Supporting Information

White *et al.* 10.1073/pnas.0803067105

SI Text

Methods Used for Counting Spermatozoa and Holes in the Perivitelline Layer (PVL). After thawing, the yolks were separated from the albumin and weighed to obtain an estimation of the total surface of the PVL. For each yolk, two pieces (≈3 cm^2) of the PVL, one from the blastodisc region and one from the opposite pole, were removed with scissors and then stained for 30 min in a 1-ml buffer solution containing 20 μl of fluorescent dye Hoechst 33342 (Sigma). Each piece was then placed on a separate slide and dried by using a hairdryer. The stained nuclei of the spermatozoa were then counted by using a Leica fluorescent microscope at X400 magnification. We counted the number of hydrolysis points (holes) caused by the acrosome reaction between the spermatozoa and the inner PVL of the ovum (1). To facilitate the counting of holes, the slides were rehydrated in a buffer solution, fixed with a 20% formalin solution, and stained with period acid/Schiff's reagent. The holes were then viewed and counted with a phase-contrast microscope at X100 magnification.

When comparing pieces of membrane from the germinal disk region and the opposite pole, we found no significant differences in the density of spermatozoa (paired *t* test, t = 0.21, $P = 0.84$, $n = 46$) or holes (t = −0.86, $P = 0.40$, $n = 20$), suggesting that these were evenly distributed in the PVL across the ovum. This pattern differs from similar studies on numerous species of birds (2) (although see ref. 3), leading us to consider any interspecific comparison of perivitelline sperm numbers with caution. However, because the same method was applied for all eggs in our study, it constitutes a valid index of relative sperm numbers according to treatment and/or incubation time. Because of their even distribution, if spermatozoa/holes were numerous, we only counted numbers in 40 fields at X400 magnification, calculated average sperm or hole density, and multiplied by the total surface of the yolk to obtain the total number of spermatozoa/holes. Numbers obtained from the animal pole and the vegetal pole of the yolk were then averaged and used as an index of the number of live spermatozoa present at the site of fertilization (4). If spermatozoa/holes were rare or lacking, density was calculated by carrying out an exhaustive count of the two pieces of membrane.

A study by Small *et al.* (5) on domestic turkeys (*Meleagris gallopavo*) showed a dramatic decrease in detectability of sperm nuclei in incubated eggs, resulting in no sperm being detected after 7 days of incubation [however, turkey sperm nuclei have been shown to exhibit a particularly high rate of decondensation (3)]. Because the eggs in our experiment were incubated for 11 days (see *Embryonic Development* in the main text), it is likely that the numbers of sperm nuclei and holes counted were underestimated. To estimate numbers of spermatozoa and holes in freshly laid kittiwake eggs and to determine the decrease in their detectability with duration of incubation, in 2006 we obtained a reference sample by collecting and opening freshly laid eggs and normally developing eggs incubated for a range of periods. We also collected and opened eggs from anti-insemination ring (AR) pairs incubated for periods ranging from 0–11 days. Furthermore, to evaluate the detectability of sperm and holes in nondeveloping eggs, we arrested development in six freshly laid eggs. This was done by pricking the yolk with a sterile needle (4 eggs) or by opening the egg and placing the yolk in a Petri dish (2 eggs). These six eggs were then incubated for various lengths of time.

Results of Counting Sperm and Holes and Estimating the Initial Number of Sperm Present. ***Number of sperm in freshly laid fertilized eggs.*** In 2006, we collected and opened 53 freshly laid nonmanipulated eggs to determine the natural range of numbers of sperm and holes present in the PVL of fertilized eggs. We found an average of 7,274.90 ± 1,883.95 spermatozoa embedded in the PVL with numbers ranging from 923–28,509, with an outlier of 97,824 (Fig. S1).

Correlation between holes and sperm. For a subsample of these eggs ($n = 9$), we also counted the points of hydrolysis (holes) produced by the spermatozoa in the inner PVL. We found an average of 4,639.49 ± 1,071.19 holes (range: 1,010–8,269, with the outlier at 22,893). There was a strong positive relationship between numbers of sperm and holes counted in the PVL (GLMM, $F_{2,62} = 39.37$, $P < 0.0001$, $R^2 = 0.56$; Quadratic relation: holes = −4E–06 $sperm^2$ + 0.6035 sperm).

Dynamics of sperm numbers in developing eggs. Because our protocol entailed opening eggs that showed no sign of development after 11 days of incubation, we also investigated the effect of this incubation time on our capacity to count sperm and holes in the PVL. We therefore opened eggs from nonmanipulated pairs after different periods of incubation. We found that time of incubation had a significant negative effect on numbers of sperm (log sperm: GLMM, $F_{1,28} = 8.41$, $P = 0.007$, $n = 29$) (Fig. S4, green circles) and holes (log holes: GLMM, $F_{1,28} = 5.45$, $P = 0.03$, $n = 29$). This rapid decline was apparently because of the disintegration of the PVL that occurs with the development of the embryo.

Dynamics of sperm numbers in incubated nondeveloping eggs. To evaluate the detectability of sperm and holes in nondeveloping eggs, we arrested development in another sample of freshly laid eggs by pricking the yolk with a sterile needle. We found significantly lower numbers of sperm (log sperm, GLMM, $F_{1,14} = 6.12$, $P = 0.028$, $n = 15$) (Fig. S4, blue triangles) and holes (log holes, GLMM, $F_{1,11} = 5.04$, $P = 0.049$, $n = 12$) in eggs incubated for 11 days than in fresh, nonincubated eggs (number of spermatozoa: 11-d incubation: 2,733 ± 583; no incubation: 7,386 ± 1,911) confirming the expected negative effect of incubation time (although the decrease was much slower than for developing eggs) and showing that in nondeveloping eggs, sperm and holes were still detectable after 11 days of incubation.

Dynamics of sperm numbers in incubated nondeveloping AR eggs. In 2006, we also incubated eggs from AR pairs for different lengths of time. The number of sperm found in the PVL depended on the length of incubation (log sperm, GLMM, $F_{1,12} = 18.95$, $P = 0.0014$, $n = 13$) (Fig. S4, red circles) with a less steep decline than for the control eggs, but was also dependent on the duration of AR wear (log sperm, GLMM, $F_{1,12} = 10.91$ $P = 0.008$, $n = 13$), suggesting that the ARs did cause fewer sperm to access the site of fertilization.

Extrapolation of initial number of sperm in incubated nondeveloping AR eggs. Using the quadratic equation obtained in the regression of the numbers of sperm and duration of incubation in the AR sample from 2006 (Fig. S4), we were able to estimate the number of sperm that were initially present in the PVL before the onset of incubation in the eggs of the main experiment (see Table S1 for method of calculation). We found that 12 of the 13 AR eggs contained numbers of sperm within the range of unmanipulated fertile eggs, 93% of which hatch (Fig. S1), providing evidence that even though the treatment reduced the number of sperm reaching AR eggs, these eggs typically received sufficient sperm to be fertilized.

1. Bakst MR, Howarth B (1977) Hydrolysis of hens peri-vitelline layer by cock sperm in vitro. *Biol Reprod* 17:370–379.
2. Birkhead TR, Sheldon BC, Fletcher F (1994) A comparative study of sperm-egg interactions in birds. *J Reprod Fertility* 101:353–361.
3. Wishart GJ (1997) Quantitative aspects of sperm:egg interaction in chickens and turkeys. *Animal Reprod Sci* 48:81–92.
4. Brillard JP, Antoine H (1990) Storage of sperm in the uterovaginal junction and its incidence on the numbers of spermatozoa present in the perivitelline layer of hens eggs. *Brit Poultry Sci* 31:635–644.
5. Small AO, Schlusser K, Ryan CJ, Jamieson IG (2000) Detecting sperm on the perivitelline membrane of incubated turkey eggs and its implications for research on fertility problems in endangered species. *Wildlife Res* 27:635–637.

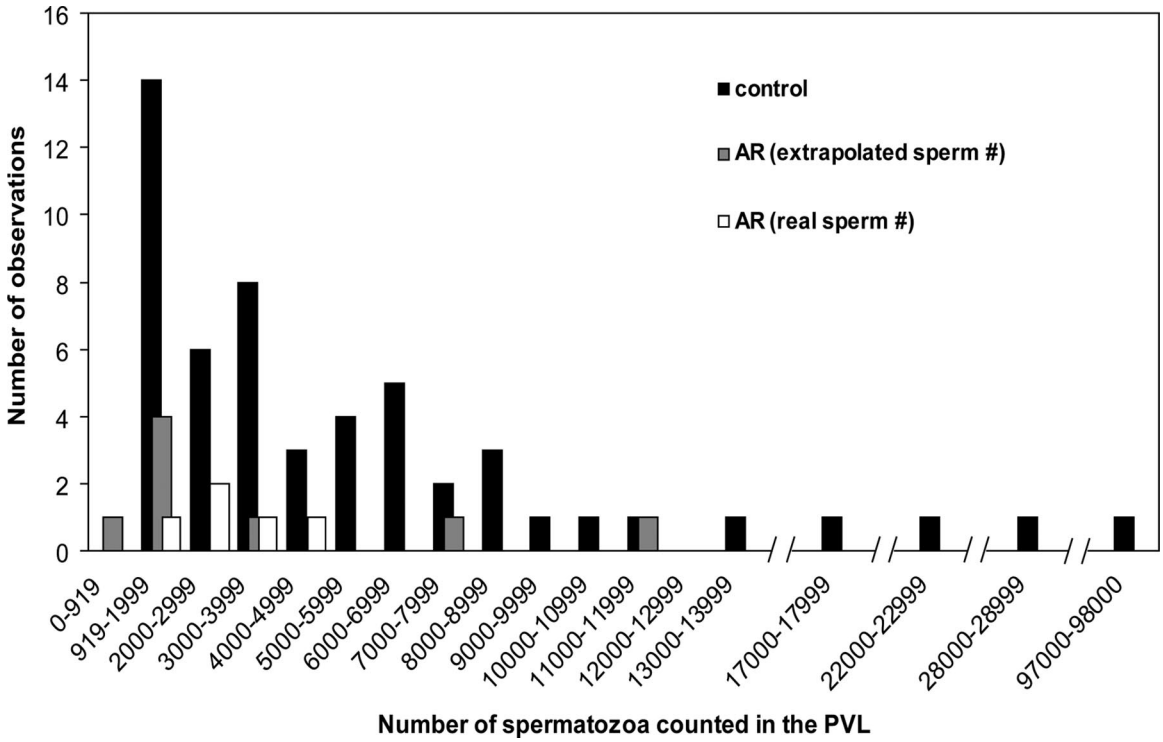

Fig. S1. Frequency distribution of the number of sperm nuclei counted in the PVL of control eggs (black bars, $n = 53$), AR eggs incubated for <5 days (white bars, $n = 5$) and AR eggs incubated for >5 days, for which initial number of sperm nuclei was extrapolated (gray bars, $n = 8$).

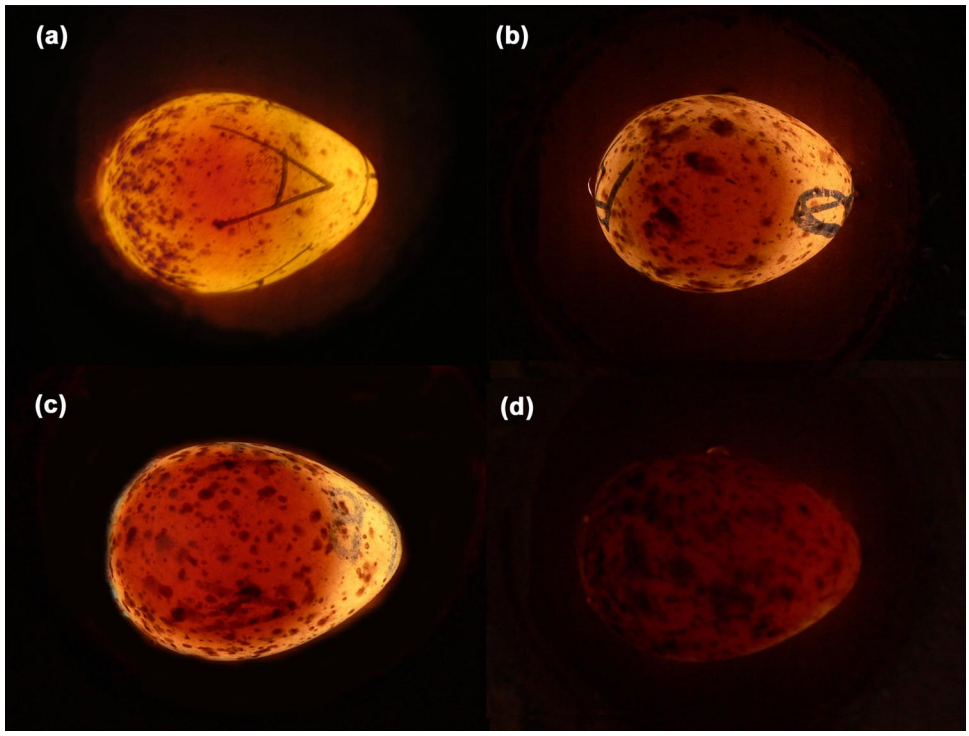

Fig. S2. Four different stages of embryonic development as revealed by the candling method. (*A*) Freshly laid or nonfertilized egg. Score 0: the egg is clear, the yolk covers only 30% of the visible surface of the egg and its contours are undefined. (*B*) Score 1: the yolk is denser, its contours are well defined, and it covers >50% of the visible surface of the egg. (*C*) Score 2: the ''yolk'' (chorioallantoic membrane) has clearly defined contours and covers >70% of the visible surface of the egg. (*D*) Score 3: the chorioallantoic membrane covers 100% of the visible surface of the egg and renders the egg opaque. Intermediary stages were scored 1−, 1+, or 2−, 2+.

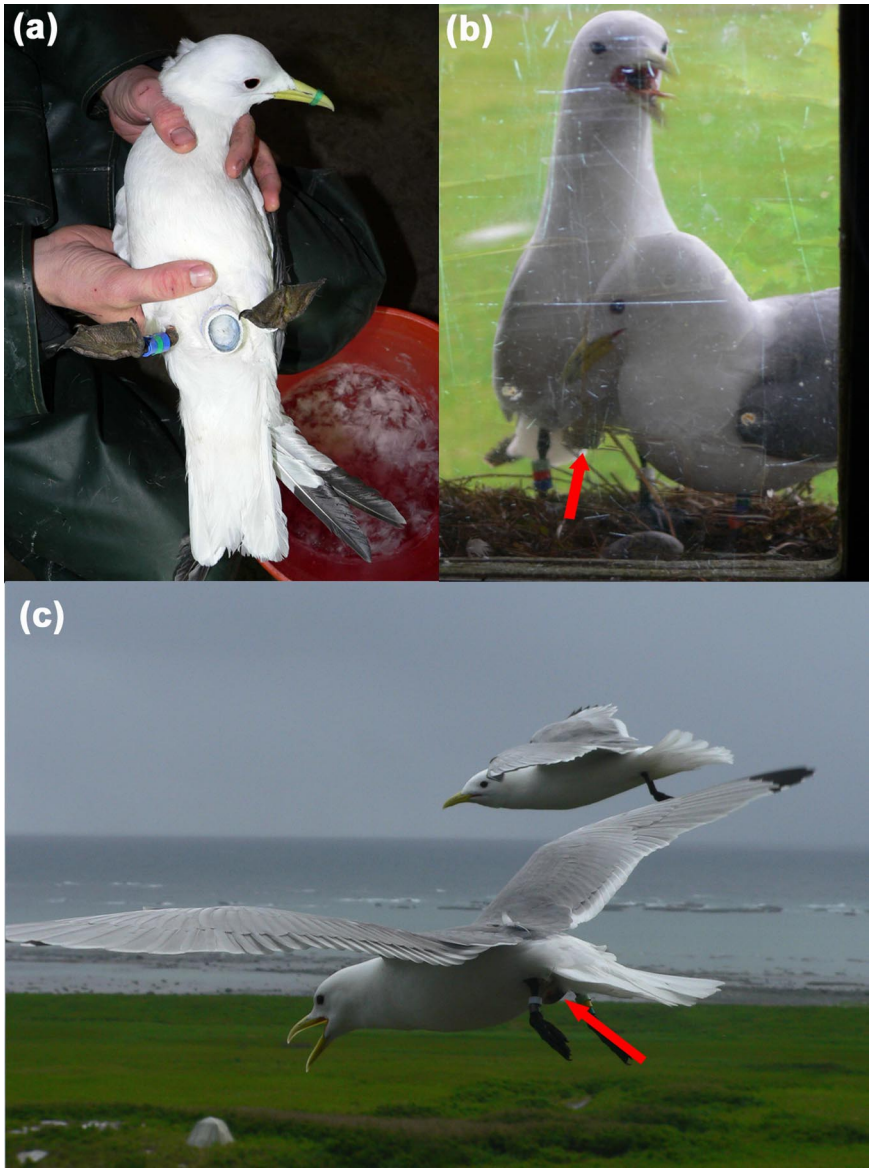

Fig. S3. Photographs of male black-legged kittiwakes wearing antiinseminator rings (ARs). (*A*) Male captured and fitted with an AR. ARs are hollow and enable normal defecation. (*B*) AR male and his mate on their nest, viewed through a one-way mirror allowing a regular and close monitoring of the fixation of the AR. (*C*) AR male in flight. Red arrows show the visible ring of the AR.

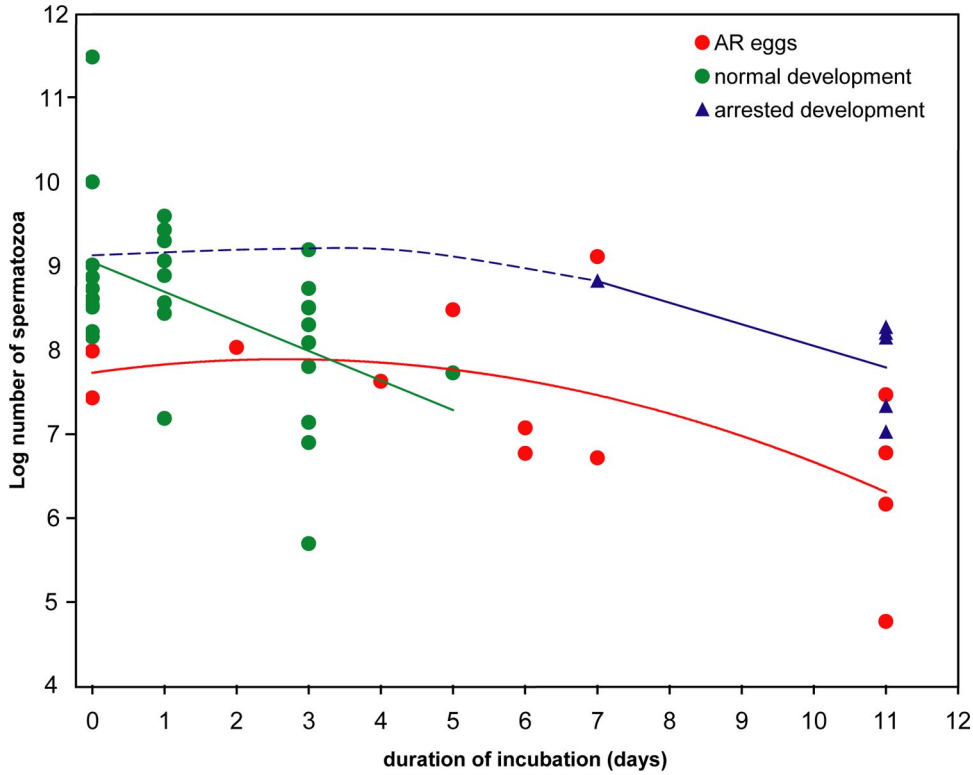

Fig. S4. Log number of spermatozoa nuclei counted on the PVL of eggs according to duration of incubation. In control eggs (green circles), no counts were possible after 5 days of incubation because of the development of the embryo. Because most AR eggs did not develop an embryo (red circles), the numbers of sperm counted started declining substantially only after 5 days of incubation (red quadratic regression curve: log spz = -0.0228(incubation time)^2 + 0.1216(incubation time) + 7.7312; $R^2 = 0.38$). The blue triangles represent control eggs in which the embryo was prevented from developing. Only eggs incubated for 7 and 11 days were obtained. The blue dashed section is the supposed curve for these eggs (starting at the same level as control and degrading at the same rate as AR eggs).

Table S1. Number of sperm nuclei counted in AR eggs incubated for a range of durations

Egg	I. Number of spz counted	II. Log number of spz counted	III. Incubation time, days	IV. Log nbr of spz predicted by model according to III.	V. Difference with predicted at day 0 (i.e. 7.73)	VI. Estimated log number of sperm present initially	VII. Estimated number of sperm present initially
A*	118	4.77	11	6.31	1.42	6.19	446
B*	476	6.17	11	6.31	1.42	7.59	1,970
C*	878	6.78	11	6.31	1.42	8.20	3,636
D*	1752	7.47	11	6.31	1.42	8.89	7,255
E	9085	9.11	7	7.47	0.27	9.38	11,854
F	825	6.72	7	7.47	0.27	6.98	1,076
G	873	6.77	6	7.64	0.09	6.86	956
H	1182	7.07	6	7.64	0.09	7.16	1,295
I	4826	8.48	5	7.77	−0.04	—	4,826
J	2055	7.63	4	7.85	−0.12	—	2,055
K	3083	8.03	2	7.88	−0.15	—	3,083
L	2944	7.99	0	7.73	0.0	—	2,944
M	1686	7.43	0	7.73	0.0	—	1,686

Using the quadratic regression obtained in Fig. S4, [log spz $= -0.0228(\text{incubation time})^2 + 0.1216(\text{incubation time}) + 7.7312$)] we calculated the log number of spermatozoa predicted by the model at 11, 7, and 6 days of incubation (column IV) and subtracted it from the value at 0 days of incubation, i.e. 7.73 (column V). We then added that difference to the log number of sperm counted (column II) and obtained the value of the estimation of the number of sperm predicted (column VI). For eggs incubated for <6 days, the model predicted a lower (or equal) value than the original number of spermatozoa; therefore, the estimated value was not used. The four eggs marked with an asterisk are the four AR eggs used in the study of sperm aging (Fig. S2), with egg A having a number of spermatozoa lower than the range obtained in control fresh eggs.