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Sperm-egg interaction assay for determination of fertile period in ostrich (*Struthio camelus*)

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ABSTRACT

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Sperm-egg interaction assay for assessment of fertile period in ostrich was carried out. A total of 24 hatching eggs collected from four female ostriches aged six years were used for this study. Significantly (P<0.01) higher number of Inner Peri-vitelline Layer (IPVL) sperm holes (18.56/mm^2) was observed in the 1st egg, which was laid on 2nd day after last mating. Subsequently, the number of IPVL-sperm holes on eggs laid on 3rd, 4th and 5th day after last mating gradually decreased and the values were 16.66/mm^2, 13.03/mm^2 and 10.27/mm^2 respectively. Initial number of IPVL-sperm holes after last mating and rate of sperm holes decline was demonstrated using a simple regression and the relationship was expressed by the linear equation as Y= -2.85 × + 24.60 (R^2=0.987). From the equation it was concluded that the number of IPVL-sperm holes declined at the rate of 2.76/mm^2 per day and expected to have 1.97 IPVL-sperm holes/mm^2 on 8th day in the germinal disc (GD) after last mating. Similarly, Outer Perivitelline Layer (OPVL) spermatozoa number observed in the 1st egg, which was laid on 3rd day after last mating was 51.66 /mm^2. Subsequently, the numbers of OPVL-sperm of eggs laid on 4th, 5th, 6th and 7th day were 45.55, 30.50, 23.00 and 21.75 /mm^2, respectively. Initial number of OPVL-sperm and rate of sperm decline pattern was demonstrated using a simple regression and the relationship was expressed as Y= -8.237 × + 75.67 (R^2=0.932). From the equation it was construed that the OPVL-sperm numbers declined at the rate of 7.99 /mm^2 per day and expected to have 2.31 OPVL-sperm per mm^2 on 9th day in the GD after last mating demonstrated that the fertile period is 9 days in ostrich hens.

Keywords: Inner and outer perivitelline layer assay, Fertile period, Ostrich.

INTRODUCTION

The fertility of eggs on commercial ostrich farms varies from 40 to 90 per cent (Schalkwyk *et al.,* 2000). Fertility is determined by candling of eggs on days 7, 14 or 21 of incubation, or at hatch. Eggs that do not show embryonic development are considered as unfertilized egg and are confirmed by the absence of embryo during the break open study. However, early reports for eggs from domestic chickens and turkeys suggested that the blastoderm of some eggs can die before or after the egg has been laid (Kosin, 1951). Furthermore, the appearance of the germinal disc (GD) may be altered as a result of storage and incubation conditions (Badley, 1997). Hence, the true status of the GD can be difficult to determine when the egg is candled or broken open.

Fertility diagnosis using 'perivitelline techniques' is based on fundamental aspects of the biology of egg fertilization in birds. For fertilization to take place, the spermatozoa must penetrate the inner perivitelline layer (IPVL), a membrane that surrounds the newly released egg. They hydrolyse the inner layer above the germinal disc with enzymes from their acrosomes, thus producing microscopic holes to swim through. These holes are direct evidence for successful penetration and fertilisation (Bramwell *et al*., 1995; Bakst *et al*., 2014). Furthermore, the unsuccessful spermatozoa in the germinal disc are covered with the outer perivitelline layer (OPVL) and inactivated. Examination of inner and outer perivitelline membranes is a useful technique to diagnose the range of female and male fertility problems (Wishart, 1988; Malecki and Martin, 2005). Hence, this study was proposed to study the sperm-egg interaction assay for assessment of fertile period in ostrich

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MATERIALS AND METHODS

Principle of sperm-egg interaction assay

Fertility diagnosis using pertvitelline techniques namely sperm holes in the inner perivitelline layer (IVPL) and sperm number in the outer perivitelline layer (OPVL) are good indicators of the probability of fertilization. (Bramwell *et al*., 1995; Bakst *et al*., 2014). By examing inner and outer perivitelline membranes, one should be able to diagnose a range of female and male fertility problems (Wishart, 1988; Malecki and Martin, 2005).

Identification of germinal disc (GD)

A total of 24 fertilized eggs collected from four female ostriches aged six years were used for this study. The egg was broken open, the germinal disc (GD) was located with the unaided eye and the albumen overlaying the yolk was removed to expose the intact yolk and the GD region. The fertilized GD (blastoderm) (Fig 1a) generally consisted of two areas, a clear concentric one in the centre, assumed to be the area pellucida, surrounded by an opaque ring that was assumed to be the area opaque (Bakst *et al.,* 1998). The unfertilized GD (blastodisc) consisted of a group of white yolk droplets

surrounded by a ring, within numerous vacuoles were seen (Fig 1b).

Isolation and processing of yolk membrane

The germinal disc (GD) was chosen because spermatozoa usually prefer this region over the rest of the ovum (Bramwell and Howarth, 1992; Birkhead *et al.*, 1994) and this was confirmed for ostrich also (Malecki and Martin 2003b). The thick egg white adhering to the yolk membrane was carefully removed using paper tissues. The yolk surface was placed on top of the yolk membrane with the GD area positioned in the centre. Then the filter paper ring adhered to the membrane and the membrane was cut along the outer ring, taken off the yolk surface and the remaining yolk was washed away with 1 per cent phosphate buffered saline (PBS) and fixed with 1 per cent neutral buffered formalin (NBF). The ring containing the membrane was placed on the glass slide for further processing.

Visualizing spermatozoa holes in the inner perivitelline layer (IPVL)

The membrane was collected and cleaned as described above and left overnight to dry on a glass slide placed on the lab bench at room temperature. After the filter ring had dried out, it was removed while sealed onto a glass slide, rehydrated with PBS, stained with Schiff's reagent (Bramwell *et al*., 1995) and allowed to dry again overnight. Once dried, the holes made by spermatozoa were viewed under normal light microscopy and the counts were expressed as spermatozoa holes per mm^2 (Fig 2a).

Visualizing spermatozoa nuclei in the outer perivitelline layer (OPVL)

A piece of vitelline membrane within the inner circle of the filter ring was stained with 1µg/ml of 4', 6'-diamidino-2-phenylindole (DPAI; Sigma, USA) in PBS (Wishart, 1987). The spermatozoa nuclei were then visualized with a fluorescence microscope using U filter cube with 372 nm excitation and 456 nm emission wavelengths. (Fig 2b).

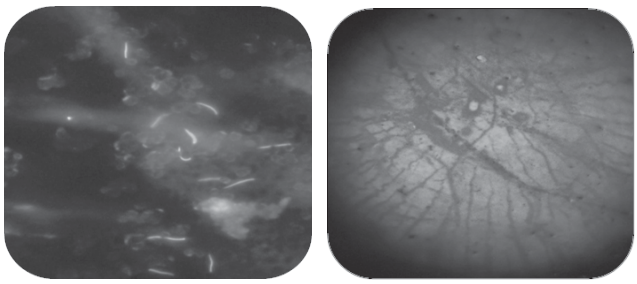

Fig. 2: a. Sperm-egg interaction assay: IPVL-sperm holes (Schiff's staining) 10x
b. Sperm-egg interaction assay: OPVL-sperm nuclei (DAPI staining) 10x

Quantifying spermatozoa nuclei in the outer perivitelline layer (OPVL)

The germinal disc (GD), a less opaque part of the vitelline membrane, need to be identified under the microscope and its centre located. The spermatozoa numbers were estimated by counting spermatozoa nuclei visible in the field of the 40× objective (an area of 0.24 mm^2) over six consecutive fields starting from the centre of the GD (Malecki and Martin, 2005) and the spermatozoa count were expressed in mm^2.

Statistical analysis

The reduction in number of spermatozoa holes in the IPVL and spermatozoa numbers in the OPVL over days was analysed using simple linear regression.

RESULTS AND DISCUSSION

Inner perivitelline layer (IPVL) spermatozoa holes

Exponential decline pattern of IPVL-sperm holes in female ostrich after mating is presented in Fig. 3. Significantly ($P<0.01$) higher number of IPVL-sperm holes (18.56 ± 4.06 /mm^2) was observed in the 1st egg, which was laid on 2nd day after last mating, this was lower than the value reported by Bramwell *et al.* (1995) in chicken (121/mm^2) and higher than that reported by Venkatesh (2005) in turkey (34.33/egg) and Hudson (2015) in guinea fowl (52.25/egg) during artificial insemination at the dose of 100 million spermatozoa. Moderately higher values were obtained by Malecki and

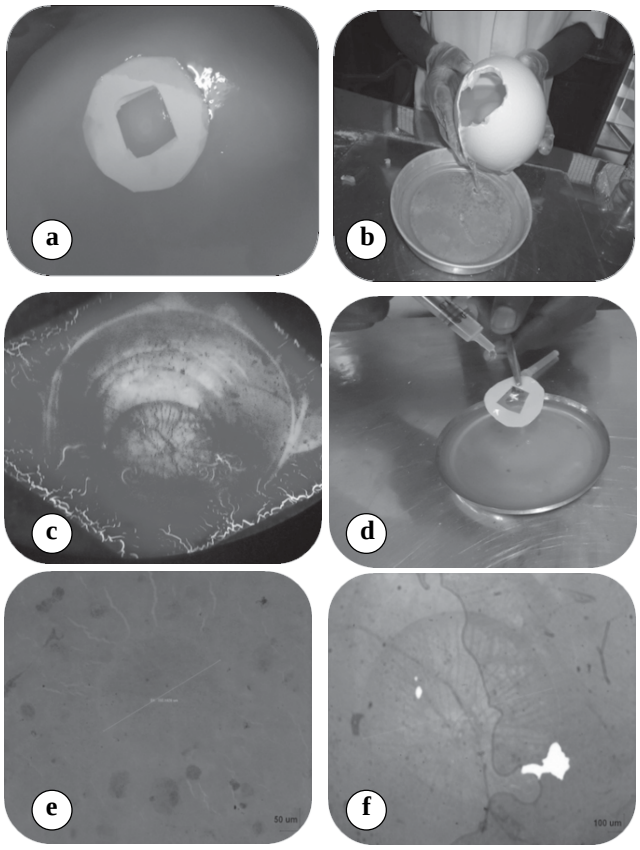

Fig. 1: Stepwise procedure for sperm-egg interaction assay
(a) Breaking open of egg ans separation of yolk from albumen
(b) Fixing the GD with filter paper
(c) Washing GD with PBS and fixing with 1 % NBF
(d) GD-stainined with Schiff's reagent
(e) Blastoderm - fertile egg
(f) Blastodisc - infertile egg

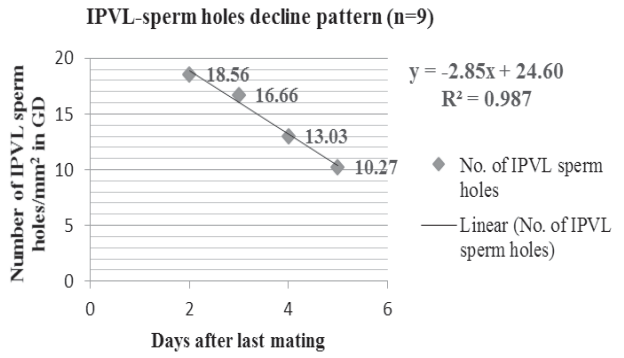

Fig. 3: Sperm-egg interaction assay: IPVL-sperm holes decline pattern

Fraser (2010) in ducks (20/mm^2) during natural mating. The number of IPVL-sperm holes observed in this study can be supported by Wishart (1997) who have noticed that eggs from chicken and turkeys had 50 per cent probability of being fertile when around three spermatozoa penetrated in the GD and maximum fertility when more than six spermatozoa penetrated this region. Comparatively higher number of IPVL sperm holes observed in the study might be due to number of spermatozoa ejaculated during mating and species difference or method of visualization. Subsequently, the number of IPVL-sperm holes on eggs laid on 3rd, 4th and 5th days after last mating was 16.66±6.33/mm^2, 13.03 ± 5.00 /mm^2 and 10.27 ± 6.23 /mm^2, respectively. The decreasing trend of IPVL-sperm holes in the eggs laid on 3rd, 4th and 5th day after last mating were comparable with that of Hudson (2015) in guinea fowl. The reduction trend may be linked to the tonicity and threshold of sperm storage tubules in regulating the number of spermatozoa to reach the area of fertilization.

Initial number of IPVL-sperm holes after last mating and decline in the rate of sperm holes pattern were demonstrated using a simple regression and the relationship has been expressed by the following linear equation:

Y= -2.85 x + 24.60 (R^2=0.987, n=9)

Where 'Y' is initial number of IPVL-sperm holes/ mm^2 after last mating and 'x' is the days after last mating.

From the equation it has been estimated that, the number of IPVL-sperm holes declined attherateof2.76/ mm^{2}perday (anticipated daily loss of 12.99 per cent) and expected to have 1.97 IPVL-sperm holes /mm^2 on 8th day in the GD after last mating. This concurs with the finding of Swan and Sicouri (1999) who have reported that spermatozoa storage occurs in ostrich hens and fertile eggs can be laid for at least five to eight days after copulation. Similar findings on linear equation was also obtained by Wishart and Staines (1999) in broiler breeder ((58+15[(log (GD IPVL holes)]) and Hudson (2015) in guinea fowl ((16.69+43.26[log (GD-IPVL holes)]) to know the fertility.

Outer perivitelline layer (OPVL) spermatozoa nuclei

Exponential decline pattern of OPVL-sperm in female ostrich after last mating, and is presented in Fig. 4. Significantly (P<0.01) higher OPVL-sperm number was observed in the 1st egg, which was laid on 3rd day after last mating, when the spermatozoa count was 51.66 ± 4.06/mm^2 this was lower than the results obtained by Malecki and Martin (2003a) and Malecki and Martin (2005) in ostrich (39 to 405/mm^2). The variation observed in this study may be due to seasonality of breeding, age, pen and the female: male ratio in the mating system. Subsequently, the number of OPVL-sperm of eggs laid on 4th, 5th, 6th and 7th days were observed to be 45.55 ± 3.00 /mm^2, 30.50 ± 0.50 /mm^2, 23.00 ± 1.00 / mm^2 and 21.75 ± 3.37 /mm^2 respectively, which was comparable with the findings of Malecki *et al.* (2004) in ostrich (118/mm^2 to 14/mm^2).

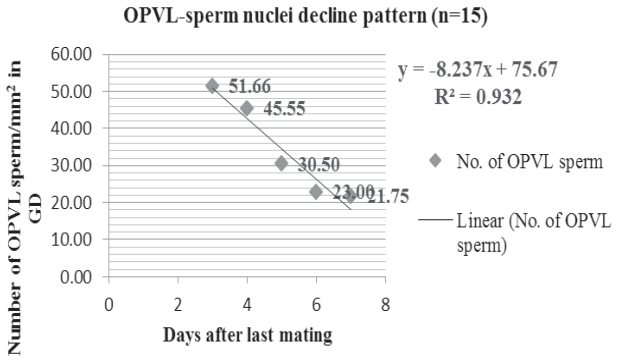

Fig. 4: Sperm-egg interaction assay: OPVL-sperm nuclei decline pattern

Initial number of OPVL-sperm and decline in the rate of spermatozoa were demonstrated using a simple regression and the relationship is expressed by the following linear equation:

Y= -8.237 x + 75.67 (R^2=0.932, n=15)

Where 'Y' is initial number of OPVL-sperm and 'x' is the days after last mating.

From the equation it is estimated that, the OPVL-sperm declined at the rate of 7.99 /mm^2 per day (anticipated daily loss of 11 per cent) and expected to have 2.31 OPVL-sperm /mm^2 on 9th day in the GD after last mating. However, a daily sperm loss of 27 per cent in emu and 17 per cent in ostrich were observed by Malecki and Martin (2002). Similar to this study, Malecki and Martin (2005) also observed a variation in the rate of sperm decline (daily loss of 15-37%) in ostrich with expected to have OPVL-sperm duration of 7.9 to 20.3 days.

From the above study it is suggested that, egg break out and number of spermatozoa trapped in perivitelline membrane overlying the GD provided a robust assessment of fertile period in ostrich. In this sperm egg interaction assay observation of 1.97 IPVL-sperm holes/mm^2 on 8th days and 2.31 OPVL-sperm/mm^2 on 9th days after last

mating demonstrated that the fertile period is 9 days in ostrich hens. Further, the sperm-egg interaction assay can be used to diagnose reproductive wastage and to identify the high fertility individual. This facility could increase the efficiency of breeding flocks either by selecting superior males or by optimizing sex ratios for the mating.

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