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Enhanced immune function does not depress reproductive output

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Costs of reproduction might be mediated by a physiological (resource allocation) trade-off between immune function and reproductive effort, and several recent studies have shown that an experimental increase in reproductive effort is associated with decreased immune function. Here we test the complementary prediction of this hypothesis: that increased immune function (specific antibody production) depresses reproductive output. Female European starlings (*Sturnus vulgaris*) were injected with a non-pathogenic antigen (sheep red blood cells) following completion of laying of their first clutch, to stimulate an *in vivo* humoral immune response (primary antibody production). We induced laying of a second clutch by removing the first clutch, and assessed changes in reproductive performance in individual females pre- and post-treatment. Injection of sheep red blood cells produced a significant antibody response in 96% ($n = 29$) of treated females, with titres comparable to previous studies (range 1–7). However, increased antibody production did not decrease primary or secondary female reproductive effort (re-laying interval, egg size, clutch size, chick growth or fledging success), compared with control, saline-injected birds ($n = 22$). These data do not support a simple resource allocation model for the cost of reproduction, based on a reciprocal, negative relationship between resources allocated to immune function and reproduction.

Keywords: immune function; reproductive effort; trade-offs

1. INTRODUCTION

In many vertebrates increased parental investment in the current reproductive attempt can negatively affect future fecundity or survival. This 'cost of reproduction' represents one of the most important, and widely studied, life-history trade-offs (Stearns 1992). Physiology must provide the mechanism(s) that underlie such trade-offs and can contribute to our understanding of how these trade-offs might constrain adaptation (Stearns 1992). However, although this fact has been explicitly recognized for over a decade (Winkler 1985), or even longer (Fisher 1930), the physiological basis of most, if not all, trade-offs remains unknown (Stearns 1992). Indeed, much current life-history theory is divorced from physiology and other components of organismal biology (Bernardo 1996).

Recently much attention has been focused on the interplay between reproductive effort and resources devoted to immune function, as a possible basis of cost of reproduction trade-offs (Gustaffson *et al.* 1994; Sheldon & Verhulst 1996). Assuming that both reproduction and immune function are costly (see § 4), and that investment in these two functions is coupled (due to finite resources), then increased reproductive effort might lead to a suppression of immune function. This could increase susceptibility to infection, consequently decreasing future fecundity or survival. Numerous studies have demonstrated a positive correlation between reproductive effort and parasite prevalence (mammals, Festa-Bianchet 1989; birds, Richner *et al.* 1995; Oppliger *et al.* 1996; insects, Konig & Schmid-Hempel 1995). More recently experimental studies involving manipulation of reproductive effort (e.g. through egg removal increasing clutch size, or through brood manipulations) have shown that increased reproductive effort is associated with decreased immune function in both captive (Deerenberg *et al.* 1997) and free-living birds (Nordling *et al.* 1998), which might mediate increased rates of infection.

Here we test the complementary prediction of the hypothesis linking reproduction and immune function: that increased immune function (specific antibody production) depresses reproductive output. We injected female European starlings (*Sturnus vulgaris*) with a non-pathogenic antigen (sheep red blood cells; SRBCs) following completion of laying of their first clutch, to stimulate a specific immune response (primary antibody production). We then induced laying of a second clutch by removing the first clutch, and assessed changes in reproductive performance in individual females pre- and post-treatment using a repeated-measures experimental design. We show that increased antibody production, during the period of egg formation, does not decrease primary female reproductive output (re-laying interval, egg size, clutch size) or secondary reproductive output (chick growth and fledging success).

2. METHODS

(a) *Fieldwork and reproductive output measures*

Fieldwork was carried out in April–June 1997 and 1998 at the Pacific Agri-food Research Center (PARC), Agassiz, British Columbia (49°N, 121°W) using a nest-box population of

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European starlings established in 1995 (approximately 130 nest-boxes in total). The experimental protocol followed the guidelines of the Canadian Council on Animal Care (SFU Project number 442B; PARC Project number 9702). Prior to onset of breeding, and during laying, all nest-boxes were checked daily to determine dates of clutch initiation, completion of laying, and clutch size. All new eggs were measured (length and breadth, ± 0.01 mm) and numbered. Fresh egg mass was calculated from the formula: $\text{mass} = 0.0009159 \times (\text{length}^{0.954}) \times (\text{breadth}^{1.877})$; (J. K. Christians, unpublished data, $r^2 = 0.98$, $p < 0.001$, $n = 175$ eggs). If no new eggs were laid on two consecutive days we assumed the clutch was complete.

(b) *Manipulation of immune function*

Upon completion of the first, pre-treatment clutch (2–4 days after the last egg was laid), breeding females were captured in their nest-box and randomly assigned to the control or treatment group. An *in vivo* humoral immune response (primary antibody production) was stimulated in the SRBC group by injecting birds with SRBCs. Females ($n = 29$) received a single intraperitoneal injection of a 5% suspension of SRBCs (ICN Biomedicals, Inc. or Sigma) in 0.5 ml of phosphate-buffered saline (PBS; 0.01 M phosphate, 0.15 M NaCl, pH 7.4). Prior to injection, SRBCs were double-washed and resuspended in PBS to achieve the desired concentration. Control females ($n = 22$) were given a single intraperitoneal injection of 0.5 ml PBS only. All birds were individually banded, weighed (± 1 g) and blood samples (< 1 ml) were taken from the brachial vein. Blood was centrifuged at 5000 rpm for 10 min and plasma stored at $-20\,^{\circ}$C until further analysis (pre-treatment antibody titre, see below). All eggs from the first clutch were removed to induce a second, post-treatment clutch and the female was released.

Following the experimental manipulation, nest-boxes were checked daily to locate experimental females. Laying date, egg and clutch size were recorded as before, and at clutch completion females were captured again (to confirm their identification), their body mass recorded, and a second blood sample obtained (post-treatment antibody titre). All females were released and allowed to incubate eggs and rear chicks from their second brood. For each female we obtained data on chick mass at 18 days post-hatching and fledging success.

Immune response (SRBC antibody titre) was measured using a haemagglutination assay (Wegmann & Smithies 1966; Hay & Hudson 1989) in 96-well microplates. Each plasma sample was assayed in duplicate using a 20 µl plasma volume. Samples were serially diluted using 20 µl PBS (1/2, 1/4, etc.) and then 20 µl of a 2% suspension of SRBC in PBS were added to all samples. Microplates were incubated at 40 °C for 1 h, and haemagglutination of 'unknown' plasma samples compared with that in negative control (PBS only) and positive control (anti-SRBC serum, Sigma) wells. Antibody titres were expressed as the $\log_2$ of the reciprocal of the highest dilution of plasma showing positive haemagglutination (Lochmiller *et al.* 1993).

(c) *Statistical analysis*

Statistical analyses were carried out using SAS (SAS Institute Inc. 1989), and power analyses for parametric tests were carried out using PASS (Hintze 1996). Immune responsiveness was analysed using the non-parametric Wilcoxon rank test (antibody titre) or χ^2 test (proportion of birds producing detectable antibodies). We compared reproductive output and body mass of females during their first clutch (re-nesting birds only), and chick mass in second clutches using general linear models (proc GLM). Otherwise, we analysed changes in reproductive output between first and second clutches in individual females using a repeated-measures ANOVA or ANCOVA, including significant covariates (e.g. laying date; proc GLM). For each analysis we first tested for a year × treatment interaction. If this was non-significant we report the main treatment effects pooling data from both years, otherwise we included year as a covariate.

3. RESULTS

(a) *Antigen-specific immune responses*

Ninety-six per cent (47 out of 49) of pre-treatment females had no detectable anti-SRBC antibodies; two females showed positive haemagglutination, both with an antibody titre of 1 (one of these birds was subsequently assigned to the treatment group and the other to the control group). SRBC treatment significantly elevated antibody titre compared with controls in both years (1997, $\mathcal{Z} = 2.86$, $p < 0.01$; 1998, $\mathcal{Z} = 4.16$, $p < 0.001$; figure 1), but there was no difference between SRBC-treated birds in the two years ($\mathcal{Z} = 1.86$, $p > 0.05$). Post-treatment, 16 out of 20 control (PBS-treated) females had no detectable anti-SRBC antibodies (four females scored with positive haemagglutination had antibody titres of 1–3), whereas 27 out of 28 SRBC-treated females showed a positive antibody response (range of titres, 1–7; $\chi^2 = 29.8$, $p < 0.001$). Including the four 'positive' PBS-treated females as controls does not affect the conclusions of subsequent analyses.

(b) *Comparison of primary reproductive output in first (pre-treatment) clutches*

There was no significant difference in body mass ($F_{1,49} = 2.79$, $p > 0.10$), mean egg mass ($F_{1,50} = 0.88$, $p > 0.30$) or clutch size ($F_{1,50} = 1.12$, $p > 0.20$) of first clutches in birds which re-nested, when comparing birds assigned to control or SRBC-treated groups (controlling for year effect; year × treatment interaction not significant). Similarly, within-treatments there was no difference in pre-treatment body mass, mean egg mass or clutch size when comparing birds which did or did not lay a second clutch, either for control females (mass, $p > 0.15$; egg and clutch size, $p > 0.70$) or SRBC-treated females ($p > 0.20$ in all cases), i.e. re-nesting birds represented a random sample of all birds initially assigned to treatment groups. Laying date of the first clutch did not differ among treatments for re-nesting birds (Wilcoxon two-sample test, $\mathcal{Z} = 0.35$, $p > 0.70$), or within-treatments comparing birds which did or did not lay a second clutch (control birds, $\mathcal{Z} = 1.49$, $p > 0.10$; SRBC birds, $\mathcal{Z} = 0.99$, $p > 0.30$).

(c) *Effect of antibody response on primary reproductive output in post-treatment clutches*

There was no difference in re-nesting probability, comparing SRBC-treated and control birds, in either 1997 ($p > 0.50$) or 1998 ($p > 0.50$), or for both years pooled ($\chi^2 = 0.017$, d.f. = 1, $p > 0.80$; table 1). Re-nesting interval (number of days between removal of first clutch and initiation of second clutches) was longer in control birds ($\mathcal{Z} = 2.00$, $p = 0.05$; table 1). Although most birds (47 out of 51) initiated their second clutch 7–11 days after removal of the first clutch, for one SRBC-treated bird the

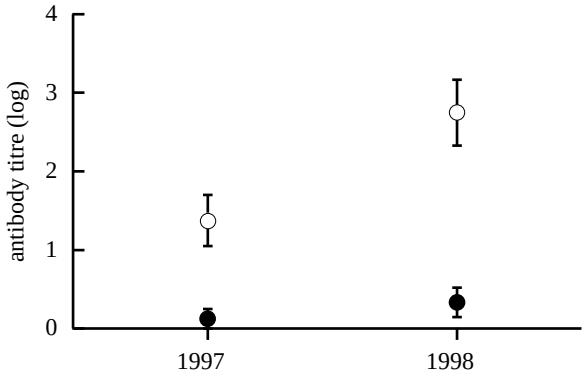

Figure. 1. Pre- and post-injection antibody titres in SRBC-treated (open circles) and PBS-treated (control; closed circles) female European starlings.

Table 1. *Comparison of primary and secondary reproductive output in SRBC-treated (n = 29) and control (n = 22) female European starlings for their second (post-treatment) clutch*

(Values are least-squares means ± s.e.)

	SRBC-treated birds	control birds
re-nesting probability	54% (29/54)	52% (22/42)
laying interval (days)	8.4 ± 0.6	10.0 ± 0.7
mean egg mass (g)	7.23 ± 0.06	7.20 ± 0.05
clutch size	5.7 ± 0.2	5.3 ± 0.2
mean 18-day chick mass (g)	76.6 ± 1.3	76.0 ± 1.7
mean brood size at 18 days	4.0 ± 0.3	3.8 ± 0.4
fledging success (%)[a]	72.2 ± 0.1	70.7 ± 0.1

[a](Number of chicks fledged/number of eggs laid) × 100.

re-laying interval was 21 days, and for three control birds it was 18, 19 and 19 days. If these birds are excluded from the analysis or re-nesting interval then there was no significant difference between treatments ($\chi = 1.60$, $p > 0.10$). We included data from these four pairs in all subsequent analyses; this did not qualitatively change any of the conclusions drawn.

There was no difference in mean egg mass ($F_{1,49} = 0.11$, $p > 0.70$) or clutch size ($F_{1,50} = 2.83$, $p = 0.10$) of post-treatment clutches, comparing SRBC-treated and control birds (table 1; controlling for variation in egg mass of first clutch, and body mass and clutch size of first clutch, respectively). Similarly, there was no significant change in mean egg mass or clutch size between pre- and post-treatment clutches in individual birds for either treatment (paired *t*-test, $p > 0.10$ in all cases), and there was no significant time × treatment interaction for either mean egg mass (repeated measures ANOVA, $F_{1,49} = 1.48$, $p > 0.20$) or clutch size ($F_{1,49} = 1.65$, $p > 0.20$). For these sample sizes, we would have been able to detect a change in egg mass and clutch size due to treatment of 3.9% (0.28 g) and 17% (0.9 eggs), respectively, with power $\beta = 0.9$ and $\alpha = 0.05$.

Body mass of females at the completion of their second clutch was marginally significantly different between treatments (control, 82.4 ± 0.8 g versus SRBC, 84.5 ± 0.7 g; $F_{1,48} = 3.88$, $p = 0.06$). However, there was no significant change in body mass between pre- and post-treatment clutches in individual birds for either treatment ($p > 0.40$ in both cases), and there was no significant time × treatment interaction for body mass (repeated measures ANOVA, $F_{1,49} = 0.89$, $p > 0.30$).

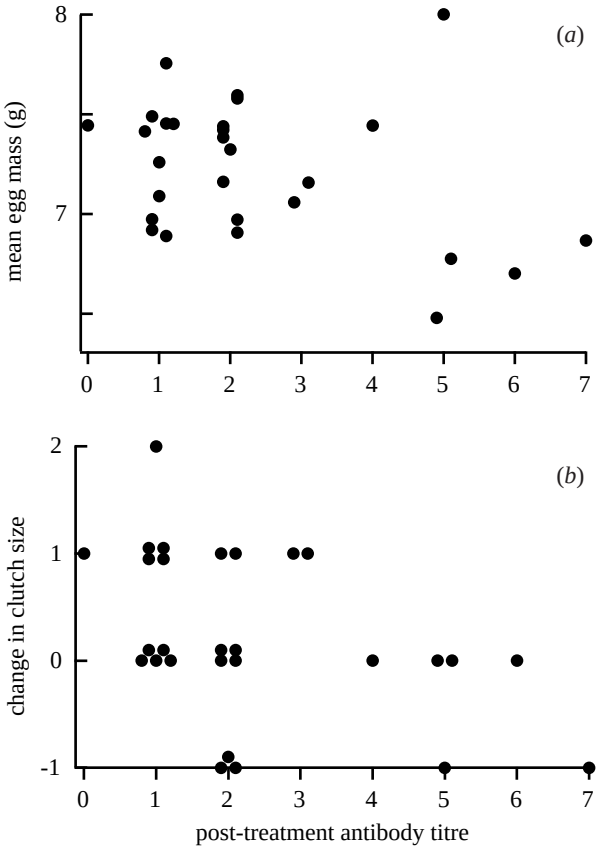

Figure. 2. Relationship between post-injection antibody titre and (*a*) mean egg mass of the second clutch, and (*b*) change in clutch size between first and second clutch, for SRBC-treated female European starlings.

(d) *Effect of antibody response on secondary reproductive output in post-treatment clutches*

Eleven control females (out of 19) and 20 SRBC-treated females (out of 26) successfully reared chicks to fledging ($\chi^2 = 1.86$, $p > 0.15$; three broods were deleted from each treatment because they were used in another experiment). There was no difference between control females and SRBC-treated females in brood size ($\chi = 0.08$, $p > 0.80$) or mean chick mass per brood ($F_{1,30} = 0.07$, $p > 0.70$) measured at 18 days post-hatching, or in fledging success ($\chi = 0.25$, $p > 0.70$; table 1). For these sample sizes, we would have been able to detect a difference in 18-day chick mass due to treatment of 9.8% (7.5 g) with $\beta = 0.9$ and $\alpha = 0.05$.

(e) *Relationship between variation in reproductive output and post-treatment antibody titre*

In SRBC-treated females, post-treatment antibody titre was independent of egg size and clutch size in the first clutch (Spearman's rank correlation, $p > 0.30$), clutch size in the second clutch ($p > 0.25$), and change in mean egg mass between the first and second clutches

($F_{1,26} = 0.08$, $p > 0.70$). Post-treatment antibody was negatively correlated with mean egg mass in second clutches, but this was marginally non-significant ($r_{28} = -0.34$, $p = 0.076$; figure 2*a*). However, post-treatment antibody titre was significantly negatively correlated with change in clutch size between the first and second clutches (defined as clutch 2 − clutch 1; Spearman's rank correlation, $r_{28} = -0.43$, $p < 0.025$; figure 2*b*).

4. DISCUSSION

There is accumulating evidence, from both correlational and manipulative studies, that increased reproductive effort can cause an increase in parasite infection, which is most likely mediated via a decrease in immune function, i.e. immunosuppression (Rosen 1996; Deerenberg *et al*. 1997). Two recent papers (Deerenberg *et al*. 1997; Nordling *et al*. 1998) have suggested a relatively simple basis for this trade-off, involving allocation decisions based on finite resources: increased reproductive effort diverts resources away from immune function. This hypothesis assumes that both reproduction and immune function are costly (Allander 1997) and also generates the complementary prediction that elevated immune function should depress reproductive effort by diverting resources away from reproduction. We tested this prediction by inducing an immune response (antibody production) to a benign antigen in female starlings and then asked the question: does increased immune function suppress primary (re-laying interval, egg size, clutch size) or secondary reproductive output (chick growth and fledging success)?

Inoculation of female starlings with SRBC induced an antibody response similar to that reported in previous studies (Deerenberg *et al*. 1997; Apanius 1998). All but one of the SRBC-treated females (96%) had positive SRBC antibody titres (range 1–7) whereas the majority of control birds (16 out of 20) had antibody titres valued at zero. Despite this marked elevation of immune function, there was no decrease in any measure of reproductive effort in SRBC-treated females, compared with either post-treatment controls or pre-treatment (first clutch) values for the SRBC birds themselves. We took blood samples from females on average 12–14 days post-SRBC injection and antibody titres were clearly elevated at this point. Most females initiated their second clutch 8–10 days after injection and, since egg development takes 4–5 days per egg in starlings (Ricklefs 1974), this means that females would have been forming eggs coincident with elevated antibody production. We are therefore confident that elevated immune function does not suppress primary reproductive effort in this species. Incubation and chick rearing take 11–12 and 21–24 days, respectively, in our population (J. K. Christians, unpublished data). It is therefore possible that antibody production had decreased again and was basal during these latter stages of breeding. Consequently, chick rearing would not have been coincident with elevated antibody production, which might explain why there was no effect on chick growth and fledging success. Nevertheless, recent studies have shown that costs incurred solely during egg production can affect subsequent chick-provisioning performance (e.g. Heaney & Monaghan 1995; Monaghan *et al*. 1998). Thus, we might still have expected to see some effect during chick rearing if there were significant costs of increased immune function earlier in the breeding cycle.

When faced with an antigenic challenge, birds may not obligatorily divert resources away from other functions (e.g. reproduction). Depending on the severity or cost of the immune challenge they may prioritize and maintain their level of investment in reproduction, maximizing that benefit, while tolerating low-level infection (Behnke *et al*. 1992). For example, Deerenberg *et al*. (1997) reported that 53% of breeding zebra finches failed to show an antibody response to SRBC, and concluded that this trait was facultatively controlled. We found no evidence for this in starlings in terms of the proportion of birds responding to SRBC (96%). However, it is possible that breeding birds produced a lower level of response (lower antibody titres) compared with non-breeders (Deerenberg *et al*. 1997). Thus, European starlings might have maintained the same level of investment in reproduction while initiating a positive, but reduced, antibody response.

The most parsimonious explanation for the lack of a negative effect of increased antibody production on reproductive effort is that antibody production itself has a negligible cost (compared, perhaps, with other components of immune function, e.g. a cell-mediated response). Most studies of immune-function mediated costs of reproduction have involved parasite-infected individuals (e.g. Richner *et al*. 1995; Oppliger *et al*. 1996; Allander 1997). Parasitized individuals have to deal with metabolic costs of parasites (Connors & Nickol 1991) in addition to the physiological costs of the immune response (leucocyte proliferation, antibody production). The SRBC is a benign antigen and so in our study female starlings only allocated resources to the immune response itself. This might represent a minor component of either the total cost of infection, or the maintenance cost of supporting a functional immune system. The costs of an antigen-specific immune response are poorly known, however; both Deerenberg *et al*. (1997) and Nordling *et al*. (1998) assumed that these costs are sufficiently high that they are negatively affected by reallocation of resources from immune function to reproduction. Klasing *et al*. (1987) showed that injection of SRBC into chicks (*Gallus domesticus*) suppressed growth, and they concluded that this response was immunologically mediated since SRBC caused no tissue damage or other pathological alterations. Rapid lymphocyte proliferation may also require increased breakdown or mobilization of protein from muscle tissues (e.g. Klasing & Austic 1984), and Deerenberg *et al*. (1997) suggested that this explained the mass loss, or lack of mass gain, in SRBC-treated finches compared with controls. However, in our study we found no difference in body mass of SRBC-treated or control starlings.

Among SRBC-treated females we did find a negative correlation between antibody titre and one measure of reproductive output (change in clutch size between first and second clutches): individuals with higher titres showed the greatest decrease in clutch size between breeding attempts. However, we believe this within-group correlation reflects 'natural' inter-individual variation in the relationship between immune function and reproduction among individuals, i.e. a phenotypic

correlation (*sensu* Lessells 1991). It does not reflect a direct trade-off based on allocation decisions between immune and reproductive function which might result from an experimental increase in the level of one of these functions.

In conclusion, the current model for the physiological basis of 'costs of reproduction' assumes (i) that both reproduction and immune function are costly, and (ii) that there is a direct resource allocation trade-off between resources committed to reproduction and those for immune function (Allander 1997; Deerenberg *et al*. 1997; Nordling *et al*. 1998). If this simple model is correct then when one of these functions is increased the other should decrease, that is, there should be a reciprocal, negative relationship between the two functions. In contrast, we found that increased immune function did not decrease reproductive effort. We suggest that more studies are needed to quantify the physiological costs of the various components of the immune response (cf. metabolic costs of parasite infection). For example, it is possible that the major cost of immune function might actually be in maintaining this system at 'basal' levels in non-infected states. Consequently, the additive costs of responding to a specific immune challenge might be minor in life-history terms.

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