Reproductive effort decreases antibody responsiveness

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SUMMARY

The prevalence and intensity of parasitic infection often increases in animals when they are reproducing. This may be a consequence of increased rates of parasite transmission due to reproductive effort. Alternatively, endocrine changes associated with reproduction can lead to immunosuppression. Here we provide support for a third potential mechanism: reduced immunocompetence as a consequence of adaptive reallocation of resources in times of increased energetic demand. In captive zebra finches *Taeniopygia guttata*, reproductive effort was manipulated through brood size. Enhanced effort was found to affect the production of antibodies towards sheep red blood cells. In addition, activity of zebra finches was manipulated independently of parental care. Experimentally increased daily workloads in activity reward schedules also suppressed antibody production. Thus, we show that not just the reproductive state, but the increased activity that accompanies reproduction is associated with immunocompetence. This mechanism may be sufficient to explain the increased parasitism observed in reproducing animals. We suggest that reduced immunocompetence as a consequence of increased reproductive effort may be an important pathway for the life history cost of reproduction.

1. INTRODUCTION

Reproductive effort entails the allocation of time and nutritional resources by parents to the production of offspring. Life history theory predicts that reproductive effort leads to negative fitness consequences for the parents in terms of reduced survival and future reproductive output, i.e. a cost of reproduction (Williams 1966; Trivers 1974). The trade-off between current reproductive effort and parental survival is particularly well documented in birds (Lindén & Møller 1989; Dijkstra *et al.* 1990). The physiological basis for a cost of reproduction is, however, poorly understood. Current explanations focus on physiological deterioration (Drent & Daan 1980), manifest as accelerated senescence (Partridge 1987), and ecological factors, such as increased risks of predation (Magnhagen 1991).

Recent studies have shown that increased reproductive effort can be associated with a greater susceptibility of the parents to parasitism (Festa-Bianchet 1989; Møller 1993; Norris *et al.* 1994; Richner *et al.* 1995; but see Merino *et al.* 1996). The mechanism underlying this association has not been studied previously and remains to be elucidated. Increased parasitism may stem from either increased exposure of parents to parasites, or from increased susceptibility to infection as a consequence, e.g. of reduced immunological defence mechanisms. Increased exposure may result from additional foraging activity or reduced maintenance activities such as preening (Clayton 1991), or from enhanced parasitic infection in the enlarged broods employed in these studies. Reduced immunocompetence can be related to: (a) the endocrine changes that accompany reproduction (Grossman 1984; Bhalla 1989; Marsh 1992); or (b) a reallocation of resources that are drawn upon to support reproduction (Deerenberg *et al.* 1996). An epidemiological analysis in wild American kestrels (*Falco sparverius*) has shown that the intensity of blood parasitism in females was negatively correlated with peripheral blood leucocyte levels and immunoglobulin G (antibody) concentration, two measures of general immune function (Apanius 1991). However, this result may be interpreted as a reflection of parental condition and is not necessarily related to the level of parental effort.

Here, we focus on a mechanism, that is, functioning of the immune system, potentially explaining the relation between reproductive effort and parasitic infection. We test the hypothesis that increased effort leads to depressed immune function, as measured by an antibody response to sheep red blood cells (SRBC). SRBC, a mixture of various antigens, can

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be classified as a generic non-pathogenic antigenic challenge. The antibody response to SRBC is considered an adequate estimator of the ability of the humoral component of the immune system to mount a response upon challenge (Bacon 1992). The relation to parasitic infection holds as far as both SRBC and parasites are complex antigens. In general, the interplay of parasites and the immune system is well established (e.g. review in Folstad & Karter (1992)). Our study does not test for causality of immune functioning in parasite resistance, which requires manipulation of the immune function itself (Norris *et al.* 1994; Sheldon & Verhulst 1996).

We first test non-breeding and non-working (control) birds for their response to SRBC and to repeated blood sampling. Subsequently, we investigate whether antigen-specific immune responses vary with parental effort, experimentally manipulated in two ways. In experiment 2, we alter reproductive effort by manipulating the number of offspring (brood sizes of two, four and six) in caged zebra finches (*Taeniopygia guttata*) and we then measure antibody responses of the parents. It has been established previously by such manipulations that brood size affects an array of behavioural and fitness-related variables: parental time budgets are altered and female body weight, offspring survival and body weight at independence are reduced by increased brood size. Parental rates of daily energy expenditure (DEE) of females (Deerenberg 1996) and the time interval till the next reproduction are increased (Deerenberg *et al.* 1996). These studies verified that brood size manipulations influenced both parental effort, current reproductive success and future reproductive decisions.

In experiment 3, we increase daily workloads of zebra finches through experimental manipulation of hopping activity outside the context of reproduction (see Deerenberg 1996). These workloads have been shown to negatively affect body mass during the experiment, and subsequent timing of reproduction (Deerenberg 1996), and the experiment thus mimics the effects of brood size manipulation.

In a fourth experiment, we provide additional nutritional resources to breeding birds with an (intermediate) brood size of four nestlings to test whether immune responses of breeding birds are affected by such additional resources. While not affecting parental time allocation (one measure of parental effort), food supplementation reduced parental weight loss during the nestling phase, as well as shortened reproductive interval, and enhanced offspring weight at independence (Deerenberg, unpublished data).

2. METHODS

(*a*) *Antigen-specific immune responses*

Reproducing birds (experiments 2 ($n = 43$ individuals) and 4 ($n = 16$)) were immunized on day 16 of the nestling period, when parental care was assumed to be at its peak level. Working birds (experiment 3 ($n = 11$)) were immunized when they had been subjected to a workload schedule for at least one week. Adult birds were injected intraperitoneally with a washed suspension of SRBC. The suspension contained 5×10^7 SRBC in 100 µl of sterile phosphate buffered saline (PBS). Blood samples and body weights were taken before immunization, and on 5 and 8 d post-immunization (control birds, experiment 1), or on 6 and 9 d post-immunization (birds in experiments 2–4). Plasma was removed. The cells were heat inactivated by incubation at 56 °C for 30 min. A 20 µl aliquot of plasma was diluted 1:1 in PBS (titre = 0) and then serially diluted (titre 1–12) in 96-well round-bottomed microtitre plates. A 20 µl aliquot of 2% SRBC-PBS suspension was then added to each dilution and incubated at 37 °C for 60 min. Titre was scored as the inverse of the dilution that contained sufficient antibodies to haemagglutinate SRBC and are based on a ^{2} log scale (Hay & Hudson 1989).

(*b*) *Corticosterone*

To explore potential interference of immunosuppressive action of stress hormones, corticosterone concentrations were measured in experiments 1 and 2. In experiment 1, 16 birds were sampled for corticosterone. In experiment 2, we pooled the blood plasma remaining after the antibody assays of day 0, 6 and 9 to obtain samples large enough for the hormone assay ($n = 41$). Corticosterone was extracted from 30 µl plasma via solid-phase extraction with acetonitrile on Baker SPE C_{18} 1 ml column (Baker, the Netherlands). Quantification of plasma hormone was performed by high-pressure liquid chromatography in combination with ultraviolet detection (HPLC-UVD; Scheurink *et al.* 1990)

(*c*) *Manipulation of parental effort*

Experiment 1. A random sample of two male and nine female zebra finches that were kept in same gender groups (*ca.* ten birds) was tested for immune responses to SRBC. These non-breeding and non-working birds were housed in $40 \times 80 \times 40$ cm cages, fed on a standard dry seed diet. Food and water was available *ad libitum*.

Experiment 2. Twenty-three pairs of captive zebra finches were housed separately in cages ($40 \times 80 \times 40$ cm), provided with a nest box and nesting material, and were kept at a constant room temperature of 25 °C and a L:D cycle of 14:10 h. They were provided with a standard dry seed mixture suitable for supporting reproduction. To manipulate reproductive effort, we transferred nestlings at 1–3 d of age to create small (two young), intermediate (four young) and large (six young) brood sizes. All experimental broods thus included both own and introduced young. Original clutch size was on average 4.4 (s.d. = 0.9) eggs. There were no significant differences in initial clutch sizes among the groups of pairs that were later given small, intermediate or large broods ($F = 0.47$, d.f. = 2, $p = 0.6$). Average manipulation (number of nestlings added to or removed from the original clutch size) of experimental brood size two was −2.44 nestlings (s.d. = 1.1); of brood size four, −0.6 nestlings (s.d. = 0.52); and of brood size six, +1.9 nestlings (s.d. = 1.2).

Experiment 3. To experimentally vary workload other than by manipulating brood size, we manipulated hopping activity of zebra finches. Non-breeding birds were operantly conditioned to hop repeatedly between perches for a food reward. Two different workloads were imposed by adjusting the number of hops required for a fixed food reward. Compared to a non-working control group with a daily activity of *ca.* 2000 hops, the mean daily number of hops was increased to on average 6158 and 8393 for

low and high workloads, respectively (Deerenberg 1996).

Experiment 4. To improve the quality of the seed diet, eight families of control (four) brood size were given a high protein food supplement from 3 d after hatching onwards. The energy content of the two diets did not differ (22.1 vs 23.6 kJ g^{-1}), while the nitrogen content was doubled (0.046 vs 0.023 g g^{-1} dry mass).

(*d*) *Data analysis*

Immune responsiveness, i.e. the fraction of birds that produced detectable antibodies, was analysed for differences among the four experiments with chi-square (χ^2) tests.

Differences in antibody concentrations (titre) among experiments, and among the three brood size categories of experiment 2, were tested with analysis of variance (ANOVA) using a repeated measurements design. We first inspected graphically whether the titre of each experiment were normally distributed and tested for normality using the Shapiro–Wilk test. The day 6 titre in experiment 3 departed significantly from normality ($W = 0.70$, $n = 11$, $p < 0.01$). All other samples were accepted as being normally distributed ($p > 0.05$). The underlying assumptions for ANOVA include, apart from normality, also homogeneity of variances, which was tested with Bartlett's test. All variances appeared homogenous (all $p > 0.18$, tests results not given).

Within each experiment, we analysed the effects of several independent variables (brood size, gender, body mass, DEE) on responsiveness using log–linear (logistic) regression, and on antibody titre using linear regression. We applied F-ratio tests on the change in deviance when a variable was added to the model to test for significance.

Effects of immunization and responsiveness on body mass changes during the immunization protocol (9 d) were analysed with repeated measures ANOVA. Of the 36 groups and subgroups used as cells in one of the ANOVAs, four samples departed from normality. Distributions of body mass of immunized parents with small broods (day 0: $W = 0.83$, $n = 16$, $p < 0.01$; day 9: $W = 0.88$, $p = 0.04$), were skewed to the left, as was the initial mass distribution of responsive birds in this group ($W = 0.82$, $n = 11$, $p < 0.02$). Mass at the end of the immunization protocol of non-immunized parents with intermediate broods also showed a distribution that was skewed to the right ($W = 0.80$, $n = 27$, $p < 0.001$). Each ANOVA consisted of cells with equal variances (all $p > 0.08$, tests results not given).

3. RESULTS

(*a*) *Experiment 1*

Non-breeding non-working zebra finches had no detectable anti-SRBC antibodies before the experiment. All birds consistently produced haemaglutinating (HA) antibodies upon immunization (100%, $n = 11$). The concentration (titre) of detectable antibodies increased during subsequent sampling days from 4.59 (s.d. = 3.52, $n = 11$) on day 5 post-immunization (PI) to 7.45 (s.d. = 2.55, $n = 11$) on day 8 PI (paired t-test: $t_{10} = -4.10$, $p < 0.01$).

Corticosterone levels of control birds were on average 1.27 ng ml^{-1} (s.d. = 0.66, $n = 16$).

Body mass declined during the immune response (day 0: 15.68 g, s.d. = 1.87; day 8: 14.26 g, s.d. = 1.97, $n = 11$). Although body weights were significantly reduced due to immunization (paired t-test: $t_{10} = 5.75$, $p < 0.001$), it is unlikely that the immunization protocol and the antibody response negatively affected the birds, because weight loss is a typical, however temporary, aspect of the immune response (Beisel 1977).

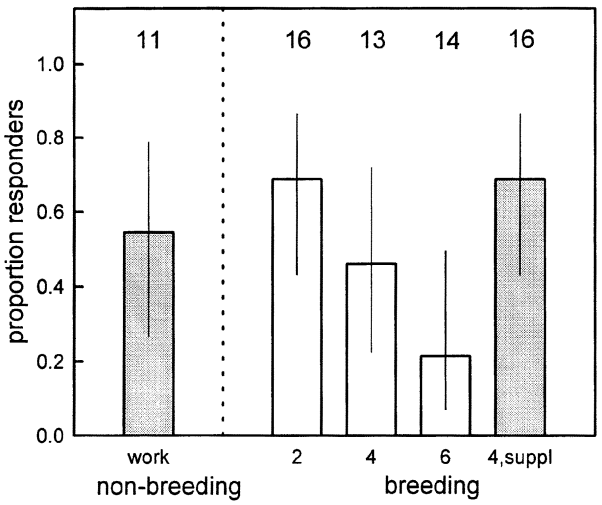

Figure 1. Proportion of zebra finches producing haemagglutinating antibodies against sheep red blood cells as a function of reproductive status, diet or work load. Control birds (non-breeding and non-working) all responded. Work load was manipulated by hopping activity; reproductive status was manipulated by brood size; diet refers to protein supplemented diet. Numbers above columns refer to sample size and error bars indicate 95% confidence intervals based on binomial expectation.

(*b*) *Experiment 2*

Of 43 parent zebra finches, 20 (46.5%) showed a positive response to immunization. Average responsiveness in breeding birds was lower than responsiveness of the non-breeding birds in experiment 1 ($\chi^2_1 = 10.3$, $p = 0.001$). The proportion of responsive breeding birds declined from 69% in parents raising two nestlings to 21% in parents raising six nestlings (figure 1). Thus, the proportion of responsive birds was affected negatively by experimental brood size ($F_{1,41} = 7.01$, $p = 0.01$). Responsiveness was not associated with gender ($F_{1,40} = 0.20$, $p = 0.7$), body mass ($F_{1,40} = 0.02$, $p = 0.9$) or DEE ($F_{1,28} = 1.94$, $p = 0.17$) before immunization.

Titre of responsive birds are given for the three experimental brood sizes in figure 2. Average titre of breeding birds were lower than of birds in experiment 1 ($F_{1,29} = 4.82$, $p = 0.04$), with again a significant effect of sampling day ($F_{1,29} = 9.70$, $p < 0.01$) and an interaction between sampling day and experiment ($F_{1,29} = 15.85$, $p < 0.001$). Thus, the change in titre was different between the two experiments. Antibody titre of responsive breeding birds neither varied with brood size, nor were they associated with gender, body mass or DEE before immunization (table 1).

Table 1. *Linear regressions of anti-SRBC titre of responsive birds on gender, body mass and, facultatively, on brood size and parental daily energy expenditure (DEE)*

	titre, day 6 (increase in)			titre, day 9 (increase in)		
	deviance	d.f.	p	deviance	d.f.	p
brood size manipulations						
null model	168.95	19	< 0.001	140.80	19	< 0.001
brood size	5.07	1	0.5	7.84	1	0.3
gender	1.25	1	0.7	1.80	1	0.6
mass, day 0 (g)	0.67	1	0.8	5.56	1	0.4
null model (DEE)	106.86	13	< 0.001	98.00	13	< 0.001
DEE (kJ d^{-1})	0.29	1	0.9	4.74	1	0.5
work schedules						
null model	19.33	5	< 0.001	46.00	5	< 0.01
gender	0.08	1	0.9	0.75	1	0.8
mass, day 0 (g)	2.51	1	0.5	10.34	1	0.3
DEE (kJ d^{-1})	8.30	1	0.16	6.80	1	0.5
food supplementation						
null model	60.18	10	0.04	92.91	10	0.001
gender	0.98	1	0.7	0.08	1	0.9
mass, day 0 (g)	4.74	1	0.4	6.83	1	0.4

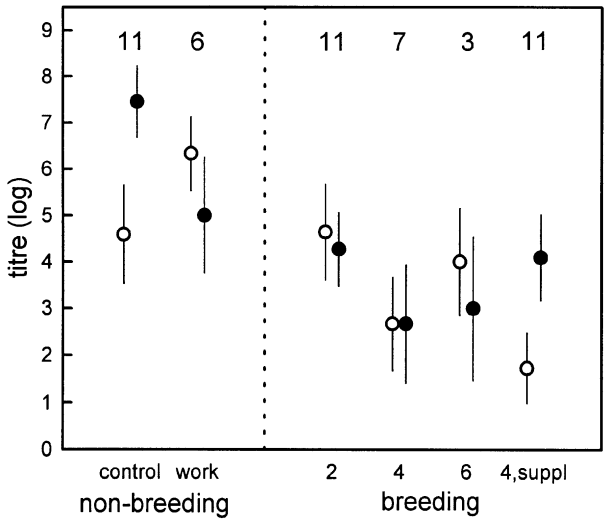

Figure 2. Intensity (titre) of antibody response against sheep red blood cells on 6 d post-immunization (PI, open symbols) and on 9 d PI (filled symbols). Means and standard error (log) titre of responsive birds in each experimental group are depicted.

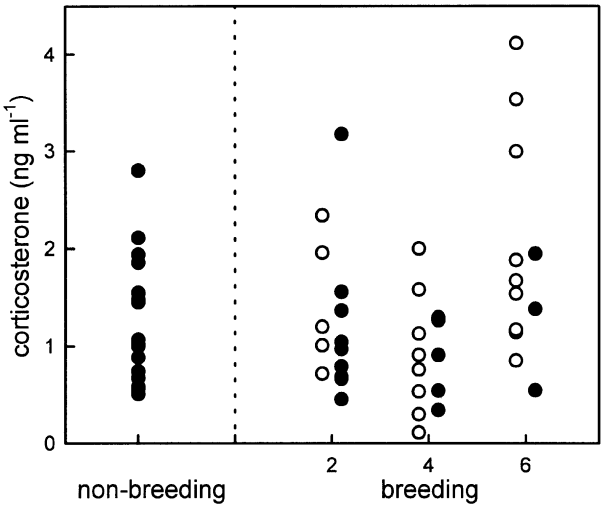

Figure 3. Plasma corticosterone concentrations of control birds (non-breeding and non-working, experiment 1) and of parents raising broods of manipulated sizes (experiment 2). Filled symbols represent birds that responded to immunization with SRBC with antibody production; open symbols indicate birds that failed to produce antibodies.

Corticosterone concentrations in plasma samples are depicted in figure 3 for responsive and non-responsive birds in each brood size category. The overall average concentration of corticosterone in breeding birds being 1.31 ng ml^{-1} (s.d. = 0.87, n = 41) was rather similar to that of the birds in experiment 1. Corticosterone varied with experimental brood size (b) in a quadratic fashion (null model: deviance = 31.46, d.f. = 40; b: change in deviance = 2.77, d.f. = 1, $p = 0.06$; b^2: change in deviance = 5.74, d.f. = 1, $p = 0.02$). Although a tendency existed towards a direct association between immune responsiveness and corticosterone plasma concentrations (c) (null model: deviance = 56.62, d.f. = 40; c: change in deviance = 2.93, d.f. = 1, $p < 0.10$), this association disappeared when we controlled for the effect of experimental brood size (b) on responsiveness (b: change in deviance = 5.73, d.f. = 1, $p = 0.02$; c: change in deviance = 1.36, d.f. = 1, n.s.).

Zebra finches were immunized at the end of the nestling period and parents usually increase in mass during the subsequent days when their young fledge (Deerenberg 1996). Intra-individual changes in body

mass after immunization ($n = 42$ birds) were therefore compared to weight changes of non-immunized birds ($n = 76$) in the same experiment (figure 4). Overall, birds that were immunized did not differ in mass from birds that were not subjected to immunization ($F_{1,112} = 2.51$, $p = 0.12$), but body mass of parent birds varied significantly with experimental brood size ($F_{2,112} = 3.90$, $p = 0.02$). There was indeed a significant change in parental body mass during this period of parental care ($F_{1,112} = 6.86$, $p = 0.01$). Immunization of birds negatively affected subsequent recovery of body mass during the 9 d of the measured immune response ($F_{1,112} = 5.43$, $p = 0.02$), while the interaction with experimental brood size bordered on significance ($F_{2,112} = 2.79$, $p = 0.07$).

To test whether changes in body mass were related to responsiveness, we compared intra-individual mass changes of immunized responsive and non-responsive birds (table 2). Average body mass did not differ between responsive and non-responsive birds ($F_{1,36} = 0.51$, $p = 0.5$), or among brood sizes ($F_{2,36} = 2.36$, $p = 0.11$), and there was no interaction between experimental brood size and responsiveness ($F_{2,26} = 0.31$, $p = 0.7$). Weight increased during the 9 d of the measured immune response ($F_{1,36} = 4.99$, $p = 0.03$), but there was no interaction of mass change with responsiveness ($F_{1,36} = 0.96$, $p = 0.3$) or with brood size ($F_{1,36} = 0.80$, $p = 0.5$). However, there was a significant interaction between those two variables in their effect on mass change ($F_{2,36} = 3.60$, $p = 0.04$). This means that the magnitude of body-mass recovery differed among subsamples of birds, grouped according to the various combinations of responsiveness and brood size.

(*c*) *Experiment 3*

Similar to breeding birds, not all zebra finches subjected to workloads produced detectable antibody titre when they were immunized with SRBC (55%, $n = 11$; figure 1). On the low workload schedule, 50% ($n = 6$) of the birds produced detectable antibodies; on the high workload schedule, 60% ($n = 5$) of the birds showed a immune response. There was no significant difference in responsiveness between the work schedules ($\chi^2_1 = 0.11$, $p = 0.7$). Responsiveness in all working birds was reduced relative to responsiveness of non-working zebra finches in experiment 1 ($\chi^2_1 = 6.47$, $p = 0.01$). Average responsiveness of working birds was similar to the average in all (non-food supplemented) breeding birds ($\chi^2_1 = 0.23$, $p = 0.6$) and did not differ significantly from the fraction responsive birds in any of the brood size manipulation categories (small: $\chi^2_1 = 0.56$, $p = 0.5$; intermediate: $\chi^2_1 = 0.48$, $p = 0.5$; large: $\chi^2_1 = 2.93$, $p = 0.09$). There was also no association between responsiveness and gender ($F_{1,9} = 2.52$, $p = 0.15$), body mass ($F_{1,9} = 2.02$, $p = 0.19$) or DEE ($F_{1,9} = 0.07$, $p = 0.8$) before immunization.

Antibody titre of responsive birds in the workload experiment are shown in figure 2. The titre of working birds were not reduced in comparison with the titre of the non-working control birds ($F_{1,15} = 0.07$, $p = 0.8$). On average, there was no change in titre between the two sampling days ($F_{1,15} = 1.44$, $p = 0.2$). The significant interaction between sampling day and experiment ($F_{1,15} = 10.81$, $p < 0.01$) may indicate an advanced timing of the antibody production or a reduced amplitude of the response of working birds. Antibody titre were not related to gender, body mass or DEE before immunization (table 1).

In this experiment, there was no control group of birds that were not subjected to immunization. Average body mass after immunization (table 2) did not differ between responsive and non-responsive birds ($F_{1,9} = 1.99$, $p = 0.2$). Among the immunized working birds, there was neither a significant change in body mass after immunization ($F_{1,9} = 0.02$, $p = 0.9$), nor an interaction of mass change with responsiveness ($F_{1,9} = 0.31$, $p = 0.6$).

(*d*) *Experiment 4*

The proportion of food-supplemented parents responding to immunization (69%, $n = 16$) was reduced in comparison with non-breeding birds ($\chi^2_1 = 4.91$, $p = 0.03$). In this experiment, we only tested parents rearing four nestlings. Antibody responsiveness was not enhanced by the diet quality enhancement relative to parents raising four nestlings on the standard seed diet ($\chi^2_1 = 1.51$, $p = 0.2$). Within the pairs that received a high-protein food supplement, responsiveness did not vary with gender ($F_{1,14} = 0.29$, $p = 0.6$) and was not associated with body mass before immunization ($F_{1,14} = 0.17$, $p = 0.7$).

Average titre of antibodies on day 6 and day 9 post-immunization of responsive birds are depicted in figure 2. Average titre of food supplemented birds did not differ from titre of birds raising broods of the same size on the seed diet only ($F_{1,15} = 0.04$, $p = 0.9$). However, the change of titre with sampling date ($F_{1,15} = 4.25$, $p = 0.06$) and the effect of food supplementation on this change bordered on significance ($F_{1,15} = 4.25$, $p = 0.06$). This suggests different patterns in the timing or amplitude of the response. Among food supplemented parents, titre of responsive birds were not associated with gender or body mass before immunization (table 1).

Similar to experiment 2, changes in body mass after immunization ($n = 16$) of parents of broods of four on either the standard seed diet or food supplemented were compared to weight changes of birds that were not subjected to immunization (food supplemented: $n = 8$; figure 4). There were no overall differences in initial body mass between immunized birds and birds that were not subjected to immunization ($F_{1,59} = 0.23$, $p = 0.6$), or between food-supplemented and standard diet birds ($F_{1,59} = 0.51$, $p = 0.5$). Subsequent changes in body mass ($F_{1,59} = 17.29$, $p < 0.001$) were not affected by the immunization protocol ($F_{1,59} = 2.68$, $p = 0.11$), but were increased by food supplementation ($F_{1,59} = 3.93$, $p = 0.05$).

Body mass changes of immunized birds are given in table 2. Average body mass did not differ between

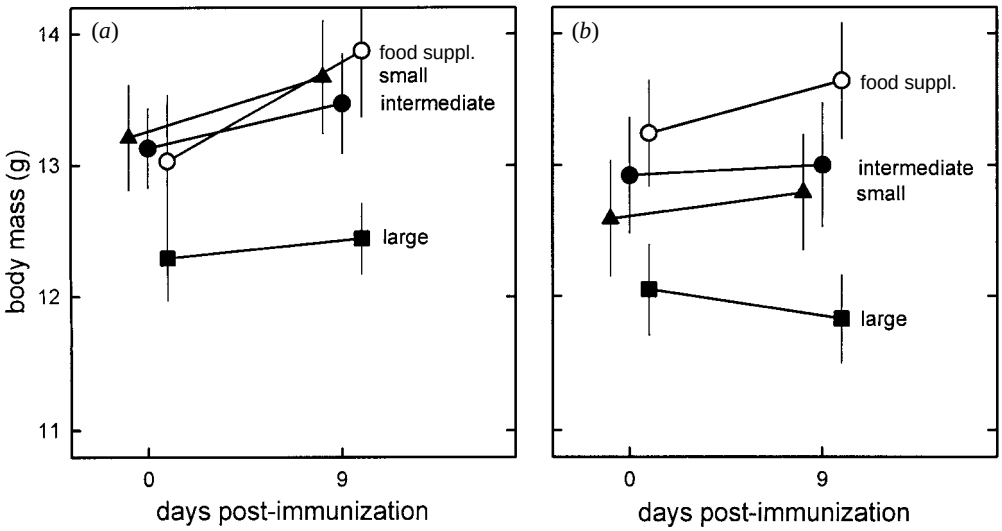

Figure 4. Body weights of breeding zebra finches (experiments 2 and 4) on day 16 and day 25 of nestling age, corresponding to day 0 of immunization (before) and day 9 PI. Means and standard errors are given for parents with experimental brood sizes of two ('small'), four ('intermediate') and six nestlings ('large'), and for parents with an experimental brood size of four that were supplemented with protein-rich food ('food suppl.'). (*a*) Breeding birds in experiments 2 and 4 that were not subjected to the immunization protocol. (*b*) Birds in experiments 2 and 4 that were immunized.

Table 2. *Changes in body mass during the immunization protocol (day 0–9) of zebra finches in the various groups of the three experiments of manipulation of parental effort*

(Birds were grouped according to whether they produced detectable anti-SRBC antibodies (responsive), or not (non-responsive). Means ± standard errors are given, with samples sizes in parentheses.)

	responsive	non-responsive
brood size manipulations		
small broods	0.11 ± 0.21 (11)	0.56 ± 0.30 (5)
intermediate broods	-0.32 ± 0.15 (6)	0.42 ± 0.17 (6)
large broods	0.60 ± 0.53 (3)	0.00 ± 0.15 (11)
work schedules	-0.12 ± 0.31 (6)	0.07 ± 0.03 (5)
food supplementation	0.44 ± 0.34 (11)	0.54 ± 0.35 (5)

standard diet and supplemented birds ($F_{1,27} = 0.57$, $p = 0.5$), or between responsive and non-responsive birds ($F_{1,27} = 0.0$, $p < 1$). Overall body-mass recovery during the immunization protocol only tended to differ from zero ($F_{1,27} = 3.84$, $p = 0.06$), with no significant effects of either responsiveness ($F_{1,27} = 1.55$, $p = 0.2$) or food supplementation ($F_{1,27} = 3.07$, $p = 0.9$). The interaction between food supplementation and responsiveness did not contribute to mass change.

4. DISCUSSION

In a group of non-breeding non-working zebra finches, it was shown that the birds responded to immunization with SRBC by producing high titre of haemagglutinating antibodies. Surprisingly, reproductive and working zebra finches did not consistently produce detectable antibodies upon immunization. It was thus demonstrated that this trait was phenotypically controlled. This study examined several ecological and behavioural factors that might influence antibody responsiveness.

The proportion of zebra finches responding to immunization with SRBC was reduced incrementally in birds raising manipulated broods of increasing size. As shown elsewhere, experimentally manipulated brood size affected the level of parental effort, as assessed by various behavioural and physiological measurements (Deerenberg 1996). Our results on immune responses provide a sufficient explanation for increased parasitism observed in birds with increasing experimentally manipulated brood sizes (Møller 1993; Norris *et al.* 1994; Gustafsson *et al.* 1994; Richner *et al.* 1995).

Non-breeding birds that were subjected to workloads also showed a reduced responsiveness. This result is consistent with studies reporting depressed humoral immunity in humans undergoing strenuous physical exercise (review in Fitzgerald 1988) and in swallows (*Hirundo rustica*) with experimentally elongated tails, a costly sexual character (Saino & Møller 1996). Comparable results for cell-mediated immune function have been obtained by König & Schmid-Hempel (1995) who experimentally withheld bumble

bee (*Bombus terrestris*) workers from foraging.

Our data show that antibody responsiveness of zebra finches depends on the demands imposed on the animal. Because all groups were treated identically (handling and blood sampling), except with respect to experimentally manipulated brood size or workload, it is unlikely that other factors can account for these results. Both breeding and working birds reduced their response rates, therefore the data presented here suggest that increased physical activity associated with parental effort influences antibody responsiveness of the parents negatively.

To our knowledge, this is the first experimental demonstration that workload associated with reproductive effort influences immune function negatively. One previous study has examined leucocyte and immunoglobulin levels in birds with manipulated broods (Gustafsson *et al.* 1994). In this review on reproduction and infectious diseases in the Collared Flycatcher, *Fidecula albicollis*, Gustafsson reported increased parasitic infection rates concomitantly with symptoms of an increased activity of the immune system in parents with manipulated brood sizes. Because the species had natural infections with blood parasites, these seemingly contradictory results may be expected. For example, the enhanced immunoactivity may have reflected a response to the increased parasitic infection, the relapse of which originally may have been due to impaired immune function, which in turn is a consequence of manipulated parental effort. To demonstrate the influence of reproductive effort on one aspect of the adaptive immune system of parents, we use the challenge imposed by a novel non-pathogenic antigen without adding additional metabolic demands.

Numerous studies have shown that immunosuppression causally increases host susceptibility to parasitic infections (review in Folstad & Karter 1992). Reproducing birds during the phase of nestling care have usually low, or increasing, levels of gonadal steroids, while circulating levels of corticosterone may be increased (Vleck & Priedkalns 1985; Hegner & Wingfield 1986*a*,*b*; Logan & Wingfield 1995). Corticosterone is associated with high metabolic demands (Wingfield 1984*a*,*b*) or metabolic stress (Siegel 1980) and exhibits immunosuppressive action (reviews in Munck & Guyre 1991; Marsh 1992). Corticosterone levels in this study were low compared to those of male and female House Sparrows (Hegner & Wingfield 1986*a*,*b*). Still, the level of this hormone was significantly affected by the brood size manipulations, with slightly increased levels in parents of both small and large broods relative to intermediate brood sizes, where one might have expected a positive linear relation. However, our results were not very specific given the fact that the analyses were not part of the experimental design and carried out on pooled samples. The only other study on brood size manipulations and hormone concentrations reported somewhat comparable results, i.e. a tendency of increased levels of corticosterone in female parents of large broods (Hegner & Wingfield 1987). Corticosterone concentrations were not different, however, between responsive and non-responsive birds within each group. These findings appear to rule out the possibility that suppressed immune responses result directly from differential stress factors imposed on the parents by manipulation of brood size.

Birds in the experiments were faced with increased energetic demands due to reproductive behaviour (nourishing young) or hopping activity. However, on average there were no corresponding changes in daily energy expenditure (DEE), except for females in the brood size manipulation experiment (Deerenberg 1996). The range of DEE values manipulated through activity (experiment 3) was similar to values of parental DEE measured in the brood size manipulation experiment (experiment 2) (Deerenberg 1996). We were unable to establish a difference in antibody responsiveness between birds maintained on low and high workloads. Because it has been shown elsewhere that DEE was slightly reduced on the high workload in spite of increased hopping activity, due to compensatory reduction of metabolic rate during inactivity (Deerenberg 1996), it was thus not unexpected that DEE was not directly associated with responsiveness, or with titre of anti-SRBC antibodies. Therefore, we suggest that the increased level of physical activity, whether or not associated with reproduction, required that the parents reallocated resources between various somatic compartments. This is dramatically demonstrated by body-mass reductions (15%) of parents during the course of the reproductive cycle (Deerenberg 1996) and by major reductions in maintenance energy (35%) of birds subjected to various workloads (Deerenberg 1996).

While parents usually increase in weight around fledging of their young, immunized parents showed suppressed weight gain. It is unlikely that reduced weight gain was entirely due to blood sampling (*ca.* 300 µl in 9 d). The existence of differences in mass change between responsive and non-responsive birds was dependent on experimental brood size. A reduced body mass, corresponding with minimized food intake, is a normal finding in immunoresponsive animals (e.g. Murray & Murray 1979; Klasing *et al.* 1987) and immunological processes thus rely primarily on stored resources (Beisel 1977). Antigen-specific immune responses require rapid lymphocyte proliferation and draw heavily on protein resources. Following an immunological challenge, there is a homeostatic response of increased protein breakdown in muscle tissue, thereby supplying the amino acids for the increased protein synthesis of the immune response in liver and other immune tissues (Klasing & Austic 1994*a*,*b*). These mechanisms explain the absence of weight gain in responsive birds only. The lack of positive body-mass changes of non-responsive birds may be explained, if non-responsive birds had insufficient body reserves both to respond to the immune challenge and to increase in weight. Apparently, there is a trade-off between maintenance and immune function, and both traded off against demands due to reproduction. This redirection of parental resources may be a significant causal component of the cost of reproduction phenomenon.

Protein malnutrition has been shown repeatedly to impair immune function (e.g. Lochmiller *et al.* 1993). In view of the crucial involvement of stored proteins in the immune response mentioned above, the supplement of high protein food was expected to enhance immune responses. Enhancing the quality of the diet of breeding birds by supplementation with high protein food increased body-mass recovery during the studied phase of parental care. Unexpectedly, food supplementation had little influence on responsiveness or weight changes of immunized birds, while responsiveness was still reduced compared to control birds. Apparently, protein availability was not limited or crucial in parents nourishing their young, or in working birds.

Why should immunocompetence decline at a time when the physiological performance of birds, i.e. parents, is at a premium? Typically, birds did or did not respond. We expected birds to adjust the quantity of their response to the amount of resources available. When responding, the intensity of the production of antibodies did not vary among experimental groups, although in the working and in the food supplementation experiments there is some indication of different patterns of antibody production between controls and experimental groups. The different patterns are suggestive, because maybe not solely the amplitude (peak titre) is important for the effectiveness of the reaction, but also the timing and the total duration of the response. All three aspects affect the total amount of antibodies produced. Because an insufficient antibody production may be of limited effectiveness, reduced responsiveness may thus be a strategy rather than an effect of insufficient resources. Conditional on general prevalence of parasites and host susceptibility, a bird that does not fight immunologically against (parasitic or other) infection may incur smaller fitness costs than when it cuts down on resources allocated to reproduction and thereby reduce its reproductive output (Forbes 1992). We surmise that the depression of immune function and a concomitant increased susceptibility to parasitism of parents is part of a larger adaptive syndrome in which resources are reallocated from self-maintenance to reproduction to maximize lifetime reproductive success.

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