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Sex-Specific Effects of Increased Incubation Demand on Innate Immunity in Black Guillemots

Lisha L. Berzins^{1,*}

H. Grant Gilchrist²

Kevin D. Matson³

Gary Burness^{4,†}

¹Environmental and Life Sciences Graduate Program, Trent University, Peterborough, Ontario K9J 7B8, Canada;

²Environment Canada, Science and Technology Branch, National Wildlife Research Centre, Carleton University, Ottawa, Ontario K1A 0H3, Canada; ³Animal Ecology, Center for Ecological and Evolutionary Studies, University of Groningen, P.O. Box 11103, 9700 CC Groningen, The Netherlands; ⁴Department of Biology, Trent University, Peterborough, Ontario K9J 7B8, Canada

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ABSTRACT

Life-history theory predicts that there should be negative fitness consequences, in terms of future reproduction and survival, for parents with increased reproductive effort. We examined whether increased incubation demand affected innate immunity and body condition by performing a clutch-size manipulation experiment in black guillemots (*Cephus grylle*). We found that plasma from males incubating experimentally enlarged clutches exhibited significantly reduced lysis titers compared with plasma from males incubating control clutches, while this was not observed in females. The increased incubation demand also impacted agglutination titers differently in males and females, although the effect of treatment was not significant in either sex. Among all birds, lysis titers increased and haptoglobin concentrations decreased from mid- to late incubation. Natural antibody-mediated agglutination titers and body condition were highly repeatable within the incubation bout and between years. This suggests that agglutination titers may serve as a reliable and resilient index of the immunological character of individuals in future studies. Overall, this study demonstrates that increased incubation demand impacts indices of innate immunity differently in males and females. The potential for different components of the immune system to

be impacted sex-specifically should be considered in future studies linking immune function and life-history trade-offs.

Introduction

Life-history theory predicts that there should be negative fitness consequences, in terms of future reproduction and survival, for parents with increased reproductive effort (Williams 1966). The concept of reproductive costs, which underlies this type of life-history trade-off, has been extensively studied in avian species (e.g., Jacobsen et al. 1995; Verboven and Tinbergen 2002; Hörak 2003; Dawson and Bortolotti 2008). Most studies investigating these life-history trade-offs have focused on the costs associated with posthatching parental care. To cover these costs of reproduction, resources may be diverted from other physiological functions, such as immune function (Deerenberg et al. 1997; Moreno et al. 1999; Ardia et al. 2003). In these studies, parents rearing enlarged broods generally mount weaker responses to an immune challenge compared with parents rearing control broods.

Relationships between incubation demand and parental quality have been less frequently considered when assessing reproductive costs (Monaghan and Nager 1997). Incubation, the process of applying heat to the eggs for embryonic development, is energetically costly (Moreno et al. 1991; Williams 1996; de Heij et al. 2007). Moreover, these energetic costs of incubation increase with clutch size (reviewed in Thomson et al. 1998). Naturally or experimentally increased energetic demands of incubation may lead to trade-offs with other physiological processes, such as immune responses, which also require energy and other common currencies (Sheldon and Verhulst 1996). Alternatively, suppression of immune defenses during incubation may function to reduce the damaging effects of oxidative stress (von Schantz et al. 1999) and/or risk of immunopathology (Räberg et al. 1998).

To date, few studies have investigated the physiological trade-offs between incubation demand and immune function; those that have are focused mainly on acquired immunity (e.g., Cichoń 2000; Ilmonen et al. 2002; Hanssen et al. 2005; Bourgeon et al. 2006). In contrast, trade-offs involving innate immunity have received little attention (but see Bourgeon et al. 2007). Innate immunity provides initial protection against pathogens and parasites (Roitt et al. 1996), and some components play essential roles in linking the innate and acquired arms of the immune system (e.g., natural antibodies [NABs] and complement; Ochsenbein and Zinkernagel 2000). Thus, given the links

* Present address: Ecosystem Science and Management Program, University of Northern British Columbia, Prince George, British Columbia V2N 4Z9, Canada.

† Corresponding author; e-mail: garyburness@trentu.ca.

between the innate and acquired immune systems, innate immunity should also be considered when examining the role of immunity in influencing life-history trade-offs.

Several components of innate immunity can be easily quantified in many bird species (Matson et al. 2005; Matson 2006). These components include nonspecific NABs, complement-like lytic enzymes, and haptoglobin-like acute phase proteins. NABs (sometimes also affiliated with acquired immunity) bind with low specificity to foreign antigens. This binding can result in opsonization, activation of the complement system, and neutralization of infection (Ochsenbein and Zinkernagel 2000). Complement molecules can also serve as opsonins, and activation of the complement cascade can lead to the rupture of bacterial cells (Ochsenbein and Zinkernagel 2000). Haptoglobin and functionally related proteins increase in concentration with an acute phase response, a suite of physiological changes associated with localized or systemic inflammation. Haptoglobin scavenges free heme, and in birds haptoglobin-like proteins are defined by this function. By binding heme, these proteins potentially eliminate an important bacterial nutrient and protect against oxidative damage. Thus, these proteins also play a role as an antioxidant (reviewed in Quaye 2008).

In this study, our objective was to explore physiological trade-offs associated with increased incubation demand in a wild population of black guillemots (*Cepphus grylle*). We hypothesized that experimentally increased incubation demand would lead to increased incubation effort by the parents, which would affect body condition and innate immunity, as is the case with acquired immunity (e.g., Hanssen et al. 2005; Bourgeon et al. 2006). Previous work in fasting eiders incubating natural clutch sizes suggests that innate immunity is maintained throughout the incubation bout (Bourgeon et al. 2007), but our experimental approach allowed us to begin separating the effects of parental quality from the effects of increased incubation demand. Specifically, we tested our hypothesis by experimentally increasing the clutch size of black guillemots. Subsequently, we measured and compared body condition and three indices of innate immunity between groups of birds incubating control (two-egg) and experimentally enlarged (three-egg) clutches. We predicted that birds incubating experimentally enlarged clutches would weigh less, after taking into account differences in structural size, compared with birds incubating control clutches. There are costs associated with maintaining immune defenses (see Klasing 2004). Thus, we predicted that birds incubating experimentally enlarged clutches would have lower NAB-mediated agglutination titers, lower complement-mediated lysis titers, and lower haptoglobin concentrations when compared with birds incubating control clutches.

Material and Methods

Study Area and Species

Research was conducted on a population of black guillemots breeding on East Bay Island (64°01'N, 81°47'W), a small (approximately 800 × 400 m) island located in East Bay, Southampton Island, Nunavut, during July and August of 2006 and

2007. The granite, bolder-strewn rock shoreline of East Bay Island provides crevice-nesting habitat and supports a colony of black guillemots (approximately 150–200 pairs annually).

The black guillemot is a long-lived seabird belonging to the Alcidae family. Clutch size is generally two eggs, although single-egg clutches do occur, and these are more likely to be laid by young females in their first breeding attempt (reviewed in Gaston and Jones 1998). Both sexes incubate the eggs, and daytime incubation shifts are shorter than 4 h in duration (reviewed in Gaston and Jones 1998). The incubation period lasts approximately 28 d (reviewed in Gaston and Jones 1998). Black guillemots typically feed inshore and close to their nesting areas. This inshore feeding behavior is hypothesized to explain how black guillemots can successfully incubate and rear two semiprecocial chicks (Mehlum et al. 1993), in contrast to most other alcids that rear only a single precocial chick.

Field Methods

Nest cavities were located before egg laying and were visited every few days to determine whether a clutch had been initiated. Once an egg was discovered, that cavity was then visited daily, weather permitting, to record the day of clutch completion (day 0). The exact clutch completion date (CCD) is known with certainty for most nests; because of weather constraints, the CCD of seven nests was estimated ± 2 d. When an egg was discovered, it was weighed with a 100-g spring balance (± 0.1 g). This procedure ensured that all eggs used in our experiment were handled before manipulation. In our experiment, we used nests in which only two eggs were laid.

In the first year of study, nest cavities were randomly assigned to either control (two-egg) or enlarged (three-egg) treatments. Because nest cavities are often reused, cavities occupied in both 2006 and 2007 were assigned to the opposite treatment in the second year (i.e., control to enlarged or vice versa). Two days (± 1 d) after clutch completion, “enlarged” nests received one additional egg, matched for lay date (typically ± 1 d) from a donor nest located elsewhere in the colony. Donor nests were not considered further. Eggs in control treatments were handled but were not cross-fostered. CCD did not differ significantly between the control ($N = 21$) and enlarged ($N = 20$) treatments ($F_{1,39} = 0.34$, $P = 0.56$).

To measure body condition and to collect blood samples for measures of immune function, incubating adults were captured on their nest at midincubation (approximately day 16) and, when possible, approximately 1 wk later at late incubation. The mean difference between mid- and late incubation was 7.2 d (± 0.8 d SD). All captured birds were weighed and measured following methods outlined by Berzins et al. (2009).

On capture of each adult, a blood sample (approximately 600 μ L) was immediately collected from the brachial vein with a 1-mL nonheparinized syringe. Approximately 25 μ L of the blood sample was used for subsequent molecular genetic sexing; the remainder of the blood was transferred into a heparinized 1.5-mL microcentrifuge tube. Blood samples were kept cool on ice and centrifuged (14,000 g for 10 min) within 10 h of col-

lection. Plasma was frozen at -20°C in the field and subsequently -80°C at Trent University until analysis.

At the mid- and late-incubation sample collection, we reweighed each egg. Using egg masses for early and late incubation, we calculated the rate of egg mass loss for control and enlarged clutches. This served as our proxy for incubation effort because adult black guillemots are monomorphic in plumage and their nests are deep within rock crevices, so reliance on behavioral observations to directly measure incubation effort was impossible. We reasoned that if black guillemots incubating enlarged clutches increased their incubation effort, we would expect to see equal rates of egg mass loss for control and enlarged clutches.

We recorded which nests had failed by the late-incubation sample collection. Failure was indicated by the absence of an incubating bird for two consecutive days or by the presence of cold, broken, or rejected eggs (eggs moved to outside the nest scrape). We were unable to follow the eggs of most nests to hatching, since our field season ended before the black guillemot breeding season concluded. In total, we studied 65 individuals that were captured in 1 or both years. We captured 33 males (17 control, 16 enlarged) and 29 females (14 control, 15 enlarged) at midincubation; we recaptured 15 males (nine control, six enlarged) and 14 females (eight control, six enlarged) at late incubation. All animals were cared for in accordance with the Canadian Council on Animal Care, and all research was approved by both the Trent University and the Environment Canada Animal Care Committees.

Molecular Sex Determination

We determined the genetic sex of incubating individuals following Berzins et al. (2009). Briefly, we extracted genomic DNA from FTA filter paper using a Qiagen kit (DNeasy Blood and Tissue kit, 69506; Qiagen Sciences, Maryland, NJ) following the manufacturer's protocol, except that we replaced AE buffer with $\text{TE}_{0.1}$ buffer (10 mM Tris and 0.1 mM EDTA [pH 8.0]). Genomic DNA was amplified by polymerase chain reaction using the P8 and P2 primers (Griffiths et al. 1998). We separated polymerase chain reaction products by electrophoresis in 1.5% agarose gels stained with ethidium bromide. All negative controls were negative for DNA contamination.

Assays of Innate Immunity

We measured the concentration of haptoglobin (mg mL^{-1}) in plasma by using a commercially available kit (phase haptoglobin, TP801; Tri-Delta Diagnostics, Morris Plains, NJ) and following the manual method instructions provided by the manufacturer (Matson 2006). In essence, this functional assay measures colorimetrically the heme-binding capacity of plasma. Absorbance was recorded at 630 nm using a Molecular Devices 96-well microplate reader (Sunnyvale, CA). Almost all samples were run in duplicate (each $7.5 \mu\text{L}$) and averaged to produce a mean concentration.

We measured the plasma levels of NAb-mediated aggluti-

nation and complement-mediated lysis by performing a hemolysis-hemagglutination assay. The assay was performed as outlined by Matson et al. (2005) at the University of Groningen in 2006 and at Trent University in 2007. Materials and supplies used in 2007 were matched as closely as possible with those used in 2006. The general assay procedure involved (1) serial dilution of eight plasma samples down the long axis (row) of a 96-well assay plate and (2) addition of a 1% rabbit red blood cell (RBC) suspension into all wells (whole rabbit blood; Harlan [B-0009D-U] 2006 and HemoStat Laboratories [RBA 050] 2007). To set up each plate, we pipetted $25 \mu\text{L}$ of each sample into columns 1 and 2 and $25 \mu\text{L}$ of phosphate buffered saline (PBS) into columns 2–12. We then performed 10 serial dilutions of the plasma and PBS mixture from column 2 to column 11 using a 1 : 2 dilution each time (for more details, see Matson et al. 2005). Following the dilution, we added an equal volume ($25 \mu\text{L}$) of a 1% RBC suspension to all wells. The plate was sealed with Parafilm M, covered with a lid, lightly shaken for 10 s, and incubated at 37°C for 90 min. Following incubation, the long axis of the plate was tilted to a 45° angle for 20 min at room temperature. At 20 and 90 min postincubation, we scanned the plate with a flatbed scanner using a positive transparency (top-lit) setting at 300 dpi. From these scans, we scored titers of agglutination (20-min scan) and lysis (90-min scan) of the rabbit RBCs. The assay and all scoring were performed blindly to sample identity by LLB. All scoring was performed in duplicate, and values were averaged to produce a mean score. Interplate assay variation, as calculated from two control samples run on each assay plate, was 7.3% for agglutination titers and 11.4% for lysis titers.

Statistical Analysis

Among the 65 individual black guillemots, we detected two outliers in the data and removed these individuals from analyses where appropriate. Because one of these individuals was captured in both years, this resulted in a sample size of 64. For the individual captured in both 2006 and 2007, we excluded the 2007 data from all analyses because CCD of this bird was particularly late (Studentized residual >3), and we wanted to avoid the possible inclusion of a renesting attempt. The second individual, captured only in 2006, was excluded from all analysis involving body condition (as either a dependent variable or a covariate) because this individual's body mass was particularly heavy (Studentized residual >3), and we suspect a transcription error in the field. To avoid any possible carryover effects of increased incubation effort, we also excluded from all analyses (except the between-year repeatability analysis) the 2007 measurements of birds that incubated enlarged clutches in 2006 (two males, two females). For the between-year repeatability analysis, we used the midincubation data for every bird captured in both 2006 and 2007 (see below for more details). Furthermore, to avoid pseudoreplication, we limited certain analyses to measurements from a single stage or a single year, depending on the analysis. For example, in a few cases birds were in the control group in 2006 and the enlarged group

Table 1: Results of linear mixed-effects models testing for differences in innate immune function and body condition between black guillemots that incubated control and enlarged clutches

	Lysis (titers)				Agglutination (titers)				Haptoglobin (mg mL ⁻¹)				Condition			
	Estimate	± SE	χ^2	P	Estimate	± SE	χ^2	P	Estimate	± SE	χ^2	P	Estimate	± SE	χ^2	P
Treatment ^a	...	...	...	...	...	...	...	...	.283	.165	3.007	.083	-2.570	4.148	.399	.527
Sex	...	...	...	...	...	...	...	...	.229	.172	1.825	.177	4.453	3.587	1.538	.215
Stage	-.872	.240	12.763	.000	-.189	.151	1.613	.204	.547	.167	10.365	.001	2.212	3.291	.425	.514
Treatment × sex	-1.037	.476	5.012	.025	1.278	.448	7.022	.008	.569	.318	3.244	.072	3.177	6.564	.229	.632
Treatment × stage	-.152	.480	.089	.766	.344	.297	1.470	.225	-.102	.348	.124	.725	-4.451	6.592	.481	.488
SCCD ^b	-.007	.055	.030	.863	.179	.068	7.158	.007	-.004	.038	.008	.931	-.433	1.057	.213	.645
Condition ^b	.002	.006	.132	.716	-.011	.006	3.357	.067	.004	.004	1.009	.315	...	...	...	...

Note. The reference categories used in the analysis were as follows: treatment = enlarged, sex = male, stage = mid. $N = 103$ total observations. Boldface values indicate statistical significance.

^aTreatment is the main effect of interest and was always retained in models.

^bStandardized clutch completion date (SCCD) and condition are covariates and were always retained in all models.

in 2007, and for these individuals we randomly selected one year to include in the within-year repeatability (mid- to late incubation), nest failure, and egg mass loss analyses. For all analyses, the α level was set to 0.05, and analyses were performed in JMP (ver. 5.0.1a; Sall et al. 2005) statistical software unless indicated otherwise.

To test the effects of clutch-size manipulation on innate immune function and body condition, we used linear mixed-effects models (lme4 package [Bates et al. 2008] in R [ver. 2.6.2; R Development Core Team 2008]) that accounted for repeated (bird and nest levels) and nonrepeated (year level) random effects ($N = 103$ total observations). We tested the effects of treatment (control/enlarged), stage (mid/late), sex (male/female), and the two two-way interactions that included treatment. The three-way interaction was also initially included in all models, but this interaction was never significant (P always > 0.12) and is therefore not discussed further. Beginning with models that included both two-way interactions, we removed the least significant terms in an iterative, stepwise fashion. Specifically, we tested significance by contrasting two hierarchical models that differed by a single main effect or interaction using log likelihood ratio tests and χ^2 statistics. Treatment and two covariates were always retained in the model.

The two covariates were CCD and body condition. The covariate CCD was included and retained in all models to control for potential variation in individual quality (Ardia 2005) and age (Hipfner et al. 1997). Before using CCD as a covariate, we standardized this parameter between years because egg laying was significantly later in 2007 (Wilcoxon two-sample test; $Z = 5.67$, $P < 0.001$). To this end, we expressed the CCD of each nest relative to the mean CCD of the colony in each year (following Winkler and Allen 1996). Body condition was estimated using the residuals from a regression of body mass on head-bill length (following Ardia 2006). As with CCD, body condition was included and retained in all models as a covariate in the analyses of the immune parameters.

The distributions of the residuals of the final models were never significantly different from a normal distribution (Shapiro-Wilk test; $W > 0.98$, $P > 0.12$). Only one dependent variable, haptoglobin, violated the assumptions of normality

and was normalized using a Box Cox transformation with $\lambda = -0.5$ (Cinchy et al. 2004). No other variables for this analysis required transformation.

We examined the repeatability of innate immunity and body condition (1) within the incubation bout (mid- to late incubation, $N = 27$) and (2) between years (midincubation 2006 to midincubation 2007, $N = 12$). To do so, we calculated the intraclass correlation coefficient following Lessells and Boag (1987). We initially performed an ANCOVA with treatment and CCD as covariates (for the within-incubation bout analysis only). Since neither covariate was significant ($P > 0.1$), all repeatability values were calculated using one-way ANOVAs with indices of immune function and body condition as the dependent variables and with bird as the main effect. For the three immune parameters, we used Box Cox transformed data (Cinchy et al. 2004) when calculating repeatabilities. Residuals for these ANOVA models (all Shapiro-Wilk test; $W > 0.96$, $P > 0.19$) were normal when using Box Cox transformed variables.

We performed a χ^2 test to examine whether nest failure differed between control ($N = 20$) and enlarged ($N = 17$) clutches. Of these 37 nests that did not fail by late incubation and for which egg mass data exist ($N = 14$), we tested the effect of clutch-size enlargement on incubation effort. As a proxy for incubation effort, we used the mean rate of egg mass loss per day for each nest (mean egg mass loss from early to late incubation divided by the number of days incubated). A Student's t -test was then used to determine whether these rates differed between control and experimentally enlarged clutches.

Results

Experimental Effects on Immunity and Body Condition

The effects of clutch-size manipulation on body condition and three indices of immune function are summarized in Table 1. First, we found sex-specific effects of treatment on lysis titers (treatment × sex: $\chi^2 = 5.01$, $P = 0.025$). Thus, we performed post hoc tests to explore the effect of treatment within each sex. In males, lysis titers were significantly reduced in birds incubating enlarged clutches compared with birds incubating

control clutches ($\chi^2 = 5.17$, $P = 0.023$; Fig. 1A). In females, lysis titers did not differ significantly by treatment ($\chi^2 = 0.73$, $P = 0.393$; Fig. 1A). Among all birds, lysis titers increased significantly from mid- to late incubation (stage: $\chi^2 = 12.76$, $P < 0.001$). Among all measurements, the mean ($\pm$ SE) of lysis was 1.74 ± 0.1 titers at midincubation and 2.59 ± 0.2 titers at late incubation. Second, we found sex-specific effects of treatment on agglutination titers (treatment $\times$ sex: $\chi^2 = 7.02$, $P = 0.008$). However, this effect of treatment was not significant within either sex (male: $\chi^2 = 1.60$, $P = 0.205$; female: $\chi^2 = 3.37$, $P = 0.067$; Fig. 1B). With agglutination titers, standardized CCD covariate was significant ($\chi^2 = 7.16$, $P = 0.007$); later CCDs were associated with higher agglutination titers. Haptoglobin concentrations decreased significantly from mid- to late incubation (stage: $\chi^2 = 10.37$, $P = 0.001$). Among all measurements, the mean ($\pm$ SE) of haptoglobin concentration was 0.17 ± 0.008 mg mL⁻¹ at midincubation and 0.13 ± 0.007 mg mL⁻¹ at late incubation. With haptoglobin, all other main effects and interactions were nonsignificant ($P > 0.07$). Body condition was unaffected by treatment, sex, stage, and CCD; all main effects and interactions were nonsignificant ($P > 0.21$).

Repeatability of Innate Immunity and Body Condition

Among individuals of both sexes, agglutination titers and body condition were significantly repeatable within the incubation bout (agglutination titers: $r = 0.92$, $F_{26,27} = 24.52$, $P < 0.001$; body condition: $r = 0.48$, $F_{26,27} = 2.84$, $P = 0.005$) as well as between years (agglutination titers: $r = 0.66$, $F_{11,12} = 4.93$, $P = 0.005$; body condition: $r = 0.61$, $F_{11,12} = 4.08$, $P = 0.012$). In contrast, lysis titers and haptoglobin concentrations were not repeatable among individuals at either timescale (lysis-incubation bout: $r = -0.11$, $F_{26,27} = 0.81$, $P = 0.703$; lysis-year: $r = 0.07$, $F_{11,12} = 1.16$, $P = 0.401$; haptoglobin-incubation bout: $r = 0.17$, $F_{26,27} = 1.40$, $P = 0.192$; haptoglobin-year: $r = -0.37$, $F_{11,12} = 0.46$, $P = 0.896$).

Experimental Effects on Nest Failure and Egg Mass Loss

Nest failure did not differ significantly between birds incubating control or enlarged clutches ($\chi^2 = 2.02$, $P = 0.156$). In total, 30% of control clutches (six of 20) and 53% of enlarged clutches (nine of 17) had failed by late incubation. Among those nests that did not fail by late incubation, for which we have egg mass data, the mean rate of egg mass loss per day did not differ significantly between control and enlarged clutches (Student's t -test, $t_{12} = 1.7$, $P = 0.121$). The mean rate of egg mass loss per day was 0.26 ± 0.02 g ($N = 8$) for eggs in control nests and 0.21 ± 0.02 g ($N = 6$) for eggs in enlarged nests.

Discussion

We initially predicted that experimentally increased incubation demand would lead to increased incubation effort by the parents, which in turn would lead to reductions in body condition

and alterations in innate immunity. The results of our experiment, however, diverged substantially from these predictions. On the one hand, birds incubating experimentally enlarged clutches did appear to increase their incubation effort relative to control birds. On the other hand, despite this increased incubation effort by the parents, we found no effects of the experimental treatment on body condition. Moreover, while we found some effects of the experimental treatment on innate immunity, these effects differed between the sexes and between indices of innate immunity. We also found that some indices of innate immunity changed over the incubation period, although these changes did not interact with treatment, as might be expected. Finally, we found that body condition and agglutination titers were highly repeatable over short and long timescales, while two other indices of innate immunity were not repeatable. Overall, these paradoxical results present something of a puzzle in terms of interpretations relating to the specific costs and benefits associated with the different components of innate immunity. Nevertheless, our results clearly identify effects that were sex specific and raise new questions about the generality of theoretical predictions relating to trade-offs between life-history events and immune function.

Birds incubating experimentally enlarged clutches appeared to increase their incubation effort relative to control birds. That is, we found no difference between control and enlarged clutches in terms of the egg-mass-loss rate from early (pre-manipulation) to late (postmanipulation) incubation. Equal rates of egg development between different clutch sizes suggest that birds incubating three eggs were expending more energy overall (i.e., the same energy per egg) to warm their clutch than birds incubating only two eggs. This finding contrasts with a study on biparental Kentish plovers (*Charadrius alexandrinus*), which found that eggs incubated in enlarged clutches lost mass more slowly than control clutches (Székely et al. 1994), and suggests that Kentish plovers did not increase the intensity of their incubation effort but rather the duration of the incubation bout. However, whether black guillemots also extended the duration of incubation is unknown, since we could not follow adults through to the hatching of their eggs.

Increased incubation demand exerted sex-specific effects on two indices of innate immunity. In the case of lysis, males incubating enlarged clutches exhibited significantly reduced titers compared with males incubating control clutches; however, this was not observed in females (Fig. 1A). Sex-specific changes in immunological defenses have been found in association with other physiological manipulations. For example in zebra finches (*Taeniopygia guttata*), increased handling time, a proxy for acute stress, leads to decreased immune function in males but not females (Berzins et al. 2008). While it is clear that the clutch-size enlargement led to sex-specific effects for two innate immune indices, the issue of why is a vexing one. Components of immunity might have different costs and benefits in males and females. However, when the theoretical costs of immunological components are considered, components are assigned a high, medium, or low cost without regard to sex (e.g., Lee 2006; Buehler et al. 2008). Thus, more evidence of sex-specific

Figure 1. Residual lysis titers (A) and agglutination titers (B) in male and female black guillemots incubating control and enlarged clutches. Sample sizes are in parentheses and represent all values for birds used in linear mixed-effects models analyses ($N = 103$). Statistical significances, but not graphed means and standard errors, account for effects of pseudoreplication and repeated measures. Asterisk indicates significance ($P < 0.05$).

cost-benefit ratios is required so that theoretical predictions regarding immune costs can be reevaluated.

An alternative explanation for the observed pattern is that when faced with increased incubation demand, one sex increased its incubation effort while the other sex either did not change its effort or in fact decreased its incubation effort. Sex-specific parental investment strategies in current reproduction have been identified in some species (e.g., Moreno et al. 1995) and inferred for other species where males, but not females,

exhibited increased parasite prevalence following experimental increases in reproductive effort (Richner et al. 1995). Although incubation is shared equally in another alcid, the little auk (*Alle alle*; Wojczulanis-Jakubas et al. 2009), this phenomenon has been little studied in black guillemots. Accordingly, sex-specific data on effort (e.g., behavioral observations, passive integrated transponder data, or daily energy expenditures) for individuals with increased workload could help support or refute this proximate explanation of the observed sex-specific effects on innate immunity. Moreover, studies on alcids show that nest/territory defense or attendance is often greater for males than for females during incubation (Wojczulanis-Jakubas et al. 2009; reviewed in Paredes and Insley 2010); thus, our results for males may reflect the combined costs of defensive behavior and increased incubation demand.

The natural progression of the incubation period was also associated with changes in indices of innate immunity in both sexes. From mid- to late incubation, haptoglobin concentrations decreased, and lysis titers increased. Agglutination titers, however, did not change. The only other study, to our knowledge, that investigated innate immunity throughout incubation found that the measured component (nitric oxide) was maintained as incubation progressed in fasting female common eiders (*Somateria mollissima*; Bourgeon et al. 2007). The observed decrease in haptoglobin concentration in black guillemots may reflect the molecule's use as a bacteriostatic or antioxidant agent (Quaye 2008), which may outstrip its production during incubation. Haptoglobin is mostly produced by the liver (Quaye 2008), and this organ atrophies in some birds during reproduction (e.g., lesser snow goose, *Chen caerulescens caerulescens*; Ankney 1977). The increase in lysis detected in both sexes over the course of incubation contrasted with the decreased levels in males incubating enlarged clutches. If both the natural progression of incubation and the addition of an extra egg reflect increased workload, then an interaction between incubation stage and treatment might have been expected. However, no such interactions were found. Although oxygen consumption increases over the incubation period (e.g., blue tits, *Parus caeruleus*; Haftorn and Reinertsen 1985), in our species the energetic costs of incubation may have been relatively constant for our mid- to late sampling interval.

Unlike indices of innate immunity, body condition was invariant. This result is consistent with a study on biparental Kentish plovers, where clutch-size manipulation had no effect on parental mass loss (Székely et al. 1994). We hypothesize that by alternating between foraging and incubating bouts within a pair, individual black guillemots may offset some of the energetic constraints of incubation experienced by uniparental incubators (see Tulp and Schekkerman 2006). Also, by feeding inshore near the colony, individuals likely experience relatively low foraging costs (Mehlum et al. 1993).

Body condition and agglutination titers exhibited a high repeatability both within incubation bouts and between years. The repeatabilities we found for agglutination (within bout, $r = 0.92$; between year, $r = 0.66$) were considerably higher than the value reported by Buehler et al. (2008) for the same

measure. In that study, agglutination repeatability ($r = 0.11$) was calculated for red knots (*Calidris canutus*) that were sampled numerous times throughout a complete annual cycle (Buehler et al. 2008). Therefore, the difference in repeatabilities between studies is likely due, at least in part, to the inclusion of different life-history stages by Buehler et al. (2008). Nevertheless, the repeatabilities calculated in both studies and the assay's relative insensitivity to inflammatory challenges (Matson et al. 2005) suggest that NAb-mediated agglutination holds the potential to serve as a reliable and resilient index of the immunological character of individuals (especially within seasons). Likewise, body condition also appears to be a fundamental quality of individuals. Since repeatability values can provide an upper limit to estimates of heritability (Falconer 1981), agglutination and body condition (at least as defined in our study) hold the potential to display evolutionary responses to selection.

In conclusion, our work demonstrates that increased incubation demand impacts innate immunity in sex-specific ways. To better understand the causes and consequences of these sex-specific effects, sex-specific data relating to reproductive effort are required. Additionally, investigating the sex-specific effects of increased incubation demand on other immunological components and responses (e.g., endotoxin-induced inflammation) would be valuable. Our work also demonstrated that body condition was not affected by experimentally increased clutch size. Thus, black guillemots were able to invest in self-maintenance despite the increased incubation demand. Finally, given the high repeatability of agglutination, we suggest that this measurement can be interpreted as a distinctive trait of individuals.

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Literature Cited

- Ankney C.D. 1977. Feeding and digestive organ size in breeding lesser snow geese. *Auk* 94:275–282.
- Ardia D.R. 2005. Individual quality mediates trade-offs between reproductive effort and immune function in tree swallows. *J Anim Ecol* 74:517–524.
- . 2006. Geographic variation in the trade-off between nestling growth rate and body condition in the tree swallow. *Condor* 108:601–611.
- Ardia D.R., K.A. Schat, and D.W. Winkler. 2003. Reproductive effort reduces long-term immune function in breeding tree swallows (*Tachycineta bicolor*). *Proc R Soc B* 270:1679–1683.
- Bates D., M. Maechler, and B. Dai. 2008. lme4: Linear Mixed-Effects Models Using S4 Classes. R Package Version 0.999375-18. <http://lme4.r-forge.r-project.org/>.
- Berzins L.L., H.G. Gilchrist, and G. Burness. 2009. No assortative mating based on body size in black guillemots breeding in the Canadian arctic. *Waterbirds* 32:459–463.
- Berzins L.L., E. Tilman-Schindel, and G. Burness. 2008. Sex-specific effects of handling time on an index of immune activity in zebra finches. *Physiol Biochem Zool* 81:383–387.
- Bourgeon S., F. Cruscuolo, Y. Le Maho, and T. Raclot. 2006. Phytohemagglutinin response and immunoglobulin index decrease during incubation fasting in female common eiders. *Physiol Biochem Zool* 79:793–800.
- Bourgeon S., T. Raclot, Y. Le Maho, D. Ricquier, and F. Cruscuolo. 2007. Innate immunity, assessed by plasma NO measurements, is not suppressed during the incubation fast in eiders. *Dev Comp Immunol* 31:720–728.
- Buehler D.M., T. Piersma, K. Matson, and B.I. Tieleman. 2008. Seasonal redistribution of immune function in a migrant shorebird: annual-cycle effects override adjustments to thermal regime. *Am Nat* 172:783–796.
- Cichoń M. 2000. Costs of incubation and immunocompetence in the collared flycatcher. *Oecologia* 125:453–457.
- Clinchy M., L. Zanette, R. Boonstra, J.C. Wingfield, and J.N.M. Smith. 2004. Balancing food and predator pressure induces chronic stress in songbirds. *Proc R Soc B* 271:2473–2479.
- Dawson R.D. and G.R. Bortolotti. 2008. Experimentally prolonging the brood-rearing period reveals sex-specific parental investment strategies in American kestrels (*Falco sparverius*). *Auk* 125:889–895.
- Deerenberg C., V. Arpanius, S. Daan, and N. Bos. 1997. Reproductive effort decreases antibody responsiveness. *Proc R Soc B* 264:1021–1029.
- de Heij M., A.J. van der Graaf, D. Hafner, and J.M. Tinbergen. 2007. Metabolic rate of nocturnal incubation in female great tits, *Parus major*, in relation to clutch size measured in a natural environment. *J Exp Biol* 210:2006–2012.
- Falconer D.S. 1981. Introduction to Quantitative Genetics. 2nd ed. Longmans, New York.
- Gaston A.J. and I.L. Jones. 1998. The Auks: Alcidae. Oxford University Press, Oxford.
- Griffiths R., M.C. Double, K. Orr, and R.J.G. Dawson. 1998. A DNA test to sex most birds. *Mol Ecol* 7:1071–1075.

- Haftorn S. and R.E. Reinertsen. 1985. The effect of temperature and clutch size on the energetic cost of incubation in a free-living blue tit (*Parus caeruleus*). *Auk* 102:470–478.
- Hanssen S.A., D. Hasselquist, I. Folstad, and K.E. Erikstad. 2005. Cost of reproduction in a long-lived bird: incubation effort reduces immune function and future reproduction. *Proc R Soc B* 272:1039–1046.
- Hipfner J.M., A.J. Gaston, and L.N. de Forest. 1997. The role of female age in determining egg size and laying date of thick-billed murre. *J Avian Biol* 28:271–278.
- Hörak P. 2003. When to pay the cost of reproduction? a brood size manipulation experiment in great tits (*Parus major*). *Behav Ecol Sociobiol* 54:105–112.
- Ilmonen P., T. Taarna, and D. Hasselquist. 2002. Are incubation costs in female pied flycatchers expressed in humoral immune responsiveness or breeding success? *Oecologia* 130: 199–204.
- Jacobsen K., K.E. Erikstad, and B. Saether. 1995. An experimental study of the costs of reproduction in the kittiwake *Rissa tridactyla*. *Ecology* 76:1636–1642.
- Klasing K.C. 2004. The costs of immunity. *Acta Zool Sinica* 50: 961–969.
- Lee K.A. 2006. Linking immune defenses and life history at the levels of the individual and the species. *Integr Comp Biol* 46:1000–1015.
- Lessells C.M. and P.T. Boag. 1987. Unrepeatable repeatabilities: a common mistake. *Auk* 104:116–121.
- Matson K.D. 2006. Are there differences in immune function between continental and insular birds? *Proc R Soc B* 273: 2267–2274.
- Matson K.D., R.E. Ricklefs, and K.C. Klasing. 2005. A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Dev Comp Immunol* 29:275–286.
- Mehlum F., G.W. Gabrielsen, and K.A. Nagy. 1993. Energy expenditure by black guillemots (*Cephus grylle*) during chick-rearing. *Colon Waterbirds* 16:45–52.
- Monaghan P. and R.G. Nager. 1997. Why don't birds lay more eggs? *Trends Ecol Evol* 12:270–274.
- Moreno J., R.J. Cowie, J.J. Sanz, and R.S.R. Williams. 1995. Differential response by males and females to brood manipulations in the pied flycatcher: energy expenditure and nestling diet. *J Anim Ecol* 64:721–732.
- Moreno J., L. Gustafsson, A. Carlson, and T. Pärt. 1991. The cost of incubation in relation to clutch-size in the collared flycatcher *Ficedula albicollis*. *Ibis* 133:186–193.
- Moreno J., J.J. Sanz, and E. Arriero. 1999. Reproductive effort and T-lymphocyte cell-mediated immunocompetence in female pied flycatchers *Ficedula hypoleuca*. *Proc R Soc B* 266: 1105–1109.
- Ochsenbein A.F. and R.M. Zinkernagel. 2000. Natural antibodies and complement link innate and acquired immunity. *Immunol Today* 21:624–630.
- Paredes R. and S.T. Insley. 2010. Sex-biased aggression and male-only care at sea in Brünnich's guillemots *Uria lomvia* and razorbills *Alca torda*. *Ibis* 152:48–62.
- Quaye I.K. 2008. Haptoglobin, inflammation and disease. *Trans R Soc Trop Med Hyg* 102:735–742.
- Råberg L., M. Grahm, D. Hasselquist, and E. Svensson. 1998. On the adaptive significance of stress-induced immunosuppression. *Proc R Lond B* 265:1637–1641.
- R Development Core Team. 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. <http://www.R-project.org>.
- Richner H., P. Christe, and A. Oppliger. 1995. Paternal investment affects prevalence of malaria. *Proc Natl Acad Sci USA* 92:1192–1194.
- Roitt I., J. Brostoff, and D. Male. 1996. Immunology. 4th ed. Mosby, London.
- Sall J., L. Creighton, and A. Lehman. 2005. JMP Start Statistics. 3rd ed. Thomson Brooks/Cole, Pacific Grove, CA.
- Sheldon B.C. and S. Verhulst. 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol Evol* 11:317–321.
- Székely T., I. Karsai, and T.D. Williams. 1994. Determination of clutch-size in the Kentish plover *Charadrius alexandrinus*. *Ibis* 136:341–348.
- Thomson D.L., P. Monaghan, and R.W. Furness. 1998. The demands of incubation and avian clutch size. *Biol Rev* 73: 293–304.
- Tulp I. and H. Schekkerman. 2006. Time allocation between feeding and incubation in uniparental arctic-breeding shorebirds: energy reserves provide leeway in a tight schedule. *J Avian Biol* 37:207–218.
- Verboven N. and J.M. Tinbergen. 2002. Nest desertion: a trade-off between current and future reproduction. *Anim Behav* 63:951–958.
- von Schantz T., S. Bensch, M. Grahm, D. Hasselquist, and H. Wittzell. 1999. Good genes, oxidative stress and condition-dependent sexual signals. *Proc R Soc B* 266:1–12.
- Williams G.C. 1966. Natural selection, the cost of reproduction, and a refinement of Lack's principle. *Am Nat* 100:687–690.
- Williams J.B. 1996. Energetics of avian incubation. Pp. 375–416 in C. Carey, ed. *Avian Energetics and Nutritional Ecology*. Chapman & Hall, New York.
- Winkler D.W. and P.E. Allen. 1996. The seasonal decline in tree swallow clutch size: physiological constraint or strategic adjustment? *Ecology* 77:922–932.
- Wojczulanis-Jakubas K., D. Jakubas, and L. Stempniewicz. 2009. Sex-specific parental care by incubating little auks (*Alle alle*). *Ornis Fenn* 86:140–148.