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Reproductive effort reduces long-term immune function in breeding tree swallows (*Tachycineta bicolor*)

Daniel R. Ardia[1*], Karel A. Schat[2] and David W. Winkler[1]

[1]*Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853-2701, USA*
[2]*Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853-6401, USA*

We examined whether strategies of reproductive allocation may reduce long-term immunocompetence through the effects of manipulated effort on secondary or acquired immunity. We tested whether increased reproductive effort leads to reduced immune function and survival by manipulating brood size in tree swallows (*Tachycineta bicolor*) and exposing breeding females to a primary and secondary exposure of sheep red blood cells to elicit a humoral immune response. Females raising enlarged broods produced fewer secondary antibodies than did females raising control or reduced broods. Most importantly, individuals with high secondary responses were more likely to survive to breed 3 years after brood manipulations, suggesting that differences in disease susceptibility may be caused by trade-offs in reproductive allocation. We also found that individual quality, measured by clutch initiation date, mediated the effects of brood manipulations, with higher-quality birds showing a greater ability to deal with increases in effort.

Keywords: acquired immunity; reproductive effort; individual quality; ecological immunology

1. INTRODUCTION

Reproductive decisions are often seen in terms of managing trade-offs between the allocation of resources to raising offspring and to maintaining parental condition (Stearns 1992). One common method of exploring reproductive decisions is to modify effort and thus to force parents to alter the trade-offs between current and future reproduction. Individuals may reduce costly expenditures that have long-term benefits to deal with a short-term but critical challenge. For example, immune-system maintenance can be downregulated during times of stress or high effort (Sheldon & Verhulst 1996; Norris & Evans 2000). Following manipulations of reproductive effort, parents show, on average, a decreased ability to respond to a specific antigen, as measured by the primary immune response (Lochmiller & Deerenberg 2000; Hasselquist *et al.* 2001; Cichón *et al.* 2001).

However, when responding to an antigen, the immune system produces both the primary immune response to fight the current threat and memory cells to fight future threats. Memory cells are antigen specific and serve to mount a secondary immune response to a second antigen exposure, which is stronger and faster than the primary immune response (Roitt 1997). This long-term component of the immune system may be critical to survival (Pitcovski *et al.* 2001); however, to date no studies have shown how short-term increases in reproductive effort might have long-term consequences for immune function and survival.

In this paper, we present the results of the first study, to our knowledge, in which the effects of reproductive effort on secondary immune response are examined in free-living animals. Because memory cells are first produced *at the same time* as the primary response, high levels of acute effort may have long-term consequences on acquired immunity. Here, we test the hypothesis that reproductive effort during primary exposure affects the secondary immune response, predicting that increases in effort during the period of memory-cell production will lead to decreased secondary immune responses. Prior work done at our research site (Hasselquist *et al.* 2001) suggested that individual quality, as reflected by laying date, affects immune responses, and we thus also tested the hypothesis that timing of breeding affects secondary responses. Secondary immune response is linked with disease resistance in poultry (Parmentier *et al.* 2001; Pitcovski *et al.* 2001), suggesting that variation in resistance should affect year-to-year survival. Thus, to explore the demographic costs of variation in secondary immune response, we tested the prediction that individuals with reduced secondary immune responses are less likely to return to breed in future years.

Memory cells are produced as a function of the processes that produce primary antibodies. Given recent suggestions of adaptive immunosuppression (Råberg *et al.* 1998; Buchanan 2000), one might predict that, during times of stress, resources to fight current threats (i.e. primary antigen exposure) might be increased at the expense of response to future risks (i.e. the strength of a secondary response). We thus tested whether primary immune response is correlated with secondary immune response. If reproductive effort causes differential immune-system trade-offs, we would predict that individuals with experimentally increased reproductive effort might decrease secondary antibody production to maintain primary antibody production. If, however, invariant processes control proliferation of both types of cell, we predict that individuals who mount stronger primary responses will also mount stronger secondary responses across all experimental groups.

* Author for correspondence (dra7@cornell.edu).

2. MATERIAL AND METHODS

(a) *Study area*

We studied tree swallows (*Tachycineta bicolor*) breeding in nest-boxes in two locations: Ithaca, Tompkins County, NY, USA (centred on 42°29′ N, 76°27′ W) and Loudon, Anderson and Knox Counties, TN, USA (centred on 35°53′ N, 84°18′ W) (see http://golondrinas.cornell.edu for further details on both sites). Tree swallows have been breeding at the Ithaca site since 1985, and here we have conducted assessments of primary immune function (Hasselquist *et al.* 2001) and long-term life-history studies (Winkler 1991; Winkler & Allen 1996; McCarty & Winkler 1999).

(b) *Experimental design*

We checked nests daily to determine the date of clutch initiation and clutch size, and as necessary to determine the hatching date. To manipulate parental effort, we randomly assigned nests with the same hatching date to one of three treatments: (i) increased; (ii) decreased; or (iii) control, to create broods that were *ca.* 50% larger or smaller than the original clutch size (e.g. on average, three, six and nine nestlings).

Chicks were individually marked with small dabs of nail polish on their claws and swapped to create the various treatments on day 3 of the nestling period (all days are referred to by days from hatching of the first nestling).

In 1999 (New York) and 2001 (Tennessee), breeding females were captured in nest-boxes while brooding day 4 nestlings. We obtained blood samples from the brachial vein to determine the presence of natural antibodies to sheep red blood cells (SRBC) in plasma. Directly afterwards, each bird was injected intraperitoneally with 5×10^7 SRBC (ICN Biomedicals, Aurora, OH, USA) suspended in 100 µl phosphate-buffered saline at pH 7.2 (PBS) (Deerenberg *et al.* 1997). All females were captured 8 days later and bled to determine post-exposure primary antibody titres in plasma. In the year following primary exposure (2000 in New York, 2002 in Tennessee), we recaptured all females that returned to breed. Because we had to wait a year, rather than the traditional 30–90 days, to recapture individuals to measure secondary response, we again obtained blood samples to determine antibody levels prior to secondary exposure. We then reinjected each bird with the same quantity of SRBC used in the primary exposure. We also recaptured and immunochallenged all individuals that returned to breed in Ithaca in 2002, 3 years after the initial experimental manipulation.

(c) *Serology*

We determined antibody titres in all blood samples using a base-2 serial dilution haemagglutination test conducted with 20 µl of plasma on 96-well microtitre plates (Roitt 1997). Samples were serially diluted starting with 20 µl PBS, and to each well 20 µl of a 2% suspension of SRBC in PBS was added. Plates were incubated at 37 °C for 1 hour. Titres are given as the $\log_2$ of the reciprocal of the highest dilution of plasma showing positive haemagglutination. For each plate, we ran both positive and negative controls. Repeatabilities are high for this procedure (pre-exposure: $R_i = 0.60$, $F = 3.91$, $p = 0.03$; post-exposure: $R_i = 0.65$, $F = 4.65$, $p = 0.01$; McGraw & Ardia 2003).

(d) *Statistical analyses*

To determine whether birds were indeed mounting a secondary immune response, we compared the proportion of individuals with pre-primary-exposure agglutination (titre greater than 1; probably a cross-reacting glycoprotein; Cotter 1998) with the proportion of individuals with pre-secondary-exposure antibodies (probably true antibodies) using a Fisher's exact test. We used a mixed model (SAS PROC MIXED) with site as a random effect and female age, clutch size, clutch initiation date standardized relative to the mean for each site and year, and brood-manipulation treatment as fixed effects to compare factors affecting the level of secondary antibody production. We used linear regression to examine the relationship between primary antibody response and secondary antibody response. We conducted stepwise logistic regression using $\alpha = 0.10$ as entry and exit probabilities (SAS PROC LOGISTIC) to examine which factors predicted the production of background antibodies and to test which variables predicted return rates. For logistic regressions, we tested the global hypothesis of no covariate effect ($\chi^2 = 0$) using changes in the likelihood ratio (–2 log likelihood) of the model with and without the effect of interest. Individual parameter estimates were tested using a Wald χ^2-test.

3. RESULTS

(a) *Presence of natural antibodies*

Few individuals had natural antibodies prior to primary inoculation (three out of 81, 3.7%), while most individuals showed low levels of antibodies prior to secondary exposure (68%, 55 out of 81; proportion showing natural agglutination versus antibodies prior to secondary exposure: $\chi^2_1 = 69.44$, $p < 0.001$). Only secondary-response levels and timing of breeding were correlated with detectable levels of antibodies prior to secondary exposure (overall logistic model: $\chi^2_2 = 9.61$, $p = 0.008$). Individuals with strong secondary immune responses had a higher probability of having circulating antibodies to SRBC prior to secondary exposure ($n = 81$, $\beta = 0.309$, Wald $\chi^2_1 = 4.04$, $p = 0.04$). Later-laying individuals in the year after but *not* in the year of the brood-manipulation treatment were less likely to have antibodies prior to secondary exposure ($n = 81$, $\beta = -0.987$, Wald $\chi^2_1 = 2.96$, $p = 0.08$). In the logistic regression, neither primary antibody response nor brood-manipulation treatment at the time of memory-cell production had an effect on the probability of producing detectable background antibodies.

(b) *Effect of reproductive effort on secondary response*

Experimentally increased brood size led to a decrease in secondary immune response relative to control and decreased brood treatments (figure 1*a*, $F_{2,71} = 4.82$, $p = 0.01$, $n = 81$). Individuals laying larger clutches tended to have stronger secondary immune responses (figure 1*b*, $F_{4,71} = 2.82$, $p = 0.03$, $n = 81$), but there was no effect of female age on immune responses ($F_{1,71} = 0.01$, $p = 0.91$, $n = 81$). Clutch initiation date in the year of the brood-manipulation treatment was the strongest predictor of secondary immune response, with early-laying individuals mounting the strongest secondary responses (figure 1*c*, $F_{2,71} = 17.41$, $p < 0.001$, $n = 81$). There were no significant interactions between variables (all $F < 0.5$, $p > 0.2$, $n = 81$). Individuals that mounted strong primary responses also mounted strong secondary responses, regardless of brood-manipulation treatment (figure 2, $R^2 = 0.29$, $\beta = 0.81$, $p < 0.0001$, $n = 81$).

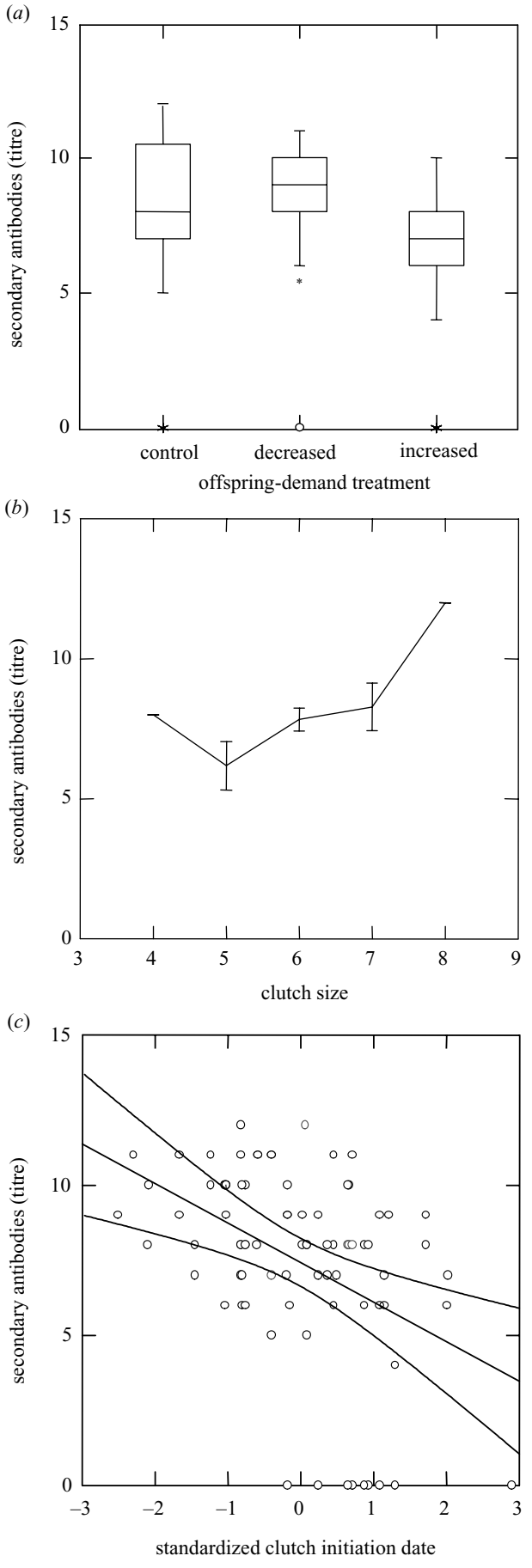

Figure 1. Effects of (*a*) reproductive effort, (*b*) clutch size and (*c*) standardized clutch initiation date on secondary antibody production to SRBC in breeding female tree swallows. (*a*) The symbols outside the boxes refer to outliers. (*b*) Means plus standard error. (*c*) Clutch initiation date standardized to a mean of 0 and a standard deviation of ± 1. Regression line plus 95% confidence interval.

(c) *Return rates*

For New York tree swallows, individuals that initiated clutches early and those that mounted strong secondary immune responses were more likely to return to breed 3 years after the initial manipulation (figure 3, $n = 50$, overall model: $\chi^2_2 = 7.51$, $p = 0.008$; effect of clutch initiation date, $\beta = -0.513$, Wald $\chi^2 = 5.67$, $p = 0.01$; effect of secondary antibody production $\beta = 0.389$, Wald $\chi^2_1 = 4.38$, $p = 0.03$).

4. DISCUSSION

Reproductive effort appears to suppress long-term immune function. Increased offspring demand during primary exposure to an antigen reduced the strength of secondary immune responses. While individual quality (e.g. timing of breeding, clutch size) also influences acquired immunity, these results indicate that increased reproductive effort decreases future immunological performance. Increasing reproductive effort has been shown to decrease primary immune function in wild passerine birds (see Lochmiller & Deerenberg 2000), but this study is the first, to our knowledge, to demonstrate that increased reproductive effort decreases acquired immunity in natural-breeding birds. However, changes in resource allocation affect antibody responsiveness to SRBC in poultry, with poultry bred for high growth having a strong reduction in immune function (Bayyari *et al.* 1997; Li *et al.* 2000). Thus, trade-offs between resource allocation and immunocompetence, both secondary and primary, may be widespread.

Our results also support the hypothesis that long-term reductions in immune responsiveness might have fitness consequences. Individuals with strong secondary responses were more likely to return to breed 3 years after brood manipulations. One possible cause of variation in survival related to acquired immunity may be disease resistance (Li *et al.* 2001; Pitcovski *et al.* 2001). Differences in secondary responses in poultry were positively correlated with susceptibility to *Eimeria acervulina*, an intestinal parasite (Parmentier *et al.* 2001). This suggests that a generalized reduction in disease resistance may be either a direct or an indirect consequence of trade-offs associated with reproductive effort. However, as in all ecological analyses of individual variation, the correlation between secondary immunity and survival may reflect common correlation with a third unmeasured indicator of individual quality.

Interestingly, our manipulation of offspring demand had strong predictive effects on secondary immunity in the year after the manipulations, but offspring-demand treatment *per se* did not have a predictive effect on return rates after 3 years. After years of intensive recapture effort in

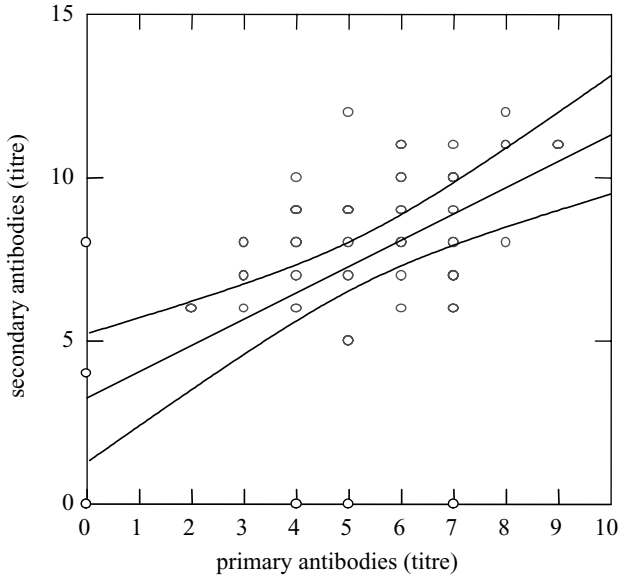

Figure 2. Relationship between the strengths of primary antibody production and secondary antibody production in breeding female tree swallows exposed to SRBC. Regression line plus 95% confidence intervals.

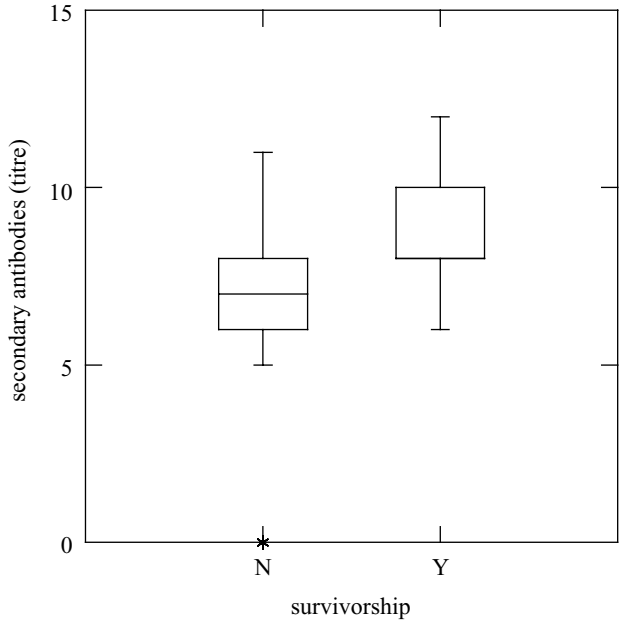

Figure 3. A box-plot summary of the secondary antibody production of female tree swallows breeding in Ithaca as a function of survivorship from 1999 to 2002. The median for group Y is the same as the boundary for the lower quartile. The symbols outside the boxes refer to outliers.

surrounding areas, we believe that return rates in tree swallows reflect survival owing to limited breeding dispersal. Differences in return rates appear to vary based purely on differences in secondary immune function and timing of breeding between individuals, suggesting that some individuals, in the face of drastic increases in offspring demand, are able to sustain both long-term and short-term immune function, whereas other individuals are not. This suggests that variation in the ability to maintain long-term immune function may be an important mediator in determining the effects of acute shortages.

As the first study to report acquired immunity in a wild bird, to our knowledge, we are confident that our protocol measured secondary responses. We found few birds with pre-primary-exposure background agglutination relative to the proportion of birds with agglutination prior to secondary exposure. Given this strong difference, we conclude that agglutination in blood samples collected from birds prior to secondary exposure indeed reflects antibodies to SRBC and that following secondary exposure these birds mounted specific secondary responses. Further correlational evidence supports this, as individuals mounting strong secondary antibody responses were more likely to have detectable background levels. In addition, our protocol should be biased against finding patterns in secondary responses. Owing to the difficulty of reliably recapturing breeding birds 30–90 days after fledging, our protocol measured secondary responses in the year after brood manipulations. As a consequence of waiting a year to resample birds, we were able to recapture only those birds that returned to breed, thus eliminating a subset of the original individuals from our study. As prior work suggests that individuals that do not return to breed in subsequent years have probably perished (Robertson *et al.* 1992), our results should be biased against finding differences, as the individuals with the weakest immune responses are likely to have been selected out of the pool in the second year, thus reducing the variance in measured secondary responses.

In assessing the effect of our brood manipulation, we assumed that the critical period affecting secondary response is during the production of memory cells (i.e. during primary exposure) not during their activation (i.e. during secondary exposure). Individuals that had strong primary responses also mounted strong secondary responses, indicating that there were no intra-individual trade-offs. Because we did not modify parental effort in the second year of the study, it is unclear what additional effect modifying parental effort during secondary exposure would have. Additional research could answer this question by manipulating parental effort during both periods, one period or neither period.

Just how increased reproductive effort decreases secondary immune function is unclear. If the production of memory cells is costly, either energetically or through limiting the availability of a trace factor such as carotenoids, then direct trade-offs associated with an acute stress might decrease their production at the time of creation. However, individuals might also be making strategic adjustments. Memory cells might not be costly, *per se*, but mounting a secondary response may be quite costly, as initiating the production of antibodies from memory cells may entail significant energetic costs (Demas *et al.* 1997; Ots *et al.* 2001). Individuals might be modulating memory-cell production as a strategy to influence the intensity of secondary production. One might predict different responses depending on the severity of the acute stress, as exposure to pathogens is probably spatially and temporally correlated. Individuals may save energy, both in the present and in the future, by downregulating memory-cell production, but they do so at the cost of impaired disease resistance and an increased risk of mortality.

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