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The Relationship of *in Vivo* Sperm Storage Interval to Fertility and Embryonic Survival in the Chicken[1]

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The effect of time after insemination on fertility and embryonic loss has been studied for each day's egg production (60–150 eggs per day) by direct observation of chicken eggs after 18 days of incubation. The results showed a significant initial increase in fertility with sperm storage from day 1 (first day of fertile eggs) to day 3, followed by a few days of high fertility which was then followed by a linear decline through 18 days. Embryonic death was higher initially, reduced to a minimum when fertility was maximum, and increased linearly with increasing age of spermatozoa until fertility was reduced to a low level.

The effect on fertility and embryonic loss was influenced significantly by both the male and female as well as time after insemination, and embryonic loss was negatively related to fertility level of the male. That embryos die at an earlier age due to fertilization by aged sperm was not conclusively confirmed in this study.

INTRODUCTION

The effect of gamete age on fertility and embryonic survival has received considerable attention in recent years. Earlier work, reviewed by Salisbury (1965), showed that spermatozoa stored *in vivo* in the female reproductive tract of some mammalian species soon lost their fertilizing capacity without an apparent increase in embryonic and fetal loss. On the other hand, storage at less than body temperature in the isolated scrotal epididymis of the male or *in vitro* in storage media resulted in an increase in embryonic and fetal loss prior to the complete loss of fertilizing capacity. In the latter two instances motility and fertility of spermatozoa are maintained for much longer periods of time than in the mammalian female reproductive tract. These findings suggest that in the female reproductive tract fertilizing capacity of sperm is lost prior to degenerative changes affecting growth and development of the resulting zygotes. The environment of the spermatozoa may vary widely in differing *in vivo* and *in vitro* situations with consequent varying effects on fertility and embryonic development. Salisbury and Hart (1970) have summarized reports covering a large number of breeding records in cattle. As the length of the *in vitro* sperm storage increased there was an initial increase in the fertility of samples as estimated by the frequency of nonreturns to service. Nonreturns reached a maximum on the second day of storage at 4 C and subsequently exhibited a linear decline with increased storage time. A comparison of the numbers of 30- to 60-day nonreturns with 150- to 180-day nonreturns has been used to yield an indirect estimate of embryonic and early fetal death. When this comparison has been applied to records of cattle a consistent observation is that the percentage of estimated deaths first decreases then increases with increasing *in vitro* storage time or age of spermatozoa.

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In a limited number of reports from direct observation of reproductive tract contents a similar effect of decreased fertility and increased embryonic loss with *in vitro* sperm age was demonstrated for swine (Dziuk and Henshaw, 1958; First *et al.*, 1963). Studies with rabbits (Miller and Blackshaw, 1968) have shown an initial increase in fertility to a maximum followed by a decreased fertility and increased embryonic loss with *in vitro* aged sperm, and Cummins and Glover (1970) found an increase in fertility 1 day after artificial cryptorchism. From direct observation of developing frog eggs, Hart and Salisbury (1967) found a higher percentage of arrestment at the gastrula stage of development due to fertilization by *in vitro* aged sperm.

In poultry, after removal of the male, a single mating with a male, or a single insemination, the female will lay fertile eggs for several days or even weeks. Although the chicken appears to be an excellent animal for studying the effect of *in vivo* aging of the male gamete, there are few published reports of the daily fertility and embryonic survival curves for this species. Nalbandov and Card (1943) have examined this problem most critically, but their results are presented by grouping 5-day intervals. Dunn (1927) reported his findings on a daily basis, but the numbers of eggs for each day were small. These two studies as well as those of Hale (1955) and Carter *et al.* (1957) and others as reviewed by Landauer (1967) suggest that embryonic survival decreases as fertility decreases with time after sperm deposition in the hens reproductive tract.

This paper reports the daily fertility and embryonic survival time for ovipositions (eggs laid) during 3-week periods after each single insemination of the female chicken with freshly collected semen from individual males.

MATERIALS AND METHODS

Leghorn-type chickens were chosen for this study because they lay at a rapid rate as well as have a high fertility and embryonic survival (hatchability). The males used were from a commercial stock used for egg production. The females were an experimental strain being used to study the effects of small body size on egg production. Semen was collected by massage from each of 15 males, and the semen from each male was used immediately to inseminate two females. Approximately 0.1 ml of semen was used for each insemination. The order of males was rotated for each replication so that no female was inseminated more than once with semen from a given male. Some males were replaced owing to their death or the inability to yield enough semen resulting in 21 different males being used in the study. Females were replaced if their previous weeks egg production was three eggs or less. The collections and inseminations were made in the afternoon, a time which has been shown to result in high fertility (Parker, 1945, 1950; Johnston and Parker, 1970).

The pedigreed eggs were collected daily beginning the second day after insemination which was the first day of fertile eggs. Thus day 1 in the tables and figure presented represents the first day fertile eggs could be laid after each insemination and not the day of insemination. The eggs were stored at 10–12 C and placed in the incubator at 37.8 C once a week. Approximately one-half of the eggs for days 1 and 2 were randomly selected for cytogenetic studies, and all eggs laid on days 9, 13, and 14 were used for this purpose. The results from cytogenetic studies will be reported in a later publication. After 18 days' incubation the eggs were examined by candling for the presence of live chicks. If no live chick was observed as determined by body movement the egg was opened and examined macroscopically for the presence of a dead embryo or infertile germinal disc. The age at death of dead embryos was estimated by the stage of embryonic development observed (Hamilton, 1952).

The effects of sperm storage time, male, and female on both fertility and embryonic survival were estimated by calculating least-square means and constants according to the method described by Harvey (1960).

RESULTS

In Table 1 are shown the number of eggs collected by days, number of fertile eggs, number of dead embryos, the least-squares percentage fertility, and the percentage of fertile eggs as dead embryos. The total number of eggs collected for each day varied from 120 to 150 except for days 1 and 2 as indicated earlier. A preliminary least-squares analysis showed a significant female effect and thus the female effect was absorbed in

TABLE 1

EFFECT OF SPERM AGE ON FERTILITY AND EMBRYONIC DEATH IN THE CHICKEN

Days[a]	No. of eggs set	No. of fertile eggs	No. of dead embryos	Fertility[b] (%)	Fertile with[b] dead embryo (%)
1	79	62	2	78.3	3.3
2	63	61	3	97.9	4.3
3	148	144	1	98.1	0
4	142	133	3	94.2	1.9
5	129	123	6	95.4	4.8
6	152	142	12	93.7	8.3
7	138	121	8	89.0	5.9
8	121	97	5	80.0	4.8
9	—	—	—	—	—
10	132	101	7	76.8	7.0
11	140	95	5	67.4	4.9
12	144	75	10	52.3	13.1
13	—	—	—	—	—
14	—	—	—	—	—
15	132	27	6	20.6	22.8
16	132	19	0	13.5	1.0
17	147	12	4	9.4	31.1
18	131	8	0	5.6	12.9

[a] Day 1 is the first day of fertile eggs.
[b] Values are least-square means.

subsequent analyses. The fertility data shown in Fig. 1 show a highly significant increase ($p < .01$) in fertility from day 1 to day 2. After maximum fertility on day 3, it dropped slightly on day 4 and remained relatively constant through day 6, and then decreased linearly through day 18. One fertile egg with a live chick was found for each of days 19, 20, and 21, but the values for these days were not included in the statistical analyses.

Also shown in Fig. 1 is the percentage of fertile eggs containing dead embryos. There was considerable day-to-day variability as the numbers on any given day (Table 1) were not large. These values in the figure were calculated from the least-squares values of live chicks as fertile eggs so consequently the value for day 3 which showed the highest percentage of live chicks becomes zero. It can be seen, however, that embryonic death was higher for days 1 and 2 than it was for day 3 (1 of 144) where it was at its minimum. From day 4 onward there was a definite trend toward increasing incidence of embryonic death with increasing age of the spermatozoa. The number of fertile eggs for days 15–18 were small resulting in a large day-to-day variability. A regression analysis showed the increase in embryonic deaths to be 0.8% per day, and the significant slope of the regression line shows that embryonic loss does increase with time after insemination.

The experiment was performed in seven replicates and the average fertility and embryonic deaths during the entire sperm storage period of 18 days for each of the replicates are shown in Table 2. These values are

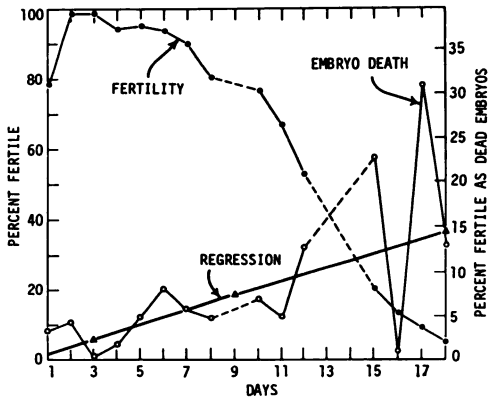

FIG. 1. Effect of sperm age on fertility and embryonic death in the chicken.

TABLE 2

VARIATION AMONG REPLICATES IN AVERAGE FERTILITY AND EMBRYONIC DEATH DURING 18-DAY SPERM STORAGE

Replicate number	Date of insemination	No. of eggs set	No. of fertile eggs	Fertility (%)	Fertile eggs with dead embryos (%)
1	9–29–69	316	195	61.7	7.7
2	10–20–69	284	173	60.9	4.6
3	11–17–69	297	189	63.6	6.9
4	12–08–69	260	178	68.5	4.5
5	1–05–70	272	160	58.8	9.4
6	1–26–70	250	173	69.2	4.6
7	2–16–70	252	153	60.7	3.3

TABLE 3
EFFECT OF MALE ON FERTILITY AND EMBRYONIC LOSS DURING 18-DAY SPERM STORAGE

Male no.	No. of eggs set	No. of fertile eggs	Fertility[a, b] (%)	Fertile as[a, b] dead embryo (%)
19	64	50	75.3	15.1
5	22	13	73.1	0
4	137	94	69.0	12.2
2	101	63	68.9	10.3
18	101	67	66.9	17.2
1	118	68	65.9	14.4
11	141	96	65.6	15.9
16	105	71	65.0	12.9
13	133	85	63.8	15.6
9	64	44	61.5	12.2
21	25	15	61.3	17.7
10	42	27	61.0	17.4
6	66	40	60.3	6.7
8	134	87	60.1	11.7
7	129	82	59.9	14.6
12	130	77	59.7	21.0
14	132	81	59.6	15.4
17	91	56	57.3	11.5
3	54	26	57.1	23.4
20	73	41	55.9	18.0
15	100	46	45.1	14.8

[a] Values are least-square means.

[b] Differences among males are significant ($p < .01$).

not least-squares means so that no adjustment for variable numbers has been made. There was some fluctuation in fertility among replicates ranging from 58.8 to 69.2%. The lowest value occurred during the coldest time of the year and during a time when egg production was declining. It is interesting to note that the highest embryonic death occurred also during the period of lowest fertility. These variations in fertility and embryonic loss with replication may be interrelated with environmental and egg production factors.

The male effects on fertility and embryonic loss over the sperm storage period are given in Table 3. The values have been ranked according to decreasing fertility of the individual males for ease of observation. Since the same males were not used in all replications for one reason or another, there are a total of 21 different males with considerable variation in the number of eggs from hens inseminated per male. The data are from the least-squares analyses with the female effect absorbed and thus the values are adjusted means. The fertility over the 18-day *in vivo* storage period ranged from a high of 75.3 to a low of 45.1% and the male effect was highly significant ($p < .01$). The embryonic loss values were obtained by subtracting the adjusted mean values from the highest adjusted mean for percentage of fertile eggs as live chicks. Thus the male with the highest mean has a zero percentage loss. The values range to as high as 23.4% of the fertile eggs as dead embryos. The correlation between percentage of fertility and percentage of embryonic loss was -0.37 which approaches significance ($p < .10$) and indicates that the males with the highest fertility produced the lowest percentage of embryonic deaths.

The average estimated age of death of the embryos is presented in Table 4. Again the small number of dead embryos for any given

TABLE 4
EFFECT OF SPERM AGE ON THE ESTIMATED AGE OF EMBRYONIC DEATH

Days	Estimated $\bar{X}$ age at death days	$\bar{X}$ Age of death days
1	2.5 (1.0–3.0)[a]	
2	7.3 (2.0–17.0)	
3	3.0	4.26
4	3.3 (1.5–5.0)	
5	3.8 (1.0–8.0)	—
6	5.0 (2.0–10.0)	
7	8.3 (2.0–17.0)	5.50
8	6.4 (2.0–17.0)	
10	2.7 (2.0–4.0)	—
11	2.6 (1.0–4.0)	
12	5.6 (1.5–17.0)	
15	6.0 (1.0–15.0)	4.68
16	—	
17	2.5 (1.0–5.0)	
18	—	—

[a] Range in estimated age at death.

day resulted in a large variation. The data were grouped for days 1–5, 6–10, and 11–18 of sperm storage to increase the number of embryonic deaths in a given period. There was no trend toward the embryos dying at a younger age with increasing age of the spermatozoa. The percentage of embryos that died after 5 days of incubation is essentially the same for the periods of 1–10 and 11–18 days of sperm storage. Thus, it would appear from this study that the age of sperm did not influence the age of death of the developing embryos.

DISCUSSION

The results from chicken eggs, collected daily after a single insemination and direct observation after 18 days of incubation show a significant increase in fertility on the day after the first day of fertile eggs. Fertility reached its maximum on day 3, i.e., 5 days after insemination. A high level of fertility was maintained from day 2 through day 6 which was followed by a significant linear decline to a low level by day 18. Embryonic loss was high initially, was reduced to a minimum at the time of maximum fertility, and increased with continued time after insemination or aging of the spermatozoa. The shapes of the fertility and embryonic loss curves are similar to those observed in mammalian species after *in vitro* storage of spermatozoa (Salisbury and Hart, 1970). The speculations for the cause(s) of the fertility and embryonic loss relationship to sperm age have been presented by Salisbury and Hart (1970) and similar speculations have been proposed for the increase in fertility after artificial cryptorchism by Cummins and Glover (1970). These speculations might well be applied to these findings for the chicken.

The results of this work confirm the trends indicated in earlier studies in which smaller numbers of eggs were observed. The increase in fertility during the first few days of sperm storage is considered to be a distinct contribution of this report. It is interesting to note that this same phenomenon has also been noted to occur in mammals (Salisbury and Hart, 1970; Cummins and Glover, 1970).

The negative correlation between male fertility and embryonic loss is similar to that reported for dairy bulls (Salisbury *et al.*, 1952) and emphasizes the need for using males of the highest fertility for maximum reproductive efficiency.

The age of death of embryos in relation to sperm storage time does not confirm the earlier data reported by Nalbandov and Card (1943). This may be due to the smaller percentage of fertile eggs as dead embryos in our study and that our values do not include those which failed to hatch.

Fertility of eggs removed for cytogenetic study on day 1, 2, 9, 13, and 14 was examined after 20 hr of incubation. When these fertility values were compared with those made on day 18 of incubation it was noted that the apparent fertility was higher among the eggs examined at the earlier time. However, only for the eggs resulting from day 14 of sperm storage were the two values materially different, being 10 percentage units. There was essentially no difference in fertility for the two incubation periods for eggs after 2 days of sperm storage. The other sperm storage periods of 1, 9, and 13 days showed fertility differences at the two incubation times intermediate to these extremes. This comparison suggests that very early deaths may have been missed during macroscopic examination of the eggs after 18 days of incubation. If these apparent early deaths were included, the estimated age of death would have been shifted toward an earlier death resulting from the use of older sperm.

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