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# Cost of reproduction in a long-lived bird: incubation effort reduces immune function and future reproduction

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Life-history theory predicts that increased current reproductive effort should lead to a fitness cost. This cost of reproduction may be observed as reduced survival or future reproduction, and may be caused by temporal suppression of immune function in stressed or hard-working individuals. In birds, consideration of the costs of incubating eggs has largely been neglected in favour of the costs of brood rearing. We manipulated incubation demand in two breeding seasons (2000 and 2001) in female common eiders (*Somateria mollissima*) by creating clutches of three and six eggs (natural range 3–6 eggs). The common eider is a long-lived sea-duck where females do not eat during the incubation period. Mass loss increased and immune function (lymphocyte levels and specific antibody response to the non-pathogenic antigens diphtheria and tetanus toxoid) was reduced in females incubating large clutches. The increased incubation effort among females assigned to large incubation demand did not lead to adverse effects on current reproduction or return rate in the next breeding season. However, large incubation demand resulted in long-term fitness costs through reduced fecundity the year after manipulation. Our data show that in eiders, a long-lived species, the cost of high incubation demand is paid in the currency of reduced future fecundity, possibly mediated by reduced immune function.

**Keywords:** incubation cost; immune function; life history; precocial; trade-off; seabird

## 1. INTRODUCTION

Cost of reproduction is a central concept in evolutionary biology, where increased investment in current reproduction is predicted to lead to a decrease in future reproductive output (Williams 1966; Charnow & Krebs 1974). Reproductive costs may be paid either directly, through reduced viability of offspring from current reproduction, or later, in terms of reduced survival or reduced number or quality of offspring in future breeding attempts (Stearns 1992). A factor mediating such delayed reproductive costs may be reduced immune function, often associated with stress and hard work, which may increase costs of infections (Sheldon & Verhulst 1996).

In birds, feeding and caring for young have been assumed to be the costliest of the reproductive phases. Empirical studies of reproductive costs in birds have therefore traditionally been done by experimentally increasing the number of young after hatching, thus increasing the offspring-feeding demands for parents, and measuring effects on various variables related to the parents' fitness. Some studies of increased brood-rearing costs have documented reduced adult survival or reduced future fecundity, although the majority of experimental studies have not found such costs (Dijkstra *et al.* 1990; Lessells 1991; Roff 1992; Stearns 1992; Golet *et al.* 1998). In contrast, incubating eggs has traditionally been

considered less costly than brood rearing (Monaghan & Nager 1997; Thomson *et al.* 1998). However, studies which have experimentally increased clutch size during incubation have documented various short-term costs to the incubating parents (Biebach 1981, 1984; Haftorn & Reinertsen 1985; Coleman & Whittall 1988; Moreno & Carlson 1989; Smith 1989; Moreno *et al.* 1991; Székely *et al.* 1994; Reid *et al.* 2000a; Engstrand & Bryant 2002; Hanssen *et al.* 2003a) and the offspring (Moreno & Carlson 1989; Moreno *et al.* 1991; Siikamäki 1995; Heaney & Monaghan 1996; Reid *et al.* 2000a,b; Engstrand & Bryant 2002; Ilmonen *et al.* 2002; Hanssen *et al.* 2003a; Larsen *et al.* 2003). Increased physical demand on the parent from increased incubation effort could also lead to delayed reproductive costs such as lower adult survival or reduced future fecundity. However, only one of the existing studies of incubation costs, known to us, has strongly indicated reduced survival (Visser & Lessells 2001), and no studies have shown reduced fecundity. One of several possible reasons for not finding such costs may be that parents are reluctant to increase effort in response to brood or clutch size manipulation, thereby jeopardizing the viability of eggs or young in enlarged broods (Siikamäki 1995; Hanssen *et al.* 2003a). That is, increased demand does not lead to the increased current parental investment that, in turn, could lead to future fitness costs for the parent (Hörak 2003). It is therefore important to investigate if increased

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demand translates into increased physical strain before expecting to find long-term costs of reproduction.

To avoid reduction of current reproductive output, parents may buffer increased reproductive demands by allocating resources from immunity to reproduction (Folstad & Karter 1992; Sheldon & Verhulst 1996). However, such allocation could result in increased susceptibility to infectious organisms and thus translate increased reproductive investment into reduced fitness. Owing to the complexity of the vertebrate immune system, it is important to evaluate more than one immune component when trying to measure immunocompetence, (Sheldon & Verhulst 1996). Hence, we measured two main types of immune cells (heterophils and lymphocytes) and the specific antibody responses against three different antigens: sheep red blood cells (SRBC), diphtheria toxoid and tetanus toxoid. The SRBC consist of whole cells with a large number of possible epitopes, which should lead to a broader activation of the immune system, whereas tetanus and diphtheria toxoids only contain a few epitopes, leading to more specific responses. These three antigens seem to elicit different humoral responses and entail different costs to the responding individual (Råberg & Stjernman 2003; Westneat *et al.* 2003; Hanssen *et al.* 2004).

In this study, we manipulated incubation demand in female common eiders, *Somateria mollissima* L., whose natural clutch size ranges from three to six eggs. By adding or removing eggs early in the incubation period, we created clutch sizes of either three (low incubation demand) or six eggs (high incubation demand). The common eider is a large sea-duck in which females incubate alone and do not feed during the entire incubation period. Hence, they lose approximately 40% of their body mass during the egg-laying and incubation period (Parker & Holm 1990). Thus, this species is suitable for exploring effects of altered resource requirements during incubation. A recent correlative study showed that low body mass and reduced immunocompetence during the incubation period in female eiders was associated with increased current and future reproductive costs (Hanssen *et al.* 2003b). Here, we investigated whether incubating a clutch of high demand (six eggs) leads to higher body mass loss (reflecting higher energy consumption) than incubating a clutch of low demand (three eggs). Additionally, we examined whether high incubation demands lead to immunosuppression. To test how females responded to the higher incubation demand, we measured if nest desertion and brood abandonment were more frequent in the high demand group. Furthermore, to evaluate if increased incubation demands lead to future fitness costs, we also measured the return rate of female eiders for the next breeding season as well as reproductive investment of the returning females in the year after manipulation. The common eider is a long-lived species (Baillie & Milne 1982; Yoccoz *et al.* 2002). Hence they have a relatively high reproductive potential, making them more sensitive to costs that jeopardize survival (Wooller *et al.* 1992). Thus, cost of reproduction in long-lived species is expected to appear as reductions in future fecundity, rather than reduced survival (Charlesworth 1980; Lindén & Møller 1989).

## 2. MATERIAL AND METHODS

### (a) *Study area and species*

This study was conducted in a common eider *S. mollissima* colony on Grindøya, Tromsø, northern Norway (69°49' N, 18°15' E). Grindøya is an island 0.65 km<sup>2</sup> in size, with a breeding colony of approximately 400 pairs of eiders. During the breeding seasons 2000 and 2001, the colony was visited daily from the start of egg-laying in mid-May in order to determine the date for onset of egg-laying and clutch size. Eiders lay one egg per day (Watson *et al.* 1993) and we assumed that the clutch was complete when no new eggs were laid during a 2-day period (clutch size range 3–6 eggs). Six eggs occur in about 5% of nests (Erikstad & Tveraa 1995).

### (b) *Experimental and general procedures*

Five days after clutch completion, each female was randomly assigned to one of two experimental groups, three (hereafter low demand) or six (hereafter high demand) eggs. The manipulations were performed by adding or removing eggs. For example, if a four-egg clutch was assigned to the high demand group, it received two eggs from a female that also had laid her last egg 5 days before. Original clutch size of the manipulated nests ranged from three to six eggs. The low demand group consisted of 31 females (16 in 2000 and 15 in 2001) and the high demand group of 34 females (19 in 2000 and 15 in 2001). No female was included in the experiment in both years. There were no differences between groups in the various measures registered at the time of manipulation, suggesting a successful randomization of treatment. (Initial clutch size: low demand, mean =  $4.3 \pm 0.1$  eggs; high demand, mean =  $4.5 \pm 0.1$  eggs,  $F_{1,64} = 1.60$ ,  $p = 0.21$ . Lay date (days after 1 May): low demand, mean =  $25.8 \pm 0.8$  days; high demand, mean =  $24.9 \pm 0.7$  days,  $F_{1,64} = 2.10$ ,  $p = 0.15$ . Body mass: low demand, mean =  $1911 \pm 21$  g; high demand, mean =  $1897 \pm 23$  g,  $F_{1,64} = 0.14$ ,  $p = 0.71$ . H/L-ratio: low demand, mean =  $-0.16 \pm 0.03$ ,  $n = 27$ ; high demand, mean =  $-0.15 \pm 0.04$ ,  $n = 30$ ,  $F_{1,56} = 0.13$ ,  $p = 0.72$ . Lymphocyte level: low demand, mean =  $1.703 \pm 0.009$ ,  $n = 30$ ; high demand, mean =  $1.685 \pm 0.009$ ,  $n = 33$ ,  $F_{1,62} = 2.25$ ,  $p = 0.14$ ). Statistics are from ANCOVA, with year and the interaction term between year and treatment as fixed factors; the interaction term was in no instances significant. There were significant mean differences between years in lay date (2000 mean =  $27.7 \pm 0.4$  days, 2001 mean =  $22.5 \pm 0.8$  days,  $F_{1,64} = 38.55$ ,  $p < 0.0001$ ), and H/L-ratio (2000 mean =  $-0.22 \pm 0.01$ , 2001 mean =  $-0.07 \pm 0.05$ ,  $F_{1,56} = 10.95$ ,  $p = 0.002$ ). We did not include any control clutches where clutch size had not been manipulated. However, a previous clutch size manipulation study conducted in the same colony found no differences in mass loss, nest desertion rates or brood abandoning rates between one group where no manipulation took place, and another group where one egg was swapped between nests without altering clutch size, suggesting that there is no effect of exchanging eggs between nests in eiders (Hanssen *et al.* 2003a). Owing to nest losses caused by desertion and predation, 24 females in the low demand and 30 females in the high demand group were initially included in the analysis of incubation costs (table 1). For some of the analyses, samples sizes were further reduced (see table 1, figure 1), as some of the serum samples were insufficient in size or quality for analyses. All birds were individually marked with foot-rings which allowed identification of birds breeding the following year. Out of the 24 females in the low demand group that incubated their eggs for

Table 1. Effects of high or low incubation demand (incubating six or three eggs, respectively) on frequency of nest loss, humoral immune responsiveness against non-pathogenic antigens (SRBC, tetanus and diphtheria toxoid), heterophil levels, incubation time, brood abandonment after hatching and return rate. (Values are expressed as mean  $\pm$  s.e. Sample sizes in parentheses.)

	low demand	high demand	test statistic	<i>p</i> -value
% nest loss	39 (31)	21 (34)	2.44 <sup>a</sup>	0.12
antibody titre against SRBC	0.21 $\pm$ 0.08 (12)	0.16 $\pm$ 0.07 (14)	0.35 <sup>b</sup>	0.56
antibody titre against tetanus	0.64 $\pm$ 0.08 (14)	0.65 $\pm$ 0.15 (10)	0.00 <sup>b</sup>	0.96
antibody titre against diphtheria	0.89 $\pm$ 0.13 (15)	0.83 $\pm$ 0.13 (9)	0.15 <sup>b</sup>	0.70
<i>H/L</i> -ratio	-0.26 $\pm$ 0.04 (18)	-0.22 $\pm$ 0.03 (23)	0.32 <sup>b</sup>	0.58
incubation time	23.2 $\pm$ 0.14 (19)	23.2 $\pm$ 0.12 (27)	0.01 <sup>b</sup>	0.92
% brood abandoning	50 (12)	36 (22)	0.63 <sup>a</sup>	0.43
% return rate	46 (24)	50 (30)	0.12 <sup>a</sup>	0.73

<sup>a</sup>  $\chi^2$  from logistic model with year as fixed factor.

<sup>b</sup> *F*-value from ANCOVA with year as fixed factor.

at least 20 days (table 1), 11 were found breeding the following year (table 1). Out of these 11 females, we were able to determine clutch size for all and date of clutch initiation (lay date) for seven (figure 2). Correspondingly, for the high demand group, of the 30 females that incubated their eggs for at least 20 days, 15 were found breeding the following year (table 1). Date of clutch initiation was registered for 10 of these and clutch size for all but one, which laid only one egg before deserting its nest (figure 2).

To induce humoral immune responses, we injected females with (i) 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 aluminum phosphate 5 mg ml<sup>-1</sup>) and (ii) 1 ml of a 2% suspension of SRBC intraperitoneally. The suspension contained  $5 \times 10^8$  SRBC in sterile phosphate-buffered saline (PBS). Further details of these methods in common eiders are presented in Hanssen *et al.* (2004). On the day of injection (5 days after laying the last egg), the females were weighed ( $\pm 2.5$  g), their wing length measured ( $\pm 1$  mm) and a blood sample ( $\sim 1.5$  ml) collected in heparinized tubes. We recaptured the birds 15 days later (2–6 days before hatching), weighed them and collected a new blood sample. Blood samples were stored on ice in the field for up to 4 h before being centrifuged (2500 rpm for 10 min). Plasma was extracted and stored at  $-20$  °C until analysis of antibody titres.

We calculated mass loss as (mass at day 5) – (mass at day 20), and the relative mass loss as the percentage of mass lost from day 5 to day 20. Mass loss and relative mass loss were highly correlated ( $r^2 = 0.79$ ,  $t = 14.70$ ,  $n = 60$ ,  $p < 0.0001$ ), and we therefore only used relative mass loss in the analyses. At the last capture, females were marked with individually colour-coded tape tags at the back of their head. This made it possible to determine if the females cared for, or abandoned, their ducklings after hatching (Kehoe 1989; Bustnes & Erikstad 1991).

### (c) Haematology

#### (i) Lymphocyte levels

Two capillary tubes with blood from each individual were centrifuged in a Compur mini-centrifuge at 11 500 rpm for 3 min and 15 s. The haematocrit value is the percentage of red blood cells in the tubes. The means from the two samples were used. One blood smear was prepared from each blood sample. The smear was immediately fixed in methanol

and stored for later analyses. Blood smears were stained using the May–Grünwald–Giemsa staining method. Smears were scanned at  $\times 1000$  magnification, and erythrocytes, lymphocytes and heterophils were counted in three independent areas. We calculated the lymphocyte/erythrocyte- and heterophil/lymphocyte-ratios (*H/L*-ratios) by averaging the ratios from the three counts of each blood smear. Estimates of the numbers of circulating lymphocytes per unit were obtained by multiplying the lymphocyte/erythrocyte-ratio with the haematocrit value (Dufva & Allander 1995; Skarstein & Folstad 1996). Repeatability (intraclass correlation coefficients according to Lessells & Boag (1987)) of this method in eiders has previously been tested and found to be generally high (*H/L*-ratio:  $r = 0.31$ , lymphocyte/erythrocyte-ratio:  $r = 0.80$  and haematocrit:  $r = 0.96$  from Hanssen *et al.* (2003b)). The *H/L*-ratio is known to increase in response to various stressors, including infectious diseases, starvation and physiological disturbance (Gross & Siegel 1983; Dein 1986; Maxwell 1993). Decreasing lymphocyte levels are indicative of immunosuppression, with a concomitant increase in susceptibility to infections (Siegel 1985; Fitzgerald 1988).

#### (ii) Haemagglutination assay

A standard haemagglutination test was performed to quantify the specific antibody concentration against SRBC. The plasma was heat inactivated at  $+56$  °C for 30 min. For each bird, 40  $\mu$ l of plasma was diluted 1 : 1 in PBS and then serially diluted in 96-well U-shaped microtitre plates (titre 1–12) in duplicates. Then, 40  $\mu$ l of 2% SRBC diluted in PBS were added to each well and the plates were incubated at  $+37$  °C for 60 min. The number of titres showing positive haemagglutination represents the SRBC-specific antibody concentration and is presented on a log scale (Hay & Hudson 1989). Repeatability of duplicates was calculated as intraclass correlation coefficients according to Lessells & Boag (1987). The average of the duplicates for each bird was used as the antibody titre for that particular capture event (repeatability of duplicates:  $r = 0.97$ ,  $n = 29$ ). The antibody titre value from the first capture (i.e. pre-injection) was then subtracted from the antibody titre obtained in samples collected 15 days after antigen injection. Twenty-six birds showed measurable responses against SRBC, whereas the remaining 26 showed no measurable responses against SRBC. In the analyses, we classified females according to whether they had measurable responses against SRBC or not. The titres of the responding

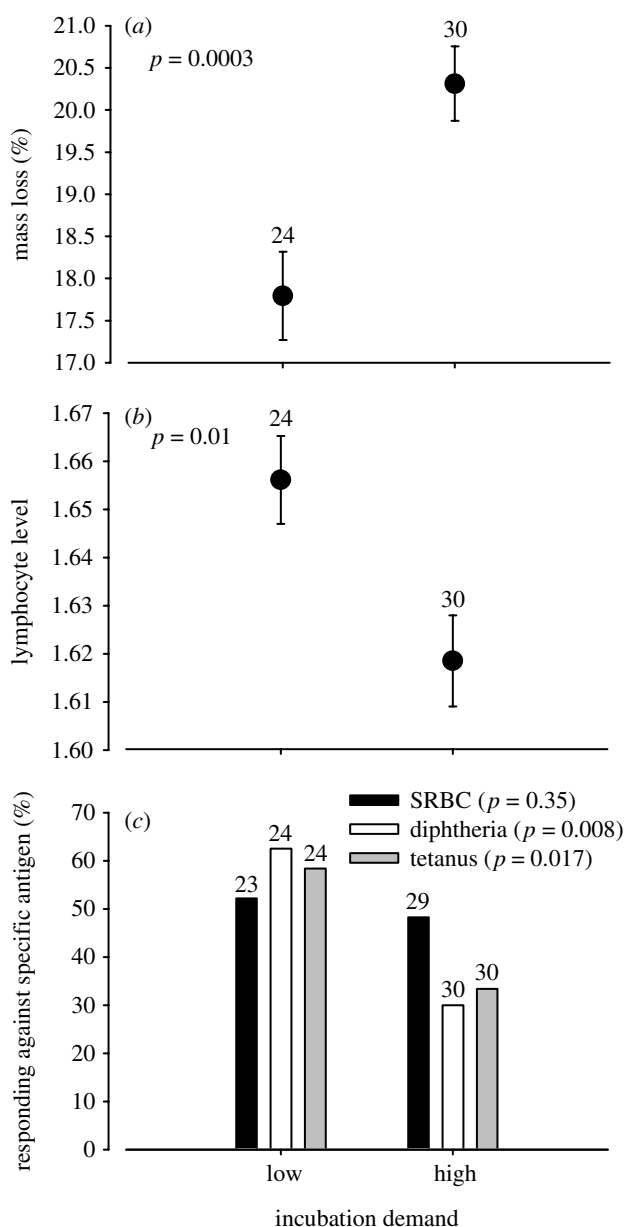


Figure 1. (a) Relative mass loss (percentage of initial mass lost), (b) lymphocyte level and (c) percentage of individuals with measurable antibody responses against diphtheria, tetanus and SRBC in relation to experimental incubation demand (incubating three or six eggs) in common eider females. Sample sizes (numbers of females) are given above each bar. See text for statistical analyses.

birds were log transformed to conform to the normality assumptions of parametric statistics.

### (iii) ELISA assay

We also measured humoral immune system activation as the antigen-specific antibody levels in the plasma of females using a standard enzyme-linked immunosorbent assay, ELISA (Hasselquist *et al.* 1999, 2001; Råberg *et al.* 2000; Hanssen *et al.* 2004). The ELISA method provides sensitive measures of the amount of antibodies that specifically bind to the coating antigen. As the secondary antibody, we used a commercial peroxidase-labelled antiduck immunoglobulin (Ig) antiserum produced in goat (item number A 6154; Sigma-Aldrich, Sweden). The dilutions used for the pre- and post-immunization 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 constituted our measure for each dilution (repeatability of duplicates as intraclass correlation coefficients; diphtheria:  $n=24$  duplicates, repeatability=0.99; tetanus:  $n=27$  duplicates, repeatability=0.96). Antibody titres against each antigen were estimated as post- minus pre-immunization values. Some individuals did not have any measurable antibody responses against one or both of the antigens (for diphtheria 30 out of 54 birds, for tetanus 30 out of 54 birds). In the analyses, we classified females as to whether they had measurable responses against the antigen (diphtheria or tetanus) or not. Among the individuals that responded, antibody levels were log transformed to conform to the normality assumption. Pre-immunization titre was 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 individual quality or response of antibody titres.

### (d) Data analysis

To test for effects of the experiment and simultaneously control for year and between-assay effects, we used year as a fixed factor in all analyses (ANCOVA and logistic regression). The first-order interaction between year and treatment was always tested, but removed if non-significant. Logistic regression was used when analysing all dichotomous independent variables (return rate, brood-tending/-abandoning, response or no response against injected antigens and nest lost/nest not lost). When analysing the long-term effects of the incubation effort experiment (laying date and clutch size in the year after manipulation), we used laying date or clutch size in the year of the experiment as a covariate to control for individual repeatability in these variables. All values are presented as means  $\pm$  s.e; all statistical analyses are two-tailed and were conducted using SAS statistical software (SAS Institute Inc. 1999).

## 3. RESULTS

Results of the incubation demand experiment are presented in table 1, figures 1 and 2. Nest loss caused by desertion and predation tended to be highest in females with experimental low incubation demand (table 1). Relative mass loss was higher in females experiencing high demand ( $F_{1,53}=14.05$ ,  $p=0.0006$ ; year effect:  $F_{1,53}=3.38$ ,  $p=0.07$ ; figure 1). In addition, fewer of the birds with high incubation demand mounted a measurable response against tetanus ( $\chi^2_{1,53}=4.54$ ,  $p=0.03$ ; year effect:  $\chi^2_{1,53}=11.66$ ,  $p=0.0006$ , figure 1; year 2000: 26% responded, year 2001: 70% responded) or diphtheria ( $\chi^2_{1,53}=5.85$ ,  $p=0.02$ ; year effect:  $\chi^2_{1,53}=1.60$ ,  $p=0.21$ , figure 1), and they also had lower lymphocyte levels ( $F_{1,51}=5.46$ ,  $p=0.02$ ; year effect:  $F_{1,51}=13.75$ ,  $p<0.0005$ ; covariate early lymphocyte level:  $F_{1,51}=4.32$ ,  $p=0.04$ ; figure 1, mean lymphocyte level: year 2000= $1.657 \pm 0.009$ , year 2001= $1.610 \pm 0.009$ ). Experimental incubation demand did not affect the tendency to mount a response against SRBC ( $\chi^2_{1,51}=0.14$ ,  $p=0.70$ ; year effect  $\chi^2_{1,51}=13.91$ ,  $p=0.0002$ , figure 1; year 2000: 28% responded, year 2001: 78% responded). Among the responding individuals, experimental incubation demand did not affect the magnitude of the antibody responses

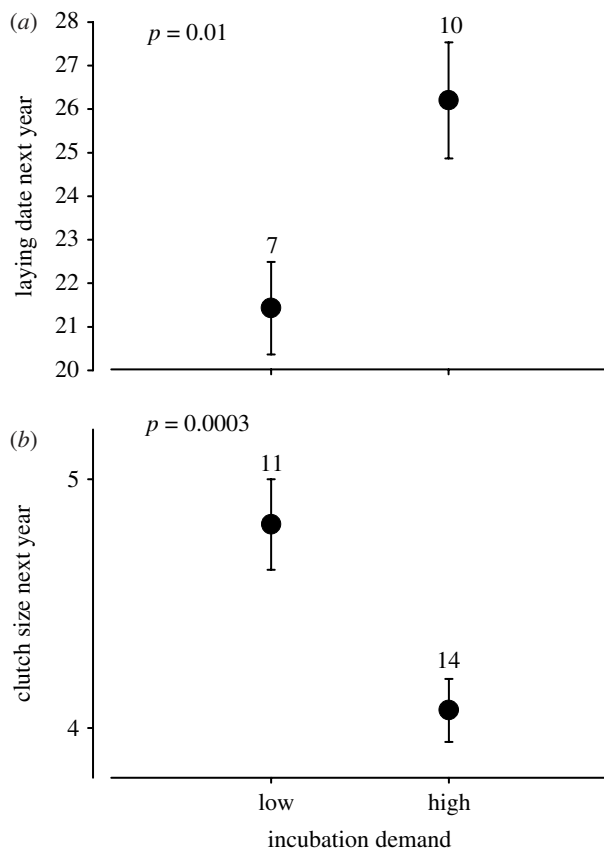


Figure 2. Long-term costs of reproduction in common eider females measured as (a) egg-laying date (date of clutch initiation in May) and (b) clutch size the year after manipulation of incubation demand. Incubation demand was manipulated by creating clutches of three (low demand) and six (high demand) eggs early in the incubation period. Sample sizes (numbers of females) are given above each bar. See text for statistical analyses.

(antibody titres) to the antigens SRBC, diphtheria or tetanus (table 1). Experimental incubation demand did not lead to changes in the *H/L*-ratio, the length of the incubation period or the tendency to abandon ducklings to other females after hatching (table 1).

We also investigated whether there were any long-term effects related to the high and low incubation demand, reflecting increased reproductive costs in the high demand group. Future reproduction was reduced in females with high incubation demand, as they laid a lower clutch size the year following the experimental manipulation (year  $n+1$ ) (incubation demand year  $n$ :  $F_{1,24}=16.72$ ,  $p=0.0006$ ; covariate clutch size year  $n$ :  $F_{1,24}=3.46$ ,  $p=0.08$ , figure 2). Also, the date of clutch initiation in the year after manipulation was delayed in the high demand group (incubation demand year  $n$ :  $F_{1,16}=6.53$ ,  $p=0.02$ ; covariate lay date year  $n$ :  $F_{1,16}=0.14$ ,  $p=0.71$ , figure 2). However, there was no difference in return rate in the next breeding season between females incubating high or low demand clutches (table 1). Even if the experimental manipulation of clutch size did not affect the ratio of brood tending versus brood abandoning in females, tending broods may be more demanding than abandoning them and we therefore also analysed models where tending versus not tending was added as a covariate. In these analyses, the sample size was reduced because not all birds were observed in the brood-tending period. Brood tending did not affect return rate

(incubation demand year  $n$ :  $\chi^2_{1,34}=0.64$ ,  $p=0.42$ ; year:  $\chi^2_{1,34}=0.29$ ,  $p=0.59$ ; brood tending year  $n$ :  $\chi^2_{1,34}=0.45$ ,  $p=0.50$ ), clutch size the year after ( $n+1$ ) (incubation demand year  $n$ :  $F_{1,15}=5.53$ ,  $p=0.04$ ; clutch size year  $n$ :  $F_{1,15}=0.81$ ,  $p=0.39$ ; brood tending year  $n$ :  $F_{1,15}=0.14$ ,  $p=0.72$ ) or lay date year  $n+1$  (incubation demand year  $n$ :  $F_{1,10}=2.26$ ,  $p=0.18$ ; lay date year  $n$ :  $F_{1,10}=0.07$ ,  $p=0.80$ ; brood tending year  $n$ :  $F_{1,10}=0.02$ ,  $p=0.89$ ).

#### 4. DISCUSSION

In this clutch size manipulation study in common eiders, *S. mollissima*, we found that high incubation demand resulted in short-term costs, in terms of higher mass loss and reduced immune function (lower lymphocyte levels and suppression of humoral immune responsiveness against both diphtheria and tetanus toxoid), compared with low incubation demand. In addition, high incubation demand also incurred long-term costs in terms of delayed nest initiation and reduced clutch size the following year. To our knowledge, such long-term effects of incubation effort have not been reported before.

##### (a) Reproductive investment

A large clutch may be perceived as more valuable because of its higher reproductive value (Trivers 1972; Coleman & Gross 1991). In eiders, nest desertion rates are reduced when clutch size, and thus clutch value, is experimentally increased (Hanssen *et al.* 2003a). In addition, large incubation cost in eiders may be compensated for by abandoning the brood to the care of other females after hatching (Bustnes & Erikstad 1991; Hanssen *et al.* 2003b) at the cost of lower survival of the adopted ducklings (Bustnes & Erikstad 1991; Eadie & Lyon 1998). In this study, neither brood abandonment nor nest desertion increased in high demand clutches, indicating that female eiders did not sacrifice eggs or young to buffer the increased incubation demand, possibly because the optimal parental investment level is higher in larger broods (Trivers 1972; Coleman & Gross 1991). It should be noted that we were unable to restore original clutch sizes after hatching (the female and brood leave the nest within 24 h), and therefore we cannot separate the effect of increased incubation costs from the effect of rearing a larger brood when analysing effects of the incubation effort experiment. However, in species where young feed themselves, costs of brood tending are probably not higher in larger broods (Lazarus & Inglis 1978, 1986). The fact that eider females readily adopt ducklings from other broods supports this assumption (Munro & Bédard 1977; Bustnes & Erikstad 1991). This assumption is also strengthened in the present study as females tending broods of six did not abandon their ducklings more often than females brooding only three ducklings. Moreover, tending ducklings did not affect survival, clutch size or lay date in the next season. Thus, it is unlikely that the long-term effects of our experiment are caused by factors other than the altered costs of incubation.

##### (b) Energetic and immune costs of incubation

Earlier studies of birds have shown that experimental enlargement of clutch size during incubation leads to increased incubation costs (Jones 1987; Moreno & Carlson 1989; Moreno *et al.* 1991; Tatner & Bryant

1993; Moreno & Sanz 1994; Siikamäki 1995; Hanssen *et al.* 2003a), and also costs to young after hatching (Heaney & Monaghan 1995; Cichón 2000; Ilmonen *et al.* 2002). A recent study on the Grindøya apopulation of eiders showed that enlarging clutch size by one egg led to reduced hatching success in low-quality females and increased mass loss in high-quality females (Hanssen *et al.* 2003a). In the present study, mass loss was highest in females incubating large clutches, indicating a higher energetic cost of incubating a large clutch. Immunocompetence may be suppressed during reproduction to free resources for reproductive investment, or to avoid immunopathology (Sheldon & Verhulst 1996; Råberg *et al.* 1998; Lochmiller & Deerenberg 2000). However, immunosuppression may result in a higher susceptibility to infections, and this trade-off has been proposed to be mediating long-term reproductive costs (Gustafsson *et al.* 1994). We found that high incubation demand was associated with a reduction of lymphocyte levels during the incubation period and a lower humoral immune responsiveness against two different antigens, diphtheria and tetanus toxoid. To our knowledge, only two studies have previously examined the effects of incubating enlarged versus reduced clutches on measures of immunocompetence (Cichón 2000; Ilmonen *et al.* 2002). These studies, on collared and pied flycatchers, did not document any effect of increased clutch size upon female condition or immunocompetence. However, unlike eiders, passerines may be able to compensate for increased incubation demands by increasing their food intake. In contrast, experimental alteration of brood size during chick feeding in passerine birds, which presumably increase nestling provisioning rate, have been found to result in a negative relationship between brood size and measures of humoral immunocompetence (Deerenberg *et al.* 1997; Nordling *et al.* 1998; Moreno *et al.* 1999; Saino *et al.* 2002; but see Bruun 2002; Ilmonen *et al.* 2003).

### (c) *Costs of incubation versus survival and future reproduction*

In the present study of a long-lived species, the return rate in the next breeding season was not affected by high or low incubation demand. However, future reproductive investment was reduced in females who experienced high incubation demand. In long-lived species, survival costs of reproduction have been documented in only four studies (Reid 1987; Pugsek & Diem 1990; Jacobsen *et al.* 1995; Golet *et al.* 1998). This may be expected, as long-lived species should only trade survival for reproduction under special circumstances (Minchella & Loverde 1981; Clutton-Brock 1984; Erikstad *et al.* 1998). Interestingly, in the present study, survival effects may indeed have been expected because high incubation demand resulted in reduced immune function. This reduced immune function may have led to increased pathogenicity from infections and ultimately, higher mortality. However, increased parasitism may reduce condition and performance without reducing survival (Lehmann 1993; Møller 1997; but see Hanssen *et al.* 2003c). In conclusion, the present study documents the importance of incubation costs by revealing immediate effects on mass loss and immune responses, and documenting, for the first time, adverse long-term effects on future reproduction.

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