BIOLOGY OF REPRODUCTION 44, 102–107 (1991)

Fertilizing Capacity of Bovine Sperm May Be Maintained by Binding to Oviductal Epithelial Cells[1]

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ABSTRACT

The ability of the bovine oviduct to maintain the motility and fertilizing capacity of bovine sperm was investigated by incubating frozen-thawed sperm with endosalpingeal epithelial cells cultured on either tissue culture plastic (nonpolarizing) or Matrigel-coated Millicell (polarizing) substrata. Sperm were also incubated in medium alone or with cultured bovine tracheal epithelial cells. Motility was determined at 6-h intervals over a 48-h period. The fertilizing capacity of sperm was evaluated after 0, 24, and 30 h of incubation by adding oocytes to the culture and determining the incidences of fertilization and polyspermy. Motility was maintained for 48 h in sperm that bound to endosalpingeal epithelial cells, but to a greater extent with polarized cells (38.4% motile) than with nonpolarized cells (0.8%). Fertilizing capacity was maintained for 30 h in sperm incubated with endosalpingeal epithelial cells on Matrigel/Millicell, but not in sperm incubated in medium alone or with tracheal cells. Only sperm incubated with oviductal cells developed hyperactivated motility. Scanning electron micrographs revealed that sperm were bound by the rostral portion of the intact acrosome to the apical surface of polarized endosalpingeal cells. These results suggest that the oviduct may not only store sperm but may also maintain sperm viability and fertilizing capacity during the preovulatory period.

INTRODUCTION

In most mammals, natural insemination of the female occurs only during estrus. The interval from the onset of estrus to ovulation may be several hours or even a few days [1] and sperm must retain their fertilizing capacity for this period. In contrast, the fertile lifespan of oocytes is generally less than half that of sperm [1]. Thus, there must be mechanisms to extend the fertile lifespan of sperm in the female reproductive tract. There is evidence that the caudal oviductal isthmus functions as a sperm storage reservoir and that sperm may be held in the reservoir by binding to the mucosal epithelium [2–7]. In cattle, approximately 8 h may be required after insemination for sufficient sperm to accumulate in the isthmic reservoir in order to ensure a high fertilization rate [8]. Since the period from onset of estrus to completion of ovulation may be as long as 30 h in cattle, sperm destined to fertilize may spend up to 22 h in the isthmus [5]. Therefore, some of the mechanisms of maintaining sperm fertilizing capacity may exist in the isthmus.

Accepted September 3, 1990.
Received March 2, 1990.
[1]Research supported by the Natural Sciences and Engineering Research Council of Canada, Agriculture Canada, Semex Canada, Florida Dairy Checkoff Fund, and the National Science Foundation of the United States of America. A preliminary report of the data was presented as an abstract titled "Sperm fertilizing capacity is maintained by binding to oviductal epithelial cells" at the Serono Symposium on Fertilization in Mammals, August 1–5, 1989, Boston, MA.
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Two recent technical advances have made it possible to examine in vitro the mechanisms involved in sperm storage in the bovine oviduct. The first of these is the ability to maintain organ-specific function in cells cultured in vitro [9], including endosalpingeal epithelial cells [10]. The maintenance of a polarized and differentiated cell population entails establishing primary cultures on components of basal laminae that have been layered onto elevated permeable membranes [11, 12]. The second advance has been the development of highly successful and repeatable in vitro maturation and fertilization techniques for bovine oocytes [13]. In this study, these techniques were used to create an in vitro system that enabled us to investigate whether the motility and fertilizing capacity of bovine sperm could be prolonged by oviductal epithelium.

MATERIALS AND METHODS

Chemicals and media were obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

Experiment 1: Maintenance of Sperm Motility

Oviducts were obtained at a local abattoir from Holstein cows that were judged to be in the preovulatory phase of a normal estrous cycle, on the basis of the presence of both a regressing corpus luteum and a dominant follicle exceeding 10 mm in diameter. The oviducts were transported at 35°C to the laboratory. There, endosalpingeal epithelium

from the oviduct ipsilateral to the preovulatory follicle was extruded as a tube by squeezing along the outside of the oviduct with watchmaker's forceps, from the uterotubal junction to the infundibulum. The extruded epithelial tube, presumably containing cells from both the isthmus and the ampulla, was disaggregated by passing it through a 30-gauge needle. The disaggregated cells were washed by 3 consecutive suspensions and centrifugations (300 × *g* for 5 min) in sterile Hanks' Balanced Salt solution supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). All epithelial cells recovered from a single oviduct were then resuspended in 5 ml of tissue culture Medium 199 with Earle's salts supplemented with heat-inactivated estrous cow serum (10% v/v), penicillin (100 U/ml), and streptomycin (100 μg/ml) (M199+). Aliquots (50 μl) of this suspension were transferred to either 450 μl of M199+ in individual wells of a 24-well flat-bottomed plastic tissue culture plate (Falcon Plastics, Los Angeles, CA) or to 150 μl of M199+ in the upper compartment of Millicell-PC culture plate inserts (Millipore Corp, Bedford, MA). The lower compartment created in the culture plate well by the Millicell insert contained 300 μl of M199+, so that the total amount of medium in the well was also 450 μl. The polycarbonate membrane of the insert had been pretreated by allowing 50 μl of the basal lamina extract, Matrigel (Collaborative Research, Bedford, MA), to gel on its upper surface. This two-compartment system, in which sperm are co-cultured with epithelial cells in the upper compartment and the epithelial cells are fed basally by medium in the lower compartment, will be referred to as "Matrigel/Millicell." Mucosal epithelial cells from bovine trachea, obtained by scraping the mucosa with a scalpel blade and disaggregating and washing the epithelial cells as described above, were also cultured on Matrigel/Millicell. All epithelial cells were cultured in M199+ at 39°C under a gas atmosphere of 5% CO_2 in humidified air. In 3 wk, all cultures formed confluent monolayers.

Semen from 3 fertile Holstein bulls was pooled, diluted in standard whole milk extender [14], and frozen in commercial 500-μl semen straws. For each experiment, a straw was thawed and motile sperm were obtained by layering the semen over a Percoll step gradient of 1.5 ml each of 25%, 50%, 75%, and 95% Percoll in HEPES-TALP medium [15] adjusted to be isosmotic to sperm when diluted with the Percoll. After centrifuging at 500 × *g* for 45 min, a pellet of sperm was collected from the bottom of the tube and resuspended in Sperm-TALP medium [15] to a concentration of 1×10^8 motile sperm/ml. Aliquants of a volume sufficient to bring the final sperm concentration to 1×10^6/ml were then transferred to cultures of endosalpingeal epithelial cells grown either (1) directly on tissue culture plastic in 24-well plates or (2) on Matrigel/Millicell or (3) on tracheal epithelial cell cultures on Matrigel/Millicell or (4) in Sperm-TALP medium alone in empty wells. Prior to the addition of sperm, the epithelial cell cultures had been rinsed and re-equilibrated with Sperm-TALP. In Matrigel/Millicell cultures, the M199+ was replaced with Sperm-TALP in both the upper and lower compartments.

Sperm motility (presence of flagellar activity) was assessed in 5 replicates of each treatment every 6 h over the course of a 48-h culture period. The experiment was performed in 5 replicates on 5 separate days and used epithelial cells from a different donor cow on each day. Cultures were examined at 400× magnification and 200 sperm from each well were classified as motile or immotile. In tracheal and endosalpingeal cultures, 100 sperm that were bound to the epithelial surface and 100 unbound sperm were classified. The data were analyzed by both CATMOD and weighted-least-squares of the General Linear Model procedures of the Statistical Analysis System [16]. The model included replicate, treatment, treatment by replicate, bound, bound by replicate, bound by treatment, bound by treatment by replicate, hour, hour by replicate, hour by treatment, hour by treatment by replicate, hour by bound, hour by bound by replicate, and hour by treatment by bound. The effect of treatment and binding on sperm motility was tested by comparing weighted-least-square means in orthogonal contrasts using both procedures. Results are reported as percent motile sperm.

Experiment 2: Maintenance of Sperm Fertilizing Capacity

Bovine oocytes were separated from the aspirated contents of follicles in ovaries also obtained at the abattoir. The oocytes were then incubated in vitro in a maturation medium for 24–30 h [17], manually removed from the expanded cumuli oophori, and washed in HEPES-TALP medium. The experiment comprised two replicates done on different days and using two epithelial cell donors. On each day, groups of 25 oocytes, selected according to the criteria of Leibfried and First [18], were transferred to one well each of (1) sperm co-cultured with endosalpingeal epithelium on Matrigel/Millicell, (2) sperm co-cultured with tracheal epithelium on Matrigel/Millicell, or (3) sperm preincubated in medium alone. Prior to the addition of oocytes, both types of sperm/epithelium co-cultures had been prepared as follows: M199+ medium was replaced with Sperm-TALP medium [15]; sperm were added and preincubated with the epithelium for 0, 24, or 30 h; then the cultures were rinsed with IVF-TALP [15] to remove unbound sperm. The 24-h time point was chosen as the maximal period that the sperm would be expected to survive in culture medium alone, and the 30-h time point was chosen as the average length of time from the onset of estrus to ovulation [14]. After the oocytes were added, the cultures were incubated in IVF-TALP for 14 h. The oocytes then were fixed in methanol:acetic acid (3:1, v/v) for 48 h and stained with 1% aceto-orcein [19]. Fixed and stained oocytes were examined by phase-contrast microscopy at 600× magnification to determine rates of sperm penetration and polyspermy. Sperm penetration was defined as the presence of either a whole

sperm, a sperm head, or a male pronucleus with associated sperm tail in the ooplasm. Polyspermy was defined as the presence of any multiple and/or combination of these sperm structures within a single oocyte. Any oocytes in which these structures could not be clearly seen were categorized as unfertilized. The effects of treatment and time were tested using the CATMOD procedure as described above.

Experiment 3: Scanning Electron Microscopy of Bound Sperm

Two replicate samples were prepared for scanning electron microscopy 24 h after addition of sperm to endosalpingeal epithelial layers on Matrigel/Millicell. Endosalpingeal cultures were obtained from two epithelial cell donors. The culture medium was replaced with protein-free Sperm-TALP prior to fixation, and the tissue was processed according to the procedures of Hunter et al. [6].

RESULTS

Experiment 1

The maintenance of sperm motility in the cultures is summarized in Table 1. After 24 h of incubation, motile sperm were not evident in medium alone, but were observed in all epithelial cultures. After 48 h, only sperm that were bound to the apical surface of endosalpingeal epithelium were motile. A higher percentage of sperm maintained motility in co-culture with endosalpingeal cells grown on Matrigel/Millicell than directly on plastic, and sperm incubated with endosalpingeal cells remained motile longer than those cultured with tracheal cells. A significant ($p <$ 0.03) epithelial cell donor cow (replicate) effect was observed; however, none of the effects by replicate interactions was significantly different.

After 12 h of co-incubation with endosalpingeal epithelium, approximately 30% of unbound sperm and a few bound sperm appeared to be hyperactivated. The unbound sperm exhibited the vigorous, nonprogressive swimming characteristic of hyperactivation [20], while the few bound sperm that were thought to be hyperactivated moved their flagellae in waves of relatively high amplitude. The flagellae of most bound sperm, however, produced low amplitude waves or were immotile. It could not be determined whether the immotile sperm were dead or only immobilized. Unbound sperm populations were observed to be replenished over the course of the incubation period by the release of sperm from endosalpingeal cells. Sperm cultured in medium alone did not show evidence of hyperactivation. Within a few hours, they agglutinated into large clumps and then became immotile.

Experiment 2

The rates of fertilization and polyspermy are presented in Table 2. Although the fertilization rates were relatively high in all groups when oocytes were added to the wells immediately after sperm addition (Time 0), only sperm preincubated with endosalpingeal cells retained a capacity for fertilization after 30 h of preincubation. Within 5 min of the addition of oocytes to 24-h and 30-h sperm/endosalpingeal co-cultures, sperm were observed to detach from epithelial cells and bind to the zonae pellucidae of the oocytes.

TABLE 1. Percentages (±SEM) of bovine sperm remaining motile during incubation with epithelial cell cultures. Each value represents a mean of 5 replicates (overall SEM = 2.7).*

	Motile sperm(%)						
		Tracheal cells		Oviductal cells (P[a])		Oviductal cells (MM[b])	
Time	Medium	Unbound	Bound	Unbound	Bound	Unbound	Bound
0	97.0 ± 0.6	97.0 ± 0.6	97.0 ± 0.6	97.0 ± 0.6	97.0 ± 0.6	97.0 ± 0.6	97.0 ± 0.6
6	80.8 ± 4.1	84.0 ± 4.7	91.0 ± 1.6	87.8 ± 2.3	93.6 ± 1.3	80.2 ± 2.8	97.8 ± 0.6
12	29.3 ± 4.2	45.6 ± 1.6	61.0 ± 4.3	80.6 ± 3.2	91.6 ± 1.3	75.6 ± 3.6	96.4 ± 0.5
18	5.2 ± 2.0	20.0 ± 5.2	39.6 ± 4.2	79.4 ± 2.7	90.6 ± 1.2	75.8 ± 3.7	95.6 ± 0.7
24	0.0 ± 0.0	9.6 ± 2.1	31.8 ± 3.5	21.8 ± 3.5	84.2 ± 4.1	35.6 ± 6.0	91.4 ± 1.0
30	0.0 ± 0.0	0.0 ± 0.0	7.2 ± 1.6	9.4 ± 2.5	63.2 ± 7.1	22.2 ± 4.1	83.0 ± 4.4
36	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.4 ± 1.2	33.6 ± 4.2	14.6 ± 3.9	57.8 ± 3.7
42	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	13.8 ± 3.7	0.0 ± 0.0	55.8 ± 4.2
48	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.5	0.0 ± 0.0	38.4 ± 9.8

[a]P, tissue culture plastic
[b]MM, Matrigel/Millicell.

	Degree of significance	
*Orthogonal contrasts	CATMOD	General Linear Model
Culture medium & trachea vs. oviduct P and MM	0.0001	0.0001
Culture medium vs. trachea	0.2562	0.0001
Oviduct P vs. oviduct MM	0.0001	0.0001
Unbound vs. bound	0.0001	0.0001

TABLE 2. Fertilizing capacity of bovine sperm after incubation with epithelial cell cultures.*

Culture	Oocytes Exposed n	Fertilized n (%)	Polyspermic n (%)
Medium alone			
0 h	50	39 (78)	7 (14)
24 h	50	0 (0)	0 (0)
30 h	50	0 (0)	0 (0)
Tracheal Epithelium			
0 h	50	27 (54)	6 (12)
24 h	50	4 (8)	1 (2)
30 h	50	0 (0)	0 (0)
Oviductal epithelium			
0 h	50	42 (84)	4 (8)
24 h	50	37 (74)	1 (2)
30 h	50	21 (42)	0 (0)

*Orthogonal Contrasts	Degree of significance Fertilized	Polyspermic
Treatments		
culture medium & trachea vs. oviduct	0.0010	0.9363
culture medium vs. trachea	0.6790	0.8441
Time	0.0001	0.0006
Treatment × time	0.0004	0.9663

Experiment 3

Scanning micrographs demonstrated that the sperm were bound to the apical surfaces of endosalpingeal epithelial cells in furrows between adjoining ciliated cells or between ciliated and nonciliated cells (Fig. 1a). Cilia and microvilli appeared to adhere to the rostral portion of the acrosome (Fig. 1b). All sperm examined by scanning electron microscopy (>200) showed an intact acrosome whenever the rostral tip of the head was visible.

DISCUSSION

These results suggest that bovine endosalpingeal epithelium may play a role in maintaining the motility and fertilizing capacity of bovine sperm during the period from the onset of estrus to the arrival of the oocyte in the oviduct. Maintenance of sperm motility appears to be related to binding with polarized endosalpingeal epithelial cells and possibly to their secretions. More than 90% of the sperm added to the endosalpingeal cultures on Matrigel/Millicell bound within minutes to areas in which actively beating cilia could be seen. It was surmised that these areas comprised the original differential epithelial cell populations that had maintained polarity. In the endosalpingeal cultures on plastic, it was apparent that there were far fewer polarized cells and far fewer bound sperm. When spent media were analyzed by polyacrylamide gel electrophoresis (unpublished observations), estrus-associated oviductal secretory proteins were only detected in the apical compartments of the endosalpingeal epithelia grown on Matrigel/

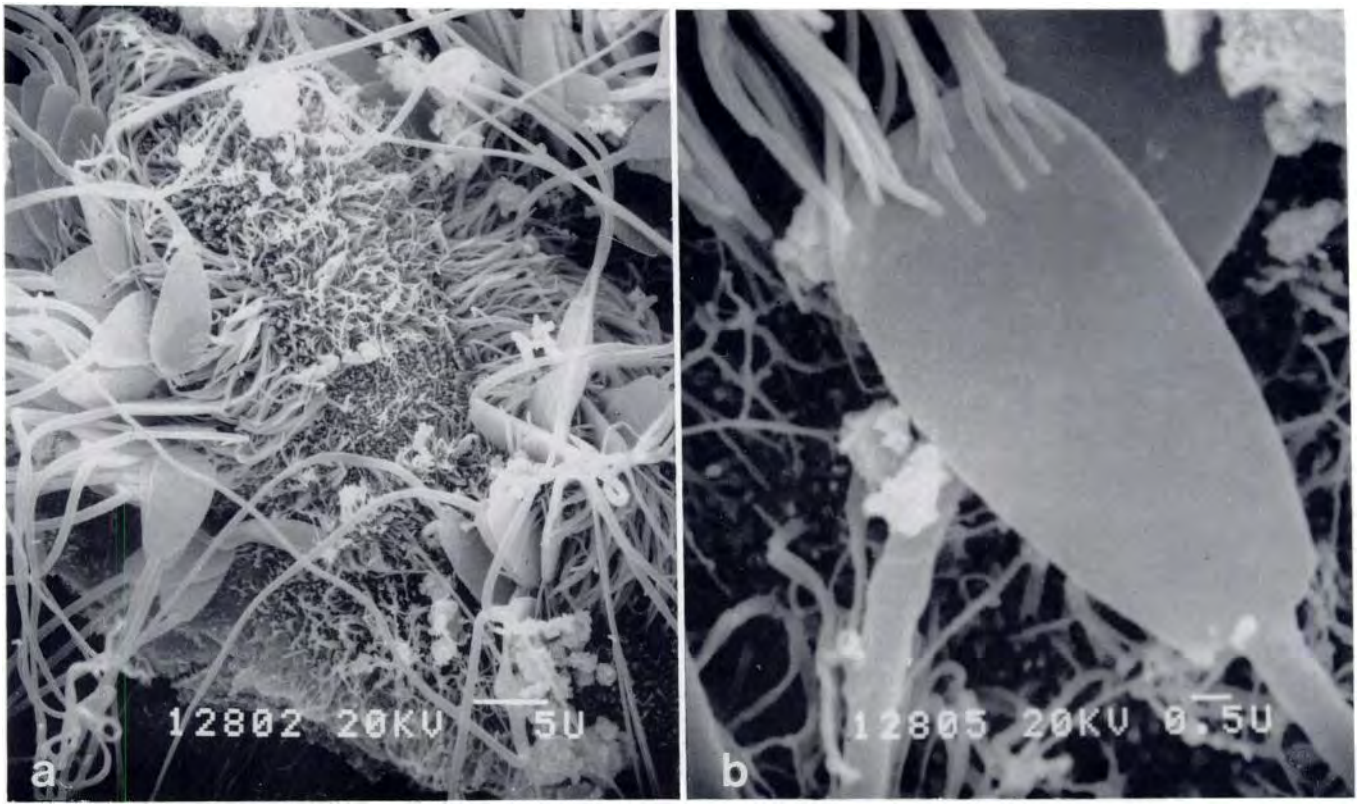

FIG. 1. Scanning electron micrographs of bovine sperm bound to cultured bovine endosalpingeal epithelial cells. a) Sperm bound to ciliated endosalpingeal epithelial cell. ×2 100. b) Cilia covering bound sperm. ×10 000.

Millicell. The tracheal cells, which were grown on Matrigel/Millicell, maintained a highly differentiated state, evidenced in the persistence of ciliated cells and the production of mucus. Nevertheless, few sperm bound to these cells and motility was not maintained as well as in the endosalpingeal cultures. The movement of sperm between bound and unbound populations could not be monitored. However, since more than 90% of the sperm were observed to bind to the oviductal epithelium initially, and since overall motility was greater in the co-cultures with polarized oviductal cells than in the other treatments, the results suggest that sperm motility could be maintained by physical interaction with polarized oviductal epithelium. It is also possible that oviduct-specific factors may add to this effect. Variation in the ability to maintain sperm motility may also exist between oviductal cell populations obtained from different donor cows. Though significant differences were observed between replicates in the current study, further experiments designed to investigate possible oviduct donor effects are required before inferences may be drawn concerning the relationship between variation in fertility observed in vitro and that observed in vivo.

The fertilizing capacity of sperm was also supported by co-culture with epithelium, especially with endosalpingeal epithelium. No doubt the fertilization rates observed were related to the numbers of motile sperm present in the cultures, yet motile sperm are not always capable of fertilizing oocytes [1]. Comparison of the results reported in Tables 1 and 2 suggests that the motile sperm had also retained their fertilizing capacity. The epithelia (and particularly the oviductal epithelium) could have prolonged the time for which sperm remained motile and capable of fertilizing in at least three ways. First, the epithelial cells may have participated in an initial "selection" of sperm destined to remain viable, perhaps because of a dependence of binding on intact plasma membranes. Second, the epithelial cells may have bound sperm over a broader spectrum of viability, eventually releasing a population with a range of viability similar to the starting population. Third, the epithelial cells may have bound sperm over the broader spectrum of viability, eventually releasing a population of select, hyperactivated sperm with high fertilizing capacity. The present study does not permit distinction between these possible mechanisms.

Before fertilization can occur, sperm must undergo certain physiological changes known collectively as "capacitation" [20]. Capacitation enables sperm to fertilize oocytes, but also reduces their lifespan [21, 22]. Bovine sperm require only 4 to 5 h to undergo capacitation in vivo [21] and in vitro [23, 24]. Since the sperm may wait 30 h in vivo before having the opportunity to fertilize an oocyte, mechanisms must exist either to delay capacitation or to extend the life of capacitated sperm. Recently, Smith and Yanagimachi [22] demonstrated that hamster sperm inseminated at the beginning of estrus require more time to capacitate in vivo than do sperm inseminated at the end of estrus. Thus, capacitation may be delayed in the oviduct in order to increase the lifespan of sperm.

In cattle, semen is deposited in the anterior vagina and most sperm spend at least 8 h outside of the oviduct [8, 25]. Therefore, the cervix or uterus could also participate in the maintenance of sperm fertilizing capacity. Bull sperm have been found in grooves in the mucosal folds of the endocervix 12 h post-coitus [26] and motile sperm have been recovered from the uterus several hours after insemination [25, 26]. Bovine sperm could be co-incubated with cultures of cervical and uterine epithelia in order to investigate the effects of these mucosae on motility and fertilizing capacity.

In addition to maintaining the fertilizing capacity of sperm, the endosalpingeal cultures appeared to induce hyperactivated motility. It is not known whether the effect was produced by epithelial secretions or by direct association of sperm with the epithelial surface. Hyperactivated sperm have been observed within the oviductal lumens of hamsters and mice [7, 28] and in the flushings from the oviducts of rabbits and sheep [29, 30]. It has been suggested that hyperactivated motility enables sperm to release themselves from the epithelial surface [7] and facilitates their passage through the highly infolded oviductal lumen [29]. In the co-cultures of bovine sperm with endosalpingeal epithelia in the present study, it appeared that hyperactivation enabled the sperm to detach from the epithelial surface.

In conclusion, these results indicate that bovine sperm motility and fertilizing capacity may be maintained, and that hyperactivated motility may be induced, by endosalpingeal epithelium. Thus, the oviduct may not function simply as a site for sperm retention, but may also participate in the physiological maintenance of sperm fertilizing capacity and consequently contribute to the fertilization of the oocyte.

ACKNOWLEDGMENTS

The authors wish to acknowledge the expert clerical assistance of Mrs. P. Fowle, the electron-microscopic core laboratory of the University of Florida interdisciplinary center for biotechnology research, Dr. Daniel Bousquet of the CIAQ for providing pooled bovine semen, and the helpful suggestions of an anonymous reviewer.

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