

Chronology of events in the first cell cycle of the polyspermic egg of the domestic fowl (*Gallus domesticus*)

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ABSTRACT The nuclear population in the polyspermic egg of the domestic hen was examined in whole-mount preparations of the germinal disc. The numbers of nuclei varied in groups of hens from averages of 5.9 to 26, depending on days from insemination. Changes in development from initial formation of pronuclei to the early mitoses of the zygote nucleus were staged according to the position of the egg in the oviduct. The findings substantiated earlier accounts on the timing of the apposition of the parental pronuclei towards the end of the first cell cycle. Additionally, analysis of the spatial distribution of accessory spermatozoal nuclei showed a slight, but significant, dispersal from a clustered arrangement at this time.

KEY WORDS: *pronuclei, polyspermy, germinal disk, domestic hen*

The progress of nuclear development in the first cell cycle, from gamete interaction to syngamy, is well known for many species in the animal kingdom. In birds, the completion of meiosis by the egg nucleus, the penetration of several spermatozoa through the perivitelline layer into the germinal disc of the egg, and their transformation into pronuclei have been described (Okamura and Nishiyama, 1978a,b; Perry, 1987). However, events occurring later in the period leading to syngamy have not been fully documented. An accurate record of nuclear features during this period, when the avian egg is most amenable to *in vitro* culture, is particularly important for work involving manipulation of the avian genome in the single-celled egg (for example, see Love *et al.*, 1994). In this report, we have examined whole-mount preparations of the germinal disc in order to obtain an overall picture of the nuclear population.

Visualization and numbers of nuclei

The transforming spermatozoal heads and the egg nuclear components fluoresced intensely after staining with the dye, 4',6'-diamidino-2-phenylindole (DAPI). By contrast, the definitive pronuclei gave a less intense fluorescence but were still distinguishable against the faint background fluorescence of the yolk-filled cytoplasm. Nuclei in the blastoderm of the laid egg (division products of the zygote nucleus), employed as controls for the staining method, gave an intense fluorescence with DAPI. In the germinal disc, most of the pronuclei were situated in a roughly similar horizontal plane, at a depth of 22-52 μm . These measurements

were made on a selection of centrally placed pronuclei in 8 germinal discs at the more advanced stages of the cell cycle. The few nuclei located at a deeper level had ill-defined outlines.

There was a wide variation between eggs in the number of nuclear bodies and pronuclei. The highest number (average 26; range 8-45) was observed in 7 eggs from a group of hens inseminated 2 and 9 days beforehand. The numbers of nuclei were lower in eggs from hens examined after an interval of 7 days from insemination. Twenty two eggs from 4 groups of hens contained a moderate number (average 11.4; range 5-22) and 8 eggs from 2 groups of hens contained few nuclei (average 5.9; range 4-11). In 3 embryos at the 2-4-cell stage, the number of pronuclei prior to syngamy was estimated to have been, respectively, 7, 8 and 13. Three eggs containing a single nucleus were designated as infertile. The question of embryonic viability versus number of accessory spermatozoal nuclei has been raised by Fofanova (1965) who concluded that a minimum number of 6 accessory nuclei was required for normal development. The present observations on the specimens in which embryonic development had commenced suggest that at least some of the precleavage eggs possessing small numbers of pronuclei were indeed viable. Furthermore, Wishart (1987), using an assay of spermatozoal heads in the perivitelline layer of the laid egg to predict fertility, found that fertility remained high even though there was a dramatic decline in

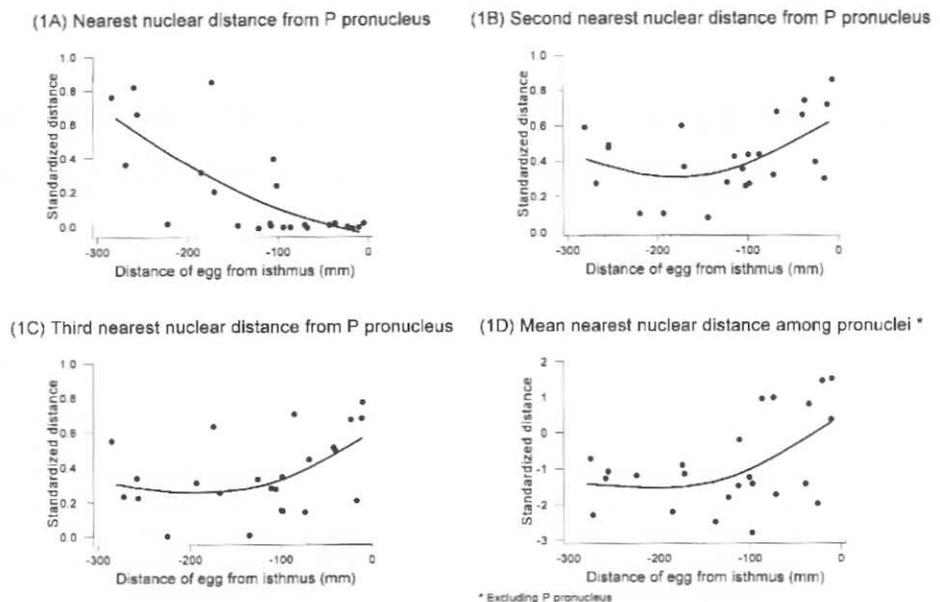
Abbreviations used in this paper: DAPI, 4', 6'-diamidino-2-phenylindole; PBS, phosphate buffered saline; DABCO, 1, 4-diazobicyclo-2, 2, 2-octane.

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Fig. 1. Curves illustrating changes in the distribution of nuclei in the germinal disk of the polyspermic egg of the domestic fowl with passage of the egg along the magnum towards the isthmus of the oviduct. In disks where neither parental (P) nucleus was identifiable, the largest nucleus was chosen as the point of reference for measurement of internuclear distances, standardized as given in *Statistical Methods*. **(A)** The arrangement of the parental nucleus and its nearest neighbor changed from a random (0.5, standardized distance) to a clustered (zero, standardized distance) pattern as the egg approached the isthmus. **(B and C)** The arrangement of the second and third nearest neighbors (accessory spermatozoal nuclei) relative to the parental nucleus showed a trend from slight clustering to a random pattern. **(D)** The arrangement of all nuclei, excluding the parental nucleus, showed a similar trend from a clustered (negative values) to a random (zero value) pattern.



the number of spermatozoal heads after 5 days following insemination.

Nuclear event sequence and distribution of nuclei

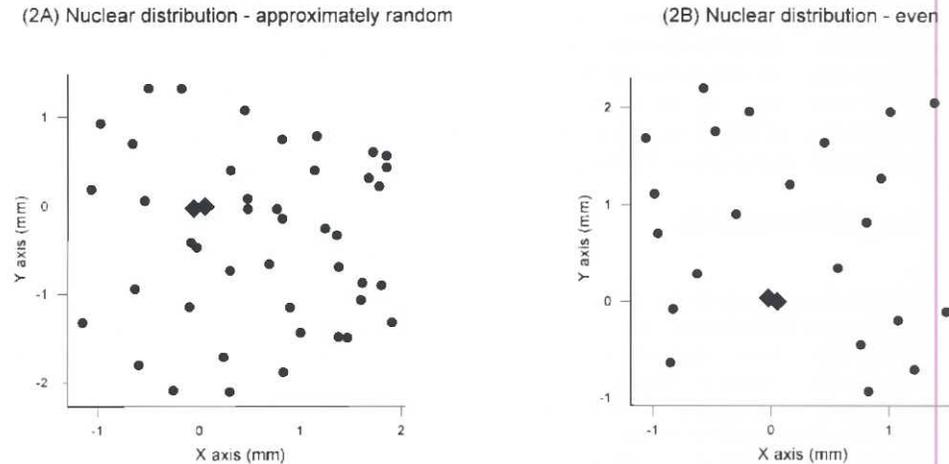
Progressive changes were noted in nuclear morphology and distribution as the egg passed along the magnum region of the oviduct towards the isthmus. On the basis of these changes, nuclear development from formation of pronuclei to the first divisions of the zygote nucleus was divided into 5 stages. The stages are listed in Table 1, giving the respective location in the magnum and times from oviposition of the preceding egg. The average length of the magnum was 360 mm (range 300-390 mm). The earlier events of gamete interaction take place in the infundibulum, at the extremity of the oviduct nearest the ovary, and in the anterior magnum. There are no sufficiently clear pictures available from the whole-mount preparations of the germinal disc in this work. Stages in Table 1 are linked to Figures in Perry (1987) where appropriate. Spermatozoal heads in the process of transformation and small pronuclei of less than 10 μm in diameter were present in the germinal discs of eggs located at 220-280 mm, and exceptionally 170 mm, from the isthmus (see Fig. 3, Perry, 1987). In 3 of these specimens, figures interpreted as the egg nucleus in telophase of the 2nd meiotic division were noted. The majority of the nuclear bodies had formed into small, spherical pronuclei containing decondensed chromatin in eggs located at 160-230 mm from the isthmus. The average nuclear diameter was 11.9 μm (range 5-21 μm). When the eggs had passed into the posterior magnum, at around 96 mm from the isthmus, the pronuclei had undergone varying degrees of swelling (average diameter 17 μm ; range 7-25 μm ; see Fig. 4 lower, Perry, 1987). Nuclear size remained relatively unchanged (average diameter 16.2 μm ; range 9-22 μm) in eggs taken from the extreme posterior region of the magnum. The diameters of the pair of pronuclei (see Fig. 4 upper, Perry, 1987) found in 7 of these last specimens were unequal. The larger of the pair had an average diameter of 19.4 μm (range 17-22 μm) and the smaller had an average diameter of 15.8 μm (range 14-17 μm).

The various nuclear bodies and definitive pronuclei were scat-

tered unevenly throughout the germinal discs. In whole-mount preparations, the area of the germinal discs ranged from 5.7 to 12 mm^2 . The analysis of nuclear distribution showed that the distance of the nearest nucleus to a parental nucleus, tentatively identified by its larger size in earlier stages, decreased to zero as the egg moved along the magnum towards the isthmus (Fig. 1A). This proximity of 2 pronuclei enabled their identification as the maternal and paternal pronuclei, and the surrounding nuclei as accessory spermatozoal nuclei. A pair of pronuclei, separated by relatively short distances of 7-25 μm , was first observed when most of the nuclei had undergone enlargement. The parental pronuclei were not always positioned at the centre of the germinal disc, neither were they centrally placed with respect to the accessory nuclei. One of the pair was amongst the largest of the nuclear population, as noted in the preceding paragraph. In several eggs, situated in the designated regions of the posterior magnum, there was no evidence of paired pronuclei. These exceptions included one egg, containing 24 pronuclei; the largest of these pronuclei, at 22 mm in diameter, was classified as the zygote nucleus having undergone syngamy (Table 1). Five other eggs had a small complement of less than 6 pronuclei. For this reason, the eggs may have been inviable though large numbers of accessory nuclei do not appear to be essential for syngamy. Alternatively, the eggs may have been retarded in development. This explanation may also account for the absence of a pair of pronuclei in 2 other specimens, containing 9 and 15 pronuclei, respectively, located at 100 mm from the isthmus.

It has been suggested, from observations on sectioned material, that the accessory nuclei gradually disperse towards the periphery of the germinal disc during the first cell cycle (Perry, 1987). To verify this proposition, the distribution of accessory nuclei was examined in the whole-mount preparations. Analyses of the distances of the second and third nearest nucleus relative to a parental nucleus showed a small, but significant increase as the egg moved towards the isthmus (Fig. 1B,C). The distances between individual accessory nuclei also increased slightly with progression through the cell cycle (Fig. 1D). Since the organization

Fig. 2. Schematic diagram of the distribution of nuclei in the germinal disk of the polyspermic egg of 2 domestic fowl, each with a pair of apposed pronuclei (diamond symbols). The axes are for scale only. (A) Egg 40 mm from the isthmus with an approximately random distribution of nuclei. (B) Egg 10 mm from the isthmus with a more even distribution of nuclei, also showing the exclusion of accessory nuclei from the ooplasm surrounding the paired parental nuclei.



of asters around the spermatozoal head following entry into the ooplasm is a common phenomenon in the animal kingdom, and these structures are associated with all accessory nuclei in polyspermic eggs (Schatten, 1994; Iwao *et al.*, 1997), astral growth may well account for the dispersal of pronuclei in the avian germinal disc. Though no general translocation of accessory nuclei towards the periphery of the germinal disc was detected, it is noteworthy that the ooplasm in the vicinity of the paired parental nuclei was seen to be devoid of accessory nuclei. Migration of accessory nuclei from this area, which was approximately 1.0 mm in diameter and was destined to form the blastomeres of the early embryo is illustrated in Figure 2, and was further highlighted by the distribution analysis. Exclusion of accessory nuclei from the cleaving blastomeres has been depicted in a report by Fofanova (1965) on the fate of these nuclei. They degenerate after undergoing one round of mitosis shortly after the division of the zygote nucleus. A more extreme example of mobility of accessory nuclei occurs in the polyspermic egg of the newt, *Cynops pyrrhogaster* (Iwao *et al.*, 1993). In this egg, which measures about 2.2 mm in diameter, all but one of the spermatozoal nuclei are displaced from their points of entry in the animal hemisphere into the vegetal hemisphere.

Chronology

The results of comparing the stage of nuclear development with location of the egg in the magnum and time of oviposition of the preceding egg indicated that the former criterion provided the more reliable estimate of stage, though there was still a broad overlap in location for a particular stage. Three eggs were at the upper limit of the time range for the category, and 2 eggs were at the lower limit. The main reason for the unpredictability in the use of time

from preceding egg is the variation in the lag period (Gilbert and Wood-Gush, 1971) between lay and the next ovulation. Also, the laying of the shelled egg can be delayed in some birds by external influences. On the other hand, passage of the egg along the oviduct occurs at a fairly constant rate, with time in the infundibulum given as 0.25-0.5 h, and in the magnum as 2.0-3.0 h (Aitken, 1971). In the current work, the interval from initial formation of pronuclei to apposition of the parental pronuclei was approximately 1.5 h. In the following 0.5 h, syngamy occurred and the resulting zygote nucleus completed one or two rounds of division. These times, for eggs obtained from a single strain of hens of uniform age, are shorter than those given by Perry (1987) for a selection of eggs from several strains having a less regular laying pattern. Experiments on *in vitro* fertilization in the domestic fowl have shown that it takes about 0.5 h for the spermatozoa to penetrate the perivitelline membrane (Bakst and Howarth, 1977), and the total time from gamete interaction to pronuclear apposition is reported to be about 4.0 h (Nakanishi *et al.*, 1990).

TABLE 1

SEQUENCE OF NUCLEAR EVENTS FOLLOWING GAMETE ASSOCIATION IN POLYSPERMIC GERMINAL DISKS DURING PASSAGE OF THE HEN'S EGG THROUGH THE MAGNUM TOWARDS THE ISTHMUS OF THE OVIDUCT

Nuclear events	Number germinal disks/total observed	Location of the egg with time	
		Average distance from the isthmus (range) mm	Average time from preceding oviposition (range) h
Transforming spermatozoa and small nuclei (<10 µm diam.)	6/6	240 (170-280)	1.5
Enlarging nuclei	8/8	193 (160-230)	1.9 (1.5-2.0)#
Pair of pronuclei separated by a distance of 7-25 µm	8/12##	96 (20-160)	2.6 (2.5-3.0)
Pair of apposed pronuclei	7/10##	55(10-110)	2.9 (2.5-3.25)
Syngamy	1	10	3.25
2-4 cell embryo	3	0	3.5

One egg taken at 3.5 h after the preceding oviposition was outwith this time range.

In 4 and 3 germinal disks respectively, a pair of pronuclei was not observed.

The findings presented here substantiate earlier accounts of pronuclear development leading to syngamy in birds. Moreover, they enable the events to be tabulated with some accuracy. They will provide a basis for experimental intervention in the first cell cycle of the avian egg. As the events are displayed in a 2-dimensional array, the avian egg would seem to be ideal material for studies on the mechanisms of pronuclear interaction.

Experimental Procedures

Materials

Hens of a laying strain, Isa Brown, in the first year of lay were housed in individual cages and maintained on a 14 h light/24 h cycle. They were artificially inseminated every 7 days. At 2 or 7 days after the last insemination, and at intervals of 1.5 to 3.5 h after laying, the hens were killed with an intravenous injection of pentobarbitone sodium ("Expiral", Sanofi Animal Health Ltd., U.K.). The eggs were located in the magnum or were partially in the isthmus. The distance of the leading edge of the egg from the magnum/isthmus boundary was measured, before immersing the egg in ice-cold, phosphate-buffered salt solution (Dulbecco's formula, without calcium and magnesium). The germinal discs were dissected from the yolk and detached from the perivitelline membrane. They were fixed in acetic alcohol (glacial acetic acid, 1 part: 100% ethanol, 3 parts) for 2 h, then hydrated through a graded series of ethanol (from 70% to 8%) for 1.5 h; and rinsed in phosphate buffered saline (PBS), pH7.2, for 2x15 min. The material was cleared and the nuclei stained in a solution of glycerol, 9 parts: PBS, 1 part, containing 2 µg/ml of the DNA fluorochrome, 4',6'-diamidino-2-phenylindole (DAPI) and 2.5 mg/ml of the antifade reagent, 1,4 diazobicyclo-2, 2, 2-octane (DABCO). The germinal discs were mounted, with the outer surface uppermost, in the DAPI/DABCO solution. The preparations were stored in the dark, at 4°C.

Microscopy

A total of 43 specimens was examined using u.v. illumination in the epifluorescent mode to detect nuclei. Measurements of nuclear diameter and internuclear distances were recorded on a Quantimet system, employing a micrometer stage. The depth of the nuclei from the upper surface of the germinal disc was measured by confocal microscopy, using the inhomogeneity between nucleus and cytoplasm to detect the nuclei, having first located their position in the u.v. mode. Attempts to stain the nuclei with several other DNA-specific fluorochromes, that can be visualised in the confocal microscope, were unsuccessful due to the affinity of the cytoplasm for these dyes.

Statistical methods

The egg nucleus, or one of the pair of parental pronuclei was selected as the point of origin in the construction of plots of the relative positions of all other nuclei in the germinal discs. At intermediate stages, in which neither of these figures could be identified, the largest pronucleus was posited as a parental pronucleus and chosen as the point of reference.

Changes in the distribution of the pronuclei as eggs moved towards the isthmus were assessed. The measurements analyzed were the distance from a parental pronucleus to its nearest, second nearest and third nearest neighbors and the average of the nearest neighbor distances amongst the accessory pronuclei. Eggs with less than 7 pronuclei and those with cleaving embryos were excluded. Neighbor distances have to be standardized to allow for different areas and shapes of germinal disc and for different numbers of spermatozoal pronuclei. Standardized distances can be interpreted as a continuously changing scale from low values indicating "clustering", through the central value showing "randomness" to high values indicating "even spacing". The distribution of the nearest neighbor to the parental pronucleus was obtained from Diggle (1983), and the distributions for the second and third nearest neighbors from the distributions of order statistics in Cox and Hinkley (1974). These have a central value of 0.5. The

mean distance amongst accessory spermatozoal pronuclei was approximately normally distributed, provided there were sufficient numbers of pronuclei (Donnelly, 1978). Subtracting the expected distance from the observed, and dividing by the standard error gives a standardized value centred about zero. Changing patterns of neighbor distances over time (using distance of the egg from the isthmus as a surrogate) were estimated by data smoothing (Hastie and Tibshirani, 1990). All the smoothed patterns presented in the results are significant at $P < 0.05$, by an approximate F-test.

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