Short Communications and Commentaries

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Numbers of Spermatozoa Attached to and Penetrating Perivitelline Layers of Japanese Quail Eggs

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In birds and some other taxa (e.g. urodele amphibians), several spermatozoa typically penetrate the ovum wall at the time of fertilization (referred to as physiological polyspermy) even though the female nucleus fuses with only a single spermatozoan nucleus (Harper 1904, Elinson 1986, Perry 1987). In birds, fertilization takes place in the infundibulum within 15 to 30 min of the ovum being shed from the ovary, at which point one or more spermatozoa penetrate the inner layer of the perivitelline membrane of the ovum (Olsen and Neher 1948). The numbers of spermatozoa in the infundibulum at the time of fertilization can be estimated by counting those trapped on the outer layer of the perivitelline membrane, which is laid down on the ovum soon after fertilization (Gilbert 1971, Wishart 1987). However, the proportion of spermatozoa that undergo the acrosome reaction and penetrate the inner perivitelline layer is not well known (Howarth 1984). The aim of this paper is to describe a simple technique that permits the identification of both: (1) holes caused by the penetration of spermatozoa into the inner perivitelline membrane; and (2) spermatozoa that have not undergone the acrosome reaction and are still attached to the outer perivitelline layer. These two simple techniques open up new possibilities for exploring the dynamics of sperm utilization and sperm competition in birds (Birkhead and Møller 1992).

Methods.—We used fertile and infertile eggs laid within the previous 48 h from naturally mated Japanese Quail (*Coturnix japonica*). Eggs were opened with scissors and the yolk and albumen separated. The yolk was placed in a petri dish with the germinal disc uppermost. A piece of the perivitelline layer approximately 1 × 1 cm around the germinal disc (diameter $\bar{x}$ = 3.75 mm) was removed with scissors and fine forceps. The piece of membrane was gently washed by shaking in phosphate buffered saline for a few seconds. A second piece of the perivitelline layer (1 × 1 cm) from the same egg, but away from the germinal disc, was also removed and washed. The two pieces were then placed on a microscope slide side by side and laid out flat. We used Wishart's (1987) technique for identifying sperm; this involved placing a drop of fluorescent Hoescht dye 33342 onto the perivitelline tissue and covering with a cover slip. Tissue samples were examined using a transmission microscope. Holes were visible using dark-field optics at a magnification of ×100 (i.e. ×10 objective; Fig. 1a). Spermatozoa were visible under ultraviolet light at a magnification of ×250 or ×400 (×25 or ×40 objective; Fig. 1b).

The method we used for visualizing holes differs from that of other authors. Bramwell and Howarth (1992b) used Schiff's reagent, and G. J. Wishart (pers. comm.) used a lectin fluorochrome to stain the perivitelline layer in order to increase the contrast between the holes and the layer. However, we found that holes were clearly visible without any stain if we simply used dark-field optics. Moreover, we could use the DNA-specific Hoescht dye 33342 to stain spermatozoa, so that both holes and spermatozoa could be counted from the same piece of perivitelline layer, under dark-field and fluorescence optics, respectively.

We examined a single egg laid by each of 15 females, recording the number of holes and spermatozoa over the area of the germinal disc and away from the germinal disc. Counts of holes were made for fields of known area (for holes 3.08 mm²; for spermatozoa 0.48 mm²) and converted to densities per square millimeter. We counted 10 nonoverlapping fields per germinal disc and 30 random fields away from the germinal disc. Densities of spermatozoa and holes were used to estimate the total numbers of spermatozoa and holes over the entire ovum. Each hole in the inner perivitelline layer was assumed to correspond to a single spermatozoa either undergoing the acrosome reaction or undergoing proteolytic activity (see below and Birkhead et al. 1994) and penetrating the layer. Spermatozoa nuclei on the outer perivitelline layer were those that had not undergone the acrosome reaction or proteolytic activity. The surface area (mm²) of the ovum (994 mm²) was calculated using the formula $4\pi r^2$ after measuring the diameter of 10 hard-boiled yolks to obtain a mean radius (8.895 mm). Densities are per square millimeter ($\bar{x} \pm$ SE).

Results.—The mean densities of both spermatozoa and holes were significantly higher in the germinal disc area than elsewhere on the ovum. The density

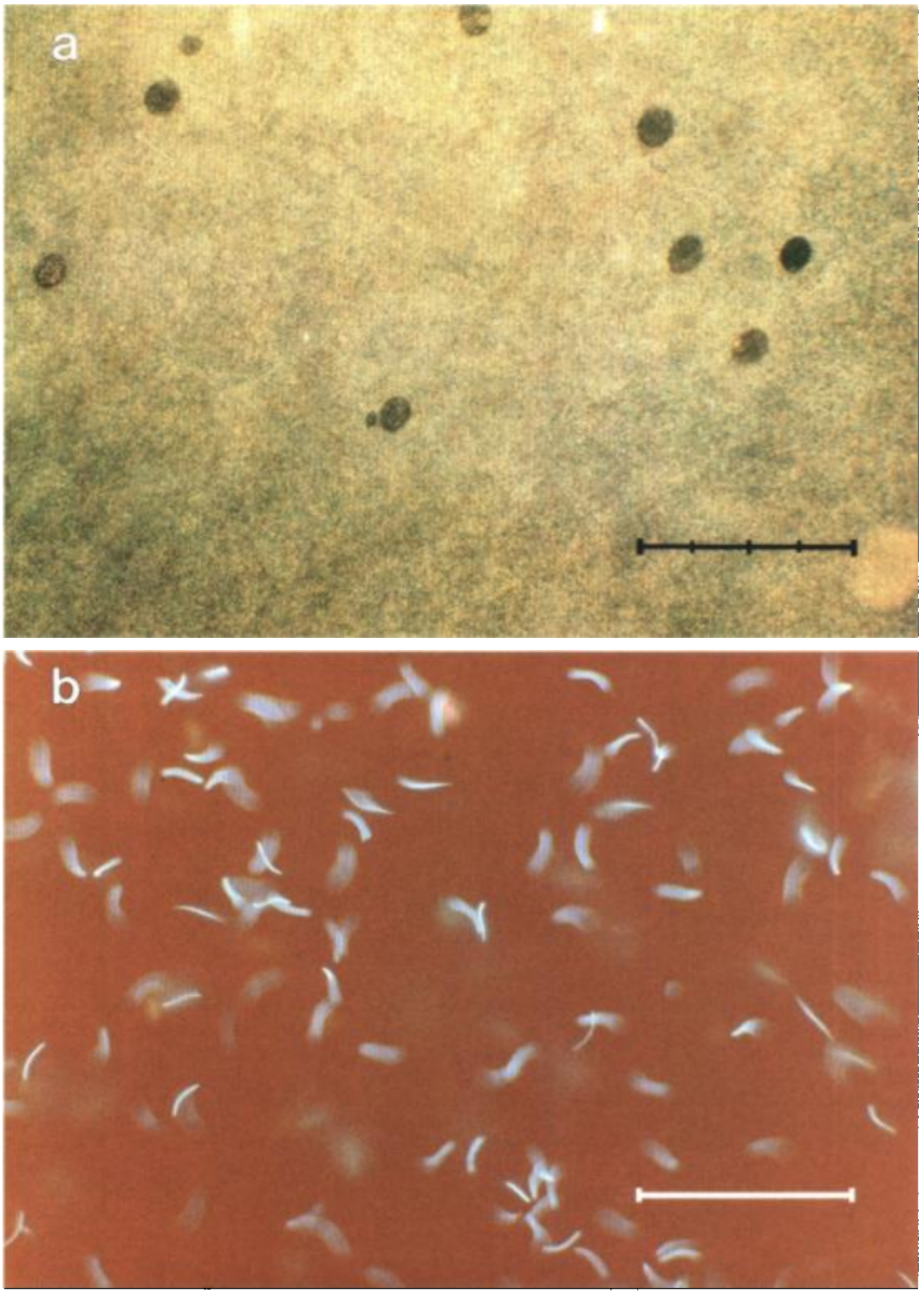

Fig. 1. (a) Holes in inner perivitelline layer of Japanese Quail egg (scale bar 200 μ with 50-μ divisions). (b) Fluorescent spermatozoa nuclei on outer perivitelline layer of Japanese Quail egg (scale bar = 50 μ).

of spermatozoa over the germinal disc (3.14 ± 0.83) was about 1.6 times higher than elsewhere on the perivitelline layer (1.90 ± 0.57; Wilcoxon matched pairs $Z = 2.10$, $P < 0.04$). Similarly, the density of holes on the germinal disc (9.12 ± 1.99) was about 1.7 times higher than elsewhere (5.34 ± 1.59; $Z = 3.41$, $P < 0.001$). The ratio of holes to sperm was 2.9: 1. The estimated total number of spermatozoa on the entire ovum was positively correlated with the estimated total number of holes (Fig. 2). The slope of this relationship ($\beta = 0.972 \pm 0.207$; $F_{1,13} = 22.14$, $P < 0.001$) did not differ significantly from 1 ($t = 0.53$, 8 df). The $\log_e$ numbers of attached spermatozoa explained 63% of the variation in the $\log_e$ number of holes on the perivitelline layer ($r = 0.794$, $r^2 = 0.63$, $P < 0.001$; $n = 15$).

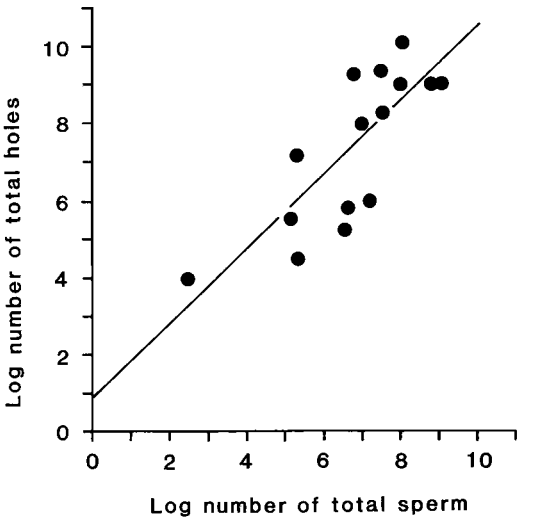

Fig. 2. Relationship between total number of spermatozoa on outer perivitelline layer and total number of holes on inner perivitelline layer of Japanese Quail eggs. Relationship is significant: $\log Y = 0.785 + 0.97 \log x$; $r^2 = 0.63$, $P < 0.001$ ($n = 15$ eggs, each from a different female).

Discussion.—The extent of physiological polyspermy in birds (i.e. number of spermatozoa undergoing acrosome reaction and penetrating inner perivitelline layer) is not well known. To some extent the number of spermatozoa penetrating the layer depends upon the number inseminated and present in the female reproductive tract. Birkhead et al. (1993) recorded a positive correlation between the numbers of attached spermatozoa and the number of holes in the perivitelline layer of Zebra Finch (*Taeniopygia guttata*) ova. Other studies have also recorded positive correlations between the numbers of spermatozoa inseminated, in the sperm storage tubules, and attached to the outer perivitelline layer (Brillard and Antoine 1990, Brillard and Bakst 1990, Bramwell and Howarth 1992a). Our results (Fig. 2) also show that the number of holes in the inner perivitelline layer of Japanese Quail eggs is positively correlated with the number of spermatozoa attached to the outer perivitelline layer. The existence of these relationships may partly explain the variation in the estimates of what have been referred to as supernumerary spermatozoa recorded in the ova of domestic fowl (*Gallus gallus*). Patterson (1910) and Olsen (1942) recorded up to 24 supernumerary spermatozoa, and Bekhtina (1966) recorded a mean of 60. In the pigeon (*Columba livia*), Harper (1904) recorded up to 25 supernumerary sperm, but Durme (1914) working on House Sparrows (*Passer domesticus*) and Barn Swallows (*Hirundo rustica*) noted that the existence of supernumerary sperm was not universal. However, in these early studies the numbers of spermatozoa penetrating the perivitelline layer was determined by locating spermatozoa within the blastoderm, rather than by counting holes, as in our study (but see below), so direct comparison is not possible.

Several previous studies of the numbers of spermatozoa attached to the outer perivitelline layer found that spermatozoa were distributed at random with respect to the germinal disc (domestic fowl [Bobr et al. 1964, Wishart 1987] and turkey, *Meleagris gallopavo* [Brillard and Bakst 1990]). It was assumed that the random distribution of spermatozoa occurred because the spermatozoa visible on the perivitelline layer were trapped there as the outer layer was laid down once penetration of the inner layer had occurred and as the fertilized ovum started to move down the oviduct (Perry 1987, G. J. Wishart pers. comm.). We found that spermatozoa on the outer perivitelline layer occurred at significantly higher density over the germinal disc area than elsewhere on the ovum in Japanese Quail. We also obtained similar results for a range of other species in another study (Birkhead et al. 1994). Overall, our findings suggest that spermatozoa are attracted to the germinal disc region of the ovum. We also found that holes in the inner perivitelline layer occurred at a higher density in the germinal-disc area than elsewhere. Harper (1904) stated that "the most favourable zone for the entrance of sperms is the fovea a zone surrounding the egg nucleus" (i.e. the germinal disc), and Bramwell and Howarth (1992b) also found that holes were concentrated over the germinal disc area in domestic fowl. Bramwell and Howarth (1992b) further noted that the holes made by spermatozoa formed a halo around the germinal disc, but that the center of the germinal disc was not penetrated. We found no "halo" effect in Japanese Quail, although we have seen it clearly in other species (Birkhead et al. 1994). The fact that we found both attached spermatozoa and holes to be more concentrated in the vicinity of the germinal disc suggests either that spermatozoa are preferentially attracted to this area, or that the germinal disc region facilitates attachment and penetration by spermatozoa (Bramwell and Howarth 1992b; see also Howarth and Digby

1973, Bakst and Howarth 1977, Okamura and Nishiyama 1978).

Given the enormous size of avian (megalecithal) ova relative to spermatozoa, it seems paradoxical that spermatozoa should penetrate anywhere other than the germinal disc (Howarth 1984); yet, we found that 98% of all holes on the ovum occurred away from the germinal disc in Japanese Quail. However, we have shown elsewhere (Birkhead et al. 1994) that some spermatozoa probably undergo proteolytic activity and penetrate the inner perivitelline layer after fertilization has taken place (i.e. at some time between fertilization and the point at which the eggs were examined). This presumably explains the occurrence of relatively large numbers of holes away from the germinal disc. It also means that the numbers of holes in the inner perivitelline layer cannot be used to assess the extent of physiological polyspermy in birds. Nonetheless, the combined number of trapped spermatozoa and holes provides a useful index of the number of spermatozoa in the female tract at the time of fertilization and, as such, provides a useful, noninvasive technique for exploring the dynamics of spermatozoa in the female reproductive tract.

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Literature Cited

Bakst, M. R., and B. Howarth, Jr. 1977. Hydrolysis of the hen's perivitelline layer by cock sperm in vitro. Biol. Reprod. 17:370–379.

Bekhtina, V. 1966. Morphological features of polyspermy fecundation in hens. *In* Summaries from Pushkin Research Laboratory of Livestock Breeding (Leningrad Region, USSR). World's Poult. Sci. J. 24:148–149 (1968).

Birkhead, T. R., B. C. Sheldon, and F. Fletcher. 1994. A comparative study of sperm-egg interactions in birds. J. Reprod. Fert. 101:353–361.

Birkhead, T. R., J. E. Pellatt, and F. Fletcher. 1993. Selection and utilization of spermatozoa in the reproductive tract of the female Zebra Finch *Taeniopygia guttata*. J. Reprod. Fert. 99:593–600.

Birkhead, T. R., and A. P. Møller. 1992. Sperm competition in birds: Evolutionary causes and consequences. Academic Press, London.

Bobr, L. W., F. W. Lorenz, and F. X. Ogasawara. 1964. Distribution of spermatozoa in the oviduct and fertility in domestic birds. II. Transport of spermatozoa in the fowl oviduct. J. Reprod. Fert. 8:49–58.

Bramwell, R. K., and B. Howarth, Jr. 1992a. Sperm-egg binding in oviposited eggs from artificially inseminated broiler breeder hens over time. Poult. Sci. 71:73.

Bramwell, R. K., and B. Howarth, Jr. 1992b. Preferential attachment of cock spermatozoa to the perivitelline layer directly over the germinal disc of the hen's ovum. Biol. Reprod. 47:1113–1117.

Brillard, J. P. H., and H. Antoine. 1990. Storage of sperm in the uterovaginal junction and its incidence on the numbers of spermatozoa present in the perivitelline layer of hens' eggs. Br. Poult. Sci. 31:635–644.

Brillard, J. P., and M. R. Bakst. 1990. Quantification of spermatozoa in the sperm-storage tubules of turkey hens and its relation to sperm numbers in the perivitelline layer of eggs. Biol. Reprod. 43:271–275.

Durme, M. V. 1914. Nouvelles recherches sur la vitellogenese des oeufs d'oiseaux aux stades d'accroissement, de maturation, de fecondation et du debut de la segmentation. Arch. Biol. 29:71–198 (cited in Romanoff, A. L. 1960. The avian embryo. Macmillan, New York.).

Elinson, R. P. 1986. Fertilization in amphibians: The ancestry of the block to polyspermy. Int. Rev. Cytol. 101:59–100.

Gilbert, A. B. 1971. The egg: Its physical and chemical aspects. Pages 1379–1399 *in* Physiology and biochemistry of the domestic fowl, vol. 3 (J. D. Bell and B. M. Freeman, Eds.). Academic Press, London.

Harper, E. H. 1904. The fertilization and early development of the pigeon's egg. Am. J. Anat. 3:349–386.

Howarth, B., Jr., and S. T. Digby. 1973. Evidence for the penetration of the vitelline membrane of the hen's ovum by a trypsin-like acrosomal enzyme. J. Reprod. Fert. 33:123–125.

Howarth, B., Jr. 1984. Maturation of spermatozoa and mechanism of fertilization. Pages 161–174 *in* Reproductive biology of poultry (F. J. Cunningham, P. E. Lake, and D. Hewitt, Eds.). Longman Group, Harlow.

Okamura, F., and H. Nishiyama. 1978. The passage of spermatozoa through the vitelline membrane in the domestic fowl, *Gallus gallus*. Cell Tissue Res. 188:497–508.

Olsen, M. W. 1942. Maturation, fertilization and early cleavage in the hen's egg. J. Morphol. 70: 513–533.

Olsen, M. W., and B. H. Neher. 1948. The site of fertilisation in the domestic fowl. J. Exp. Zool. 109:355–366.

Patterson, J. T. 1910. Early development of the hen's egg. J. Morphol. 21:101–134.

Perry, M. M. 1987. Nuclear events from fertilisation to the early cleavage stages in the domestic fowl (*Gallus domesticus*). J. Anat. 150:99–109.

Wishart, G. J. 1987. Regulation of the length of the fertile period in the domestic fowl by numbers of oviductal spermatozoa as reflected by those trapped in laid eggs. J. Reprod. Fert. 80:493–498.

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