routinely used as an assay of T-lymphocyte cell-mediated immune function in studies of poultry (Goto *et al.* 1978; Tsiagbe *et al.* 1987; Cheng & Lamont 1988). It is being increasingly used also in ecological studies (Sorci *et al.* 1997; Saino *et al.* 1997; Moreno *et al.* 1998) given its benign character compared with other methods used to evaluate immunocompetence. Typically in birds, one wing-web is challenged with PHA while the opposite is injected with phosphate buffered saline (PBS), and the difference between the swellings attained during 24 h is used as response. We have used the simplified protocol proposed by Smits *et al.* (1999), which avoids the injection of PBS in the opposite wing-web as a control. Smits *et al.* have examined the results from PHA skin tests on 608 birds in seven studies representing passerines, waterfowl, upland game birds and raptors. Correlations between 'PHA–PBS' and 'PHA only' responses within each study showed a very close association. They conclude that 'overall, the response to PHA was so much greater than the (non)response to PBS, that the PBS control may be considered an effort illspent'. The advantages of eliminating the PBS injection are a decrease by half in the handling time of the birds (and thereby in stress), a reduction in the probability of errors when injecting into such a thin structure as the wing-web of a passerine and a decrease in the coefficient of variation that is due to measurement inaccuracies (Smits *et al.* 1999). Smits *et al.* show convincingly that this protocol reduces inaccuracies inherent in the technique.

Twelve days after hatching, females were captured and weighed with a Pesola spring balance to the nearest 0.1 g. Subsequently they were injected with 0.05 ml of a solution of PHA in

Table 1. *Effects of clutch manipulation on parental provisioning, parental masses and offspring mass and tarsus length on day 13*

(Means $\pm$ s.d. (*n*) under different letters (A, B, C) are significantly different according to Bonferroni a posteriori tests. All *p*-values less than 0.05 are significant according to the sequential Bonferroni test (Rice 1989).)

	reduced	control	enlarged	<i>F</i>	<i>p</i>
female provisioning day 3 (feeds h ⁻¹)	A 4.2 $\pm$ 1.4 (23)	B 7.1 $\pm$ 3.7 (21)	B 8.9 $\pm$ 3.9 (21)	13.26	0.0000
male provisioning day 3 (feeds h ⁻¹)	A 9.8 $\pm$ 4.1 (23)	B 13.8 $\pm$ 5.0 (21)	B 15.6 $\pm$ 7.2 (21)	6.12	0.0038
female provisioning day 13 (feeds h ⁻¹)	A 10.2 $\pm$ 4.8 (18)	A 12.0 $\pm$ 4.7 (17)	B 18.8 $\pm$ 10.3 (16)	6.69	0.0027
male provisioning day 13 (feeds h ⁻¹)	A 11.3 $\pm$ 4.0 (18)	AB 15.2 $\pm$ 7.9 (17)	B 17.7 $\pm$ 6.6 (16)	3.80	0.0295
female mass day 13 (g)	A 12.4 $\pm$ 0.5 (19)	A 12.6 $\pm$ 0.5 (19)	B 12.0 $\pm$ 0.4 (20)	8.11	0.0008
male mass day 13 (g)	A 12.4 $\pm$ 0.5 (20)	A 12.2 $\pm$ 0.3 (22)	B 12.2 $\pm$ 0.6 (16)	1.62	0.2074
chick mass (g)	A 14.2 $\pm$ 0.6 (23)	A 14.0 $\pm$ 0.6 (24)	B 13.8 $\pm$ 0.8 (22)	2.02	0.1401
chick tarsus length (mm)	A 17.6 $\pm$ 0.3 (23)	A 17.4 $\pm$ 0.4 (24)	B 17.3 $\pm$ 0.4 (22)	2.84	0.0654
no. of fledged young	A 3.7 $\pm$ 0.7 (23)	B 5.2 $\pm$ 1.3 (24)	C 6.9 $\pm$ 1.4 (22)	42.62	0.0000

PBS (5 mg of PHA in 10 ml of PBS) in the left wing-web after measuring its thickness at the point of injection. Five measures were taken with a digital spessimeter with constant pressure (Mitutoyo 7/547, Tokyo, Japan) to the nearest 0.01 mm to calculate the repeatability of wing-web measurements. We recaptured most females 24 h after the first capture to take five new measurements of the thickness of the left wing-web at the point of injection. Immune response was estimated as the difference between initial and final measurements.

(c) *Provisioning rates*

We have recorded provisioning rates on days 3 and 13 after the chicks hatched. The first day represents the period when females have to brood ectothermic nestlings and males perform most of the feedings, while the second day represents the plateau in provisioning rates of almost fledged young by both parents. On day 3, nests were filmed for 1 h with a video camera to count the number of feeding trips performed by both mates. During the 24 h period between capturing and recapturing females (day 13 after hatching) we filmed the entrance of the nest-boxes with a video camera for 1 h to count the number of feeding trips performed by both mates, and to check that female behaviour at the nest was not markedly altered. The video camera was placed 5–10 m away from the nest-box. Handling of female pied flycatchers has little effect on their subsequent provisioning behaviour (Moreno *et al.* 1997). However, all sessions in which one pair member that we knew was present in the study area did not turn up at the nest, have been excluded from analyses, as the absence could be due to the birds being disturbed by the camera or other unknown factors. There was no effect of time of day when films were made on provisioning rates (correlations for males and females, days 3 and 13, $p > 0.15$). Provisioning

rates were square-root transformed to obtain normality in the data.

3. RESULTS

The brood manipulation treatment had a significant effect on provisioning rates of males and females on day 3, with parents feeding reduced broods significantly less than control and enlarged broods (table 1). Treatment had a significant effect on provisioning rates of males and females on day 13, with females feeding enlarged broods significantly more than control and reduced broods (table 1). For males, only the difference between the reduced and enlarged treatments was significant (table 1). Females caring for enlarged broods weighed less on day 13 than the rest, while there was no difference for males (table 1). Provisioning rates on days 3 and 13 were highly correlated both for males ($r_{47} = 0.34$, $p = 0.019$) and for females ($r_{47} = 0.38$, $p = 0.008$). Enlarged broods resulted in more chicks fledged than control broods, and those more than reduced broods (table 1). Differences among treatments in mean chick size and mass were not significant (table 1).

Repeatability of wing-web measurements was derived from the intra-class correlation coefficient in an ANOVA (Lessells & Boag 1987). Wing-web thickness measures were significantly repeatable, both for initial measures ($r = 0.69$, $F_{36,142} = 11.81$, $p < 0.001$) and for final measures ($r = 0.71$, $F_{36,148} = 13.43$, $p < 0.001$). Female mass on day 13 was negatively correlated with immune response ($r_{36} = -0.33$, $p = 0.047$). Brood size was negatively correlated with response when controlling for female mass in a

Figure 1. T-cell-mediated immune response to PHA injection (wing-web swelling in mm) according to experimental treatment. Means (points), s.e. (boxes) and s.d. (bars) are presented. Sample sizes above error bars.

multiple regression ($\beta_{31} = -0.37$, $p = 0.040$). When controlling for the effects of female mass, experimental treatment was significantly associated with response (ANCOVA: model $F_{3,30} = 4.25$, $p = 0.013$, $r^2 = 0.30$; factor, $F_{2,30} = 4.07$, $p = 0.027$; covariate, $F_{1,30} = 8.65$, $p = 0.006$; figure 1). A posteriori linear contrasts show that the response decreased from the reduced to the control and from the control to the enlarged group ($F_{1,30} = 8.14$, $p = 0.008$). In a multiple regression controlling for female mass, response was significantly associated with female provisioning rate on day 3 ($\beta_{33} = -0.34$, $p = 0.036$). This was not the case for female provisioning rate on day 13 ($\beta_{33} = -0.29$, $p = 0.081$).

4. DISCUSSION

Parents caring for enlarged broods provisioned their broods at a higher rate than parents of the control and reduced treatments on day 13, while males and females caring for reduced broods were less active in provisioning on day 3 than those of the control and enlarged treatments. If these simple measures of effort involved in parental care can be considered as representative of parental exertion (see Moreno *et al.* (1995) for caveats about the use of simple provisioning rates), the experimental treatment had an effect on the parental workload of both males and females. Moreno *et al.* (1995) showed no effect of brood manipulations for females with respect to parental daily energy expenditure (DEE) in the same population in another year, while there was a weak effect for males. However, DEE measures the total energy budget, and not that part devoted to exclusively parental activities. DEE appears to be constrained in birds (Moreno *et al.* 1999), suggesting that it is not a good measure for detecting survival costs. Survival costs may be due either to a reallocation of energy resources among different functions within a constant total budget, or due to different mechanisms not directly involving energetic costs (Svensson *et al.* 1998). There was also consistency in male and female workload between the first and second halves of the brood rearing period. Chicks did not differ among treatments in either size or mass, indicating that parents were able to adjust their effort to brood demand.

Thus, increased parental effort seemed capable of compensating for increased brood demand in enlarged broods.

However, maternal workload may have been increased already from incubation in our experiment, as the daily energy expenditure of incubating females increases with clutch size in this species (Moreno & Sanz 1994). Including the incubation phase in experimental manipulations of parental effort is necessary, given the increasing evidence that the optimal clutch size of birds could be influenced by the number of eggs the parents can afford to incubate (Heaney & Monaghan 1996; Monaghan & Nager 1997; Thomson *et al.* 1998). The practice of manipulating parental effort only after hatching of the young in altricial birds (e.g. Nordling *et al.* 1998) seems at present to be well-entrenched among field workers, despite the fact that it removes the potentially limiting incubation phase (Moreno *et al.* 1991).

Our results confirm the evidence of a link between parental effort and immunity (Deerenberg *et al.* 1997; Nordling *et al.* 1998) by focusing on a different aspect of the immune response, namely T-cell dependent immunocompetence. By using a simplified protocol of the skin testing technique (Smits *et al.* 1999) we are able to show that cellular proliferation after the injection of a mitogen depends on experimental clutch manipulation. Females caring for enlarged broods had a significantly lower response. These females had a higher provisioning rate, suggesting that maternal effort is directly involved in immunosuppression. Also, response was negatively associated with brood size on day 13 and with provisioning rate on day 3. Thus, maternal workload during the brooding phase may be as or even more important than during the late nestling phase (Moreno 1989; Sanz 1997). Parental effort should be measured during this crucial first half of the nestling period in altricial birds, as its effects on general condition may be more crucial than effort expended at a later stage (Moreno & Hillström 1992). Our results and those of Nordling *et al.* (1998) are the first to relate immune response to parental effort in a wild population in the context of a field experiment.

The negative independent effect of female mass on immune response has to be factored out in all analyses of parental effort, as it runs against the effect of maternal workload (a greater effort leads to lower mass and lower response, while a higher mass is associated with a lower response). It has been shown that mass loss is a typical aspect of the immune response (Beisel 1977; Deerenberg *et al.* 1997). Thus, lighter females may be those investing more in immune function independently of parental effort. A greater investment in immune function would negatively affect body reserves. This effect would obscure the impact of maternal effort on immunocompetence, given the independent effects of treatment on body reserves and on immune response. The physiological implications of a negative association between cellular immunocompetence and body mass remain to be explored.

To conclude, we have been able to show a negative effect of maternal effort, measured as experimental treatment, experimental brood size or provisioning rate, on T-cell-mediated immune function in a field situation, thus providing support for the hypothesis that immune suppression caused by reproductive effort may be an important mechanism mediating the life-history cost of reproduction.

We thank Cristina Acosta for help in the field, and Pepe Tella, who forwarded an unpublished accepted manuscript. Two anonymous reviewers contributed to improve a first version of the paper. The study was financed by project PB97-1233-C02-01 of the DGES (Spanish Ministry of Education and Culture). The Dirección Gral. para la Conservación de la Naturaleza donated the nest-boxes, while Javier Donés, director of 'Montes de Valsaín' (Organismo Autónomo Parques Nacionales) gave permission to work in the study area. Finally the Dirección General del Medio Natural of the Junta de Castilla y León gave permission for capturing, ringing and handling birds.

REFERENCES

- Allander, K. 1997 Reproductive investment and parasite susceptibility in the great tit. *Funct. Ecol.* **11**, 358–364.
- Beisel, W. R. 1977 Magnitude of the host nutritional responses to infection. *Am. J. Clin. Nutr.* **30**, 1236–1247.
- Cheng, S. & Lamont, S. J. 1988 Genetic analysis of immunocompetence measures in a white leghorn chicken line. *Poultry Sci.* **67**, 989–995.
- Daan, S., Deerenberg, C. & Dijkstra, C. 1996 Increased daily work precipitates natural death in the kestrel. *J. Anim. Ecol.* **65**, 539–544.
- Deerenberg, C., Apanius, V., Daan, S. & Bos, N. 1997 Reproductive effort decreases antibody responsiveness. *Proc. R. Soc. Lond. B* **264**, 1021–1029.
- Drent, R. H. & Daan, S. 1980 The prudent parent: energetic adjustments in avian breeding. *Ardea* **68**, 225–252.
- Goto, N., Kodama, H., Okada, K. & Fujimoto, Y. 1978 Suppression of phytohemagglutinin skin response in thymectomised chickens. *Poultry Sci.* **57**, 246–250.
- Gustafsson, L. & Sutherland, W. J. 1988 The cost of reproduction in the collared flycatcher. *Nature* **335**, 813–817.
- Heaney, V. & Monaghan, P. 1996 Optimal allocation of effort between reproductive phases: the trade-off between incubation costs and subsequent brood rearing capacity. *Proc. R. Soc. Lond. B* **263**, 1719–1724.
- Hoffman-Goetz, L. & Pedersen, B. K. 1994 Exercise and the immune system: a model of the stress response? *Immunol. Today* **15**, 382–387.
- Hungerford, D. A., Donnelly, A. J., Nowell, P. C. & Beck, S. 1959 The chromosome constitution of a human phenotypic intersex. *Am. J. Hum. Genet.* **11**, 215–235.
- Lessells, C. M. & Boag, P. T. 1987 Unrepeatable repeatabilities: a common mistake. *Auk* **104**, 116–121.
- Lindén, M. & Möller, A. P. 1989 Cost of reproduction and covariation of life history traits in birds. *Trends Ecol. Evol.* **4**, 367–371.
- Lochmiller, R. L., Vestey, M. R. & Boren, J. C. 1993 Relationship between protein nutritional status and immunocompetence in northern bobwhite chicks. *Auk* **110**, 503–510.
- Lundberg, A. & Alatalo, R. V. 1992 *The pied flycatcher*. London: T. & A. D. Poyser.
- Monaghan, P. & Nager, R. G. 1997 Why don't birds lay more eggs? *Trends Ecol. Evol.* **12**, 270–274.
- Møller, A. P. 1996 Parasites and the evolution of host life history. In *Host-parasite evolution: general principles and avian models* (ed. D. Clayton & J. Moore), pp. 105–127. Oxford University Press.
- Moreno, J. 1989 Variation in daily energy expenditure in nesting northern wheatears (*Oenanthe oenanthe*). *Auk* **106**, 18–25.
- Moreno, J. 1993 Physiological mechanisms underlying reproductive trade-offs. *Etologia* **3**, 41–56.
- Moreno, J. & Hillström, L. 1992 Variation in time and energy budgets of breeding wheatears. *Behaviour* **120**, 11–39.
- Moreno, J. & Sanz, J. J. 1994 The relationship between the energy expenditure during incubation and clutch size in the pied flycatcher *Ficedula hypoleuca*. *J. Avian Biol.* **25**, 125–130.
- Moreno, J., Gustafsson, L., Carlson, A. & Pärt, T. 1991 The cost of incubation in relation to clutch size in the collared flycatcher *Ficedula albicollis*. *Ibis* **133**, 186–193.
- Moreno, J., Cowie, R. J., Sanz, J. J. & Williams, R. S. R. 1995 Differential response by males and females to brood manipulations in the pied flycatcher *Ficedula hypoleuca*: energy expenditure and nestling diet. *J. Anim. Ecol.* **64**, 721–732.
- Moreno, J., Potti, J. & Merino, S. 1997 Parental energy expenditure and offspring size in the pied flycatcher *Ficedula hypoleuca*. *Oikos* **79**, 559–567.
- Moreno, J., de León, A., Fargallo, J. A. & Moreno, E. 1998 Breeding time, health and immune response in the chinstrap penguin *Pygoscelis antarctica*. *Oecologia* **115**, 312–319.
- Moreno, J., Merino, S., Potti, J., de León, A. & Rodríguez, R. 1999 Maternal energy expenditure does not change with flight costs or food availability in the pied flycatcher: costs and benefits for nestlings. *Behav. Ecol. Sociobiol.* (In the press.)
- Nordling, D., Andersson, M., Zohari, S. & Gustafsson, L. 1998 Reproductive effort reduces specific immune response and parasite resistance. *Proc. R. Soc. Lond. B* **265**, 1291–1298.
- Råberg, L., Grahm, M., Hasselquist, D. & Svensson, E. 1998 On the adaptive significance of stress-induced immunosuppression. *Proc. R. Soc. Lond. B* **265**, 1637–1641.
- Rice, W. R. 1989 Analyzing tables of statistical tests. *Evolution* **43**, 223–225.
- Roff, D. A. 1992 *The evolution of life histories*. New York: Chapman & Hall.
- Saino, N., Calza, S. & Möller, A. P. 1997 Immunocompetence of nestling barn swallows in relation to brood size and parental effort. *J. Anim. Ecol.* **66**, 827–836.
- Sanz, J. J. 1997 Clutch size manipulation in the pied flycatcher: effects on nestling growth, parental care and moult. *J. Avian Biol.* **28**, 157–162.
- Sheldon, B. C. & Verhulst, S. 1996 Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317–321.
- Smits, J. E., Bortolotti, G. R. & Tella, J. L. 1999 Simplifying the phytohemagglutinin skin testing technique in studies of avian immunocompetence. *Funct. Ecol.* **13**. (In the press.)
- Sorci, G., Soler, J. J. & Möller, A. P. 1997 Reduced immunocompetence of nestlings in replacement clutches of the European magpie (*Pica pica*). *Proc. R. Soc. Lond. B* **264**, 1593–1598.
- Svensson, E., Råberg, L., Koch, C. & Hasselquist, D. 1998 Energetic stress, immunosuppression and the cost of an antibody response. *Funct. Ecol.* **12**, 912–919.
- Thomson, D. L., Monaghan, P. & Furness, R. W. 1998 The demands of incubation and avian clutch size. *Biol. Rev.* **73**, 293–304.
- Tsiagbe, V. K., Cook, M. E., Harper, A. E. & Sunde, M. L. 1987 Enhanced immune responses in broiler chicks fed Methionine-supplemented diets. *Poultry Sci.* **66**, 1147–1154.
- Williams, G. C. 1966 Natural selection, the cost of reproduction and a refinement of Lack's principle. *Am. Nat.* **100**, 687–690.
- Winkel, W. & Berndt, R. 1972 Beobachtungen und Experimente zur Dauer der Huderperiode beim Trauerschnäpper (*Ficedula hypoleuca*). *J. Orn.* **113**, 9–20.