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Nuclear events from fertilisation to the early cleavage stages in the domestic fowl (*Gallus domesticus*)

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INTRODUCTION

The avian ovum is fertilised in the infundibulum, the anterior region of the oviduct. Several spermatozoa penetrate the inner perivitelline layer and enter the germinal disc, the small area of cytoplasm at the surface of the yolk mass where development is initiated. As it traverses the oviduct, the ovum is progressively invested with the outer perivitelline layer, the albumen and the shell. The first cleavage division of the zygote broadly coincides with the onset of shell membrane formation. Initially, the cleavage furrows are incomplete, forming open blastomeres (Patterson, 1910; Kochav, Ginsburg & Eyal-Giladi, 1980) and, from the second division onwards, cleavage tends to be asynchronous (Patterson, 1910; Eyal-Giladi & Kochav, 1976). The fine structure of the early cleavage furrows has been described by Gipson (1974) and Bellairs, Lorenz & Dunlap (1978).

There have been few accounts of the sequence of nuclear events in the polyspermic avian ovum since Harper (1904) published his classic study on the pigeon. In the domestic fowl, Olsen (1942) and Bakst & Howarth (1977) examined the early stages of the penetration of spermatozoa, Bekhtina (1966) extended these studies to cover the period to the time of the first cleavage division, and Patterson (1910) and Bekhtina (1960) described the early cleavage nuclei. In some reports (Patterson, 1910; Bekhtina, 1966; Fofanova, 1965) particular attention was paid to the fate of the supernumerary spermatozoal nuclei.

This study was undertaken to clarify certain controversial points. The association of male and female pronuclei is stated to occur shortly after ovulation by Olsen (1942) and several hours later by Bekhtina (1966). Moreover, it is reported that mitosis in the early cleavage nuclei is aberrant (Emanuelsson, 1965), though normal mitotic figures were observed by Bekhtina (1960).

The present observations were made on material stained with the fluorochrome, 4′,6′-diamidino-2-phenylindole (DAPI) or by the Feulgen method. The former dye selectively stains DNA with a greater sensitivity than the Feulgen technique (Coleman, Maguire & Coleman, 1981) and enables nuclei to be readily discerned in the yolk-laden cytoplasm of the germinal disc. The results essentially confirm some of the earlier findings and provide more precise information on the events of spermatozoal transformation, pronuclear apposition and mitosis.

MATERIALS AND METHODS

Materials

Hens of 3 laying strains, Light Sussex and White Leghorn (High Sex Strain, Euribrid Co.) in the first year of lay, and Golden Comet (Hubbard) in the second year of lay, were housed in individual cages and maintained on a 14 hours light/24 hours cycle. A total of 61 hens, taken within 10 days of artificial insemination, were used for the investigation. At selected intervals after the time of lay, birds were killed with an intravenous dose of pentobarbitone sodium (Anaesleep-Forte: Dales Pharmaceuticals Ltd, Skipton, UK). The ovum was rapidly removed from the oviduct and plunged into an ice-cold, phosphate-buffered salt solution (Dulbecco's formula without calcium and magnesium: Flow Laboratories, Irvine, UK). Some ova were also taken from virgin hens.

Microscopy

After stripping off the albumen and shell membrane, if present, the germinal disc was excised from the ovum. The remainder of the yellow yolk and, in some preparations, the composite perivitelline layer was removed, then the germinal disc was drained and allowed to flatten with its outer surface uppermost. The preparation was fixed in acetic alcohol (glacial acetic acid, 1 part: 100% ethanol, 3 parts) for 2 hours, rinsed briefly in 100% ethanol, then in 95% ethanol. It was lightly stained with an alcoholic solution of light green for ease of visualisation in the paraffin block. After dehydration in 100% ethanol (3 × 30 minutes), the material was cleared in chloroform and infiltrated with paraffin wax. Serial sections, 10 μm thick, were cut parallel to the surface of the germinal disc and mounted on gelatine-coated slides.

For the DAPI method, the sections were routinely hydrated and stained following the procedure given by Coleman *et al.* (1981). They were soaked in 200 mM-KCl for 1 hour, drained and lightly blotted. The slides were flooded with DAPI (Sigma, London, UK) at a concentration of 1 μg/ml in McIlvaine's buffer, pH 4·0, and a coverslip was placed on the preparation. After 2 hours, the excess stain was drained from the slide and the coverslip sealed into position. The sections were examined within 24 hours using ultraviolet illumination in the epifluorescent mode. Other sections were stained by the standard Feulgen technique, dehydrated and mounted in DPX.

RESULTS

Embryonic age

For hens laying in long sequences of one egg per day, the time of lay can be used as a guide to predict the age of the embryo in the oviduct. Oviposition is followed by the next ovulation at an average interval of 24 minutes (Morris, 1973). The ovum is immediately engulfed by the infundibulum and it takes an average time of 5·1 hours to traverse the magnum and reach the uterus (Morris, 1973). These timings may vary between strains and in individuals, for instance, Olsen (1942) noted that the first external change in the germinal disc – the formation of the cleavage furrow – occurred within a range of 4·8–6·7 hours after oviposition of the preceding egg. In the following account the interval from the time of lay of the preceding egg is given to indicate the age of the embryo at particular developmental stages.

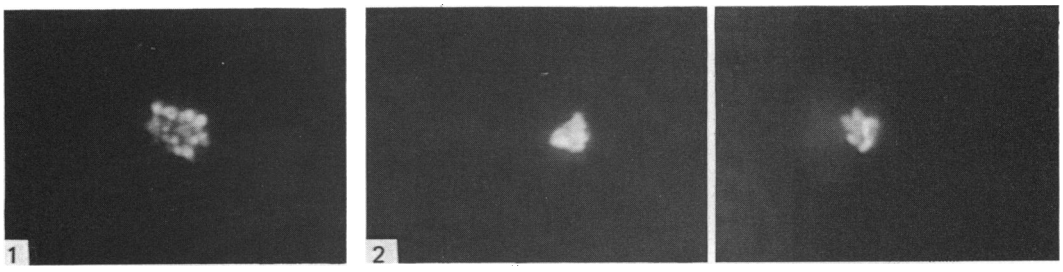

Fig. 1. Female nucleus at the metaphase stage of the second meiotic division in a fertilised ovum recovered from the anterior magnum. DAPI. × 1200.

Fig. 2. Late telophase chromosomes, observed in adjacent sections, at the completion of the second meiotic division of the female nucleus. The figure on the right had been extruded with the second polar body. DAPI. × 1200.

Precleavage stages

0·9–1·1 *hours*

Ova surrounded by no or little albumen were recovered from the infundibulum or anterior magnum, respectively. The preparations were stained by the Feulgen method. An irregular, Feulgen-positive body, 4·0 μm in diameter, was detected in the superficial cytoplasm near the centre of three germinal discs. Similar bodies have been identified by Bekhtina (1960, 1966) as the female nucleus in the metaphase stage of the 2nd meiotic division. Spermatozoa were discerned in the superficial cytoplasm of one of these specimens, and in the perivitelline layer of another specimen. It was common to find small groups of cells with intensely-stained nuclei trapped in the perivitelline layer. These groups were the remnants of the granulosa from the ovarian follicle.

1·2–1·6 *hours*

The ova were in the anterior region of the magnum and were enveloped in a thin layer of albumen. In these preparations the perivitelline layer was removed to eliminate the granulosa cell nuclei, and DAPI was employed as the stain. Female nuclei in metaphase (Fig. 1) were detected in four germinal discs; in a fifth, the division had progressed to telophase (Fig. 2). Spermatozoal heads, numbering from 3 to 9, were observed in the cytoplasm of each of these specimens. In a sixth specimen, six definitive nuclei were observed in addition to four spermatozoal heads. The transformation from spermatozoon to small pronucleus (Fig. 3) appeared to take the following course. On entering the cytoplasm the elongated head swelled slightly over its entire length. It became ellipsoidal, then pear-shaped with a long 'tail' which gradually disappeared. Its further expansion to form a spherical nucleus was accompanied by granulation and dispersion of the chromatin, indicated by a decrease in intensity of the fluorescence.

1·8–3·5 *hours*

The ova were in the mid- to posterior region of the magnum and were surrounded by a thick layer of albumen. The number of pronuclei observed in each germinal disc ranged from 6 to 15. During this period they exhibited a gradual increase in diameter from 7 to 17 μm. At the earlier times, the nuclei were scattered, but later, two nuclei came to occupy a central position while the remainder tended to be displaced towards the periphery of the germinal disc. In one specimen, taken at 3 hours,

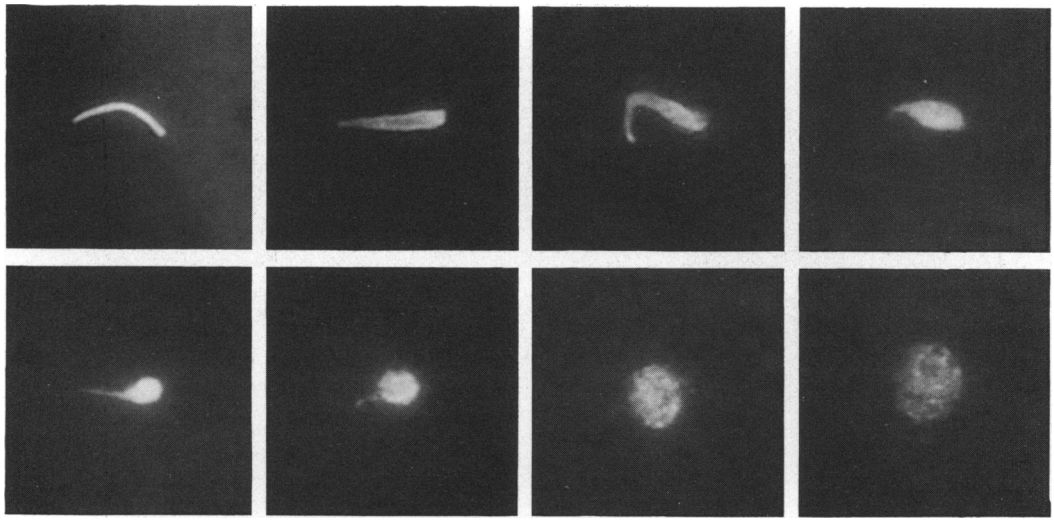

Fig. 3. The sequence shows the steps in the transformation of the spermatozoal head (top left) to small pronucleus (bottom right). The specimens were selected from ova obtained from the anterior magnum. DAPI. × 1200.

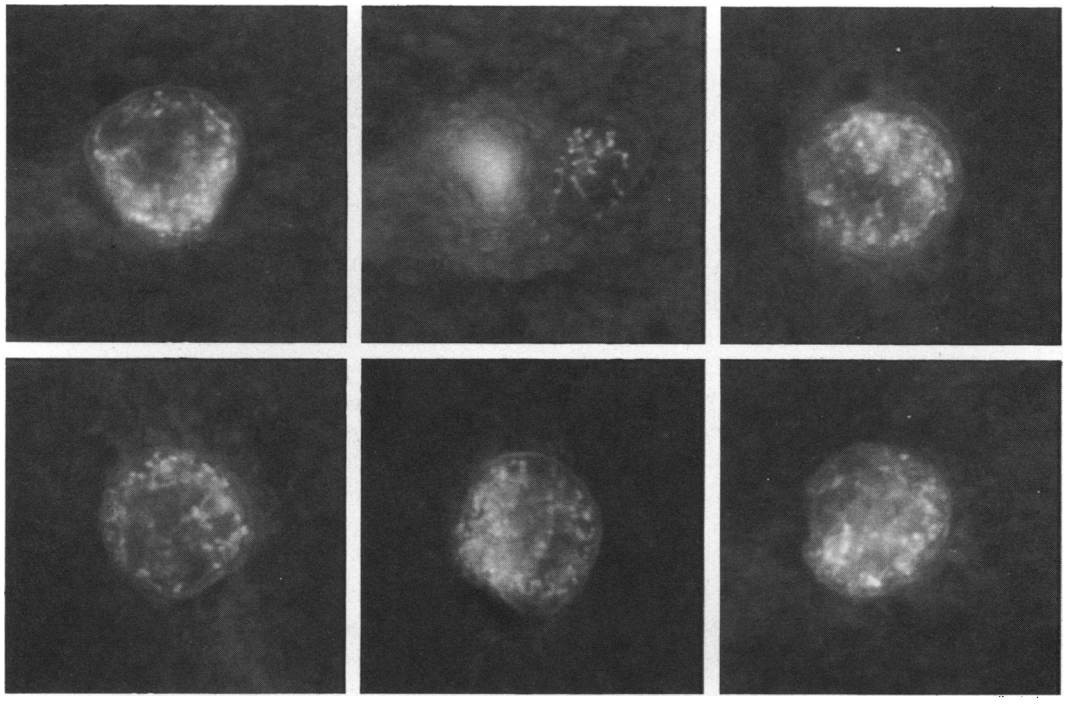

Fig. 4. Five of nine pronuclei in a germinal disc of an ovum from the posterior magnum. Top row, serial sections through two apposed nuclei. DAPI. × 1200.

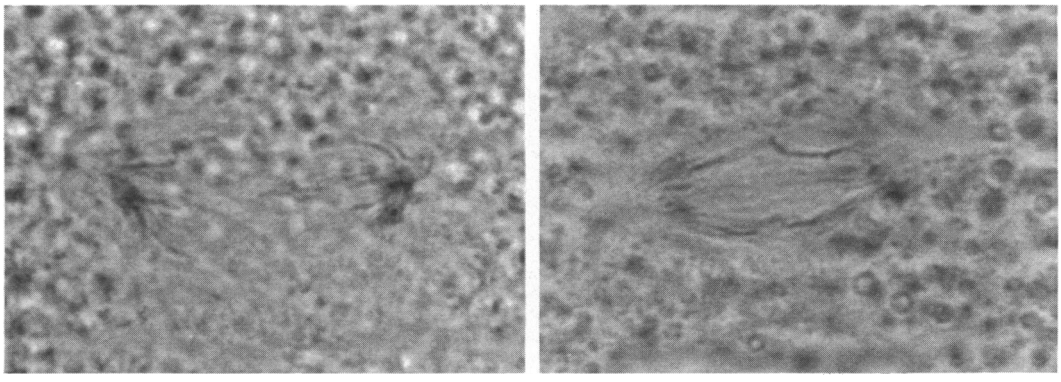

Fig. 5. Anaphase chromosomes in an ovum from the posterior magnum. Left, an anaphase figure located near the centre of the germinal disc is possibly the zygote nucleus. Feulgen method. × 2000.

two nuclei were separated by a relatively short distance of 10 μm. In another, taken at 3·5 hours (Fig. 4), two nuclei were in apposition at the centre of the germinal disc and 25 μm beneath the external surface. These were tentatively identified as the male and female pronuclei. Their distance of separation from the supernumerary nuclei in the same section was about 450 μm.

3·5–4·5 *hours*

The ova were in the isthmus, the transition zone between the magnum and uterus. The shell membrane was either partially or completely formed and in a flaccid condition. Mitotic figures were observed in one germinal disc fixed at 3·7 hours and in two germinal discs fixed at 4·5 hours. In the specimen from which the most complete series of sections was obtained, there were three sets of anaphase chromosomes (Fig. 5), five interphase nuclei and two very swollen nuclei (Fig. 8). One of the anaphase figures was centrally placed and was likely to be the zygote nucleus.

Early cleavage stages

Observations were made on 9 embryos (Stage I, Fyal-Giladi & Kochav, 1976) in eggs recovered from the isthmus or uterus at 5–7·5 hours. All the eggs were enclosed in a shell membrane with one exception, in which the embryo was at the first cleavage stage.

Cleavage nuclei

Daughter nuclei derived from the zygote nucleus were observed at a distance of 175–180 μm from the first cleavage furrow. In one embryo they were in interphase (Fig. 6), and in another they had divided again but had not yet migrated polewards. The third, and probably the fourth, nuclear divisions also occurred in synchrony. All the nuclei in any one embryo up to the 12–16 blastomere stage were identical, those in interphase (Fig. 6, inset) being of uniform size, and those in division (Fig. 7) being in the same mitotic phase. On focussing through the plane of the section, numbers of elongated and spherical chromosomes were evident in the metaphase and anaphase figures. During interphase the nuclei enlarged from 8 μm to about 17 μm in diameter. They stained intensely with DAPI, but gave an extremely faint reaction with the Feulgen method unless they were in mitosis.

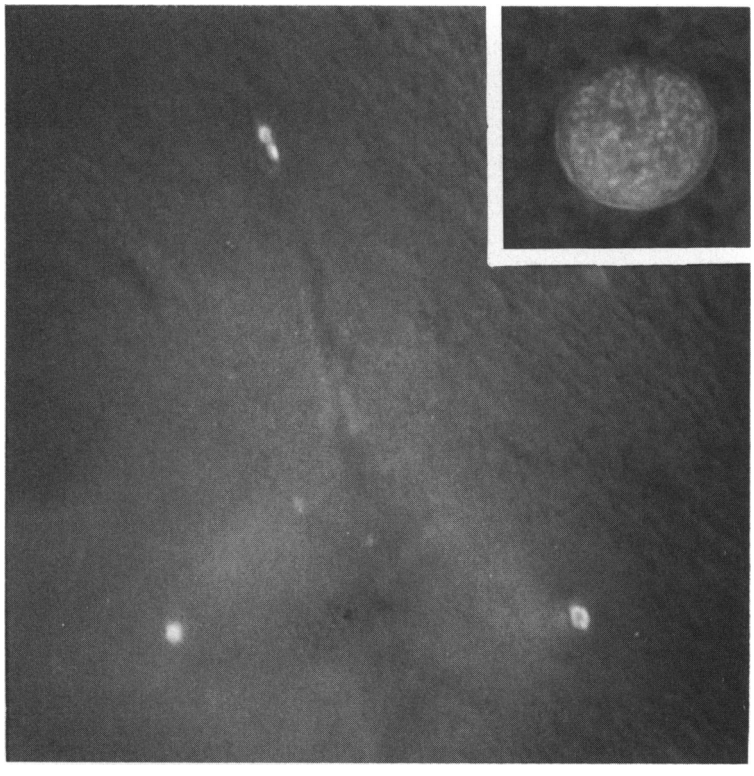

Fig. 6. A section, parallel to the surface of the germinal disc, through an embryo of two blastomeres. The two daughter nuclei are visible on either side of the cleavage furrow; at one end (right) of the furrow is a pair of supernumerary nuclei. × 155. Inset. One of 8 interphase nuclei in an embryo of 6 blastomeres. DAPI. × 1200.

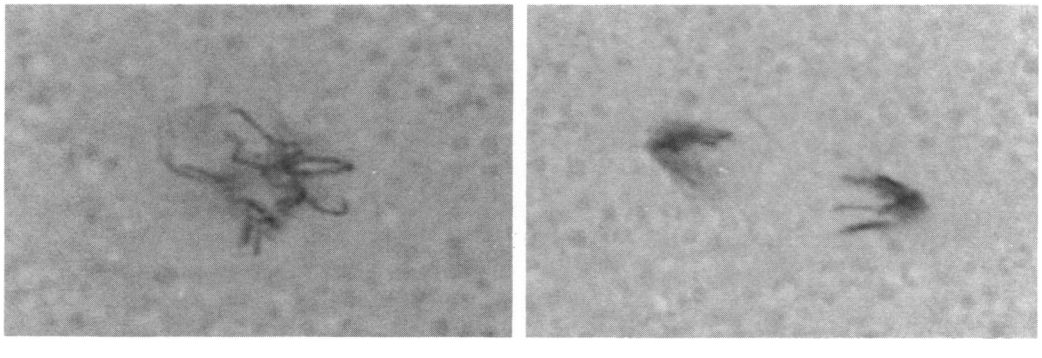

Fig. 7. Cleavage nuclei in mitosis. Left, one of 12 metaphase figures in an embryo of 12–16 blastomeres; right, one of 6 anaphase figures in an embryo of 6–8 blastomeres. Feulgen method. × 2000.

Supernumerary nuclei

The nuclei that had undergone one round of mitosis did not separate after division and remained in pairs (Figs. 6, 8). As with other supernumerary nuclei observed prior to first cleavage (Fig. 8), they were extremely swollen and their chromatin was in the form of a meshwork at the centre of the nucleus. At later stages the chromatin condensed into an irregular body that stained intensely with the

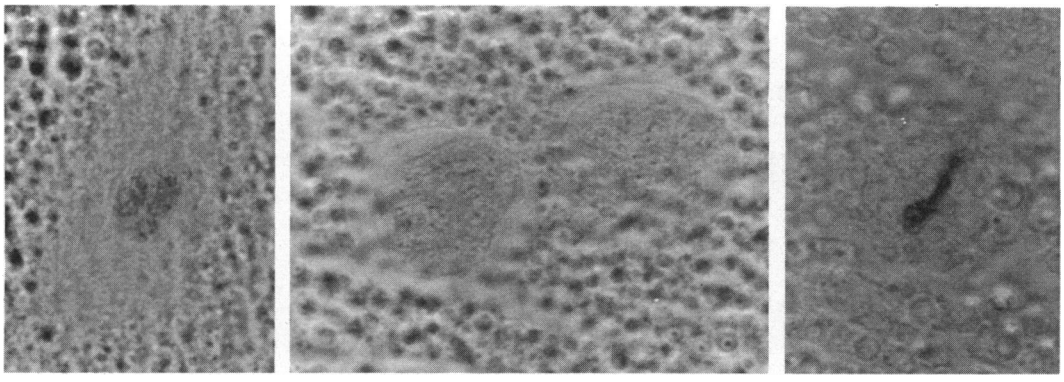

Fig. 8. Degenerating supernumerary nuclei. Left, a swollen nucleus from the germinal disc containing the anaphase figures shown in Figure 5; centre, a pair of nuclei in an embryo at the late 2-blastomere stage; right, a nucleus with clumped chromatin in a 4-blastomere embryo. Feulgen method. × 1200.

Feulgen method (Fig. 8). Such degenerated nuclei were evident in regions of the germinal disc peripheral to the blastomeres at the 4–16 blastomere stages.

Infertile ova

Since the domestic fowl exhibits limited parthenogenetic development, and numbers of viable and degenerating nuclei are present in the germinal disc of the unfertilised, laid egg (Kosin, 1945), it was of interest to examine infertile, oviductal eggs. Two nuclei, in fairly close proximity, were found in each of three germinal discs. One pair, 9 μm in diameter, was in an ovum fixed at 3·5 hours from an inseminated hen; two other pairs, 12·5 μm in diameter, were in ova fixed at 6·5 hours from virgin hens. The presence of two nuclei in infertile ova several hours after ovulation suggests that, in this instance, the second meiotic division is completed and that both daughter nuclei remain in the germinal disc. Their subsequent cycling appears to be retarded in comparison with the nuclear divisions in fertilised ova.

Some nuclear anomalies were also noted. The germinal vesicles were intact in two ova recovered from virgin hens at 6·5 hours. Another ovum obtained from an inseminated hen at 1·0 hour contained a fairly large nucleus of 15 μm in diameter. This nucleus may have failed to enter the second meiotic division.

DISCUSSION

A summary of the observations on fertilisation and early cleavage in the fowl egg is illustrated diagrammatically in Figure 9. A rough estimate of the actual age of the embryo is obtained by deducting 0·5 hour, the average interval between oviposition and the next ovulation, from the times given. Though this value is not known for the strains of hen used for the investigation, the timing of the cleavage stages was comparable with that reported by Olsen (1942) and Eyal-Giladi & Kochav (1976).

The initial stages in the interaction of spermatozoa and ovum have been examined by Olsen (1942), and in greater detail in an *in vitro* system by Bakst & Howarth (1977) who found that it took about 0·5 hour for the spermatozoa to gain access to the perivitelline space in these circumstances. The outer component of the perivitelline layer was considered to act as a barrier to excessive polyspermy, as the spermatozoa were incapable of penetrating this layer (Bakst & Howarth, 1977). The present

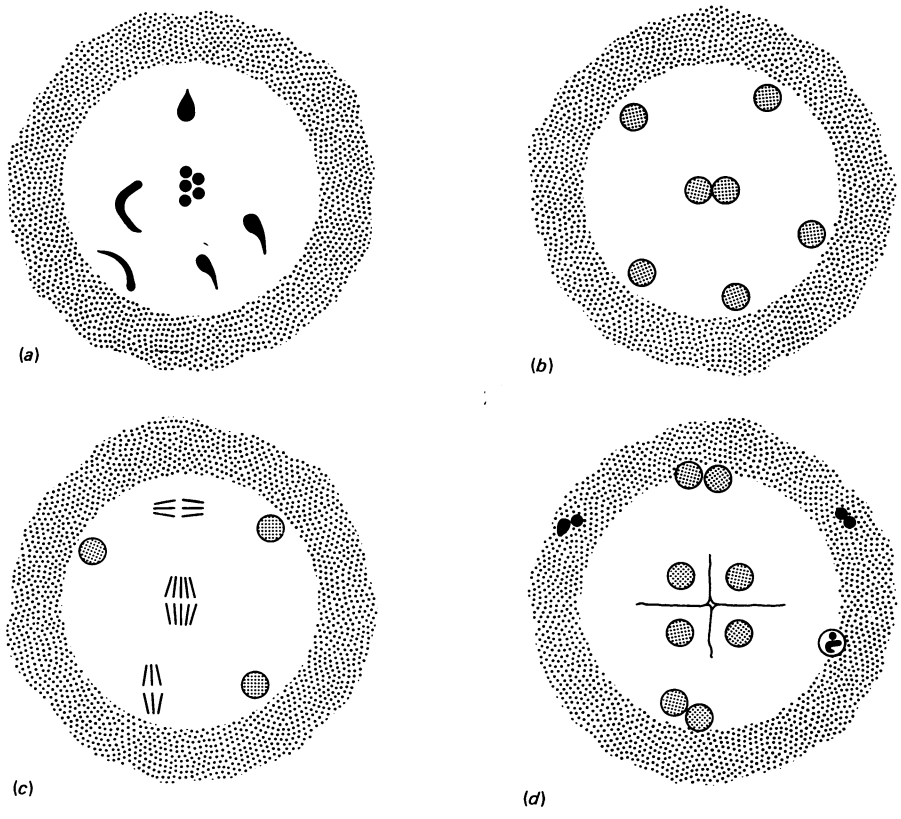

Fig. 9(*a–d*). Schematic diagram of the germinal disc of the chick embryo to illustrate the sequence of events during fertilisation and early cleavage at approximate times after oviposition of the preceding egg. (*a*) At 1·4 hours, the female nucleus (centre) is in metaphase of the second meiotic division and the spermatozoal heads are in various stages of transformation; (*b*) at 3·5 hours, the pronuclei have enlarged and two centrally placed pronuclei are in apposition; (*c*) at 4·5 hours, the zygote nucleus and several supernumerary male nuclei are in mitosis; (*d*) at 5·5 hours, a cleavage nucleus derived from the zygote nucleus is in each of the 4 open blastomeres, and degenerating supernumerary nuclei are present at the margin of the germinal disc with the yellow yolk (stippled areas). The drawing is not to scale.

observations show that by the time the ovum has passed a short distance into the magnum, approximately one hour after ovulation, the spermatozoa have migrated into the cytoplasm and have transformed into pronuclei. In confirmation of earlier findings (Harper, 1904; Bekhtina, 1960, 1966), the female nucleus remains in an arrested metaphase state for most of this period, and the second meiotic division is completed when spermatozoal transformation is at an advanced stage. This evidence is contrary to the claims (Olsen, 1942; Olsen & Fraps, 1944) that processing of the gamete nuclei and their juxtaposition takes place by 0·25 hour after ovulation. As shown here, and by Harper (1904) and Bekhtina (1966), the male and female nuclei come into apposition towards the end of the period from the time of gamete interaction to the first mitotic division of the zygote nucleus. Thus, the relative timing of pronuclear association in aves is comparable to that for amphibia (Ubbels, Hara, Koster & Kirschner, 1983).

On present evidence, the events of fertilisation in the fowl appear to conform to the general pattern of spermatozoal transformation (Longo, 1973) and pronuclear

enlargement (Gurdon & Woodland, 1968). Though the steps in the morphological alteration from spermatozoa to pronucleus have been depicted in diagrammatic form by Harper (1904), the observations reported here provide a more definitive record of chromatin dispersion. During this process, the nuclei of the spermatozoa exhibit a 50-fold increase in volume, a value comparable to that obtained for *Xenopus* spermatozoal nuclei (Gurdon & Woodland, 1968). The mode of association of the maternal and paternal sets of chromosomes was not noted in the present work. However, from the account of Bekhtina (1966), this event seems to be like that described for other species (Longo, 1973) in which chromatin condensation precedes the breakdown of the respective nuclear envelopes.

Due to technical considerations, the number of pronuclei (5–15) observed in the germinal disc was an underestimate of the total number. Figures quoted in the literature for the number of supernumerary pronuclei are: 4–24 (Patterson, 1910), 20–60 (Bekhtina, 1966) and 1–60, average 10 (Fofanova, 1965). Before the time of pronuclear association most of the supernumery nuclei are indistinguishable from those destined to form the zygote. They undergo the same morphological changes and many of them enter mitosis in synchrony with the zygote nucleus. In experimental systems similar changes have been shown to occur in spermatozoa introduced into amphibian eggs (Graham, 1966) or extracts of egg cytoplasm (Lokha & Masui, 1983). That the supernumerary nuclei undergo one or even two rounds of division is indicated by the presence of groups of two and of four supernumerary nuclei at the 2–4 blastomere stage (Patterson, 1910; Fofanova, 1965). The finding on their degeneration at this stage is in accord with these studies. Patterson (1910) reported their disappearance by the 32-cell stage, and Fofanova (1965) found remnants of fragmented chromatin persisting in a 200-cell embryo. The onset of the process seems to involve an excessive swelling of the nucleus and concomitant chromatin condensation. Because these nuclei are distributed at the periphery of the germinal disc, in the vicinity of the massive stores of yellow yolk, they may be removed from the sphere of influence of cytoplasmic factors (Gurdon & Woodland, 1968) required for the maintenance of cyclic nuclear activities. In this regard, aberrant nuclear divisions are common in the peripheral blastomeres at later stages of development (Emanuelsson, 1965). The haploid state of the supernumerary nuclei is unlikely to offer an alternative explanation for their degeneration, for they appear to be capable of supporting normal development for a limited period. There is a high incidence of haploidy in embryos that die in the first few days of incubation, and many of these embryos are considered to be androgenetic (Fechheimer, 1981). On the other hand, nuclei of maternal origin, in the unfertilised egg, degenerate within 24 hours of incubation in most strains of the domestic fowl (Kosin, 1945).

The observations on the cleavage stages provide conclusive evidence for the presence of nuclei in the blastomeres. Though Patterson (1910) and Bekhtina (1960) described nuclei and mitotic figures at the one cell to four blastomere stage, Bellairs *et al.* (1978) were unable to detect nuclei in embryos with fewer than 12 blastomeres. The cleavage nuclei are located at points equidistant from the borders of the respective blastomeres, as noted by Patterson (1910) for the daughter nuclei of the first division. They enlarge during interphase to about the same extent as the pronuclei and divide three or four times in synchrony at roughly 40 minute intervals. Nuclear enlargement is a prerequisite for DNA synthesis (Gurdon & Woodland, 1968), and some evidence for DNA synthesis at the 32-cell and later stages of quail embryos has been obtained by Callebaut (1983). Contrary to the suggestion that mitosis is atypical in

the early cleavage stages of the chick embryo (Emanuelsson, 1965), the configurations of the metaphase and anaphase chromosomes described here and by Bekhtina (1960) do not appear to deviate from the normal forms of avian chromosomes.

In conclusion, the nuclei that are destined to participate in the development of the chick embryo display a pattern of behaviour during fertilisation and early cleavage which is characteristic for animals in general. The supernumerary nuclei follow the same sequence until the early cleavage stages when they degenerate.

SUMMARY

The changes in nuclear morphology from fertilisation to the 16-blastomere stage of development in the domestic fowl were examined in sections of the germinal disc stained with DAPI (4′,6′-diamidino-2-phenylindole) or with the Feulgen technique. By one hour after the estimated time of ovulation, the second meiotic division of the ovum was completed and the spermatozoal heads (3–9) in the germinal disc had transformed into pronuclei. During the following two hours all the pronuclei enlarged, and two of them became juxtaposed. Mitotic figures and large pronuclei were observed at 4 hours. The zygote nucleus went through 3–4 division cycles in synchrony. The supernumerary male nuclei divided once, at most, and all showed evidence of degeneration by the 4-blastomere stage, at about five hours. Therefore, the nuclei destined to form the future chick embryo follow a pattern of behaviour during fertilisation and early cleavage similar to that of animals in general.

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