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# Costs of immunity: immune responsiveness reduces survival in a vertebrate

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Immune defences are undoubtedly of great benefit to the host, reducing the impact of infectious organisms. However, mounting immune responses also entails costs, which may be measured by inducing immune responses against artificial infections. We injected common eider (*Somateria mollissima*) females with three different non-pathogenic antigens, sheep red blood cells (SRBC), diphtheria toxoid and tetanus toxoid, early in their incubation period. In the group of females that mounted a humoral immune response against SRBC, the return rate was only 27%, whereas the group of females that did not mount a response against SRBC had a return rate of 72%. Moreover, responding against diphtheria toxoid when also responding against SRBC led to a further reduction in return rate. These results are repeatable, as the same effect occurred independently in two study years. The severely reduced return rate of females producing antibodies against SRBC and diphtheria toxoid implies that these birds experienced considerably impaired long-term survival. This study thus documents severe costs of mounting humoral immune responses in a vertebrate. Such costs may explain why many organisms suppress immunity when under stress or when malnourished, and why infections may sometimes be tolerated without eliciting immune responses.

**Keywords:** sheep red blood cells; diphtheria; tetanus; toxoid; local survival; eiders

## 1. INTRODUCTION

Parasites have the potential to reduce the fitness of their hosts severely (Price 1980; Loye & Zuk 1991; Hanssen *et al.* 2003a); therefore hosts have developed elaborate immune defences to combat infections. That parasites and infections still prevail despite these defences may be attributed to rapid evolution of infectious organisms to evade the host's defences (Stearns 1999; Frank 2002). Interestingly, many of the costs associated with disease, for instance fever, inflammation and septic shock, are not direct costs of the infectious organism but rather the result of the activation of the immune defence (Janeway *et al.* 1999; Levin & Antia 2001). Thus, the immune defence itself may have costs making it a two-edged sword that hosts thus have to use with caution (Råberg *et al.* 1998; Westneat & Birkhead 1998). Costs of activated immune systems have been measured as reductions in parental effort and in offspring quality and numbers (Ilmonen *et al.* 2000; Råberg *et al.* 2000). In an invertebrate, the bumble-bee (*Bombus terrestris*), it has even been shown that immune-system activation of heavily resource-limited (i.e. starved) individuals can result in impaired survival (Moret & Schmid-Hempel 2000). These demonstrations of costs of immune defences may suggest that suppression of immune responses would be adaptive when the risk of experiencing such costs is higher than the expected costs

from the infection itself. Accordingly, we can predict an adaptive strategy of immunosuppression during periods of stress and resource limitation. Several studies have shown that stress and resource limitation result in suppressed immunocompetence both within and outside the reproductive season (Lochmiller *et al.* 1993; Saino *et al.* 1997; Svensson *et al.* 1998; Cichón *et al.* 2002).

We have explored the costs of mounting immune responses against three non-pathogenic antigens in breeding female common eiders, *Somateria mollissima*, a long-lived sea-duck. Female eiders are capital breeders that do not eat during the egg-laying and incubation periods and hence lose 40% of their pre-breeding body mass during this time (Korschgen 1977; Parker & Holm 1990). Body reserves are hence crucial and females with low body reserves more frequently abandon their clutch or brood (Bustnes & Erikstad 1991; Erikstad *et al.* 1993; Hanssen *et al.* 2003b), presumably to avoid reaching a level of energy depletion where self maintenance and ultimately survival are reduced.

Thus, breeding female eiders are likely to face considerable resource limitations, as they rely solely on stored body reserves, making them excellent study objects when investigating the costs of immune defence. Early in the incubation period, we captured female eiders and inoculated each with sheep red blood cells (SRBC) and with diphtheria–tetanus vaccine. We thereafter studied whether the inoculation led to antibody responses specifically directed against each of the antigens and, if present, the magnitudes of these responses. Using this design as opposed to a design with a sham-injected control group allows us to measure any cost of humoral immune-system

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activation and at the same time it excludes the possibility that any observed effects are a result of something else in the injected solution of SRBC or vaccine other than the antigens (e.g. the adjuvant). Moreover, our design also maximizes sample size allowing examination of the effects of qualitative and quantitative variation in the antibody responses against each injected antigen. We measured short-term costs of immune responses as the loss of body mass after inoculation, and long-term costs of immune responses as the return rate in subsequent breeding seasons.

## 2. MATERIAL AND METHODS

### (a) *Treatment and general procedures*

The study was conducted in a breeding colony (ca. 400 pairs) of common eiders on the island of Grindøya, northern Norway (69°49' N, 18°15' E). The breeding biology of eiders at our study site has been described previously (Erikstad *et al.* 1993). We captured females (29 females in 2000 and 28 in 2001) 5 days into the incubation period, weighed them ( $\pm 2.5$  g) and injected them intraperitoneally with 1 ml of a 2% suspension of SRBC. SRBC in sterile phosphate-buffered saline are a standard complex antigen commonly used in immunological studies (Hay & Hudson 1989). The females were also injected with 150  $\mu$ l diphtheria–tetanus vaccine in the pectoral muscle (SBL Vaccin AB, Stockholm; diphtheria toxoid 38 Lf (flocculation entities) and tetanus toxoid 7.5 Lf, mixed with the adjuvant aluminium phosphate 5 mg ml<sup>-1</sup>). Before vaccination we collected a blood sample of ca. 1.5 ml from the brachial wing vein to control for possible initial levels of antibodies against SRBC, diphtheria toxoid and tetanus toxoid. We recaptured females 15 days in 2000 and both 7 and 15 days in 2001 after antigen injection. At each recapture females were weighed and a blood sample was collected. We calculated mass loss from vaccination to recapture (15 days later) and also relative mass loss (mass loss per mass at vaccination). Mass loss and relative mass loss are strongly correlated ( $r^2 = 0.79$ ,  $F_{1,56} = 210.91$ ,  $p < 0.0001$ ), hence we used relative mass loss in all analyses. The entire colony was intensely searched for breeding females in the years following the treatment (2001–2003). All breeding females were captured and their ring numbers registered to determine the between-year return rate up to 2 years after the treatment. The Norwegian Animal Research Authority approved the treatment and field study.

### (b) *Immunological procedures*

Humoral immune-system activation against SRBC was measured using a standard haemagglutination test (Cichón 2000). Humoral immune-system activation against diphtheria and tetanus was measured using a standard enzyme-linked immunosorbent assay (ELISA; Hasselquist *et al.* 1999, 2001; Råberg *et al.* 2000). As a secondary antibody we used a commercial peroxidase-labelled anti-duck immunoglobulin (Ig) antiserum produced in goats (cat. no. A 6154; Sigma-Aldrich, Sweden). The dilutions used for the pre- and post-injection plasma were 1:400 for the tetanus plates and 1:200 for the diphtheria plates. To reduce the effects of between-plate variation, we ran all samples from each year simultaneously in the same batch. For each individual, pre- and post-injection serum samples were added to the plate in duplicate and the average antibody titre constitutes our measure for each dilution (repeatability for duplicates as intraclass correlation coefficients: diphtheria:

$n = 24$  duplicates, repeatability = 0.99; tetanus:  $n = 27$  duplicates, repeatability = 0.96; SRBC:  $n = 29$  duplicates, repeatability = 0.97). Antibody titres against each antigen were estimated as post- minus pre-injection values. Pre-injection titres were zero for all except 10 individuals (three diphtheria, one tetanus and six SRBC). These individuals did not differ from the other individuals with respect to quality or to response-antibody titres. Pre-injection responses were not related to return rate. Some individuals did not have any measurable antibody responses against one or more of the antigens (diphtheria, 33 out of 57; tetanus, 30 out of 57; SRBC, 28 out of 57). In the analyses, we classified females according to whether they had measurable responses against each of the antigens or not. The titres of the responding birds were log transformed to conform to the normality assumptions of parametric statistics. To keep disturbance at a minimum, in 2000 we measured primary antibody titres only after 15 days. This timing is close to that taken to attain the peak titres for primary IgG responses against non-pathogenic antigens in birds (Hasselquist *et al.* 1999). However, it may be somewhat later than in studies measuring responses to SRBC with a haemagglutination test (i.e. 6–9 days; e.g. Peters 2000). To check whether this had any effect on our measure of antibody titres, we therefore recaptured females twice (7 and 15 days after injection) in 2001. We found that 75% of birds (18 out of 24 birds) showed a humoral response against SRBC on both day 7 and day 15. Moreover, there was a positive correlation between immune response on day 7 and that on day 15 ( $F_{1,23} = 8.81$ ,  $r^2 = 0.29$ ,  $p = 0.007$ ). Only four out of the 22 birds that showed a response at day 7 had 'lost' it at day 15. Moreover, only one of the birds that did not respond at day 7 showed a response at day 15. Thus, we conclude that measuring antibody responsiveness towards SRBC at day 15 does not underestimate the frequency of responding birds.

### (c) *Statistical procedures*

To get a measure of individual quality in female eiders, we used a principal component analysis including date of egg laying (fitter females lay their eggs earlier in the season; Spurr & Milne 1976), body mass (fitter females have larger body reserves; Bustnes & Erikstad 1991; Erikstad *et al.* 1993; Hanssen *et al.* 2003b) and clutch size (fitter females produce larger clutches; Erikstad *et al.* 1993; Hanssen *et al.* 2003b). The first principal component (PC1) explained 48% of the variation in the data. PC1 was negatively related to egg-laying date ( $r = -0.78$ ) and positively related to both clutch size ( $r = 0.58$ ) and body mass ( $r = 0.71$ ), hence higher values of PC1 reflect higher quality. To control for any effects of individual quality, we included PC1 as a covariate in all analyses. When analysing the effects of the humoral immune-defence activation, we kept experimental clutch size (as part of an ongoing study of incubation costs females were randomly assigned a clutch size of three or six eggs when captured and inoculated) and year as random factors and then added quality and response/no response against each of the three antigens to the models. When analysing binary response variables, such as return rate and the tendency to mount or not mount an immune response, we used logistic regression models. All values are presented as means  $\pm$  s.e. and all tests are two tailed.

## 3. RESULTS

### (a) *Immunosuppression*

We found that suppression of humoral immune responses was frequent among incubating female eiders.

Table 1. Return rate in common eider females in relation to the tendency to mount a humoral immune response against SRBC, diphtheria toxoid and the interaction between the two.

(Responding or not against tetanus toxoid, year, experimental clutch size, quality and other two-way interactions were initially included in the logistic regression model but were removed as they did not contribute significantly to the model. The deviance of the null model was 79.00 with 56 degrees of freedom, while after fitting the final model the deviance was reduced to 57.87 with 53 residual degrees of freedom. Results are type III sums of squares.)

variable	$\Delta D$	$\Delta d.f.$	$p$
+ SRBC response or not	17.99	1	< 0.0001
+ diphtheria response or not	4.38	1	0.04
+ diphtheria response or not $\times$ SRBC response or not	7.82	1	0.005
rejected terms			
+ year	0.21	1	0.65
+ quality	0.005	1	0.95
+ experimental clutch size	0.11	1	0.74
+ tetanus response or not	0.07	1	0.79

Among the 57 inoculated individuals, 42% responded against diphtheria, 47% responded against tetanus and 51% responded against SRBC (21% against no antigens, 33% against only one antigen, 14% against SRBC and tetanus only, 12% against diphtheria and tetanus only, 4% against SRBC and diphtheria only and 16% against all three antigens). Moreover, responses against the different antigens were only partly correlated within individuals. Females responding against diphtheria had a larger probability of also responding against tetanus ( $\chi^2_{1,56} = 11.30$ ,  $p = 0.0008$ ; controlling for experimental clutch size:  $\chi^2_{1,56} = 2.07$ ,  $p = 0.15$ ; year:  $\chi^2_{1,56} = 6.17$ ,  $p = 0.01$ ; quality:  $\chi^2_{1,56} = 0.55$ ,  $p = 0.46$ ). Also, the strength of the response against tetanus tended to be higher in individuals also responding against diphtheria ( $F_{1,26} = 3.96$ ,  $p = 0.06$ ; controlling for experimental clutch size:  $F_{1,26} = 0.21$ ,  $p = 0.65$ ; year:  $F_{1,26} = 6.28$ ,  $p = 0.02$ ; quality:  $F_{1,26} = 0.72$ ,  $p = 0.41$ ). Responding against SRBC was not associated with an increased tendency to respond against either diphtheria or tetanus ( $p > 0.73$ ). Among the females responding against two or more antigens, the strengths of the responses (antibody titres) were not correlated ( $p > 0.19$ ). Quality was not related to the strengths of the responses against any antigens (all  $p$ -values greater than or equal to 0.35) or to the tendency to respond or not (all  $p$ -values greater than or equal to 0.28).

#### (b) Mass loss and immune responses

Neither response/no response nor the magnitude of antibody production in responding birds had any effect on female relative mass loss (all  $p \geq 0.15$ ). Because mass loss is apparently a very good predictor of energy consumption in female eiders (as they are fasting during the incubation period; Parker & Holm 1990), this suggests that mounting a humoral immune response is not associated with increased energetic requirements. Furthermore, responding females did not show any increased propensity for clutch or brood desertion ( $p \geq 0.18$ ).

#### (c) Return rate and immune responses

The proportion of females returning to breed the year after the injections was significantly lower among those that mounted an antibody response against SRBC during incubation (28% versus 71%; table 1, figure 1a), and this was consistent in both years (response against SRBC or

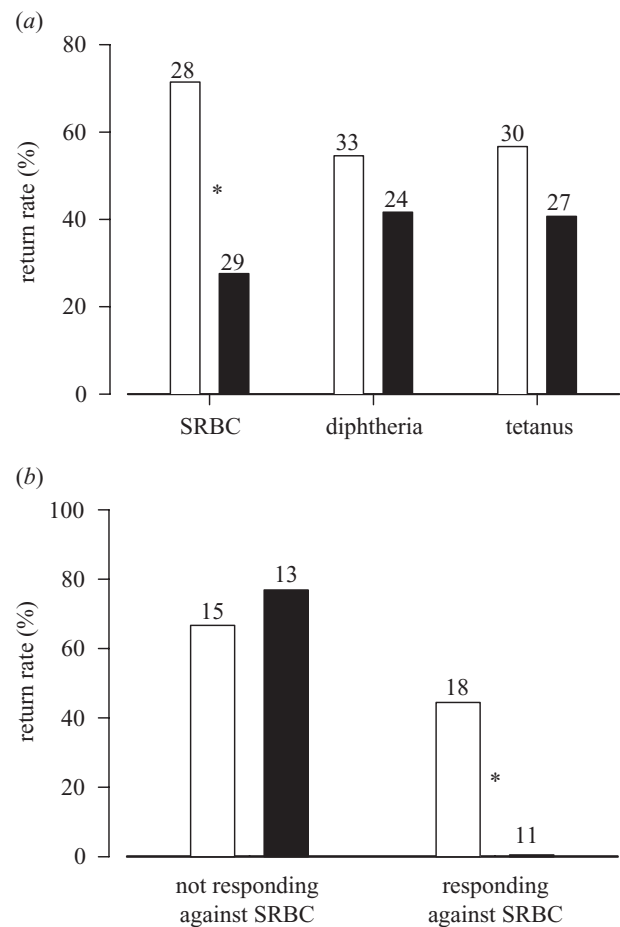


Figure 1. (a) Return rates of female common eiders that did and did not mount humoral immune responses against injected SRBC, diphtheria toxoid and tetanus toxoid in a 2 year study (2000–2001). Sample sizes are shown above each bar. Open bars, not responding; filled bars, responding. (b) Return rates of female common eiders that did and did not mount humoral immune responses against SRBC, depending upon whether or not they also mounted a response against diphtheria toxoid. Sample sizes are shown above each bar. Open bars, not responding against diphtheria; filled bars, responding against diphtheria. \* Symbolizes significant differences; see table 1 for statistical details.



not:  $\chi^2 = 7.41$ ,  $p = 0.007$ ; year effect:  $\chi^2 = 0.27$ ,  $p = 0.60$ ; year  $\times$  response to SRBC;  $\chi^2 = 0.65$ ,  $p = 0.42$ ). The difference between responders and non-responders to SRBC remained when we analysed return rate over the next 2 years (41% versus 86%; logistic regression:  $n = 57$ ,  $\chi^2 = 12.72$ ,  $p = 0.0004$ ).

Among the responding individuals, return rate the next year was not related to the antibody titres against any of the antigens ( $p$ -values in the range of 0.25–0.68). This suggests that it was not the amount of antibodies produced, but rather whether the humoral immune system was activated or not, that caused the severe reduction in return rate. Furthermore, in females already responding against SRBC, also mounting a humoral response against diphtheria had a strong additional negative effect on return rate ( $p = 0.005$ ; table 1, figure 1*b*), whereas responses against tetanus did not affect return rate ( $p = 0.79$ ; table 1, figure 1*a*).

#### 4. DISCUSSION

The observed reduction in between-year return rate in female eiders responding to SRBC and diphtheria toxoid could not be attributed to any toxic or other adverse effect of the antigen injections, as all individuals received the same treatment (two injections each, one with SRBC and one with diphtheria–tetanus vaccine). That the same effect occurred in two consecutive study years strengthens the general validity of the result.

##### (a) *Return rate and dispersal*

The low return rate of female eiders that mounted humoral responses against SRBC and diphtheria could be caused by high mortality, high dispersal or a high incidence of abstinence from breeding. In female eiders, between-year survival is high, and in our study colony it has been estimated at *ca.* 80% based on capture–recapture data (Yoccoz *et al.* 2002). This value is very close to the value we found for females that did not mount an antibody response against SRBC in our study (86% over 2 years), but much higher than the value for females responding to this antigen (41% over 2 years). This suggests that mounting humoral immune responses against these non-pathogenic antigens resulted in impaired survival. Moreover, site fidelity in female eiders is very high: 98% of re-sighted females repeatedly return to their birth colony (Baillie & Milne 1989; Swennen 1990) and even in cases of disturbance and reduced breeding success only 2.5% of the exposed females change breeding colony (Wakeley & Mendall 1976). Thus, it is very unlikely that the considerably reduced return rate could be explained by high dispersal. Female eiders can abstain from breeding every third or fourth year, before returning to breed in the same colony (Yoccoz *et al.* 2002). This, however, did not affect our results, as the proportion of females not breeding the first year after the antigen injection but returning in the second year (14–15%) did not differ between responding and non-responding females. Apparently, the long-term effects of mounting humoral immune responses against certain antigens had severe negative effects on survival in female eiders.

##### (b) *Individual quality*

Different return rates could be explained by other factors influencing the tendency to mount a humoral immune response. Immune responsiveness may be related to individual quality (Sheldon & Verhulst 1996; Saino *et al.* 1997; Hanssen *et al.* 2003*c*). Quality, however, was not related to the tendency to mount a response against SRBC, diphtheria toxoid or tetanus toxoid. Furthermore, individual quality was not related to the antibody titres against SRBC, diphtheria toxoid or tetanus toxoid among the responding individuals. Hence, there was a large variation, both in the response/no response to the antigens and in the strength of the immune response, which is not related to current status or condition. It is possible that there are underlying genetic causes for the observed variation in immune responsiveness and specificity, possibly related to the highly variable major histocompatibility complex genes (Apanius *et al.* 1997; Penn & Potts 1999) or other variable genes important for variation in immune responsiveness (Puel & Mouton 1996).

##### (c) *Costs of immune-system activation*

One important factor that may help to explain why female eiders suffered much higher costs of mounting adaptive immune responses than other investigated bird species is the fact that the female eiders relied solely on stored fat, protein and nutrient reserves when their immune defence was challenged. Thus, costs inducing higher mortality may be related to the conversion of fat and protein to energy or to the inability to compensate for energy or nutrient losses during the antibody response by altering food intake. Energy limitations have been suggested to be an important explanation for why immunity is suppressed in low-quality individuals and during strenuous work (Sheldon & Verhulst 1996; Nelson *et al.* 2002). We did not find, however, that mounting a humoral immune response against SRBC, or SRBC and diphtheria, was associated with increased resource expenditure (*i.e.* higher mass loss). Moreover, the rate of clutch and brood desertion did not differ between responders and non-responders suggesting that short-term costs such as energetic constraints are low. Thus, energy limitations could not have been the major factor causing the observed pattern of severely reduced return rate in antibody-producing females.

Our study is, to the best of our knowledge, the first to show that a vertebrate is exposed to such severe negative fitness consequences as effects on survival caused by immune-system activation *per se* (*i.e.* when immune responses are elicited by non-pathogenic antigens) may lead to reduced survival in a vertebrate. Only one previous study, of an invertebrate (bumble-bees) kept under laboratory conditions, has demonstrated negative survival effects of mounting an immune response (encapsulation response; Moret & Schmid-Hempel 2000). In investigated birds, inoculation with SRBC, or diphtheria and tetanus toxoids, has not caused any negative effects on survival (Saino *et al.* 1997; Råberg *et al.* 2000; Cichón *et al.* 2001). It is therefore surprising that such severe costs are induced in female eiders. One explanation may be that the combined effect of being challenged simultaneously with several antigens caused the dramatic effect. Moreover, properties of the antigen itself (*e.g.* number of epitopes,

risk of cross-reactivity and autoimmunity) may have induced higher costs for some antigens than others. Injection with SRBC introduces whole cells with many different possible epitopes, which may activate a wider spectrum of the immune system. By contrast, diphtheria–tetanus vaccine contains just the toxoids of these two antigens, thus causing a more specific activation of the humoral immune system. However, the immune responses to diphtheria and tetanus toxoids may differ considerably. In a recent study of blue tits (*Parus caeruleus*), stabilizing selection occurred on the primary humoral immune response against diphtheria, suggesting that individuals producing the highest primary antibody responses paid a cost in terms of reduced survival (Råberg & Stjernman 2003). By contrast, for tetanus there was no selection on primary responses and a positive directional selection on secondary responses, suggesting that there is no cost to a high response to this antigen. This is in accordance with the findings from the present study that responses against tetanus did not lead to survival costs whereas responses against diphtheria did.

#### (d) *Fasting and immune-system activation*

Another important difference between female eiders and most other birds is that female eiders are fasting during the incubation period. Passerines, in which all comparable studies have been conducted (Saino *et al.* 1997; Råberg *et al.* 2000; Cichón *et al.* 2001), in general have access to food throughout breeding. This may suggest that some important nutritional component may be limited in eiders, which are prevented from restoring their nutritional resources during incubation, for example antioxidants that scavenge free radicals produced by an activated immune system (von Schantz *et al.* 1999). In this respect, it is interesting to note that in the study by Moret & Schmid-Hempel (2000) the bumble-bees had to be starved for the severe consequences of immune-system activation to appear. Fasting also prevents the introduction of new food-transmitted infections and results in a natural reduction in the numbers of intestinal parasites (Thompson 1985), suggesting that multiple challenges to the eider immune system during incubation are rare. Hence, under normal conditions the humoral immune system may seldom be highly and multiply activated in incubating eiders, which could explain why such detrimental effects as we found in the present study are seldom observed under natural conditions. Also, recent studies have proposed a link between fasting and immunosuppression through a reduction in levels of the hormone leptin, which regulates food intake, body weight, stress response and T-cell-mediated inflammatory responses (Lord *et al.* 1998; Harris 2000; Kuchroo & Nicholson 2003). This may explain the high frequency of immunosuppression in fasting eiders and possibly also why no short-term effect of immune-system activation was found.

## 5. CONCLUSIONS

In the present study, it is likely that we have induced an unusual situation in incubating eiders by simultaneously providing several potent antigens in high concentrations. This treatment may have mimicked multiple severe infections and therefore induced seemingly maladaptive

humoral immune responses in the highly stressed and malnourished females. This treatment allowed us to reveal the very high costs that can be induced by a humoral response under certain conditions; however, we argue that female eiders are seldom exposed to such severe immune challenges under natural breeding conditions. Thus, the present study implies that immune-system activation *per se* can have severe negative consequences on fitness in vertebrates. It also underlines the fact that activation of an induced defence such as the vertebrate immune system may entail severe long-term costs despite the fact that no short-term energetic costs are observed. Our study may also have implications for vaccination programmes in humans, as it suggests that caution should be exercised when planning preventive vaccination programmes in humans that are under severe nutritional stress, in particular if several antigens are injected simultaneously.

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