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DISTRIBUTION OF SPERMATOZOA IN THE OVIDUCT AND FERTILITY IN DOMESTIC BIRDS

VI. THE RELATIONS OF FERTILITY AND EMBRYO NORMALITY WITH SITE OF EXPERIMENTAL INSEMINATION

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Summary. Effects on embryonic development of inseminating normally into the vagina and directly into the anterior oviduct were compared in the domestic hen. Insemination into the anterior oviduct led to a higher incidence of early mortality, severe retardation and other abnormalities of the embryo. These effects were overbalanced by an increased fertile period, so that a larger number of live chicks was obtained following a single insemination into the anterior oviduct than following an insemination into the vagina.

INTRODUCTION

Van Krey, Ogasawara & Lorenz (1966) described an effect of site of artificial insemination (A.I.) on embryonic viability: intramagnal A.I. which filled the infundibular sperm-host glands of the hen's oviduct while leaving the utero-vaginal host glands essentially empty, greatly increased the incidence of apparent pre-oviposital mortality while significantly prolonging the fertile period. Several hypotheses were proposed to explain this effect, but no experimental results were adduced to test them. The effect itself was incompletely explored because of the nature of the experimental procedure, i.e., since all eggs were opened for examination after no more than 24 hr of incubation, only pre-incubation or very early mortality could be detected.

Further experiments have thus been performed on the effects on embryonic viability of inseminating into the anterior oviduct. Improved techniques of differentiating embryonic mortality and retardation of development were studied for this purpose.

MATERIALS AND METHODS

Evaluation of fertility and embryonic viability

Kosin (1944, 1945a) and Munro & Kosin (1945) recognized that fertility could not be determined unequivocally by macroscopic examination of the blastodisc, since some embryos died before gross change was sufficiently apparent to the unaided eye. The Kosin test (Kosin, 1944), which involves

superficial staining of the blastodisc *in situ* and examination under the microscope, detects a number of very early dead embryos, but in our hands it has not been successfully performed except on unincubated eggs. Thus, whenever the Kosin test has been used, information on viability of the embryos has necessarily been foregone. Also, embryos may die too early to be detectable by the Kosin test because often after intramagnal A.I. (Van Krey *et al.*, 1966) very early dead embryos were detected only by sectioning and staining paraffin-embedded blastodiscs.

Munro & Kosin (1945) also examined sectioned blastodiscs microscopically to demonstrate otherwise inapparent embryos; like us, they used the technique only on unincubated eggs. It has been the practice to designate such detectable embryos as pre-ovipositally-dead, but neither this technique nor the Kosin test demonstrates that embryos which appear infertile to the naked eye are incapable of development, and for this reason such blastodiscs are designated 'micro-embryos' in this study. Also, neither technique allows evaluation of the potential for the development of other embryos in the same lot of eggs, since all will have been opened unincubated. Obviously, a technique is needed which allows both incubation and microscopic examination. It appeared likely that such a technique might be developed through use of the procedure first described by King (1936) and Olsen & Knox (1938) for the detection of embryonic development by candling during the first day of incubation, provided changes did not take place during incubation to obscure subsequent microscopic evidence of pre-ovipositally dead embryos in paraffin sections.

A focused candler (Bundy Incubator Co., Springfield, Ohio), containing a 500-watt incandescent lamp filtered by a pale blue-green lens (King, 1939), was used in experiments as follows: eggs were set on their sides in the incubator, and 22 hr later the trays returned to level; the eggs were left undisturbed in this position for 2 hr so that the blastodiscs approached the uppermost portion of the shell as closely as possible. Each egg was removed from the tray and presented to the candling-lamp aperture without rotation, so as not to disturb this orientation. A developing blastodisc was seen through the translucent shell as a dark spot, usually about 4 mm in diameter, which moved under the shell if the egg was rocked gently.

This method was capable of being used with considerable precision. However, occasionally a mottled spot on the yolk or an abnormal chalaza resembled a blastodisc. Also, 'glassy' shells (Almquist & Burmester, 1934) and air bubbles from broken air cells usually obscured the blastodisc, but such eggs would not be expected to hatch. A few slow-developing embryos were so small after incubation for 24 hr that they could be seen only with difficulty.

The procedure developed for testing experimental inseminations (and used in Experiment 2) was as follows: eggs were set and candled as described above. Apparent infertiles, and eggs with glassy or cracked shells or broken air cells were opened. Such eggs showing any obvious development were classified and discarded, but yolks with apparently infertile and doubtful blastodiscs were fixed in 4% formaldehyde, embedded in paraffin wax, serially sectioned, stained with haematoxylin and eosin, and searched for cell nuclei at $\times 400$ magnification. Meanwhile, the remaining eggs were returned to the incubator,

set normally on end and incubated to hatching, but additional candling examinations were made at 4, 11 and 18 days of incubation for removal and examination of dead embryos. Eggs with retarded embryos, or ones that could not be classified, were marked distinctively and also returned to the incubator; those that showed no development at 4 days were fixed and sectioned as above. All eggs were eventually broken for examination unless they hatched normally. The entire procedure is referred to in this paper as the 'combination technique'.

Although this procedure was new by virtue of its unusual completeness, its only previously untested feature was the histological examination of eggs incubated for 24 hr. Kosin (1945b) warned that the histological picture of infertile eggs (examined for parthenogenetic development) degenerated very rapidly after the eggs were laid. Consequently, in the two experiments described below, alternate eggs from each hen were opened unincubated, and all except obviously fertile eggs were examined microscopically, as described above. Then, if incubation for 24 hr did obscure the evidence of early cell development, the percentage designated infertile would increase in incubated as compared with unincubated eggs, at the expense of percentage micro-embryos.

Methods of insemination and experimental procedures

Experimental animals were Single Comb White Leghorn fowl maintained in individual cages. All were from the same hatch though the males were unrelated to the hens. The hens had been in production for approximately 6 months. Each received a single insemination of 0·05 ml semen by one of the techniques described below; eggs produced starting the 2nd day thereafter—for 22 days in Exp. 1 and for 29 days in Exp. 2—were collected twice daily and stored for up to 1 week at 13° C.

The insemination procedures were as follows: (1) midvaginal and (2) intramagnal, both as described in Van Krey *et al.* (1966); (3) intra-infundibular, in which, as in intramagnal, the oviduct of the anaesthetized hen was exposed through a short mid-abdominal incision; but instead of puncturing the oviduct with a hypodermic needle, a plastic tube containing the semen was inserted into the infundibulum through the ovarian pocket, and the semen was expelled by gentle blowing. As also with intramagnal A.I., all hens subjected to this technique had a hard-shelled egg in the uterus, ensuring that all spermatozoa would remain in the infundibulum.

All inseminations were made with pooled semen, collected from several males chosen at random so as to reduce the effects of individual cocks, but, because the surgical procedures were time-consuming, each semen pool was used for a maximum of three hens.

In Exp. 1, data were obtained from twelve hens that received midvaginal A.I. and nineteen that received intramagnal A.I. (Two hens that laid only infertile eggs after midvaginal A.I. were excluded.) Eggs laid on odd-numbered days were broken unincubated; those laid on even-numbered days were incubated 24 hr and were then broken without candling.

In Exp. 2, intra-infundibular A.I. was performed, in addition to mid-

vaginal and intramagnal A.I., with fifteen hens per group. Incubated eggs were subjected to the successive candlings of the combination technique as described above. Alternate eggs for fresh breakout were sampled only following the 8th day after A.I., because of the low egg production of surgically inseminated hens during the 1st week, and because after midvaginal A.I. few eggs are not obviously fertile during this period. This restriction thus preserved as many eggs as possible for the test of embryonic development while ensuring an adequate sample size for testing microscopic detection of early development. The test was discontinued when so few eggs were fertile that further comparisons would not be worthwhile—on the 15th day following intravagainal A.I. and the 24th day following intramagnal or intra-infundibular A.I.

RESULTS

The results of Exp. 1 (Table 1) were unexpected and, at the same time, confirmed some observations of Van Krey *et al.* (1966) on the effects of intramagnal A.I. During the 3-week test period 39% of the eggs broken fresh

TABLE 1

FERTILITY AND EMBRYONIC ABNORMALITIES IN UNINCUBATED AND 24-hr-INCUBATED EGGS LAID DURING 22 DAYS FOLLOWING MID-VAGINAL AND INTRAMAGNAL A.I. (EXPERIMENT 1)

A.I. site	*Egg treatment*	*Total no. eggs*	*Infertile eggs* (%)	*Fertile eggs*		
				*Micro-embryos** (%)	*Gross†*	
					Abnormal (%)	*Normal* (%)
Vagina	Unincubated	88	57	4	—	39
	Incubated	89	58	0	2	39
Magnum	Unincubated	88	9	39	—	52
	Incubated	85	9	13	22	55

* Micro-embryos were infertile-appearing blastodiscs on naked-eye examination, in which clusters of cells could be demonstrated in fixed and stained sections.

† No attempt was made to classify the normality of grossly fertile-appearing unincubated blastodiscs. Most of the abnormal-appearing 24-hr-incubated blastodiscs were undersized or appeared to have developed little or not at all in the incubator.

contained micro-embryos, compared with only 4% from the hens inseminated midvaginally. Following 24 hr of incubation there was a striking reduction in blastodiscs assigned to this category, respectively 13% and 0%, but the number of apparent infertiles was not increased as would be expected if incubation obscured the blastodermal development. Instead, the entire difference was due to additional grossly apparent fertility. The only possible conclusions are that: (1) development was not obscured by incubation; and (2) many of the micro-embryos were not dead but were capable of development in the incubator.

Of exceptional interest was the close correspondence between the number of micro-embryos lost and the number of embryos that appeared grossly abnormal after 24 hr of incubation. These were typically small. This correspondence is

thus strong, though circumstantial, evidence that some of the micro-embryos observed in unincubated eggs were retarded rather than dead, a retardation that typically persists, or at least remains apparent, through the first 24 hr of incubation. What the subsequent development of these embryos would be was not answered in this experiment.

Experiment 2 was designed to attain several objectives in a single trial: (1) to explore the validity of the combination technique described above, and, especially, to assess the confidence with which micro-embryos, both living and dead, could be detected in eggs incubated for 24 hr; (2) to investigate the subsequent development and hatching potential of live micro-embryos; (3) to test a hopefully improved method, intra-infundibular A.I., whereby infundibular host glands could be filled with spermatozoa while keeping the utero-vaginal glands empty (also accomplished with intramagnal A.I.), but without damaging the oviduct; (4) to examine the hatching potential of eggs fertilized by spermatozoa stored in infundibular glands; and (5) to improve the accuracy of the estimates, previously obtained (Van Krey *et al.*, 1966), of the duration of fertility in relation to route of insemination by prolonging the egg-examination period through 29 days after A.I. These objectives are necessarily interlinked, and cannot be taken up and disposed of in any logical order.

TABLE 2

EGG PRODUCTION, FERTILITY AND HATCHABILITY FOLLOWING INSEMINATION INTO VARIOUS SITES (EXPERIMENT 2)

A.I. site	*Average egg production*				*Average duration of:*	
	1st week (No.)	*2nd week (No.)*	*3rd week (No.)*	*4th week (No.)*	*Hatchability (days)*	*Fertility (days)*
Vagina	4·3	4·9	4·6	4·7	6·2	11·3
Magnum	1·5	3·3	4·0	4·9	17·3	20·8
Infundibulum	1·5	3·3	4·3	4·8	13·9	21·7

Fifteen hens/group.

Table 2 shows the weekly egg production, the length of the fertile period and the period during which eggs were laid that could hatch, following mid-vaginal, intramagnal and intra-infundibular A.I. Intra-infundibular and intramagnal A.I. gave very similar results, at least for these parameters. Egg production was as strikingly reduced by both surgical treatments as had previously been observed with intramagnal A.I., and fertility was similarly prolonged by both treatments to 20·8 and 21·7 days, compared with 11·3 days following intravaginal A.I. These averages are respectively 2·0 and 2·9 days longer than the previously published estimate following intramagnal A.I. (Van Krey *et al.*, 1966), which is certainly due to the more complete sampling period, since terminal fertility in thirteen of the thirty hens occurred between Days 23 and 27 after A.I. Production of eggs containing embryos capable of hatching was also strikingly prolonged by surgical A.I., from an average of 6·2 days following midvaginal A.I. to 17·3 days following intramagnal A.I. The period following intra-infundibular A.I. was somewhat less (13·9 days),

but the difference was due entirely to three hens that failed to produce any chicks. If these hens were disregarded, the average duration was 17·4 days. The estimates of average period of production of eggs capable of hatching were less precise, of course, than were the average duration of fertility figures, since the former were based on alternate eggs only.

The combination technique proved, as expected, to be a tool of considerable precision for assessing the consequences of an insemination. Of 369 eggs set, 152 were removed from the incubator after 24 hr as presumptively infertile. Only three of these had grossly visible embryos when opened, and of these one egg had a crack which obscured the germ spot and one had been classified as questionable.

Eggs classified by candling as retarded, a total of thirty-three, contributed important new information. All but six of these were removed from the incubator when recandled at 4 days: eleven appeared grossly infertile on breakout (the classification was in error and was presumably brought about by mistaking a small yolk blemish or an abnormal chalaza for a retarded blastoderm); three showed abortive development without embryo formation; and thirteen contained embryos most of which were dead and all of which were retarded. The few that were alive appeared to consist of a tiny beating heart and a few blood vessels. Five of the six eggs that remained in the incubator hatched, but the chicks were abnormally small and weak and would certainly have been culled in normal hatching practice. Thus, a number of abnormal embryos, which were incapable of developing into healthy chicks, were identified by candling after 24 hr of incubation by the small size of the germ spot.

A comparison of the results of using the combination technique with examination of blastodiscs of alternate eggs opened unincubated is made in Table 3. Incubated eggs were classified as retarded if they had been provisionally so classified when candled at 24 hr and if any grossly visible embryonic development was subsequently demonstrated (including hatching of dwarfed chicks as described above). Only one embryo that appeared normal at 24 hr was included in this category; it was dead and clearly retarded at 4 days. The column headed 'Otherwise abnormal embryos' includes all other forms of abnormal and dead embryos detected by the 4-day candling, but which had appeared normal after 1 day.

The data in Table 3 suggest that the embryos which appeared retarded on candling after 1 day were mainly live micro-embryos. The percentage of these when added to that of the remaining micro-embryos is nearly the same as the percentage of micro-embryos in the unincubated eggs in this comparison (13% compared with 15%). Since the 'retarded' classification by candling was to some extent subjective, it is likely that some of the 'otherwise abnormal embryos' could also be included in this category.

In the second experiment, in contrast to Exp. 1 (Table 1), an effort was made to classify the unincubated blastodiscs. Those that had noticeable lacunae or were unusually small were classified as 'Abnormal' and so recorded in Table 3, but whether or not they would have developed abnormally if incubated could not, of course, be determined. Quite possibly some of the small blastodiscs might have proved to be retarded, though less so than the micro-embryos.

TABLE 3

COMPARISON OF EMBRYO EVALUATIONS IN INCUBATED AND UNINCUBATED EGGS FOLLOWING INSEMINATIONS INTO VARIOUS SITES (EXPERIMENT 2)

A.I. site	*Period after A.I. (days)*	*Egg treatment*	*Total no. eggs*	*Infertile eggs (%)*	*Fertile eggs*					
						Blastodisc		*Embryos*		
					Micro-embryos (%)	*Abnormal (%)*	*Normal (%)*	*Retarded (%)*	*Otherwise abnormal at 4 days (%)*	*Normal at 4 days (%)*
Vagina	8 to 15	Unincubated	40	50	15	8	28			
		Incubated	36	69	3			6	0	22
Magnum	8 to 24	Unincubated	66	21	15	11	53			
		Incubated	62	27	3			5	5	60
Infundibulum	8 to 24	Unincubated	69	22	14	9	55			
		Incubated	65	14	5			17	6	58
Unweighted average		Unincubated		31	15	9	45			
		Incubated		37	4			9	4	47

TABLE 4

CLASSIFICATION OF EMBRYOS IN INCUBATED EGGS FOLLOWING INSEMINATION INTO VARIOUS SITES (EXPERIMENT 2)

A.I. site	Period after A.I. (days)	Eggs examined (No.)	Infertile eggs (%)	Fertility[1] uncertain (%)	Fertile eggs					Hatched	
					Micro-embryos[2] (%)	Retarded[3] embryos (%)	Otherwise dead		Development[5] not known (%)	Culls[6] (%)	Vigorous chicks (%)
							To 4 days (%)	Later[4] (%)			
Vagina	2–8	64	20	0	2	2	0	9	9	3	55
	9–15	32	66	9	0	6	0	0	6	0	12
	16–22	34	94	0	6	0	0	0	0	0	0
	23–29	33	94	0	0	3	0	3	0	0	0
	2–29	163	60	2	2	2	0	4	5	1	24
Magnum	2–8	23	9	0	17	9	13	0	0	4	48
	9–15	23	9	0	4	4	0	4	0	0	78
	16–22	30	27	10	3	10	3	3	3	0	40
	23–29	35	86	3	0	0	7	0	0	0	7
	2–29	111	38	4	5	5	5	2	1	1	39
Infundibulum	2–8	19	0	0	0	26	5	0	5	11	53
	9–15	24	0	0	4	8	8	12	0	8	58
	16–22	32	12	6	9	16	6	6	0	0	44
	23–29	34	82	6	3	3	3	0	0	0	3
	2–29	109	29	4	5	12	6	5	1	4	36

[1] Eggs with infertile-appearing blastodiscs that were accidentally destroyed during preparation for microscopic examination are designated 'Fertility uncertain'.

[2] Micro-embryos tabulated here (in contrast to those in unincubated eggs, Tables 1 and 3) were all dead; these probably correspond to the previously designated 'pre-ovipositally-dead embryos' (Munro & Kosin, 1945).

[3] Embryos recorded here as 'Retarded' omit those included in Table 3 that hatched, and which are tabulated here as 'Cull' chicks (see footnote 6).

[4] The category 'Later dead' includes chicks still alive at 21 days of incubation but unable to hatch.

[5] Eggs with apparently normal embryos for which incubation had to be terminated prematurely, either because of imperfect glassy or cracked shells or because of accidental breakage, were designated 'Development not known'.

[6] Dwarfed and semi-dwarfed hatched chicks were designated 'Culls'. Most of these were demonstrably retarded at 1 day of incubation. No teratology was encountered in this experiment.

At best, however, this classification appears to suffer from ambiguities, and indicates the weakness of using unincubated eggs for assessing the results of experimental matings or A.I.

Since the numbers of micro-embryos in unincubated eggs were accounted for without significant loss in eggs incubated for 1 to 4 days, the condition posed above for validity of the 'combination technique' appeared to have been met, and its use for a detailed examination of the effect of the site of A.I. on embryonic development justified. An examination of temporal patterns of embryonic abnormality in eggs, to which this technique was applied, was thus made; weekly percentages are recorded in Table 4.

Although the amount of embryonic abnormality in these eggs could not be compared directly with previous observations (Table 1 and Van Krey *et al.*, 1966) because of the longer period of egg collection after A.I., as well as the longer period of incubation, the same pattern of difference was apparent between results of anterior-oviduct and midvaginal A.I. Total verified embryonic abnormalities during 4 weeks following midvaginal, intramagnal and intra-infundibular A.I. respectively were 9, 18 and 32% of all eggs incubated and 26, 33 and 45% of all fertile eggs. Of these, only the dead micro-embryos, the retarded embryos and the hatched chicks classified as culls in this experiment (Table 4) were probably related to the micro-embryos encountered here (Table 3) and previously in unincubated eggs. These abnormalities were respectively 5, 11 and 21% of all incubated eggs.

As previously observed, there was a difference in temporal distributions of embryonic abnormality. Following midvaginal A.I., 20% of fertile eggs laid during the 1st week and 80% of those laid in the 2nd week were abnormal. In contrast, during the 1st and 2nd weeks following intramagnal A.I., 47 and 13% of fertile eggs were abnormal, respectively. Incidence of abnormality remained unchanged during the first 2 weeks following intra-infundibular A.I. (42 and 40%, respectively), but the kind of abnormality differed. There was twice the incidence of retardation found after intramagnal A.I., and 4 times that obtained following midvaginal A.I.; also, fewer of these were preovipositally dead, and some retarded embryos actually hatched, though the chicks were defective.

It is noteworthy that, in spite of the increase in early incidence of embryonic abnormality, and in spite of the lowered early egg production following both forms of surgical A.I., both were capable of yielding more chicks from a single insemination than could be obtained by the midvaginal route. If all eggs laid had been set, a calculated seventy-five and sixty-eight vigorous chicks would have been obtained from the fifteen hens each that received intramagnal and intra-infundibular A.I., respectively, as compared to forty-three chicks that would have been obtained by midvaginal A.I. The difference is, of course, due to the prolonged fertile periods that followed the surgical routes (Table 2).

DISCUSSION

The present experiments were an extension of the work of Van Krey *et al.* (1966) and they have yielded a more complete description of the phenomenon

reported by those workers. However, the greatly increased incidence of embryonic retardation in eggs fertilized by spermatozoa from abnormally-filled infundibular glands has still not been explained. Van Krey *et al.* (1966) suggested that the large numbers of spermatozoa at the site of fertilization might have led to excessive polyspermy and thus to interference with normal development. Results of Fofanova (1964), which showed that either excessive or deficient numbers of supernumerary spermatozoa led to abnormal early development of the blastodisc, support this hypothesis.

Moreover, if the abnormal early cleavage patterns observed by Fofanova (1964) foreshadow abnormal subsequent development and/or embryonic death, these results may have a more general significance. Poor hatchability of fertile eggs laid towards the end of the fertile period has commonly been attributed to the senescence of spermatozoa (see Lorenz, 1959) but it may well be due to deficient numbers of spermatozoa. If so, this could explain also the prolongation of the period of production of eggs capable of hatching, following intramagnal and intra-infundibular A.I. when large numbers of spermatozoa are available; all of the few fertile eggs obtained by midvaginal A.I. were non-viable during this period. Experiments designed to explore the relationship between numbers of spermatozoa and embryo viability are in progress.

One of the possible alternative hypotheses that might be put forward to explain the high incidence of embryonic abnormality after anterior-oviduct A.I. presupposes a 'sperm-selection mechanism' operating on spermatozoa deposited in the vagina, which might fail or be bypassed with spermatozoa deposited anterior to the uterovaginal junction. Results suggestive of such a mechanism were obtained with semen from low-fecundity cocks by Ogasawara, Lorenz & Bobr (1966), but such a mechanism remains to be demonstrated conclusively.

The present experiments demonstrated that an embryo of the type previously designated 'pre-oviposital death' (Munro & Kosin, 1945), was sometimes capable of development in the incubator, but observed development was abnormal; those that hatched were small and weak. Embryonic retardation has, of course, been long recognized. Olsen & Knox (1938) observed a small percentage of 'small, slow-developing' embryos and considered it 'questionable whether any of them would hatch'. They were unable to detect those embryos by candling (using a less powerful and less sophisticated lamp than the one used here) after 16 to 20 hr of incubation.

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