

# Differentiating Between Parthenogenetic and “Positive Development” Embryos in Turkeys by Molecular Sexing

G. CASSAR,\* M. MOHAMMED,\* T. M. JOHN,\* P. GAZDZINSKI,† and R. J. ETCHES\*,1

\*Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada, N1G 2W1, and †Cuddy Farms, Strathroy, Ontario, Canada, N7G 3H6

**ABSTRACT** In mated or inseminated turkeys, 5 to 15% of eggs set for incubation show only rudimentary development. Most of these embryos die during the first 24 to 48 h of incubation and contain only unorganized sheets of tissue. This abnormal development is termed “positive development” (PD). Turkey eggs also show incidence of parthenogenesis and the resulting progeny is believed to be always male. As both types of embryos are morphologically similar at the early stage of incubation, it has been speculated that PD embryos may in fact be parthenogens. By identifying the sex at the blastodermal stage with the help of DNA markers, we have differentiated between the PD embryos and parthenogens.

Parthenogenetic embryos were obtained from eggs laid by uninseminated or virgin Beltsville Small White (BSW) hens, and the PD embryos were obtained from

eggs of inseminated Nicholas and British United Turkeys of America (BUTA) hens. DNA was extracted from blastoderms of parthenogenetic and PD embryos. Turkey W-chromosome specific DNA probe and primers were used to detect females in all samples by Southern blot and polymerase chain reaction (PCR), respectively.

No female was detected among the 35 parthenogens examined, whereas there were 3 females among the 11 PD embryos. The presence of both males and females among PD embryos suggests that they are products of fertilization, and that at least these 3 female embryos, if not all the 11 PD embryos, are not of parthenogenetic origin. It is concluded, therefore, that PD embryos result from errors in fertilization or from early embryonic mortality following successful fertilization, and that they are unlikely to be of parthenogenetic origin.

(Key words: parthenogenesis, turkey, positive development, W-chromosome, embryonic mortality)

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## INTRODUCTION

In commercial turkey hatcheries when eggs from mated or inseminated hens are incubated, 5 to 15% of the embryos exhibit rudimentary development and often die within the first 24 to 48 h of incubation. Typically, the development in these embryos is unorganized and the embryonic tissue is a sheet of epithelial cells overlying the yolk. The presence of cells is taken as positive evidence of development and therefore, these embryos are commonly referred to by poultry breeders as positive development (PD) embryos, or as retarded or moribund embryos. Kosin (1951) has described the gross and microscopic appearance of such moribund turkey blastoderms.

Parthenogenesis is another phenomenon observed relatively frequently in turkeys. Normally, parthenogenetic development is initiated in 1 to 30% of unfertilized eggs (Olsen and Buss, 1967; Olsen and Marsden, 1953, 1954a; Schom *et al.*, 1982; Savage and

Harper, 1986; Cassar *et al.*, 1998), although development is completed only in a small percentage of parthenogens (Olsen, 1975). In Beltsville Small White (BSW) turkeys, selective breeding increased the incidence of parthenogenesis from 16.7% to over 40% (Olsen, 1975). In most cases, the embryo, which dies within 24 h of incubation, is an unorganized sheet of epithelial cells. The morphology of parthenogenetic embryos during different developmental stages has been described by Savage and Zakrzewska (1997). Parthenogenetic embryos begin their development from haploid ova (Olsen, 1975; Harada and Buss, 1981), and they are thought to be able to survive to hatching only if a diploid lineage is established and becomes predominant. Hatched parthenogenetic poulters hitherto reported have invariably been males, and more than 87% of their cells have been found to be diploid (Cassar *et al.*, 1998).

Based on morphology and histology, parthenogenetic embryos and PD embryos are indistinguishable. Further-

<sup>1</sup>To whom correspondence should be addressed: retches@aps.uoguelph.ca

**Abbreviation Key:** BSW = Beltsville Small White; BUTA = British United Turkeys of America; PCR = polymerase chain reaction; PD = “positive development”.

more, in commercial flocks, they normally occur at approximately the same frequency. Because of the apparent similarities between the two phenomena, there have been speculations as to whether these two are alternate classifications of the same phenomenon. The ability to diagnose whether or not the embryonic mortality and poor hatchability are due to parthenogenesis or to PD would be an asset for improving reproductive management. Because existing information suggests that parthenogens are always males (Poole and Olsen, 1957), a sex-based identification was considered to be a useful diagnostic tool for differentiating between the parthenogens and the PD embryos.

## MATERIALS AND METHODS

All eggs used for procuring parthenogenetic embryos were derived from noninseminated or virgin hens of the BSW line, which has an established record for high incidence of parthenogenesis (Olsen, 1975). These birds originated from a flock kindly provided by Hans Abplanalp (University of California, Davis, CA 95616). Eggs were kept overnight at 15 C and were set for incubation the following morning at 37 C. Embryos exhibiting signs of development were collected after candling on Day 5 of incubation. Of 76 infertile eggs collected, 35 parthenogenetic embryos were obtained. Pooled samples of two parthenogenetic embryos each (except for one sample that contained three), were prepared for DNA extraction. Pooling of samples was considered to be less laborious, particularly in view of the fact that the primary objective was to examine whether or not W-chromosome is present in any of the parthenogenetically produced embryos.

Eggs for PD embryos were collected from inseminated turkeys from the Nicholas and British United Turkeys of America (BUTA) lines raised at Cuddy Farms (Strathroy, ON, Canada, N7G 3H6). Eleven PD embryos from presumably fertilized eggs were collected after 5 d of incubation and prepared individually for DNA extraction. Blood samples from male and female BSW turkeys were used as standards for sex identification. Embryos were stored in calcium-magnesium-free phosphate-buffered saline at -70 C until further use.

### Isolation of Genomic DNA

The whole embryonic tissue from parthenogenetic and PD embryos were used for genomic DNA extraction. DNA from the embryonic tissue samples and the adult turkey blood was extracted according to the protocol of Gross-Bellard *et al.* (1972). Briefly, tissues or erythrocytes were homogenized with the help of a Wheaton

homogenizer<sup>2</sup> in a buffer solution containing 0.1 M NaCl, 0.1 M Tris, 0.2 M sucrose, 0.05 M EDTA, and 0.5% SDS. After overnight incubation at 37 C with proteinase K, two phenol chloroform extractions were done. These extractions were followed by incubation with 3.0 M sodium acetate and cold isopropanol at -20 C for 3 h. After centrifugation, the pellets were washed with 70% cold ethanol and then dissolved in Tris-EDTA buffer.

### Preparation of W-Chromosome Specific Probe

DH5 $\alpha$  competent *Escherichia coli* cells were transformed with pUC119 plasmid vector carrying a 0.4 kb W-chromosome specific (Saitoh *et al.*, 1989) insert (kindly provided by S. Mizuno, Department of Agricultural Chemistry, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981, Japan) at the *Pst*I restriction site (pUMG0401). After growth of the cells, an alkaline lysis preparation (Birnboim, 1983) was carried out to isolate the plasmid DNA, which was digested with *Pst*I and run on a 0.9% agarose gel to recover the 0.4-kb insert. The DNA insert was extracted from the gel by removing the agarose that contained the band, and subsequently purifying with the help of an Agarose Gel DNA Extraction Kit.<sup>3</sup> The probe was then labeled using the DIG System<sup>3</sup> via random-primed labeling. The W-chromosome probe was stored at -20 C in Tris-EDTA buffer until further use.

### Southern Blot Analysis

The genomic DNA of the parthenogenetic embryos and standards as well as the plasmid DNA from pUMG0401 were analyzed by Southern blotting to determine the sex of the embryos. Because the primary objective in carrying out Southern blotting was to determine whether any female existed among the parthenogenetic embryos, no PD embryos were included. The DNA was digested at 37 C with *Pst*I restriction endonuclease and then separated on 0.8% agarose gel. The gel was washed twice in 0.5 M NaOH + 1.5 M NaCl, and twice in 0.5 M Tris-HCl + 3 M NaCl at room temperature for 20 min each. The DNA was transferred overnight to nylon membrane<sup>3</sup> by capillary flow in 10 $\times$  SSC (from a 20 $\times$  SSC stock made up of 3 M NaCl and 0.3M Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>·2H<sub>2</sub>O, with pH adjusted to 7 using 1 M HCl). DNA fixation by baking the membrane at 100 C for 45 min in a vacuum oven was followed by prehybridization in 5 $\times$  SSC + 0.1% sarcosyl + 0.02% SDS + 1.0% blocking reagent at 65 C for 1 h. Hybridization was performed in prehybridization solution containing 0.6 ng DIG-labeled probe overnight at 65 C, after which the membrane was washed four times for 5 min each in 2 $\times$  SSC + 0.1% SDS. After binding, the probe with anti-DIG-alkaline phosphatase, bands were detected with lumigen PPD, and visualized on Kodak X-Omat film (Cat. No. 165 1454).

<sup>2</sup>Wheaton, Millville, NJ 08332.

<sup>3</sup>Boehringer Mannheim Canada, Laval, PQ, Canada, H7V 4A2.



**FIGURE 1.** Southern blot analysis of parthenogenetic embryos to detect the sex. Lane 1:  $\lambda$ DNA/*Hind*III standard; Lane 3: DNA from blood of female turkey; Lane 6: pUMG0401; Lanes 9 to 23: parthenogenetic embryos; Lane 24: DNA from blood of male turkey. Lanes 2, 4, 5, 7, and 8 are blank. Bands in Lanes 5, 7, and 8 are "over-flow" from Lane 6. Note the absence of bands in Lanes 9 to 23 (parthenogenetic embryos) and in Lane 24 (male genomic DNA).

### ***Amplification of Genomic DNA***

A PCR was employed to increase the sensitivity of detection of the W-chromosome sequence in the DNA derived from the embryos. In addition to parthenogenetic embryos, PD embryos were also included in the PCR analysis in order to determine whether or not the PD embryos were different from the parthenogens with respect to sex. The DNA samples were amplified according to the protocol accompanying Taq DNA polymerase<sup>4</sup> using a PTC-100 thermal cycler.<sup>5</sup> The amplification was carried out by denaturation at 94 C for 3 min, followed by 35 cycles of melting at 94 C for 45 s, annealing at 55 C for 30 s, and extension at 72 C for 1.5 min. The amplified product was incubated at 72 C for 10 min before storing at 4 C until further use. The final reaction volume of 100  $\mu$ L contained 1 $\times$  PCR buffer,<sup>4</sup> 0.2 mM of each dNTP, 1.5 mM MgCl<sub>2</sub>, 0.5 mM of each primer, and 2.5 units of Taq DNA polymerase. Each sample was run with turkey W-chromosome specific primers<sup>4</sup> (sequences: 5'-TCAACCAGAAATAGGACGT-3' and 5'-CATATTTATTCGATAAAACT-3') for sex detection.  $\beta$ -Actin primers<sup>4</sup> (sequence: 5'-GTTTGAGACCTTCAACACC-3' and 5'-CCAAGAAAGATGGCTGGAAG-3') were used as

positive controls for the presence of genomic DNA. The PCR products were analyzed by agarose gel electrophoresis on 1.0% agarose gel containing 0.5  $\mu$ g/mL ethidium bromide final concentration. Gels were then visualized and photographed under exposure to ultraviolet light.

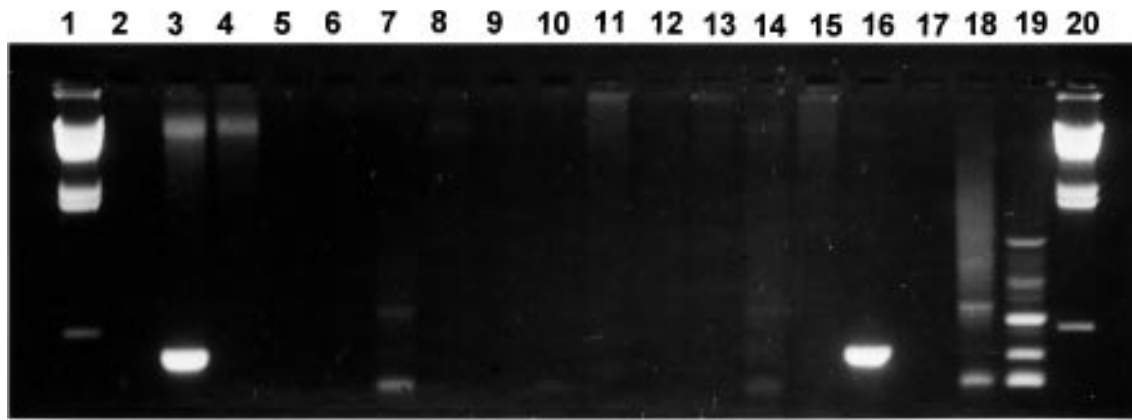
### **RESULTS**

Southern blot analysis revealed no females among parthenogenetically developing embryos (Figure 1). The female genomic control and pUMG0401 plasmid DNA in (Figure 1, Lanes 3 and 6, respectively) showed multiple bands signifying the repetitive nature of the turkey W-chromosome sequence. Bands seen on Lanes 5, 7, and 8 are the result of an "overflow" of excessive concentration of pUMG0401 plasmid DNA loaded to the well in Lane 6. Male genomic DNA (Figure 1, Lane 24) showed no such bands due to the absence of W-chromosome in males. No bands representing W-chromosome were observed in Lanes containing parthenogenetic embryo DNA (Figure 1, Lanes 9 to 23) indicating that these embryos were not females.

Sex identification of PD and parthenogenetic embryos using PCR (Figures 2 and 3) was done to increase the sensitivity of detection of the W-chromosome. Primers designed to amplify  $\beta$ -actin sequences were used as positive controls for the presence of genomic DNA.  $\beta$ -Actin amplification was observed in genomic DNA

<sup>4</sup>Gibco-BRL, Burlington, ON, Canada, L7P 1A1.

<sup>5</sup>MJ Research Inc., Watertown, MA 02172.



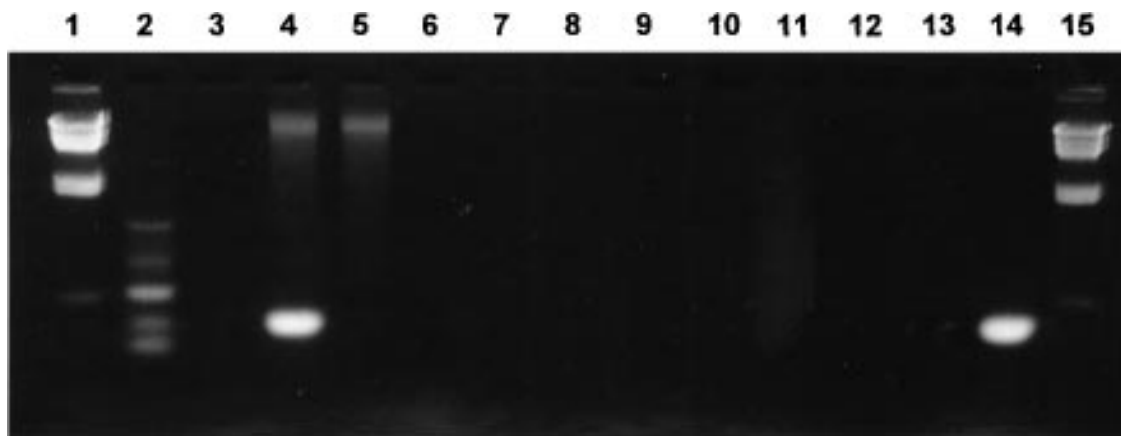
**FIGURE 2.** Ethidium bromide stained 1% agarose gel of polymerase chain reaction (PCR)-amplified positive development (PD) embryo DNA for sex detection. Lanes 1 and 20:  $\lambda$ DNA/*Hind*III standard; Lane 2: no template; Lane 3: DNA from blood of male turkey with  $\beta$ -actin primers; Lane 4: DNA from blood of male turkey; Lanes 5 to 15: PD embryos; Lane 16: PD embryo with  $\beta$ -actin primers; Lane 17: blank; Lane 18: DNA from blood of female turkey; Lane 19: pUMG0401. Note the presence of bands in Lanes 7, 10, and 14, which correspond to the W-chromosome bands in Lanes 18 and 19.

prepared from a male blood sample (Figure 2, Lane 3; Figure 3, Lane 4) but amplification of W-chromosome sequence by pUM primers was not observed (Figure 2, Lane 4; Figure 3, Lane 5). No W-chromosome sequence amplification by pUM primers was observed in any of the parthenogenetic embryos (Figure 3, Lanes 6 to 13), whereas three cases in which the W-chromosome sequence was amplified by pUM primers were observed among the 11 PD embryos (Figure 2, Lane 5 to 15). Amplification of the plasmid DNA containing the W-chromosome sequence by pUM primers indicates the repetitive nature of the W-chromosome sequence as observed by the multiple bands of amplification.

## DISCUSSION

The origin of positive development embryos has been a contentious issue. Kosin (1951, 1958) postulated that

underdeveloped or retarded embryos that represent cases of early death or of moribund blastoderms are the products of essentially normal fertilization, whereas some investigators (Olsen and Marsden, 1954b; Olsen, 1962) have suggested that they could be of parthenogenetic origin. The resolution of this difference of opinion depended upon finding recognizable differences, if any, between the two types of development. Olsen and Marsden (1954b) provided circumstantial evidence to support the hypothesis that parthenogenetic development occurs among apparently nonfertilized eggs from mated turkeys. In their study, eggs from mated turkeys were incubated for 24 to 72 h and then separated by candling into fertilized and unfertilized groups. Further incubation resulted in 18% of these "unfertilized eggs" manifesting development of embryonic tissue. Because this development occurred apparently in "unfertilized" eggs, it was considered to



**FIGURE 3.** Ethidium bromide stained 1% agarose gel of polymerase chain reaction (PCR)-amplified parthenogenetic embryo DNA for sex detection. Lanes 1 and 15:  $\lambda$ DNA/*Hind*III standard; Lane 2: pUMG0401; Lane 3: no template; Lane 4: DNA from blood of male turkey with  $\beta$ -actin primers; Lane 5: DNA from blood of male turkey; Lanes 6 to 13: parthenogenetic embryos; Lane 14: parthenogenetic embryo with  $\beta$ -actin primers. Note that no bands corresponding to the W-chromosome bands in Lane 2 are present in Lanes 6 to 13.

be parthenogenetic. In another experiment (Olsen, 1962), a genetic color marker was used as a tool to identify parthenogenesis in eggs of mated turkeys. In this experiment, recessive white female BSW turkeys were inseminated with semen from either Dark Cornish chicken or from New Jersey Buff turkey males, whose plumage color was dominant to the recessive white of BSW turkeys. The resulting progeny produced some advanced embryos/poults with the recessive white down color, suggesting that they had developed from unfertilized eggs. The disadvantage of a genetic color-marker method, however, is that it could establish the parentage only in those embryos that are old enough to show down color. Kosin's (1958) experiments, on the other hand, demonstrated that the frequency of retarded or moribund embryos could be altered by preincubation storage time, which would not have influenced the rate of parthenogenesis. Based on this difference in the developmental response to storage, Kosin concluded that parthenogenesis is less likely to be a factor in the occurrence of retarded early embryos.

In the present study, the sex of the embryos served as a marker for distinguishing between parthenogenetic and PD embryos, because it has been previously established that all parthenogens are males (Poole and Olsen, 1957). As there is no sexual dimorphism in growth pattern in early embryonic stages in these birds, most conventional sex identification methods based on morphological differences (see review, Basrur *et al.*, 1998) are of no use in situations such as the present one, in which sexing has to be done in embryos at very early developmental stages. In view of this, the diagnosis of sex, based on chromosomal make-up, was considered to be the best alternative. In birds, female is heterogametic sex, with a heteromorphic pair of chromosomes referred to as Z and W, and the male is homogametic with a ZZ chromosome pair. Female birds can, therefore, be identified by the presence of a W-chromosome. Although the two techniques employed in the present investigation, i.e., Southern blot and PCR (using turkey W-chromosome specific DNA probe and primers, respectively), are both reliable in comparison to conventional morphological methods, it may be cautioned that in Southern blot, an insufficient amount of DNA to probe may result in low limit of detection.

Assuming that parthenogenetically developing embryos are always males, the presence of W-chromosome can preclude a parthenogenetic origin, but in the absence of it, the differentiation between the PD embryos and parthenogenetic embryos is inconclusive. In the present investigation, the lack of evidence for a W-chromosome in the parthenogenetically developed group of embryos confirms that those embryos were males. The presence of 3 females among the 11 PD embryos indicate that PD embryos developed from fertilized eggs, and that at least these 3 female embryos, if not all of the PD embryos included in this study, are products of normal fertilization and not of parthenogenetic origin.

The results of the present investigation corroborate the suggestion that parthenogenesis in turkey occurs through spontaneous activation of haploid eggs (Olsen, 1975; Cassar *et al.*, 1998). The absence of W-bearing cells in parthenogens indicates that only Z-bearing ootid survive repeated mitotic divisions following the activation of a haploid female pronucleus. Although the present study does not totally rule out the possibility of parthenogenesis in mated turkeys, it does provide evidence to prove that the PD embryos are not universally of parthenogenetic origin. Our observation that at least some of the early embryonic mortality in eggs from mated turkeys is not due to parthenogenesis suggests that errors in fertilization or postfertilization cleavages may be the cause for some of the failures in embryo survival, and that further improvement in fertilization techniques could alleviate at least some of the losses through embryo mortality.

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