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Frequency and Cytological Aspects of Diploid Parthenogenesis in Turkey Eggs

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Summary. Naturally occurring parthenogenesis in unfertilized turkey eggs was encountered at this station in 1952. Since its discovery, selective breeding on a family basis has been practiced yearly in an attempt to further intensify this trait in certain lines of turkeys and chickens.

Cytological studies likewise have been conducted and various experimental procedures employed in an attempt to develop a clearer picture of just where and the manner by which diploidy is restored. Available cytological data and indirect evidence secured in related studies indicate that restoration of diploidy in unfertilized turkey ova is due to suppression of the second polar body. It would appear that in the absence of sperm, meiosis II is not completed. Chromosomes of the second polar body and those of the egg pronucleus probably never completely separate and subsequently the unfertilized ovum resumes development with a diploid complement of chromosomes.

Four potential uses of unfertilized turkey eggs and parthenogenetic poults in biological research are given.

A naturally occurring, diploid type of parthenogenesis was encountered in unfertilized eggs of nonmated Beltsville Small White (BSW) turkeys at the Agricultural Research Center, Beltsville, Md., in 1952. The original observations revealed that 156 or 16.7% of 934 unfertilized turkey eggs, laid 60 to 224 days following removal of males, underwent an abortive type of embryonic development upon being incubated (Olsen and Marsden, 1953). The development encountered at that time consisted solely of an unorganized growth of extra embryonic membranes with no evidence of either blood or embryo formation. Since these initial observations, much progress has been made toward the development of a strain of BSW turkeys where virgin hens can reproduce without benefit of a male. This report will deal with various aspects of parthenogenetic development in eggs of BSW turkeys.

The onset of parthenogenetic cleavage of turkeys takes place within the oviduct a few hours following ovulation. In one sense, however, parthenogenetic development may be looked upon as starting 4 or 5 hours prior to ovulation since two cell divisions (reduction divisions) are initiated before the ovum is released from the ovary and in the absence of sperm, even in mated birds.

The first mitotic division occurs in unfertilized turkey eggs some 5 or 6 hours after time of ovulation and after the ovum has entered the uterus (Haney and Olsen, 1958). This time interval is approximately the same as that given by Olsen and Fraps (1944) for the first cell division of normally fertilized eggs, indicating little or no delay in the onset of the first cell division in eggs developing parthenogenetically.

Parthenogenetic cleavage in eggs of both chickens and turkeys is characterized by the seeming lack of organization on the part of early blastomeres. The first cleavage furrow tends to be irregular, giving rise to daughter blastomeres which vary greatly both in size and shape. As viewed microscopically in cross section, cells in blastoderms of newly laid, unfertilized eggs have formed in multiple layers, with little evidence for formation of a segmentation cavity. Nearly all blastoderms examined thus far have shown degenerative changes, as indicated by numerous vacuoles appearing throughout the protoplasmic disc (Olsen and Marsden, 1954; and Olsen, 1965a). Many of the existing cells also have lost their staining ability hematoxin and eosin) by the time of lay, indicating that many of them by that time are either moribund or already dead (Kosin and Nagra, 1956).

Unfertilized turkey eggs, upon being placed in an incubator do not undergo development immediately. There is a characteristic 2–3 day delay before growth of the blastoderm can be detected in intact eggs by candling, or by visual inspection of the germinal area on being broken out (Olsen and Marsden, 1953; and Olsen, 1965a). This initial time-lag has been attributed to the time necessary for certain cells of an unorganized, multilayered blastoderm to reorganize and form a normal one (Olsen, 1965a). Incubation time for parthenogenetic poults, in consequence, is approximately 2 days longer than for poults from fertilized eggs.

The characteristic multilayered arrangement and seemingly lack of organization of cells developing parthenogenetically, both within the hen's oviduct and in the incubator, raise the question of possible viral involvement.

It has been well established that certain viruses in tissue culture can cause the piling up and fusion of cells (Harris, 1970). Broadly speaking, the unfertilized turkey egg may be looked upon as a sterile, preseeded tissue culture media containing all necessary ingredients for cell growth and development. It is not inconceivable that cells multiplying within unfertilized eggs may react to latent virus infections similar to those in tissue culture tubes.

In support of this hypothesis, it has been found at ARC, Beltsville, Maryland, that subcutaneous injections of live Rous sarcoma, Newcastle disease and fowl pox viruses administered to virgin BSW hens can enhance parthenogenesis in their eggs (Olsen, 1956, 1961; Olsen and Poole, 1962; Gill and Stone, 1962, personal communication). Spontaneous parthenogenesis in unfertilized White Leghorn chicken eggs also has been noted at Beltsville, following a natural outbreak of visceral lymphomatosis (Olsen, 1966b). When live Rous sarcoma, Newcastle disease and fowl pox viruses were inactivated with beta-propriolactone and then injected subcutaneously into virgin BSW hens, no enhancement of parthenogenetic development occurred (Olsen, 1962b).

Differences have been noted in the character of parthenogenetic development following the employment of different live viruses. The chief effect of the DNA fowl pox virus appears to be in promoting greater organization of cells so that there is an increase in number of well-defined embryos. The effect of this virus also appears to be associated with twinning. In one test, 15% of all parthenogenetic embryos encountered in eggs of BSW turkeys (unselected for parthenogenesis) had two or more embryos on a single blastoderm (Olsen, 1962a). As many as 12 parthenogenetic embryos have been found on one blastoderm following a booster shot of fowl pox virus administered to virgin hens. Kosin and Sato (1960) also reported finding a significant number of twins (17%).

It is significant that at Pennsylvania State University (PSU) where no live fowl pox virus vaccine is employed, no recognizable parthenogenetic turkey embryos have been encountered in eggs from their own flock (Buss, personal communications). When virgin Pozo Gray turkey hens originating at PSU were transferred to the Agricultural Research Center (ARC) at Beltsville and vaccinated for fowl pox, one parthenogenetic embryo was encountered among the 780 eggs laid by this imported stock. During the following 4 years, a total of 3,402 unfertilized Pozo Gray eggs were incubated at Beltsville. Thirteen parthenogenetic embryos appeared among these eggs. In contrast, "four years of intensive selection for an increased incidence of parthenogenesis at PSU failed to give rise to a single hen with the ability of producing eggs capable of forming true embryos. This was so in spite of a marked increase at PSU in the overall average percentage increase of unorganized parthenogenetic membranes (16 fold) in 5 years" (Olsen and Buss, 1967).

When hatching eggs from mated, vaccinated BSW turkeys from the ARC were transferred to PSU and the poults hatched and reared to maturity in the absence of live fowl pox virus, the virgin hens produced eggs which yielded a high percentage of parthenogenetic embryos.

In contrast to the organizing effect of the live DNA fowl pox virus, employment of the three live RNA viruses, Rous sarcoma, Newcastle disease, and leukosis resulted only in an increased incidence of an unorganized type of tissue growth.

Restoration of Diploidy

The exact mode and place of restoration of diploidy in cells of unfertilized turkey eggs have been a subject of much discussion among investigators. Beatty (1957) gives several possible routes which cells might follow. Briefly, these are: 1. Suppression or re-entry of the first polar body, 2. suppression or re-entry of the second polar body, 3. a nuclear division at or after mitosis I without a corresponding cytoplasmic division, and 4. the fusion of two haploid cells sometime after mitosis I.

Sex inheritance in birds is the reverse from that found in mammals. The germ cells of the turkey male carry the two XX sex chromosomes, while the heterogametic female has the XY pair. Suppression or re-entry of the first polar body should, theoretically, give rise to some female parthenogens. However, all sexually mature parthenogens encountered thus far have been males. Furthermore, inspection of the gonads of 67 fully developed parthenogenetic embryos revealed that all were males (Poole and Olsen, 1957). Sato and Kosin (1960) also established cytologically that cells found in the unorganized membranes of unfertilized turkey eggs carried the two XX sex chromosomes. On the basis of their observations, they concluded that the genetic sex of the membranes growths and of embryos was determined to be male. It seems unlikely, therefore, that the first polar body is involved in the restoration of diploidy.

Suppression or re-entry of the second polar body is another route by which diploidy could be restored. This route to diploidy would produce parthenogens which could show heterozygosity to some degree. Preceded by normal meiotic reduction and crossing over, it would produce the autosomal, diploid equivalent of a fertilized egg containing chromosomes heterozygous within crossover regions for any loci at which the dam was also heterozygous. It should be pointed out that in birds neither the first nor the second polar bodies are actually extruded from the egg. Polar bodies, when encountered, are always found in shallow depressions of the protoplasm, just beneath the vitelline membrane at the upper surface

and near the center of the germinal disc (Olsen, 1942; Olsen and Fraps, 1944, 1950). Their location is, therefore, always in close proximity to the female pronucleus.

The two remaining routes to diploidy, namely: 3. a nuclear division at or following mitosis I without a corresponding cytoplasmic division and 4. the fusion of two haploid mitotic cells, if followed, should give rise to parthenogens completely homozygous at every loci. Data will be presented subsequently indicating that some turkey parthenogens are heterozygous, at least at some segregating loci. Heterozygosity in parthenogens raises serious doubts that restoration of diploidy in unfertilized turkey ova is being accomplished through either routes 3 or 4.

A number of studies, involving a variety of approaches, have been conducted in an attempt to obtain additional information on the genotype of the parthenogen, especially as it relates to the probable time and place of chromosome doubling.

Cytological Approach

In both chicken and turkey ova, onset of the two reduction divisions is preceded by changes that occur in the large germinal vesicle or nucleus of the ovarian egg. These changes are indicated by the disintegration and subsequent disappearance of the germinal vesicle. The nuclear changes begin some 3—5 hours prior to ovulation (Olsen and Fraps, 1950) irrespective of whether or not ova are destined to be fertilized or to develop parthenogenetically. All the preovulatory nuclear changes are initiated by or at least associated with the release of the luteinizing hormone of the fowl's pituitary gland. These changes have been induced experimentally in chickens following appropriate injections of the ovulation-inducing hormone (Olsen and Fraps, 1950). Thus, we see that both physiological and genetic factors are involved in the initiation of the two reduction divisions.

The first polar body is extruded approximately 2 hours before ovulation. The initial stages in the formation of the second polar body also occur before the ovum is released from the ovary, but the process is not completed until after ovulation and, in mated birds, following entrance of the sperm (Olsen and Fraps, 1944).

A number of cytological studies have been conducted to determine ploidy of cells in embryonic tissues from unfertilized turkey eggs. Yao and Olsen (1955), Sato and Kosin (1958, 1960), Sarvella (1970, 1971) made chromosome counts of cells of embryonic membranes from eggs of BSW, Washington State White and Broad Breasted Bronze turkeys. They encountered cells having haploid, diploid and tetraploid numbers of chromosomes. Poole (1959) examined cells of pinfeathers of adult parthenogens and found that they were diploid.

Darcey and Buss (1968) and Darcey *et al.* (1972) determined the ploidy of cells of 16 blastoderms of newly laid BSW turkey eggs at 5—7 hours of incubation. Haploid and diploid cells, in about equal numbers were encountered in these unfertilized eggs.

Sato and Kosin (1958, 1960), Darcey and Buss (1968), and Darcey *et al.* (1972) postulated that the ovum starts mitotic division as a haploid cell. After a few divisions, some, but not all, mitotic cells either fuse or else blocked mitoses occur in certain haploid mitotic cells, thus restoring the diploid number of chromosomes. Diploid mitotic cells in subsequent divisions would then presumably divide normally producing only daughter cells having the diploid number of chromosomes.

Blood Antigen Approach

Comparative studies were conducted on the antigens present in the blood of parthenogens and their dams. No evidence was found for the presence of hemagglutinogens in turkey parthenogens not present in the blood of their dams (Law, 1962, personal communications).

Skin Grafting Approach

Studies involving skin grafting were designed to test known principles of histocompatibility as well as to test for heterozygosity in the parthenogen. Reciprocal skin exchanges were made between 14 BSW turkey hens and their parthenogenetic sons. All 14 grafts, including second sets from parthenogens to their dams, were accepted. All grafts from dams to their parthenogenetic sons were uniformly rejected (Healey *et al.*, 1962). These results were to be expected since each parthenogen received only a sample of the genes of his heterozygous dam. Each dam, however, possessed all the antigens of her parthenogenetic son, and she therefore accepted his skin.

In further tests for heterozygosity in the parthenogen, skin grafts were made between parthenogenetic males and their normal offspring. If the parthenogenetic male is completely homozygous at every loci, then each of his sons and daughters should have received a full complement of his genes, and, therefore, should possess all the antigens of the sire. The offspring, therefore, should not reject their sire's skin. This theory formed the basis for the following tests.

Three BSW parthenogenetic males (P-10573, P-2087 and P-NB) were mated to unrelated BSW hens to produce male and female progeny. Skin exchanges were subsequently made between each parthenogen and his sons and daughters. All six offspring of parthenogen P-10573 given grafts rejected their sire's skin. Of seven offspring of parthenogen P-2087 receiving grafts, six rejected, and one accepted, skin of their sire. Parthenogen P-NB had only one offspring, a female. She accepted the skin of her par-

thenogenetic sire. Thus, among 14 skin exchanges between parthenogens and their progeny by unrelated hens, only two accepted their sire's skin (Poole *et al.*, 1963).

In further tests for heterozygosity, two parthenogens (P-2789 and P-386) were backcrossed to their own dams to produce sons and daughters. Skin exchanges were subsequently made between the parthenogenetic sire and his offspring by his own dam. Skin from P-2789 was found to be compatible with three and incompatible with four of the backcross progeny. The skin of P-386 was found to be compatible with two and incompatible with two of his backcross progeny. On the basis of skin grafting tests, Poole *et al.* (1963) and Poole (1965) concluded "that parthenogens can be heterozygous at at least one of the segregating genetic loci assumed to control histocompatibility in turkeys."

Genetic Color Marker Approach

Employment of a genetic marker for down color showed that normal meiotic segregation of chromosomes occurs. Beltsville Small White hens were mated to homozygous bronze males to produce virgin females that were heterozygous (Cc) for bronze plumage color. Down color of parthenogenetic embryos and poults from these virgin heterozygous bronze females was recorded. Seventeen bronze and 16 white embryos and poults were encountered. This 1:1 ratio of bronze to white indicated that the gene for bronze down color was segregating as would be expected had the same heterozygous hens been mated to BSW (cc) males (Olsen, 1966a). More extensive data are now available on segregation of the bronze gene. Of 620 parthenogenetic embryos and poults, 334 (53.8%) were colored and 286 (47.2%) were white. These supplementary data also approximate a 1:1 ratio, verifying the fact that genes controlling down color are segregating independently and randomly during meiosis in ova that eventually give rise to parthenogens (Olsen and Buss, unpublished data).

From the unfertilized eggs of heterozygous bronze hens, a number of bronze parthenogenetic males were hatched and raised to maturity. Four of these bronze parthenogens were mated to virgin BSW (cc) females and observations recorded on down color of embryos and poults. All poults sired by three of the bronze parthenogens were colored, indicating that each of these sires was homozygous at the C loci. On the other hand, the fourth parthenogen sired 137 poults of which 59 were white and 78 bronze, showing that this particular bronze male was heterozygous at the C locus (Olsen, 1966 and unpublished data).

Both skin grafting and color marker tests for homozygosity showed that some parthenogens were heterozygous at certain loci, indicating that cytological routes 3 and 4 are not the ones being followed by turkey germ cells in the restoration of diploidy. As previously noted, if duplication of chromosomes occurs at or after mitosis I, the individual arising from such germ cells theoretically, at least, should be homozygous at every loci.

Restoration of diploidy at meiosis II also explains why high fertility is attainable when hens, whose unfertilized eggs have exhibited a high incidence of parthenogenesis, are mated. When viable sperm are present in the infundibulum, meiosis II is completed, allowing for normal fusion of haploid male and female pronuclei. In the absence of sperm, chromosomes of the second polar body and those of the female pronucleus probably never separate completely and, subsequently, the unfertilized ovum resumes development as a diploid cell.

Although data presented indicate that the primary route to diploidy followed by turkey ova is through suppression of the second polar body, the possibility is recognized that occasionally an ovum might take an alternate route. The fly, *Drosophila mercatorum*, might be cited as one example. It has been shown with the aid of genetic markers, that 94% of the parthenogenetic flies are completely homozygous and arise due to postmeiotic fusion of two haploid cells. Six percent of the flies, however, are heterozygous and probably arise as a result of fusion of two meiotic products (Carson *et al.*, 1969). Bergerard (1962) also reported that in eggs of Phasmida (Stick Insect) developing parthenogenetically, diploidy was restored following blocked mitoses in haploid mitotic cells.

In the parthenogenetic lizard, *Cnemidophours uniparens*, the somatic number of chromosomes is doubled early in oogenesis, presumably by a premeiotic endoduplication and the 3 N level is restored by two subsequent maturation divisions (Cuellar, 1971).

Potential Uses of Parthenogenetic Development

A special strain of BSW turkeys whose unfertilized eggs show a high incidence of parthenogenesis has been developed at ARC, Beltsville, Maryland. This has been accomplished through family selection, in the presence of live fowl pox virus, for a high incidence of parthenogenetic embryos. The frequency of parthenogenetic development in eggs is now sufficiently high enough to allow use of this stock in biological research. Some potential uses of these birds and their eggs follow:

1. For embryological studies, particularly those dealing with differentiation, gastrulation, primitive streak and embryo formation.

2. As a biological system for demonstrating principles of tissue histocompatibility.

3. For the study of mutations and also as a tool for the identification and elimination of detrimental genes. It is now possible to state, with certainty, whether or not a given abnormality found in parthe-

nogenetic embryos is inherited if pedigreed stock is employed.

4. Parthenogenetic sires are ideal birds for use in the rapid development of isogenetic lines of turkeys. Parthenogens are probably homozygous at most loci, thus making them not only highly inbred individuals but also birds that are essentially free of known embryonic lethal genes. At Beltsville, we have been successful in obtaining viable adult F_1 progeny from a backcross mating of parthenogen 2542 to his dam 1822, also from matings of F_1 and F_2 inbred sons of parthenogen 2542 to their respective F_1 and F_2 inbred sisters (Olsen, 1969).

By using inbred sires (sons and grandsons of a parthenogenetic male, 2542), we have greatly intensified the capacity for the development of parthenogenetic embryos in our special line of BSW turkeys. Data presented illustrate the rapid progress made recently.

During 1952—1964, we incubated 68,879 unfertilized BSW turkey eggs, of which 6,063 (8.7%) gave rise to embryos and 305 to hatched parthenogens (Olsen 1960; 1965b). In contrast, during the 1971 season 8,883 unfertilized eggs were incubated, of which 1,986 (22.4%) gave rise to embryos and 296 to hatched parthenogens. Approximately 65 young parthenogens produced in the winter of 1971 have survived to November 1, 1971. We attribute much of this rapid progress in our selection program to an overall reduction of embryonic lethal genes through inbreeding. Fewer lethals, theoretically, should allow more parthenogenetic embryos to form and develop to the hatching stage.

It is problematical whether or not continued selection of turkeys, based upon their ability to produce eggs giving rise to parthenogenetic embryos, would eventually lead to the development of a variety of turkey capable of reproducing with a high degree of efficiency without benefit of a male. In 1971, about 3.2% of the unfertilized turkey eggs incubated gave rise to hatched parthenogens. Some animals, however, through natural selection have developed species capable of producing unfertilized eggs having much higher hatchabilities. For example, the fly, *Drosophila mangabeirai Malogolowkin*, according to Carson *et al.* (1957) produces unfertilized eggs, about 60% of which hatch. Furthermore, more than 80% of the flies which emerge survive to the adult stage. It would be interesting to know how many generations of natural selection were required for this species of fly to attain this degree of reproductive proficiency and whether or not a similar incidence can be achieved in turkeys.

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