Measuring Sperm:Egg Interaction to Assess Breeding Efficiency in Chickens and Turkeys

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ABSTRACT Systems used to measure fertility in poultry have themselves presented a major impediment to progress in maintaining or improving fertility. Generally, these systems have been time-consuming, quantitatively inadequate, or both. A simplistic illustration of the basis of the problem is that if six fertile eggs were laid by a turkey hen during 1 wk after insemination, then all we know is what happened to six sperm: they fertilized the eggs. If 100 million sperm were inseminated, then information on the other 999,999,994 is missing. A better approach for quantitating breeding efficiency is to estimate the numbers of sperm that interact with the egg in the infundibulum. These can be identified in laid eggs, as sperm in the outer perivitelline layer (OPVL sperm), or holes produced by sperm in the inner perivitelline layer (IPVL holes). Eggs can contain up to 250,000 OPVL sperm, so the scale improves on binary estimation of fertilization status. The number of spermatozoa interacting with the perivitelline layer is related to the artificial insemination (AI) dose, the number of oviducal sperm, and the probability of fertilization, not just for one egg, but for subsequent eggs laid by the same hen. Practical applications of sperm:egg interaction measurements include: replacement of fertility trials for evaluation of semen; general fertility evaluation; and monitoring breeding efficiency of commercial turkey and broiler breeders. Furthermore, studies of sperm transfer into eggs raise interesting questions about the efficiency of turkey hens’ response to AI or mating frequency of broiler hens in commercial flocks.

(Key words: chicken, turkey, fertility, sperm-in-eggs)

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INTRODUCTION

When a hen is inseminated, she will lay fertilized eggs for a period of time without need for further artificial insemination (AI) or copulation. This period is known as the fertile period and is brought about by oviducal storage of sperm (Lake, 1975). Length of the fertile period is characteristic of each species and, in nature, is slightly longer than the time taken for a hen to lay her clutch, so that all eggs laid have the opportunity to be fertilized. Thus, the fertile period for the pigeon is around 8 d whereas that of the capercaille is 24 d (Birkhead and Möller, 1992). In poultry, genetic selection and environmental regulation have enabled hens to lay continuously over prolonged periods. However, their ability to store sperm—and thus their fertile period—has not increased equivalently and poultry hens will lay unfertilized eggs unless they are inseminated periodically. In the poultry breeding industry, the primary goal is to ensure that breeding hens lay only fertilized eggs and considerable effort is expended on maintaining fertility at the highest level possible. The purpose of this paper is to review methods for quantifying breeding efficiency in poultry and the application of these techniques for monitoring commercial breeding flocks of chickens and turkeys.

MEASURING FERTILITY

A typical pattern of laying fertilized and unfertilized eggs after a single AI of domestic fowl (chicken) hens with around $50 \times 10^6$ million sperm is shown in Figure 1. During a given postinsemination interval, hens will lay different numbers of eggs, as a series of fertilized and then unfertilized eggs; although the switch from fertilized to unfertilized may be staggered. The fertile period or fertility of a single hen can be described in terms of interval until last fertilized egg is laid, until the first unfertilized egg is laid, or as the mean of these parameters (Lake, 1975; Wishart, 1987; Brillard and Antoine, 1990). However, this definition does not take

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Abbreviation Key: AI = artificial insemination; IPVL = inner perivitelline layer; OPVL = outer perivitelline layer; SST = sperm storage tubules.
FIGURE 1. Pattern of lay of fertilized (shaded) and unfertilized (open) eggs by artificially inseminated hens. The proportions of fertile eggs laid by hens A, B, C, D, and E are 92, 75, 30, 77, and 58% and the fertile periods are 14, 12, 5, 12, and 9 d each, respectively.

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into account the numbers of eggs laid by a particular hen, so, more usually, we measure percentage fertilized eggs in a given period of time, such as Days 2 to 8 or 2 to 15 after insemination (e.g., Wishart, 1985).

There is considerable variation between hens in their response to AI. Thus, statistical interpretation of the fertility of a group of hens usually is provided as the mean proportion of fertilized eggs laid by 12 to 15 hens inseminated with aliquots of the same sample of semen (e.g., Lake et al., 1981). Unfortunately, most statistical comparisons of such groups are inappropriate because the fertility data are not normally distributed (Kirby and Froman, 1990). The basis of this problem is the complex sigmoidal relationship between the number of spermatozoa inseminated and the proportion of fertilized eggs laid by hens (Taneja and Gowe, 1962; Van Duijn, 1965). An alternative way to compare fertilizing abilities of two samples of semen is to inseminate a range of sperm numbers and to describe comparative fertilizing ability of samples in terms of the number of sperm that enable fertilization of 50% of eggs laid by groups of inseminated hens (Wishart, 1985). Whereas this method provides a better measure, the number of hens required makes it impractical for routine use.

Kirby and Froman (1990) introduced a hybrid of the temporal and percentage fertile eggs systems in which they determined the percentage fertilized eggs produced by groups of hens on each of the days following AI, to produce a composite duration of fertility. This nonlinear distribution is then subjected to logit transformation and the time in days corresponding to 50% fertilized eggs derived as the group parameter for statistical comparison. This method is practical, but has no adjustment for the number of inseminated spermatozoa; which will cause problems at high insemination doses if there is a maximum oviducal storage capacity (Bakst et al., 1994; McDaniel et al., 1997), and will, in turn, determine a maximum length of the fertile period beyond which no fertilized eggs will be laid.

Each of the above methods for measuring fertility has practical or theoretical disadvantages. One problem common to all is that data are collected during an interval when the birds are not inseminated or, if naturally mated, are removed from the males, which involves 2 to 3 wk in chickens (longer in turkeys) during which the production of fertilized eggs will fall to zero. Depending on the husbandry system and commitment to produce fertilized eggs, these requirements are at least inconvenient and often impossible. Our proposal is that parameters derived from data based on an assessment of whether or not an egg is fertilized can be replaced with more robust measurements of breeding efficiency. These are based on the measurement of sperm transfer into hens and eggs. To understand these methods, we first need an outline of oviducal sperm transport and storage.

**SPERM IN THE FEMALE REPRODUCTIVE TRACT**

Little is known of the mechanisms of cellular interaction between avian sperm and the epithelial or fluid environment within different regions of the oviduct. However, research over the past 10 yr has provided insights into the number of spermatozoa at different oviducal locations, how these change with time, and how they relate to the probability of a fertilized egg being laid (Bakst et al., 1994).

We are obliged to start this inventory with AI, so that a known number of sperm are deposited in the proximal vagina. Less than 2% of an inseminated dose of 100 to 200 million spermatozoa are found in the uterovaginal sperm storage tubules (SST) of either turkeys (Brillard and Bakst, 1990) or chickens (Brillard, 1993; McDaniel et al., 1997). The majority of the sperm are extruded from
the vagina within 30 to 60 min of insemination in both chickens (Allen and Grigg, 1958) and turkeys (Howarth, 1971). Upward transport of sperm is inhibited by a magnal egg (Bobr et al., 1964), although a hard-shelled egg in the uterus may not be an impediment to upward transport of sperm (Bakst, 1981). Less than 0.02% of the inseminated dose reaches the infundibulum in chickens (Brillard, 1990) or turkeys (Bakst, 1981). Sperm in the infundibulum may enter the infundibular SST, but this number is low following intravaginal insemination (Bakst et al., 1994). In the absence of an infundibular egg, some sperm undoubtedly pass through into the body cavity (Brillard, 1990) and others may be engulfed by macrophage (Koyonagi and Nishiyama, 1981). However, significant numbers are trapped in the forming outer perivitelline layer (OPVL) of the egg (Bobr et al., 1964).

The above studies mostly involve sacrifice of birds to gain access to sperm within the oviduct, so only an inventory of sperm numbers at a fixed point in time can be made for any one bird. Attempts have been made to quantify changes in numbers of oviducal sperm by examining hens at different stages of the oviducal cycle (Bakst, 1981) or different intervals after AI (Brillard, 1990), but hen-to-hen variation limits the value of these observations.

Earlier studies on the number of sperm interacting with the egg in the infundibulum were more qualitative (Bobr et al., 1964), but recent quantitative studies have shown that their numbers, at 40,000 (see Wishart, 1996), are similar to the number of spermatozoa washed from an empty infundibulum (Brillard, 1990). Most importantly, evidence of these sperm can be quantified in the laid egg, which provides a valuable noninvasive reporter of the numbers of oviducal sperm.

**Sperm: Egg Interaction**

Sperm encounter the egg in the infundibulum within approximately 15 min of ovulation. They interact with the inner perivitelline layer (IPVL), which is the outer investment of the egg at this time, release their acrosomal enzymes, and hydrolyse a small hole in the IPVL (IPVL hole), through which they pass to reach the oocyte (Bakst and Howarth, 1977). Most of the holes are concentrated in a 2.6-mm diameter circle of IPVL over the germinal disc, where they are approximately 20 times more frequent than in the IPVL from other regions of the egg in both chickens (Bramwell et al., 1995) and turkeys (Wishart, 1997a). The total IPVL holes over the germinal disc can number several hundred. However, because this represents a small area (approximately 5 mm$^2$) of the IPVL, there are approximately $50 \times$ more total holes found evenly distributed in the IPVL from other regions of the egg (Wishart, 1997a). The mechanisms responsible for the increased density of IPVL holes at the germinal disc remain unknown, despite considerable interest and speculation (Bramwell and Howarth, 1992; Steele et al., 1994).

Within a few minutes of fertilization, as the egg enters the magnum, the outer perivitelline layer (OPVL) is laid down around the IPVL (Bellairs et al., 1963). Sperm which are free in the lumen of the infundibular/magnal interface at this time become trapped in the proteinaceous mesh of the OPVL. These can be demonstrated in the laid egg (Bobr et al., 1964; Bakst and Howarth, 1977), where they are found evenly distributed throughout all regions of the OPVL of both chickens (Wishart, 1987) and turkeys (Wishart, 1997a). There are approximately $10 \times$ more sperm trapped in the outer perivitelline layer (OPVL sperm) than IPVL holes (Wishart, 1997a).

Both OPVL sperm and IPVL holes can be demonstrated in the laid egg and there is an approximately linear relationship between IPVL holes at the germinal disc (GD IPVL holes); IPVL holes from other regions of the egg (non-GD IPVL holes), and OPVL sperm (Bramwell et al., 1995; Wishart, 1997a). A logarithmic relationship between these parameters and the probability of a chicken egg being fertilized showed that approximately 0.1 OPVL sperm per square millimeter corresponded to a 50:50 chance of the egg being fertilized (Wishart, 1987). Later studies provided a figure of 0.5 OPVL sperm per square millimeter, equivalent to 3 GD IPVL holes (Wishart, 1997a). This appears to be in agreement with data derived from Bramwell et al. (1995), who determined directly the frequency of GD IPVL holes, and is in agreement with the direct measurements of Wishart (1997a). Similar relationships have been found between IPVL holes, OPVL sperm, and fertility in turkey eggs (Wishart, 1997a).

The number of OPVL sperm in laid eggs was, at first, assumed to relate to the number of oviducal sperm in the infundibulum at the time of fertilization (Wishart, 1987). Certainly the number of OPVL sperm is of a magnitude similar to the number of sperm that can be washed from the infundibulum (Brillard and Antoine, 1990), but it remains impossible to assay both concurrently in the same bird. However, techniques developed to extract sperm from the SST have shown that the number of SST sperm is linearly correlated with OPVL sperm in oviducal eggs from turkeys (Brillard and Bakst, 1990) and chickens (Brillard, 1993). As the SST are the major, and often the sole, site of oviducal sperm storage (Bakst et al., 1994), the number of sperm that can be demonstrated in laid eggs to have interacted with the perivitelline layers are indeed indicative of the actual number of oviducal sperm.

**Regulation of the Fertile Period by Oviducal Sperm**

The finding that numbers of OPVL sperm and thus, in turn, IPVL holes, are correlated with the numbers of oviducal spermatozoa, as well as with the fertility status of laid eggs, encouraged studies of the changing levels of these parameters in eggs laid during the fertile
The OPVL sperm found in the first fertilized egg laid by chicken hens after AI was found to be linearly (Wishart, 1987; Wishart et al., 1992) or logarithmically (Brillard and Antoine, 1990) correlated with the dose of inseminated sperm. For GD and non-GD IPVL holes, results were more biased towards a linear than logarithmic distribution (Bramwell et al., 1995). In these studies, the relationship is obscured by individual hen variation, but the likelihood is that, at higher doses of inseminated sperm, the OPVL sperm and IPVL holes will tend to asymptote, as the storage capacity of the SST are reached, a concept explored by McDaniel et al. (1997).

Several authors have concluded that in chickens, the number of sperm getting through to the first fertilized egg laid after AI is an indicator of the length of time that the hen will continue to lay fertilized eggs, and that individual hen variation in the rate of decrease in the numbers of OPVL sperm (Wishart, 1987; Brillard and Antoine, 1990) or IPVL holes (Bramwell et al., 1995) is of lesser importance in determining fertility.

A major problem in all of these studies has been the large variation in the number of OPVL sperm found in eggs from different hens following AI with aliquots of the same sperm suspension (Wishart et al., 1992). In fact, the problem seems to be a variation in response to AI, as the order of individuals within a group of hens ranked on the basis of OPVL sperm in laid eggs changed following equivalent inseminations made a few days apart (Wishart, 1996). The low repeatability within hen
for number of OPVL sperm should be considered in the design of studies. Despite this variation, the number of sperm inseminated into the proximal vagina is correlated (linearly at low doses) with the number of OPVL sperm or IPVL holes found in laid eggs.

Enumerating sperm-in-eggs is an excellent way to assess sperm transfer into the hens' oviduct, the fundamental parameter of breeding efficiency. Furthermore, as length of the fertile period is determined by the number of sperm interacting with the first fertile egg laid after AI, only this one egg needs to be assessed to estimate the fertility of inseminated hens.

**ENUMERATING SPERM:EGG INTERACTION TO ASSESS SPERM FUNCTION IN VIVO AFTER AI**

A major reason for the limited application of sperm quality assays has been the poor correlation between the test parameter and resultant fertility status of eggs laid by inseminated birds (Wishart, 1996; Donoghue, 1998). Part of this problem has been the nature of the method for assessing fertility, rather than the sperm quality test itself; large differences in the number or quality of inseminated spermatozoa will show as small differences in the number of fertilized eggs laid by inseminated hens (Wishart, 1989), which has been demonstrated using sperm:perivitelline layer interaction (Robertson et al., 1998). For differences in the quality of sperm from different individual roosters, as assessed by sperm motility, adenosine triphosphate content, and sperm: perivitelline interaction in vitro, the correlation with percentage fertile eggs was nonlinear and obscure. However a linear relationship ($r^2 = 0.81$) was found between parameters of sperm quality and IPVL holes from eggs laid by inseminated hens (Robertson et al., 1998). Thus, enumeration of IPVL holes provided a clearer validation of sperm quality tests as a basis for selection of breeding males.

The fertilizing ability of cryopreserved chicken sperm is $< 2\%$ of fresh sperm (Wishart, 1985), but this has been difficult to reconcile with most fertility data, which show that hens inseminated with cryopreserved sperm will lay $\geq 50\%$ fertilized eggs (e.g., Lake et al., 1981). However, as the OPVL sperm in eggs from hens inseminated with cryopreserved spermatozoa was 1 to 2% of the OPVL sperm found in eggs from hens inseminated with unfrozen spermatozoa (Alexander et al., 1993), the lower fertility figure was validated.

During the first few weeks after AI, the number of OPVL sperm in eggs laid by turkey hens inseminated with sperm which had been stored at 5 C for 24 h, was reduced to approximately 24% compared to eggs laid by hens inseminated with fresh sperm. However, the proportion of fertilized eggs laid by hens inseminated with fresh or stored sperm was not significantly different during this time period, although reduced fertility in the stored-semen group was apparent in later weeks (Donoghue et al., 1995). This greater discerning power of OPVL sperm estimations to distinguish between the quality of fresh and stored turkey sperm also has been found for IPVL hole estimations (Donoghue, 1996).

Effects of dietary energy intake on sperm function have been demonstrated by enumerating GD IPVL holes in eggs laid by inseminated hens (Bramwell et al., 1996a). Eggs from groups of hens inseminated with semen from males with increasing dietary energy content had GD IPVL hole means of 6.6, 42.9, and 62.3, whereas the proportions of fertile eggs from same the three groups were 69, 77, and 77 (Bramwell et al., 1996a). A similar approach has been used to demonstrate the effect of heat stress on fertility of broiler breeder males (McDaniel et al., 1996).

Thus, the quantitative advantage of using sperm:egg measurements for differentiating between fertility of semen samples from different males, from groups of males subjected to different dietary or environmental regimes, or after liquid or frozen storage, seems convincing. A practical advantage, particularly true for turkeys, is that hens do not have to be kept until they consistently lay infertile eggs before fertility can be assessed. Indeed, the effects of a second insemination can be assessed before any infertile eggs are laid—by enumerating perivitelline sperm numbers on a before-and-after basis. Furthermore, because the length of the fertile period, in chickens at least, may be correlated with the OPVL sperm in the first fertile egg (Wishart, 1987; Brillard and Antoine, 1990), this single estimation will provide a usable parameter of sperm fertilizing ability.

**APPLICATION OF SPERM:EGG INTERACTION TESTS FOR MONITORING AI EFFICIENCY IN COMMERCIAL TURKEY FLOCKS**

The sperm:egg interaction test offers insight into efficiency of AI in commercial turkey flocks. Figure 2 shows the distribution of GD IPVL holes in samples of 60 eggs from three flocks. The most striking feature of the distributions is the $>200$-fold range of IPVL holes in eggs. As all eggs were gathered on the same day, they are from different hens, and represent the number of spermatozoa transferred to the oviduct of each hen following the same AI regimen. A similar distribution was found for OPVL sperm in eggs from turkey flocks (Wishart et al., 1995; Wishart, 1996). We found the same skewed distribution in non-GD IPVL holes; medians of the distributions of GD and non-GD IPVL holes from seven turkey flocks were linearly correlated ($r^2 = 0.79; n = 7; P < 0.01$), but the means of these parameters less so ($r^2 = 0.50$).
The distribution of IPVL holes was more skewed in flocks in which the proportion of fertilized eggs was lower. The median number of GD IPVL holes for samples of eggs tended to be linearly correlated with the fertility of the whole flock ($r^2 = 0.70$; $n = 5$; $P < 0.10$).

The main value of these estimations over candling fertility is that they are done on unincubated eggs, so results can be obtained quickly (although the eggs can be stored for several weeks before evaluation). Although it is possible to estimate fertility status of unincubated eggs from the morphology of the blastodisc (Kosin, 1945), the sperm:egg interaction assay involves fewer errors, because viewed features are less equivocal and there are more data points. The large number of data points means that greater detail can be obtained from a smaller number of units, potentially enabling derivation of whole flock fertility from samples of eggs. Additionally, sperm:perivitelline interaction can differentiate fertility between groups of turkeys early in the season, long before this is manifested as differences in the proportion of fertilized eggs (Donoghue et al., 1995).

With the large range in the number of IPVL holes in eggs (Figure 2), even in cases of severe subfertility, the problem appears to be one of differing sperm transport among hens, rather than insufficient sperm/AI. Of course, semen quality and insemination technique are factors which can be linked to poor fertility in turkey flocks. However, there may be fundamental physiological aspects of oviducal sperm transport/storage and physiological/behavioral responses to AI that remain to be elucidated.

**APPLICATION OF SPERM-EGG INTERACTION TESTS FOR MONITORING MATING EFFICIENCY IN BROILER BREEDER FLOCKS**

The distribution of GD IPVL holes in samples of 60 eggs from three broiler breeder flocks (Figure 3) is skewed and covers a 400-fold range. Similar distributions were reported for OPVL sperm (Wishart and Staines, 1995) and for non-GD IPVL holes (Staines et al., 1998). The median numbers of OPVL sperm and non-GD IPVL holes from samples of eggs were linearly correlated (Staines et al., 1998). For 15 broiler flocks, we also found a linear correlation between the median numbers of GD and non-GD IPVL holes (Figure 4); correlations of means were lower.

Logarithmic transformation of data for OPVL sperm and non-GD IPVL holes in eggs from broiler breeder flocks revealed two populations of eggs: one, a normal distribution, representing hens with different numbers of oviducal spermatozoa; and second, a group of eggs outside of this distribution, which contained no sperm and thus represented hens that had no oviducal sperm (Wishart and Staines, 1995; Staines et al., 1998). Figure 5 shows the logarithmically transformed data for GD IPVL holes from turkey (Figure 2) and broiler (Figure 3) eggs. Clearly, the two populations seen in broiler flocks are not apparent in turkey flocks. A further difference between the distributions of GD IPVL holes in eggs from the two species is that coefficients of variation of IPVL holes in four turkey flocks were approximately 0.6 times those for broiler flocks.
For floor-mated broiler flocks, it was clear that subfertility is a problem of sperm allocation among hens rather than total number of sperm transferred by the roosters. The striking feature of the distribution of IPVL holes in eggs from broiler flocks was the enormous variation (Figure 3). Although some eggs contained sufficient sperm to ensure fertilization of 100 eggs, 25% of eggs from the same flock were unfertilized. Thus, it seems that the main cause of subfertility in floor-mated broiler flocks is that many hens rarely, if ever, mate.

In floor-mated broiler flocks, dose and quality of sperm deposited are variable and, as with AI, sperm transfer and a hen’s oviducal storage capacity affect outcome. Additionally, the hen’s response, behavioral or physiological, to mating itself and whether or not she has, or accepts, the opportunity to mate are relevant. The latter factor may be the basis for the greater spread of IPVL holes in eggs from floor-mated broilers, compared here to turkey flocks mated via AI. The major difference between the distributions of log GD IPVL sperm in chicken and turkey flocks (Figure 5) is the demonstration that a distinct population of broiler eggs contain no sperm. Logically, AI reduces the range in response since AI dose is controlled. The standard deviation for number of OPVL sperm in eggs from naturally mated broilers was twice that with AI (Wishart et al., 1992).

Despite this variation, statistical parameters derived from distributions of OPVL sperm (Wishart and Staines, 1995) and non-GD IPVL holes (Staines et al., 1998) in samples of 60 eggs were shown to be linearly correlated with broiler flock fertility (the proportion of fertilized eggs produced by the whole flock). Results in Figure 3 show that the distribution of GD IPVL holes becomes more skewed and the median of the distribution is lower in samples from flocks producing a lower proportion of fertilized eggs. Figure 6 shows the relationship between flock fertility and number of GD IPVL holes in samples of 60 eggs from eight flocks in which GD IPVL holes was assessed directly and another 16 flocks in which number of GD IPVL holes was derived from non-GD IPVL holes, using the relationships described by Wishart (1997a). The curve depicted fits a logarithmic curve.
FIGURE 6. Relationship between median for points of hydrolysis in the inner perivitelline layer over the germinal disc (GD IPVL holes) for samples of 60 eggs and flock fertility in broiler breeder flocks ($r^2 = 0.90; n = 24; P < 0.05$). Equation is: percentage fertilized eggs = 58 + 15[$\log$(GD IPVL holes)].

If we assume that in a broiler flock less than 85% fertilized eggs is undesirable, then a median GD IPVL of < 55 can predict this with 100% accuracy (12/12) and >55 GD IPVL holes can predict a proportion of > 85% fertilized eggs in 83% of flocks (10/12). Further refinement of this relationship with more data should enable more accurate links between different levels of flock fertility and sperm-in-egg data. Bramwell et al. (1996b) studied the relationship between flock fertility and mean GD IPVL holes in samples of 15 eggs from flocks, but found only a limited correlation between these parameters. A significant nonlinear (or linear) relationship was shown for flock age and mean GD IPVL holes. The difference between these and our data may be due to the smaller sample size and use of the mean rather than the median.

The advantages of assessing broiler flock breeding efficiency by quantifying perivitelline sperm (or holes) are the same as those discussed for turkey flocks. In practice, these methods can differentiate between flocks in which there is no apparent difference in fertility (Bramwell et al., 1996b) and can highlight problem flocks (Wishart and Staines, 1995; Staines et al., 1998). Enumeration of perivitelline holes or sperm provides a tool for monitoring both fertility and also aids understanding of breeding efficiency in broilers.

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