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"Hirosima" Method of Artificial Insemination of the Domestic Fowl[1)]

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(With 16 figures & 10 tables)

CONTENTS

INTRODUCTION

Poultry husbandry in Japan is now turning from a sideline of crop farming to more commercial type; forms of enterprise or cooperation under cage management keeping thousands to ten thousands of birds are increasing year after year. It is natural that the demands for hatching eggs from superior stocks should be greatly

1) This investigation was aided by a grant from the Japanese Ministry of Education.

expanding. Under such a circumstance, it is considered that the application of the artificial insemination to the poultry breeding work is of great significance.

The artificial insemination of the fowl has greatly been enlightened by the works of BURROWS and QUINN (1935, 1937) who have paved the way for collecting fowl semen by massage method. Since then, a large number of researches have been made: some of them centering upon the collection of semen, some upon the introduction of semen, and others upon the making-up of semen dilutors. As a whole, however, the artificial insemination of the fowl cannot yet be seen as fully established as that of mammalian farm animals.

In view of this, experiments for improving the techniques of artificial insemination of the fowl have been carried out during the period from 1960 to 1964 at the Zootechnical Laboratory of Hiroshima Agricultural College. Although there is much room for more improvement in preparing the semen dilutor, the techniques here presented for collecting and introducing the semen appear to be fairly fitted to the practical purposes. The object of this paper is to show the experimental results hitherto obtained.

PART I
TECHNIQUE OF SEMEN COLLECTION

It was a great achievement of BURROWS and QUINN (1935) in the breeding of the poultry to find out the massage method for collecting semen from the cock. The first method of these authors was to collect the semen by massaging the keel and the soft sides of the abdomen above the gizzard and below the pelvic bones of bird held by an assistant in a headdown position. The same authors (1937) devised later a bird holder for applying massage without assistant and proposed a new method of "milking" by gripping the base of the copulatory organ when the organ is protruded by a slight stimulation of the cock mounted on the holder; upon ejaculation, the semen is caught in a small beaker which is held under the vent with the hand. Ever since, this second method of BURROWS and QUINN seems to have been of general use among the workers of artificial insemination of chikens with more or less modifications.

Undoubtedly the foundamental technique of artificial insemination is to collect the semen from the male animal with the least change both qualitatively and quantitatively, not to mention of the least soiling and contamination. In respect to this, a striking development of artificial insemination of large farm animals during the last quarter of the century can be mainly attributed to the invention and improvement of artificial vagina. In birds, however, the use of artificial vagina is not practical as in mammals because of the chracteristic structure of the copulatory organ of the bird.

From this standpoint of view, a trial of improving the massage method of collecting semen of the fowl has been conducted at this laboratory, stress being laid on obtaining the semen without changing its genuine nature. It has been preliminarily

reported by TSUKUNAGA and TAKAHASHI (1960) that the use of a bird holder suspending cock's feet is very practical for applying the massage to the bird; they constructed a wooden model together with a semen receptacle attached to the bird body. After testing with this and several other models, the present authors were able to get a new type of holder as well as of semen receptacle finished which can be employed with much expediency and regular success for any size bird (YAMANE, TSUKUNAGA and TAKAHASHI, 1962 b).

1. DESCRIPTIONS OF INSTRUMENTS

(1) "Hiroshima" pattern bird-holder.

The holder consists of a pair of wooden stands upholstered with a vinyl cushion and a pair of iron legs which can be folded up like a camp chair (Fig. 1, 2). Each

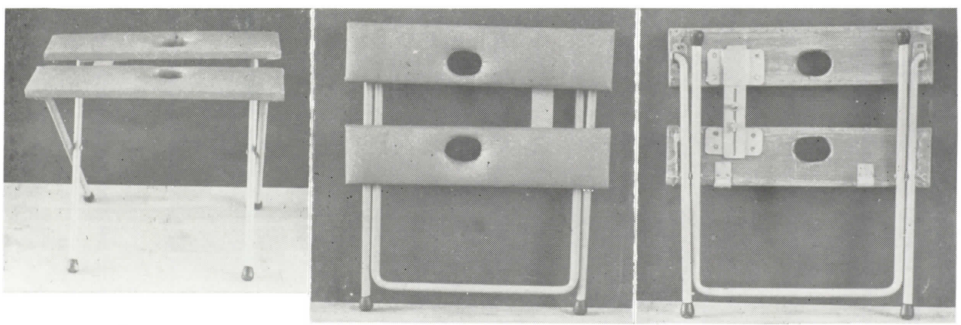


Fig. 1. "Hiroshima" pattern bird holder adaptable to any size bird.
1. Side view of the holder unfolded.
2. Surface view of the holder folded.
3. Reverse side of the holder folded.

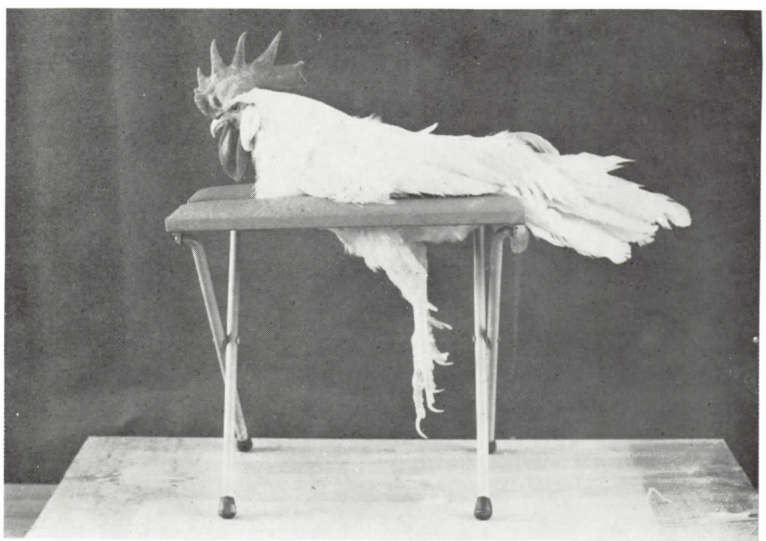
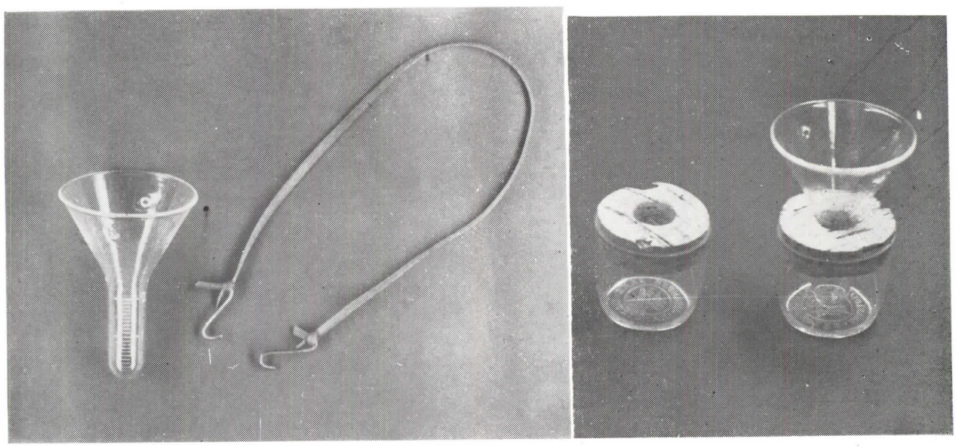


Fig. 2. A White Leghorn cock on a holder.

stand is 40 cm. long and 9 cm. broad; it is set apart from each other leaving so wide a space that the body of bird can be fixed into it. This interspace can be regulated according to the size of bird to be operated by means of an iron plate which connects both the stands, the range of adjustment being 4 to 11 cm. For the White Leghorn cock a 10 cm. interspace is appropriate. Besides, each stand has an elliptic hole of 4 by 5 cm. diameter for inserting the cock's leg; the hole situates approximately at the middle of the stand and 1 cm. apart from its inner margin. The legs of the holder are constructed from iron pipes of 15 mm. diameter; when they are unfolded, the holder becomes 35 cm. in height and when folded up, the holder is portable as a board of 40 cm. square. The total holder weighs about 2.6 kg.

(2) "Hiroshima" model semen receptacle.

Fixing of bird on the holder enables the attachment of a semen receptacle over the vent of the bird with great ease. The receptacle has been slightly modified in shape from that previously reported in order to let the semen rapidly flow down (TSUKUNAGA and TAKAHASHI, 1960). It is a pyrex funnel of 65° angle, 3.5 cm. in diameter and 6.0 cm. in depth; its tube part is calibrated and closed at the bottom, the whole content being about 2.0 cc. At the upper margin of the funnel two small holes are symmetrically made for hooking with a pair of rubber suspenders. Since the temperature in the abdominal cavity of the fowl represents 41° to 42°C, more precaution for temperature shock should be taken in cock sperms than in mammalian sperms. Hence, it is recommended during the cold season to use the receptacle in combining with a polyethylene cup which contains warm water of 41°– 42°C; the stem of the receptacle is put into the container through a cork stopper. For a temporary use at semen collection, the cup is more practical than vacuum flask in view of its shape and weight (Fig. 3, 4).



a b c

Fig. 3. A semen receptacle, "Hiroshima" model.
a. A funnel-shaped receptacle with a pair of rubber suspenders.
b. A container holding warm water of 41° to 42°C.
c. A receptacle put into the container.

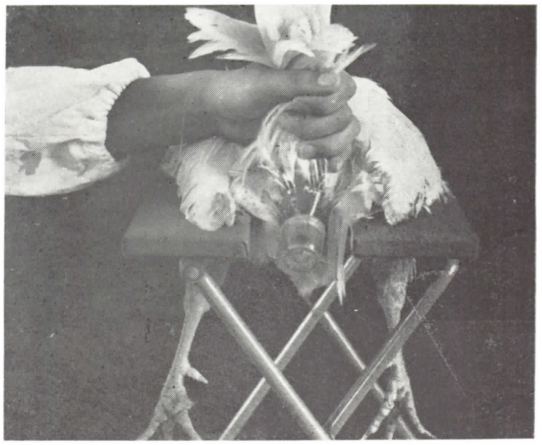


Fig. 4. A semen receptacle with a container attached over the vent of a cock, the receptacle being suspended from cock's shoulders.

2. MANIPULATION OF SEMEN COLLECTION

In the cock from which semen is to be recovered, the featherlings growing around the vent should be beforehand plucked. At manipulation, the cock is brought up to the holder above described by putting each of bird's legs fully into a hole of each stand; the trunk of the bird is kept in a horizontal position between both the stands. In this pose the cock can never attempt to escape because of completely pending of its feet (Fig. 2). Hereupon, a tampon of thumb-size is inserted about 3–4 cm. deep into the rectum for the prevention of soiling of semen with faeces; then, the vent and its surroundings are cleaned with a tampon moistened with sterilized physiological saline solution. Following the cleaning of the cock's body, a sterilized semen receptacle is attached over the vent by means of a rubber cord suspended from the shoulders, i.e. roots of the both wings. It goes well as the pygostyle and the posterior ends of both the pubic bones hold the receptacle in position during the operation. The operator faces to the cock's head, either on standing or on bended knees according to the height of the holder i.e. whether the holder is put on a table or on the ground, applies massage to the abdomen of the bird by inserting his right hand beneath the holder and slightly keeping the roots of the wings with the thumb and fingers of his left hand. On massage, grasp the ischio-pubal regions of the bird, then apply repeatedly fast strokes over the abdomen up to the breast. Within a few seconds, sometimes almost momentarily, an ejaculatory response occurs in the bird showing the reflex movement in its head, neck and tail similar to that which takes place in natural matings. It was observed that the cock ever mated with hen can hardly response upon massage as has been already reported by LAKE (1957); the ejaculation upon massage seems in part to be a conditioned reflex of the cock brought up to the holder.

3. CHARACTERISTICS OF SEMEN COLLECTED BY THE FOREGOING TECHNIQUE

For the experiments of semen collection, 15 Single Comb White Leghorn cocks were used. After treatment with vermicides and vaccines, they were separately kept in individual cages throughout the years and supplied with an ordinary forage of breeding ration. The age of the cocks when they were experimented with varied from 8 months to 2 years but all of them were trained at the age of 8 months for collecting semen by massage.

All sperms in the fresh semen collected by the foregoing manipulation exhibit in the majority of cases most active motility, scored with 5, inducing characteristic swirly currents. Fresh semen is commonly milky white in appearance but it varies considerably from viscous white to watery yellowish-grey according to a relative amount of the content from the *vasa deferentia* and the transudate or secretions from the accessory reproductive organs (vascular bodies and lymph folds) described by NISHIYAMA (1955) and LAKE (1957).

There are so many factors controlling semen characteristics as already shown by many workers; breed, individuality and age of bird, frequency of ejaculation, season of the year, and other environmental conditions are main factors to be considered. Nevertheless, the technique of collecting semen too should not be left out of account. According to PARKER, MCKENZIE and KEMPSTER (1942), the mean volume of semen collected by the massage technique was 0.88 ml., whereas the mean volume per ejaculate obtained in the semen collector was 0.36 ml.

In respect to this, Table I recording from three White Leghorn cocks kept in individual cages at this laboratory may give ample view of characteristics of semen collected by the technique herein described during the whole year.

Of these cocks, semen examination took place two or five times every month beginning with November; the duration of examination extended for cock No. 53 and 73 from the second breeding year to the third breeding year while this lasted for cock No. 99 from the first to the second breeding year. In tabulating an arbitrary classification was made into three seasons, namely, March to June, July to October and November to February, for the production of semen in these seasons appeared most active, less active and fairly active, respectively.

A comparison of the data in the table may possibly be done with those figures obtained by KATSURAGI and SAEKI (1958) who have observed in White Leghorn and Nagoya cocks at the National Institute of Agricultural Science, Chiba City, since the locality is not much different from the place of this college in view of the climate as well as the length of the day. These authors collected semen twice a month by means of old massage technique of BURROWS and QUINN (1935) and WHEELER (1948) holding a cock between the thighs of the operator. They found that the largest amount of semen was obtained in spring, March to May, showing 0.23 ml. and the smallest in summer, June to August, with 0.18 ml. The average volume of 152 semen samples from 16 cocks was 0.21. per collection. On the contrary, the present

Table I. Seasonal fluctuations of qualities of semen collected by "Hiroshima" method in three White Leghorn cocks.

Season of the year	Semen qualities on average	Cock No. 53	Cock No. 73	Cock No. 99	Seasonal average per ejaculaate
March to June	Volume per ejaculate ml.	0.50	0.34	0.59	0.48
	Sperm concentration per cu. mm.	3,852,000	4,182,000	2,938,000	3,657,000
	Total number of sperms per ejaculate	1,455,110,000	1,433,370,000	1,713,520,000	1,534,000,000
	pH-value of semen	7.30	8.00	7.50	7.60
July to October	Volume per ejaculate ml.	0.28	0.12	0.64	0.35
	Sperm concentration per cu. mm.	2,224,000	2,091,000	1,505,000	1,940,000
	Total number of sperms per ejaculate	678,960,000	219,260,000	1,024,080,000	640,770,000
	pH-value of semen	7.05	7.08	7.30	7.14
November to February	Volume per ejaculate ml.	0.50	0.42	0.68	0.53
	Sperm concentration per cu. mm.	2,384,000	1,421,000	1,308,000	1,728,000
	Total number of sperms per ejaculate	1,202,190,000	585,760,000	869,650,000	885,870,000
	pH-value of semen	7.25	7.20	7.03	7.20
Yearly average per bird	Volume per ejaculate ml.	0.42	0.29	0.64	0.45
	Sperm concentration per cu. mm.	2,820,000	2,564,000	1,917,000	2,441,000
	Total number of sperms per ejaculate	1,112,086,000	746,130,000	1,202,410,000	1,020,213,000
	pH-value of semen	7.20	7.42	7.27	7.31

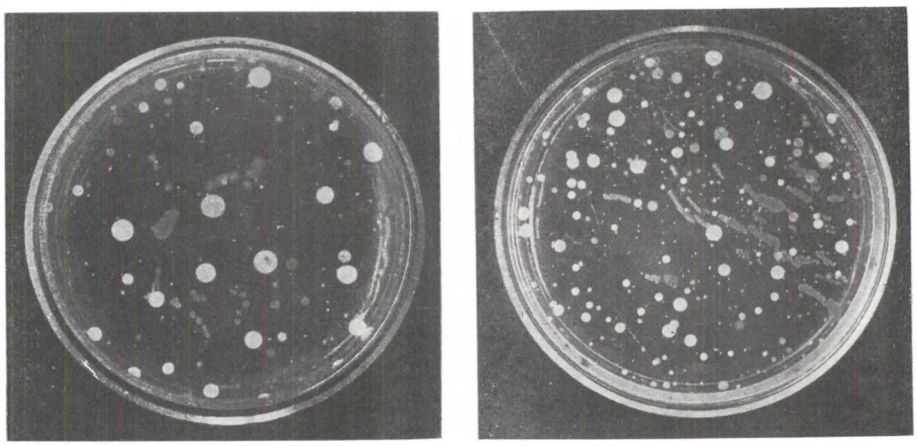
method has shown the average volume of 0.48 ml. in the best season, 0.35 ml. in the worst season and 0.45 ml. on the yearly average. As a whole, these amounts of semen are about two times larger than those collected by the old massage method regardless of the sampled season.[1)]

Another characteristics to this method is that semen can be recovered in the least contaminated condition. During the first half period of this work in which semen collection was performed without inserting a tampon into the rectum, 24 samples out of 160 or 15% had to be discarded due to soiling to faeces. Later, the rectum properly being stoppered, the discarded samples could be reduced to 6 : 126 or about 5%.

More pronounced are the results of bacterial counts on each ten trials in which

1) When the seasonal fluctuations are not considered, the examinations of 286 semina collected from 15 White Leghorn cocks have shown that the volume per ejaculate averaged 0.448 ml. ranging from 0.05 to 2.00 ml., the sperm concentraiton per cu. mm. averaged 2,576,900 ranging from 114,000 to 5,544,000, the total number of sperms per ejaculate averaged 1,121,605,000 ranging from 26,220,000 to 2,309,400,000.

semen has been alternately collected from four cocks by both the present method and the old massage method using neither bird holder nor semen receptacle attached to the bird. The bacterial counts were conducted by plating semen samples on "standard method" agar (Fig. 5). Although in the old method only clean portions were attentively recovered, the number of bacteria per ml. semen amounted 259,700 on the average with a range of 67,000 to 648,000, whereas in the present method it revealed an average count of 37,800 ranging from 10,000 to 159,000; the "cleanness" of semen in the latter case was almost seven times higher than in the former.



A B

Fig. 5. Bacterial cultures of two semen samples from a White Leghorn cock which was subjected to the "Hiroshima" method (A) and the old method of massage (B). The difference of both the methods in contamination is obvious.

4. DISCUSSION

Up to date, the first and second method of BURROWS and QUINN (1935 : 1937) in collecting semen from the cock have been generally used for the purpose of artificial insemination. Especially the application of bird holder in the second method have greatly facilitated the operation. The holder devised by BURORWS and QUINN (1937) is a wooden dock-like stand stalled on a base. The stand is fitted for a bird trunk while the head, escutcheon and legs of bird are put outside of it. According to these authors the stand is high enough to prevent the bird from obtaining a footing on the base but it appears the suspension of the feet is no main object of this holder; in the figures given by them the cock is standing on tiptoe. The same pose of the cock is seen in the figures of KAMAR (1958) who applied a holder similar to that of BURROWS and QUINN. As a matter of fact, this type of bird holder seems to require another equipments for holding the head and the wings by means of pillory and strap, respectively. The object of employing this holder appears rather to sub-

stitute operator's hands.

Since the massage inducing the orgasm should be of quite comfortable nature, it is desirable to put the bird at a free state as much as possible. Considering this, for the holder herein proposed, special emphasis has been placed upon the complete suspention of cock's feet with the aim of putting the bird almost in "powerless" condition without being compelled by any form of restraint. In this pose the ejaculatory response rapidly takes place accompanying with a complete spasm. This rapid response natually gives less opportunity of admixture of faeces and urates. Further, the holder considered, in contrast to the dock-type holder, permits the abdominal massage in a greater extent of bird's body in favor of the interspace of its stands.

The application of the holder is most advantageous in the point that it enables the attachment of semen receptacle over the vent of the cock. In the use, the receptacle resembles to the semen collector devised by PARKER (1939) in the face that semen upon ejaculation can be directly caught into it without giving room for contamination and dust mixing, but it differs greatly in the point that there is no need of mating. The first fact is specially of great importance as compared with the massage methods hitherto applied in which the operator has to hold a beaker or funnel beneath the vent of bird in time just before ejaculation. Also, there is no such worry of intermingling of exudates, blood materials and any other cellular debris, as in the method of "milking" the copulatory organ. LAKE (1956) is of opinion that the fluids from the lymph folds and vascular bodies effect detrimentally upon the sperms in the artificial fowl semen which is obtained by squeezing the copulatory organ. If it be so, such a detrimental effect due to the milking is completely eliminated by the present method.

As for the semen characteristics, it was found that the semen thus obtained was about two times greater in volume and seven times smaller in bacterial contamination as compared with that collected by the old methods while the motility of sperms were always most active. It may, therefore, be concluded that the genuine nature of fowl semen can be maintained at the collection by the abdominal massage, when the "Hiroshima" pattern bird holder is employed in combining with the "Hiroshima" model semen receptacle.

5. SUMMARY

A new model of bird-holder in combination with a semen receptacle was constructed for the purpose of collecting the fowl semen without changing its genuine nature when the massage method is applied. The results obtained are summarized as follows:

The design of the holder here presented is to keep a bird completely in suspending pose without footing on the base whereas its abdomen is fully exposed to the operator's hand. The bird mounted on this holder can never attempt to escape; upon massage it shows an ejaculatory response within a few seconds, presumably in

part due to the conditioned reflex. This rapid response naturally gives less opportunity of admixture of faeces and urates.

The application of the holder of this type enables a funnel-shaped receptacle to be attached over the vent of the bird for catching the semen directly from the bird. During the cold season, the receptacle can be equipped with a container holding water of 41° to 42°C for protecting sperms from temperature shock.

On examining, the semen thus collected was approximately two times larger in volume per ejaculate and seven times smaller in bacterial counts as compared with that obtained by the method lacking these equipments. Since no "milking" or squeezing process is necessary in this method, occasional admixture of transudates, blood cells etc. can be completely eliminated; thus, the fowl semen can be collected in a more natural state than by the massage methods without using them.

PART II
TECHNIQUE OF SEMEN INTRODUCTION

In the artificial insemination of the fowl, JULL and QUINN (1931) found that the placing of semen on the external orifice of the oviduct, resulted only in 19.1% fertility of 969 eggs incubated. Later. QUINN and BURROWS (1936) devised a methed of semen introduction which consisted of exposing the oviduct by pressing the abdomen and the direct introduction of semen by using a common 1-cc. tuberculin syringe at a distance 2 to 3 cm. deep into the everted vagina. Ever since, this technique has come into general use. Since the syringe of such a type has very short nozzle, some workers subsequently equipped it with a glass extention tube for the purpose of deeper injection. Nevertheless, viewed from the length and form of the attached tube, the semen cannot be discharged into the uterus even by such an extension ; hence the vaginal insemination seems to be a common technique for the fowl at present.

In this connection, the data obtained by ALLEN and BOBR (1955) are worthy of note. These authors attempted intrauterine insemination with the aim of keeping the fertilizing capacity of the semen diluted with 15%-glycerol containing buffers which were found to be completely infertile, if glycerol was not removed by dialysis. The results of the experiments by using a syringe connected with a glass cannula about 10.5 cm. long showed a remarkable difference between vaginal and uterine inseminations in the fertility of eggs produced during the first week after handling; the uterine insemination exhibited 73% fertility of 66 eggs whereas no fertile eggs were secured among 64 eggs produced by the vaginal insemination. On the other hand, there are many workers who have pointed out the disturbances of ovarian and oviducal function caused by various manipulative treatments of the posterior regions of the egg tract of hens (GILBERT and LAKE, 1963). Hence, there appears that very little attention has been drawn to the effect of intrauterine insemination for the domestic hen.

From this point of view, the experiments were undertaken to find out the effects

of uterine and vaginal methods of insemination upon the fertility of hens, stress being laid first on the design of suitable inseminator for deep insemination, secondly on the evaluation of results of fertility tests.

1. "HIROSHIMA" PATTERN, AVIAN INSEMINATOR AND ITS MANIPULATION

The inseminator here presented consists of three parts: 1-cc. glass tuberculin syringe, 9 cm. long polyethylene tube and 1.5 cm. long round-tipped and side-holed glass tube (Fig. 6). This inseminator differs from those used by previous workers (PARKER, MCKENZIE and KEMPSTER, 1942; ALLEN and BOBR, 1955; LAKE, 1960) in the point applying a polyethylene tube with a holed hollow glass knob instead of a glass tube for extension. Since the uterine and vaginal parts of the hen's egg tract represent no direct passage from the vent, a certain flexibility of the inseminator is necessary at manipulation especially when a deep insemination is to be done. For this purpose, the polyethylene tube has proved to be most suitable because of its flexibility with some hardness. In addition, the hollow sidely-holed glass knob plays as a path-finder sliding along the inner wall of the egg tract. Of course, any danger to the bird from broken glass is completely eliminated by this construction.

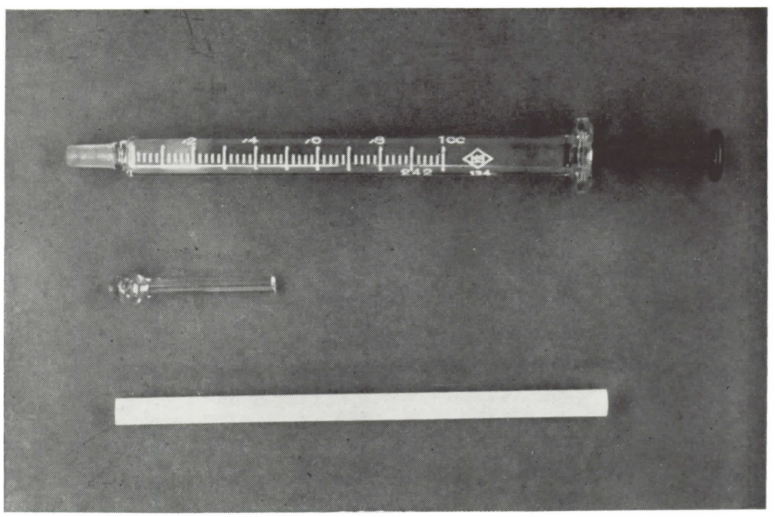


Fig. 6. An avian inseminator consisting of 1-cc. glass tuberculin syringe, 9 cm. long polyethylene tube and 1.5 cm. long round-tipped glass tube.

Before use, the inseminator was sterilized as usual by boiling or steam. The insertion of the inseminator was performed as follows: The hen for insemination is first brought up to a bird holder in the same pose as the cock for semen collection (YAMANE, TSUKUNAGA and TAKAHASHI, 1962, b). The holder is placed either on floor or on table with a slight inclination for ascending the hen's rear.

An assistant, keeping gently the hen's back with the left hand, applies a pressure to her abdomen beneath the ilio-pubal regions with the right hand from caudal to oral. This manipulation induces an eversion of the vagina and enables the inseminator to be inserted with great ease (Fig. 7).

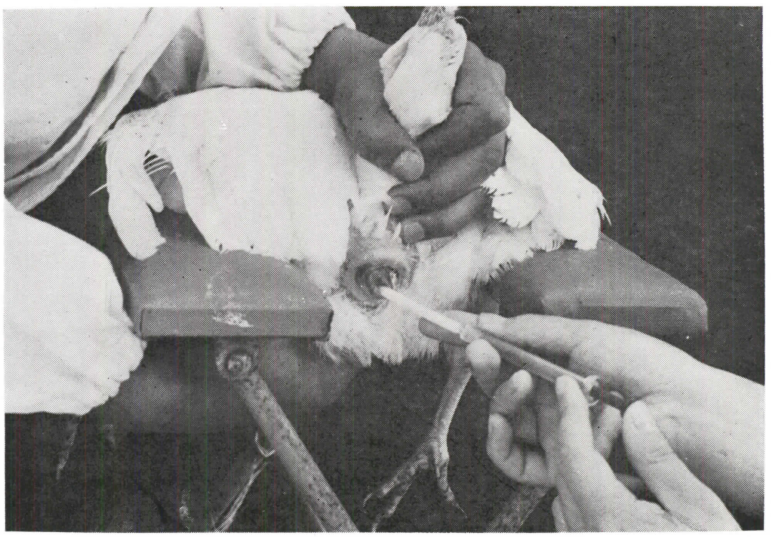


Fig. 7. Insertion of inseminator tube into the everted vagina at insemination.

As seen in the diagram given by GILBERT and LAKE (1963) and in the description of FUJII (1963), the posterior part of the hen's egg tract in natural position exhibits nearly a sigmoid form which is firmly held by a serous membrance and connective tissue.

When the inseminator is introduced a little left downward through the everted vaginal orifice, a feeble resistance is first felt probably due to a small flexure of the vagina. On farther insertion of the inseminator, the second but strong resistance is met due to the flexture of the anterior part of the vagina. Now, the semen is discharged on this point at the vaginal insemination. For the uterine insemination, the inseminator is farther propelled gently right upward. Then the inseminator, passing through the utero-vaginal junction and the third flexture proximal to it, is admitted freely into the caudal end of the uterus where the semen is to be deposited. Measured in the everted state of the vagina, these points situate in the White Leghorn hen approximately 4 to 5 cm. and 7 to 8 cm. deep from the vent in the vaginal and uterine method, respectively. The topographic relation of these two points of semen discharge is shown in Fig. 8 in which the posterior regions of the egg tract is stretched out and incised.

It must also be noticed that difficulties and ease of introducing the inseminator greatly depends upon the amount of rectal content; the rectum filled with feces often makes a barrier in much greater degree than does the structure of the utero-vaginal regions.

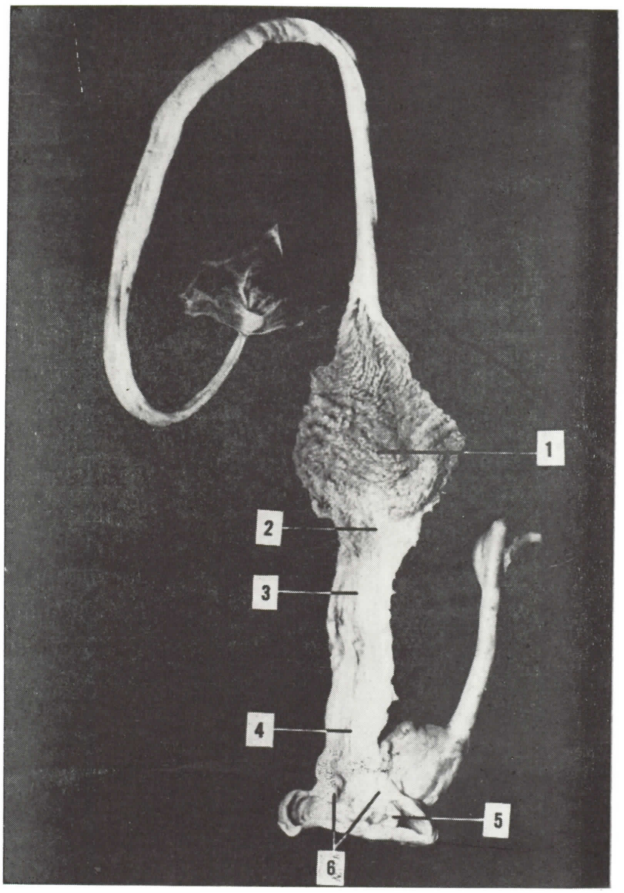


Fig. 8. Topographic relation of the sites of semen deposition in the egg tract of the hen; uterus, vagina and cloaca are extended and incised.
1. Site of uterine insemination. 2. Utero-vaginal junction. 3. Site of vaginal insemination. 4. Site where a feeble resistance is felt at inserting the inseminator tube. 5. Anus. 6. External vaginal orifice, incised.

Just before insemination, the hand pressure applying to the hen's abdomen is released while the inseminator syringe should follow the retraction of the vagina by keeping slight pressure on it. After injection of semen, the inseminator is quickly withdrawn.

2. EXPERIMENTAL METHOD

Two lots of experiments, viz., uterine and vaginal inseminations, were carried out by employing the inseminator described above. The semen samples were collected at 2.00 to 3.00 p.m. by abdominal massage from eight 2-year old White Leghorn cocks applying a bird holder and a semen receptacle previously reported (YAMANE, TSUKUNAGA and TAKAHASHI, 1962, b). The semina used shortly ($\frac{1}{2}$–4 hours)

after collection were kept at room temperature which might have varied from time to time, either diluted or undiluted, but the stored samples were always diluted with a yolk-citrate buffer at the rate 1 : 4 and kept at 2–5°C in a refrigerator after gradual cooling. This dilutor consists of one volume 6.5% $Na_3C_6H_5O_7 \cdot 2H_2O$ solution, one volume 9.0% glucose solution and two volumes fowl egg yolk. As for the details of the dilutor here used see Part III.

Uterine and vaginal inseminations were simultaneously performed upon two flocks of hens with fractions of one and the same ejaculate for eliminating the errors due to the individuality of the males and fluctuations in qualities due to the different samples. A dose of semen per injection was 0.2 ml. with diluted samples but it amounted 0.05 ml. in in black letters condition.

The results of inseminations were examined from the two points of view: the flock fertility viz., percentage of fertilized hens on simultaneously inseminated hens with fractions of the same ejaculate, and the "first-week egg fertility" viz., percentage of fertile eggs on eggs produced by each flock during the first week reckoned from the second day following insemination.[1] Fertility of eggs were tested by candling on the fifth or sixth day of incubation.

3. EXPERIMENTAL RESULTS

The results of experiments obtained are summarized in Table II to IV in which the uterine and vaginal methods are abbreviated as U and V respectively, and the figures in blackletters indicate a superiority of one method to the other.

First, on exmining Table II and III, it is noticeable that these is a tendency of the superiority of the uterine insemination in fertility per flock as well as in fertility per eggs produced in the first week. As for the flock fertility, 10 uterine inseminations out of 11 have exhibited a full fertility level, whereas in the vaginal method this level has been attained by 6 inseminations, the other 5 cases being varied only from 25 to 50% fertility. Thus, the average flock fertility has shown 96.9% in the uterine method and 75.0% in the vaginal method. In fertility of eggs of the first week, the frequency of cases where a higher fertility has been secured is 6 out of 11 in the uterine method against 4 in the vaginal method, the average showing 75.0% and 62.6% respectively.

The preponderance of the uterine insemination is more obvious in the trial tested with the semen which were kept at 2 to 5°C for 48 and 72 hours, so to speak, with the "environmentally stabilized" semina. As seen in Table IV, the uterine inseminations with 48- and 72-hour old semina have given 81.0% and 83.0% in flock fertility, respectively, whereas the vaginal inseminations have shown correspondingly only 40.0% and 66.0%.

1) The egg laid on the first day after insemination is unexceptionally infertile when the hen is inseminated afternoon.

Table II. Feritilty of Flocks in Parallel Inseminations by Uterine and Vaginal Methods with Semen kept at Room Temperature.

Flock No.	Hens inseminated		Hens fertilized		Fertility per flock		Semen condition
	U	V	U	V	U	V	
1	3	3	3	3	100	100	Undiluted; ½-hour old.
2	5	8	5	8	100	100	〃 〃
3	5	4	5	4	100	100	〃 〃
4	3	3	3	3	100	100	〃 〃
5	2	2	2	1	**100**	50	Diluted; 〃
6	2	4	2	1	**100**	25	〃 〃
7	5	2	5	2	100	100	Diluted; 1-hour old.
8	6	4	6	2	**100**	50	〃 2-hour old.
9	3	4	3	2	**100**	50	〃 〃
10	3	2	2	2	66	**100**	〃 〃
11	4	4	4	2	**100**	50	〃 4-hour old.
	Total				Average		
	41	40	40	30	96.9	75.0	

N. B. For explation of the tables, see the text.

Table III. First Week Fertility of Eggs in Parallel Inseminations by Uterine and Vaginal Methods with Semen kept at Room Temperature.

Flock No.	Eggs produced		Eggs fertilized		First week fertility of eggs %		Semen condition
	U	V	U	V	U	V	
1	12	13	11	12	91.7	92.3	Undiluted; ½-hour old.
2	23	31	13	25	56.5	**80.6**	〃 〃
3	18	17	15	7	**83.3**	41.2	〃 〃
4	10	13	9	10	**90.0**	76.9	〃 〃
5	12	5	12	2	**100.0**	40.0	Diluted; 〃
6	7	3	6	2	**85.7**	66.0	〃 〃
7	21	11	20	6	**95.2**	54.5	Diluted; 1-hour old.
8	23	9	13	3	**56.5**	33.3	〃 2-hour old.
9	13	11	8	7	61.5	**63.6**	〃 〃
10	11	10	6	6	54.5	**60.0**	〃 〃
11	12	10	6	8	50.0	**80.0**	〃 4-hour old.
	Total				Average		
	162	133	119	88	75.0	62.6	

Table IV. Fertility of Flocks in Parallel Inseminations by Uterine and Vaginal Methods with Semen Stored at 2 to 5°C.

Flock No.	Hens inseminated		Hens fertilized		Fertility per flock %		Semen condition
	U	V	U	V	U	V	
12	16	5	13	2	**81.0**	40.0	Diluted; 48-hour old.
13	6	3	5	2	**83.0**	66.0	Diluted; 72-hour old.

Table V. First Week Fertility of Eggs in Parallel Inseminations by Uterine and Vaginal Methods with Semen Stored at 2 to 5°C.

Flock No.	Eggs produced		Eggs fertilized		First week fertility of eggs %		Semen condition
	U	V	U	V	U	V	
12	72	8	58	3	**80.5**	37.5	Diluted; 48-hour old.
13	26	8	15	3	**57.7**	37.5	Diluted; 72-hour old.

This distinction is likewise remarkable in the first week fertility of eggs which has shown 80.5% with 48- hour old semen and 57.7% with 72- hour old semen for the uterine insemination, respectively, in contrast to 37.5% with both kinds of the semen for the vaginal insemination.

At last, it may not be out of place to state here that no significant difference in egg production could be observed in subsequent laying records between the hens treated with both methods of insemination.

Table VI. Average Egg Production per Hen during the Period from 2. to 15. Day Following a Single Inseminathon.

Hens inseminated		Eggs produced biweekly		Biweekly egg production per hen	
U	V	U	V	U	V
57	44	505	392	8.88	8.90

As shown in Table VI, the average number of eggs per hen produced during the period from the second to the fifteenth day following a single insemination was 8.88 and 8.90 in the uterine and vaginal method, respectively.

4. TIME OF INSEMINATION

PARKER (1945) is of opinion that the fertility and hatchability of eggs can be promoted by inseminating hens in the afternoon. MOORE and BYERLEY (1942) obtained better fertility, during days 2–6 inclusive, if an egg was present in the shell gland at the time of insemination than if one was present in the albumen-secreting part of the oviduct (cit. from LAKE, 1962, p. 348). LAKE and WOOD-GUSH (1956) have also found that the largest yield of seemen of the cock occurs late afternoon. On the other hand, SCHINDLER and his co-workers (1957) have shown that there is no difference in fertility between hens inseminated while containing a shell-egg in their uterus and those not containing such an egg. Aside from the problem of fertility, however, there is technical reason to believe that the afternoon is preferable to the forenoon for insemination, needless to say of avoiding the alarm of other hens in the same house during the egg-lying time. It must be taken in consideration that hen's vagina some hours after egg-laying, i.e. in the early afternoon is strikingly

loosened and very liable to evert, consequently it permits the insertion of inseminator tube with ease whereas later towards the evening this becomes more difficult.[1] From these points of view, the appropriate time of insemination for hens seems to be about 2.00 to 3.00 p.m. with some range.

5. DISCCUSSION

In the foregoing experiments, it was aimed to study comparatively the effects of uterine and vaginal inseminations on the fertility of hens. For this purpose, a new model inseminator was devised, the use of which made the deposition of semen possible both beyond or this side the utero-vaginal junction without giving injury to the bird nor lowering the egg production.

In the artificial insemination, there are innumerable factors influencing the fertility of hens, the interaction of which can often mislead the conclusion. Hence, every precaution was taken against these factors in comparing the two methods of insemination. First of all, possible errors due to the individuality of males and the fluctuation in qualities of different semen samples were completely eliminated by using the fractions of the same ejaculate and allotting the same amount of semen to each hen of the lots of uterine and vaginal inseminations. The fluctuation of fertility caused by the individuality of hens due to the heredity and external conditions were partly inevitable but the comparison of the fertility rates of eggs produced during the first week, strictly speaking, the second to the eighth day after treatment in both the lots would make them avoidable to a certain degree if this is done by considering the bird fertility per flock simultaneously treated. It must also be remembered that any environmental factors inducing the decline in viability of sperms may affect the fertility of eggs more conspicuously than does the site of semen injection. This fact is to be seen in Table II and III where the vaginal insemination has exceptionally surpassed the uterine method in fertility. In the cases where the semen was used in diluted form but after keeping at room temperature some hours long (Flock No. 9 to 11), reverse effects have appeared since such conditions can affect the viability and fertility of sperms in varying ways. However, the constant condition in which semen was subjected to the dilution with the egg yolk-citrate buffer and to the temperature 2 to 5°C has excluded this failure; such a condition has been proved to be very favorable for keeping the fertility potential of semen as long as about two weeks after insemination (YAMANE, TSUKUNAGA and TAKAHASHI, 1962, a).

All things considerd, the data in Table III and IV are consistent in the fertility rates per flock treated as well as per eggs produced during the first week following insemination, and a higher fertility has been obtained by the uterine insemination; this plainly indicates its superiority to the vaginal method. It is therefore deduced

1) The introduction of semen over a shell-egg deep into the uterus can be done by application of the inseminator above deseribed but it requires much time and skill.

that the uterine part of the egg tract is the proper place where the sperms can keep their fertility potential for a long time. The uterine insemination is nothing else but a direct introduction of sperms into their natural environment whereas the vaginal insemination is merely a deposition of sperms on a path to their proper site; the distinction in the effects of both the methods is self-evident. In reference to this finding, the puzzling data of ALLEN and BOBR (1955) is of considerable interest. By nature, fowl sperms in dilutors giving a final concentration of 15% glycerol had been known to be completely infertile. Despite of this, relatively a high fertility was secured by introducing such sperms into the uterus. This demonstrates clearly a rapid extrication of the sperms from the unfavorable suspending medium to their proper environment.

In the hen, the uterus and utero-vaginal junction appear to be a very sensitive place since these regions, according to GILBERT and LAKE (1963), are well innervated and abundant of nerve cells. In fact, there are numerous works reporting the disturbances in either ovarian or oviducal function caused by various manipulative treatments of the distal regions of the oviduct. Under the experimental conditions described above, however, no serious effect upon the laying activity of hens were observed; a momentary insertion of inseminator does not seem to act so irritatingly, if properly manipulated.

As for the time of insemination, there have been two opinions of controversy, either forenoon or afternoon. From the technical point of view, however, the afternoon insemination is preferable to the forenoon, if the uterine method should be applied, basing upon the fact that the vaginal orifice remains relaxed for a while in the afternoon; it permits the inseminator tube to be inserted with easiness.

6. SUMMARY

In the artificial insemination of the Whie Leghorn hen, comparative experiments were conducted to determine the effective site of semen deposition upon fertility. An inseminator eqipped with a 10 cm. long polyethylene catheter ending in a round-tipped sidely-holed short glass tube was designed for deep insemination. Parallel inseminations were performed into the uterus and vagina for different hens with the same amount of fractions of the same ejaculate, either diluted or undiluted. Higher fertility rates per flock and per first-week eggs were obtained by the uterine insemination than by the vaginal method. No significant distinction in subsequent egg production was observed between both the methods, if properly manipulated.

PART III
PROBLEM OF DILUTOR FOR FOWL SEMEN

1. SIGNIFICANCE OF DILUTOR FOR FOWL SEMEN

Hitherto, the use of semen dilutor has been generally believed to be of little significanc for the artificial insemination of the fowl in view of the fact that a single ejaculate can be allotted for ten hens or more even in undiluted condition, and that the long-term storage of semen is not practicable because of easiness of shipment of birds or eggs. However, a considerable development in the battery or cage management of poultry has taken place in this country and enormous demands for hatching eggs of the strains of high performance are increasing year after year; the artificial insemination of the fowl has become more important than in earlier times. As a consequence, the semen dilutor is not less important for the insemination of the fowl compared with that for mammalian farm animal. Nevetheless, at present, the fundamental knowledge on the physiology of fowl sperms is so meager that the technician of poultry breeding is apt to be preoccupied with the experiences of mammalian sperms. One should know first a great difference in physiology between avian and mammalian sperms which shall be described in the following articles.

2. PHENOMENON OF NECK-BENDING OF FOWL SPERMS

During the course of the research on the characteristics of fowl semen, a peculiar morphological change of the sperm came to the present authors' notice which manifested in bending of the neck of sperm in natural semen *in vitro* (Fig. 9). The bending occurs not only at the junction between the midpiece and the head of sperm but also at every point through the midpiece; it can take place in an acute or oblique angle and in an extreme case the sperm can be doubled, its head and tail being firmly adhered each other (Fig. 10, 1–6).

The occurence of sperm abnoramalities in fowl semen has already been noticed by numerous authors as SAMPSON and WARREN (1939), PARKER, MCKENZIE and KEMPSTER (1942), TAKEDA (1961) and others, but most authors have indiscriminately treated the neck-bending from other types of sperm abnormalities; sometimes it has been taken for normal (PARKER et al., 1942). Perhaps, SAEKI (1960) was the first worker who centered upon the defect termed "croock-neck" of fowl sperms and confirmed its relation to low fertility of fowl semen though no explanation was made about its causal factor.

Since no sperm within the *vasa deferentia* and in the fresh ejaculate shows neck-bending, this defectiveness must be taken for a posto-ejaculatory phenomenon.[1)]

1) Observations on the semina squeezed from the *vasa* of sacrifized cocks showed no out-break of neck-bending of sperms though they were most active in movement even at the storage of 48 hours or more *in vitro*.

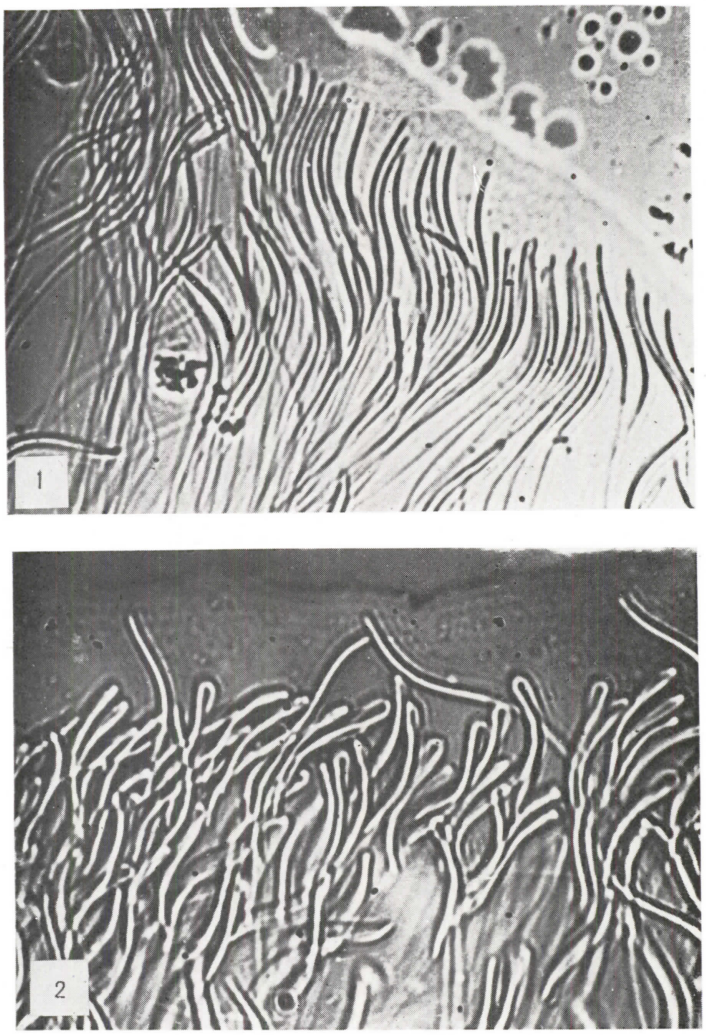


Fig. 9. 1, fowl sperms showing the normal form with the most active motility in a hanging drop of semen immediately after collection; 2, the same semen sample 6 hours after collection exhibiting the remarkable neck-bending of sperms throughtout the optical field.

In fact it occurs in the ejaculate more and more with the lapse of time *in vitro*. Fig. 9 illustrates a sample of semen under hanging condition just after collection containing most active sperms (1) and the same sample six hours after collection exhibiting the remarkable neck-bending of sperms (2).

The locomotive action of the neck-bent sperms appears mostly to be a forward movement, very often misleading as if they were normal or short-headed sperms.

Concerning the qualities of seminal plasma of the cock, NISHIYAMA (1951) reported that upon ejaculation a considerable amount of "transparent fluid" is secreted from either the phallus or the lymph folds bilaterally attached to the former or

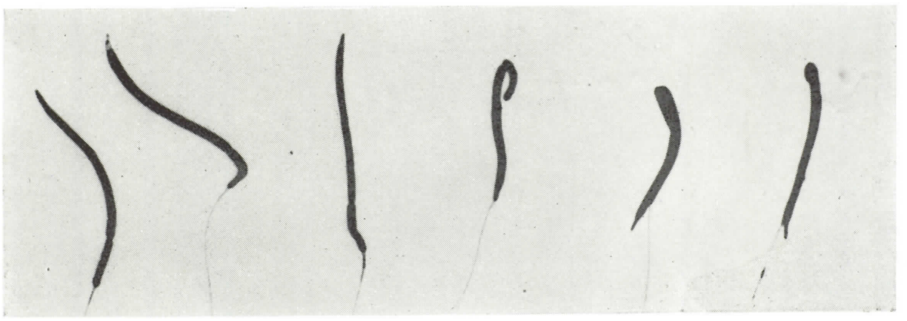


Fig. 10. Individual sperms showing the neck-bending of different degrees. 1, a normal sperm; 2-6, neck-bent sperms.

from both the organs and added to the dense semen ejected from the *vasa*. In view of this, it was suggested that the neck-bending of sperms must be closely related to the properties of seminal plasma for which the transparent fluid plays a great role. This presumption was further proven by the following experiment. Every 3 semen samples were repeatedly collected from 3 cocks with an interval of 30 minutes for each bird. The determination of percentage of neck-bent sperms were carried out with smeared semen. A drop of semen on a slide was fixed in formalin vapor for

Table VII. Characteristics of semen collected with an interval of every 30 minutes.

No. cocks	No. ejaculates	Valume per ejaculate (cc.)	pH	Apperarance	Sperm concentration per cu. mm. (millions)	Sperm activity (scores)	Percentage of neck-bent sperms at storage (hrs.)			
							0	6	24	48
73	1	1.1	6.8	milky white	2,624,000	100	5	14.5	16.5	41.0
	2	0.3	6.8	thin watery	1,132,000	77.5	60	90	95	95
	3	0.25	7.0	greyish watery	924,000	95	65	90	95	100
73	1	0.5	7.6	milky white	3,168,000	100	16	25	47	85
	2	0.2	7.0	〃	2,112,000	100	12	30	56	74
	3	0.15	7.0	light colored watery	1,256,000	92.5	37	58	71	90
52	1	0.35	7.6	milky white	3,432,000	100	7	16	19	24
	2	0.3	7.4	〃	2,568,000	100	4	14	34	80
	3	0.075	6.4	light colored watery	936.000	100	5	17	26	73
53	1	0.2	7.4	milky white	3,812,000	95	30	30	30	35
	2	0.45	7.6	〃	1,364,000	100	10	15	23	30
	3	0.2	7.5	light colored watery	892,000	50	50	62	65	70

1–2 hours, then smeared and put into methanol for a few minutes; after washing in distilled water it was simply stained with carbol fuchsin or in combination with Giemsa solution. Microscopic estimations of neck-bent sperms were made every time for 500 sperms in the random fields by use of the oil immersion lens and its percentage to normal sperms were computed (Table VII).

As seen in Table VII, a considerable difference in the appearance of semen among the ejaculates which were collected with an interval of every 30 minutes; it changed from thick milky white to thin watery consistency (see also Fig. 11). This is caused not only by the decrease in sperm concetration, but also by the increase in relative amount of transparent fluid because a greater part of semen stored within the *vasa* is discharged at the first ejaculation. The data showed that the thinner was the seminal plasma, i.e., the greater was the amount of transparent fluid mixed, the greater was the number of neck-bent sperms. It is, therefore, natural that the repeatedly followed ejaculation inducing thinner seminal plasma and lower sperm concentration has associated with a higher percentage of defective sperms.

Since a high percentage of neck-bent sperms was observed both in the semen with high and low pH, the pH seems to have little concern with the neck-bending of sperms.

It is very peculiar that the temperature shock induces a remarkable increase of neck-bent sperms within a short time the mechanism of which will be discussed later in another place.

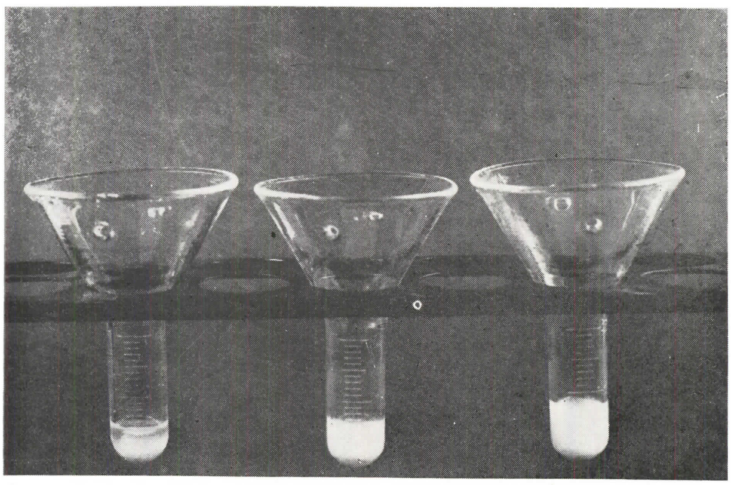


Fig. 11. Three ejaculates from a cock repeatedly collected with an interval of 30 minutes; right to left the first, second and third ejaculate. Compare the turbidity of seminal plasma and the volume of sedimented sperms in three ejaculates.

3. DECLINE IN FERTILITY OF FOWL SEMEN *IN VITRO*

As shown above, fowl sperms in natural semen during storage are very liable to a peculiar defect, i.e. neck-bending, progressively with the lapse of time. It is unlikely that such defective sperms can penetrate into the egg cell though they could

Table VIII. Results of a single insemination per hen with natural semen stored at 2-5°C.

No. hens inseminated	Storage period of semen	Eggs laid	Eggs fertilized	Average 2-week fertility per hen %
		during 2 weeks after insemination		
I_1		4	3	75.0
I_2		8	7	87.0
I_3		11	0	0
A_5		4	3	75.0
A_6		8	3	37.5
A_7	½—1 hr.	11	4	36.3
A_8		3	2	66.6
H_1		13	8	61.5
H_2		7	6	85.7
H_3		4	4	100.0
H_4		11	3	27.2
Flock average		7.6	4.3	65.18
A_6		12	0	0
A_7		12	2	16.6
A_8	2—2½ hrs.	4	3	75.0
A_9		10	1	10.0
A_{10}		4	2	50.0
Flock average		8.4	2.0	37.9
H_6		1	1	100.0
H_7		12	1	8.3
H_8		10	4	40.0
H_9	6 hrs.	2	1	50.0
H_{10}		4	1	25.0
H_{11}		3	2	66.6
Flock average		5.3	1.7	48.31
D_1		9	0	0
D_2		9	0	0
D_3	24 hrs.	10	1	10.0
D_4		7	0	0
D_5		10	4	40.0
Flock average		9	2.5	25.0
D_7	48 hrs.	9	0	0
D_8		7	0	0
Flock average		8	0	0

retain high motlity, moreover when considered that the neck-bending might be only a part of damages over all the sperm visible under the light microscope.

A series of insemination experiments with semen kept at 2–5°C *in vitro* has shown a marked decrease in fertility, although the insemination was done by uterine method. Average biweekly fertility on eggs per flock resulted in 65, 38, 48 and 25% at the storage of $\frac{1}{2}$ to 1 hour, 2–2$\frac{1}{2}$ hours, 6 hours and 24 hours, respectively, and null at all after 48 hours storage (Table VIII).

Since the motility of sperms did not decline during the span of storage above mentioned, it seems to be justifiable to infer that the neck-bending of sperms partly responsible to the decrease in fertility. In this connection, the experimental data obtained by SAEKI (1960) are of great importance; the author, using the semen diluted with only fructose which was known to be most effective to sperm longevity, has secured 85, 59 and 37% fertility, respectively, when the percentage of neck-bent sperms were 15, 24 and 37. The correlation between the percentage of the neck-bent sperms and the fertility has revealed −0.77.

4. NATURE OF NECK-BENDING OF FOWL SPERMS

In view of the fact that the extensive occurrence of neck-bent sperms is a posto-ejaculatory constant phenomenon peculiar to fowl semen, a close relation of the structure of sperms to the quality of seminal plasma in the fowl has to be considered. Since the bending takes place only at the midpiece, i.e., the junction between the head and the tail, this part of the sperm must be the *locus minoris registentie.* If any stress exerts upon this weak point, the bending of the sperm will be a natural consequence in which the movement of the tail plays a great role. It is plainly a vital phenomenon because no neck-bending takes place in the immobile sperm. Hence, the more beneficial is the medium for the viability of sperms suspended, the higher is the percentage of neck-bent sperms in it. The data presented by SAEKI (1960) definitely substantiate this assumption; the author found that 5.2% fructose was a suitable dilutor for storaging sperms but it showed the highest percentage of neck-bent sperms compared with 0.9% NaCl- and Tyrode solution or semen under undiluted condition.

As for the stress caused by the seminal plasma, its hydrating effect on the midpiece is first to be considered. Microscopical examination of the midpiece of neck-bent sperms at high magnification revealed that this part was more or less swollen up or disrupted. The figure resembled the damage to be observed in the sperm when immersed into distilled water though in a less degree; in the latter case a remarkable bending of the neck, eventually looping of the tail, was observed in association with the segmentation or disintegration of the midpiece (Fig 12).

At present, our knowlegde of the structure of the fowl sperm has been greatly extended by use of the electron microscope. According to GRIGG and HODGE (1949), in the fowl sperm treated with distilled water, the thin outer membrance is disrupted and a number of granules are liberated. For the most part of the granules,

Fig. 12. Morphological changes of fowl sperms immersed into distilled water. Note a neck-bending associated with more or less segmentation of the midpiece.

which are themselves swollen and broken down to some extent by distilled water. BONADONNA (1954) reports also that the wrapper surrounding the midpiece appears to be the most sensitive to the action of distilled water.

All things considered, in the fowl the seminal plasma seems to act upon the sperm in the same way as does distilled water, by hydrating the midpiece; in other words, the seminal plasma appears to be hypotonic to the midpiece at least. For this assumption a series of experiments conducted by TSUKUNAGA and TAKAHASHI (1961) was evidential when glucose solution of varying concentrations were applied ranging from 4.0 to 12.0%. The advantage of using glucose, beside its anaerobiotic effect upon the sperm, lies in the elimination of ion actions (YAMANE, 1921). Every semen sample was diluted 1 : 4 with a glucose solution and stored at 2–5°C. Examinations of sperm activity took place every 24 hours; it was expressed in a sum total of percentages of each motility grade, which was scored with as follows: very active 1.00, active 0.75, weak 0.50 and very weak o.25. The determination of percentage of neck-bent sperms were carried out with smeared semen as above described.

As seen in Fig. 13, it was found that in 4.0 g/dl glucose solution, the activity of sperms was lowest accompanying the greatest number of neck-bent sperms both at 24- and 48- hour storage. With increase of glucose concentration up to 9,0 or 10 g/dl, the activity became higher whereas the number of neck-bent sperms almost reciprocally decreased. Thus, the optimum concentration of glucose solution for suppressing the neck-bending of the sperm and keeping the highest activity at both 24– and 48- hour storage lies between the concentration 9.0 to 10.0 g/dl., corresponding to $\Delta = -0.93$°C to -0.105°C. This indicates that in the fowl the seminal plasma is decidedly hypotonic to the midpiece of the sperm; its hydrating effect induces the disintegration or disruption of the midpiece which manifests in the neck-bending due to the vibration of the tail. It follows, therefore, that any medium more hypo-

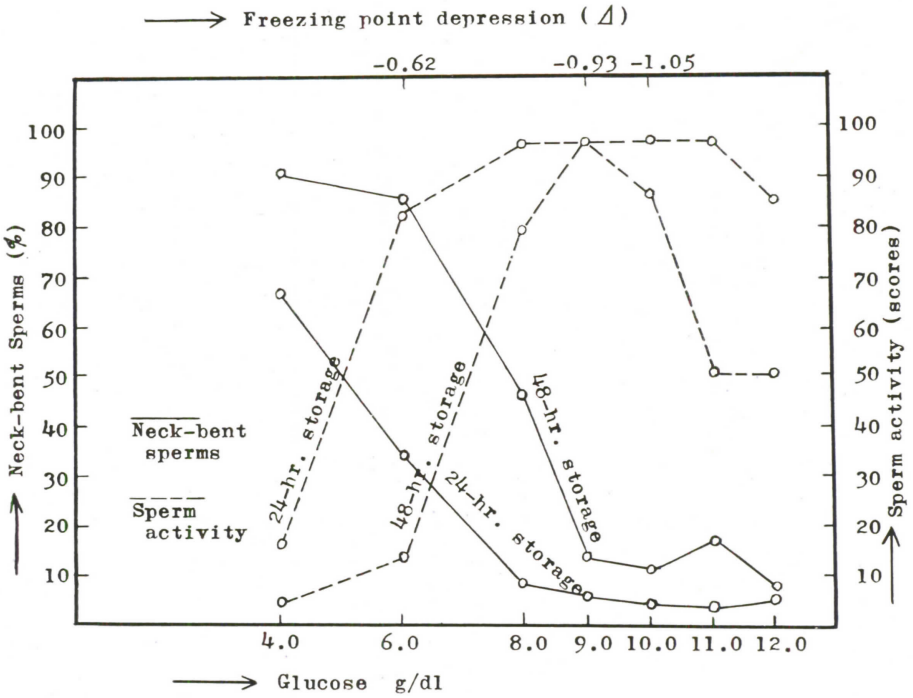


Fig. 13. Relationship between the percentage of neck-bent sperms, activity scores of sperms and the concentration of glucose solution.

tonic than that corresponding to the Δ above given causes high percentages of neck-bent sperms. Since the Δ of fowl seminal plasma shows only $-0.58^{\circ}C$,[1] it is no wonder that even the natural semen exhibits neck-bent sperms.

5. COMMON OCCURRENCE OF NECK-BENDING IN AVIAN SPERMS

The neck-bending is not confined to the fowl sperm. SAEKI and BROWN (1962) have already observed the same phenomenon in the semen of turkey. The present authors were also able to find the neck-bending of sperms in the ejaculate obtained by massage method from a Japanese Green pheasent. Each drop of the semen sample was fixed in the vapor of osmic acid: the first drop 2 hours, the second 6 hours, and the third 8 hours after collection. While the 2-hour old sperms remained quite intact, the 8-hour old sperms began to bend their necks (Fig. 14). It was of great interest to find out that the 6-hour old sperms exhibited a vague segmentation of the midpiece resembling that is observable in the sperms immersed into distilled water (Fig. 12).

1) Determined by ordinary cryoscopic method on a composite semen sample collected from a White Leghorn cock. This value empirically coincides with $-0.59^{\circ}C$ and $-0.60^{\circ}C$ found by LAKE (1960)

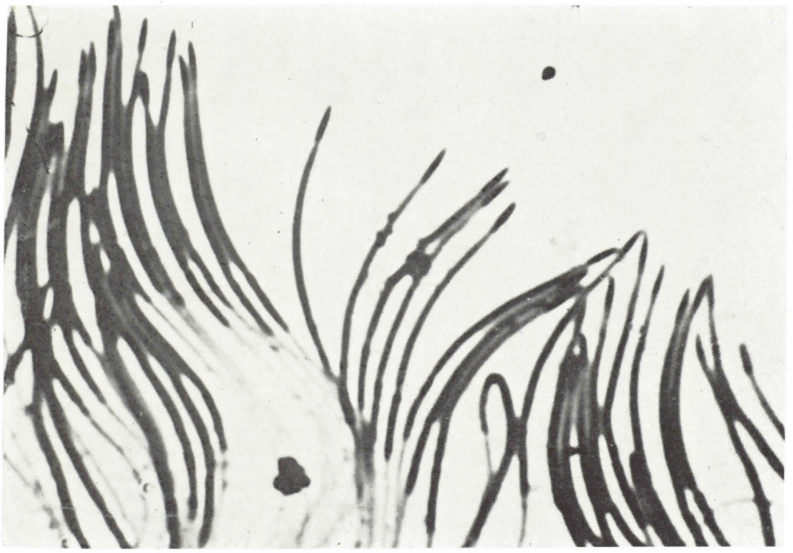


Fig. 14. Hanging drop of a Japanese Green pheasant semen sample stored 8 hours 2-5° C; some sperms on the right side show the neck-bending.

There is reason, therfore, to believe that the neck-bending can take place in any avian sperm which is morphologically similar to that of the fowl.

6. BASIC PRINCIPLE OF MAKE-UP OF THE DILUTOR FOR FOWL SEMEN

As above described, avian sperms, when they are kept *in vitro*, are fatally liable to the neck-bending due to the osmotic effect of the seminal plasma. It follows, therefore, that the dilutor for the fowl semen must be such one which is most effective not only in keeping the activity of sperms for a longest possible term but also in suppressing the neck-bending of sperms. Tests were at first made to find the effects of the dilutors commonly used upon the activity and longevity of sperms without notice of neck-bending. As shown in Fig. 15, no dilutors applied, except the yolk-citrate buffer, were favorable to both the activity and longevity of sperms, resulting in more decrease of live sperms in contrast to undiluted semen. On the contrary, the yolk-citrate buffer, revealed a remarkably beneficial effect of dilution showing its usefulness to cock semen.

On the basis of foregoing results, the relation of the yolk-citrate buffer to the neck-bending of fowl sperms was further examined considering its osmotic effect. Fig. 16. shows clearly that, within a limit, the neck-bending of sperms always takes place in the yolk-citrate buffer but the percentage of neck-bent sperms decreases almost reciprocally to the concentration of sodium citrate. At the concentrations of 6.0 to 6.5%, its occurrence is practically negligible.

In view of the freezing-point depression, this finding empirically coincides with

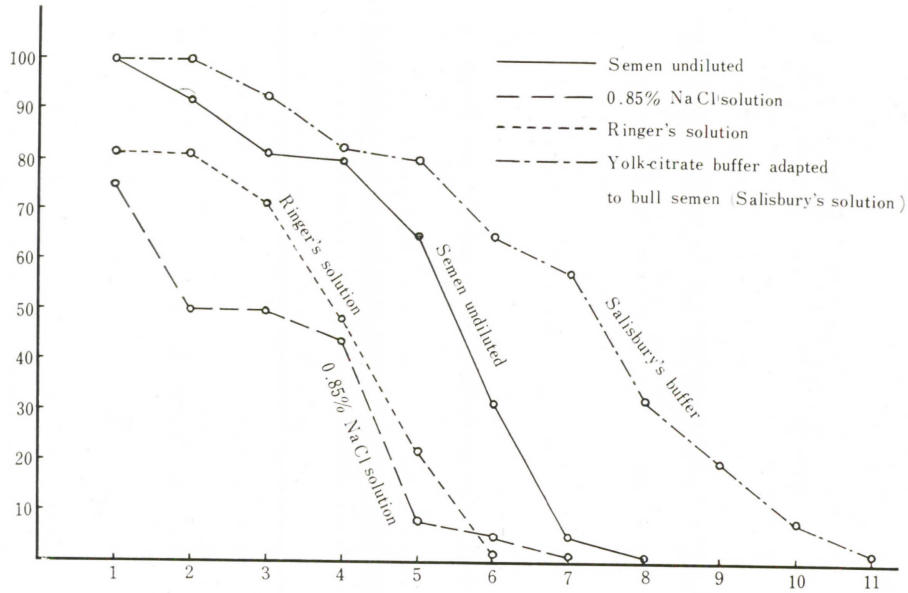


Fig. 15. Effects of some dilutors on the activity and longevity of cock sperms.

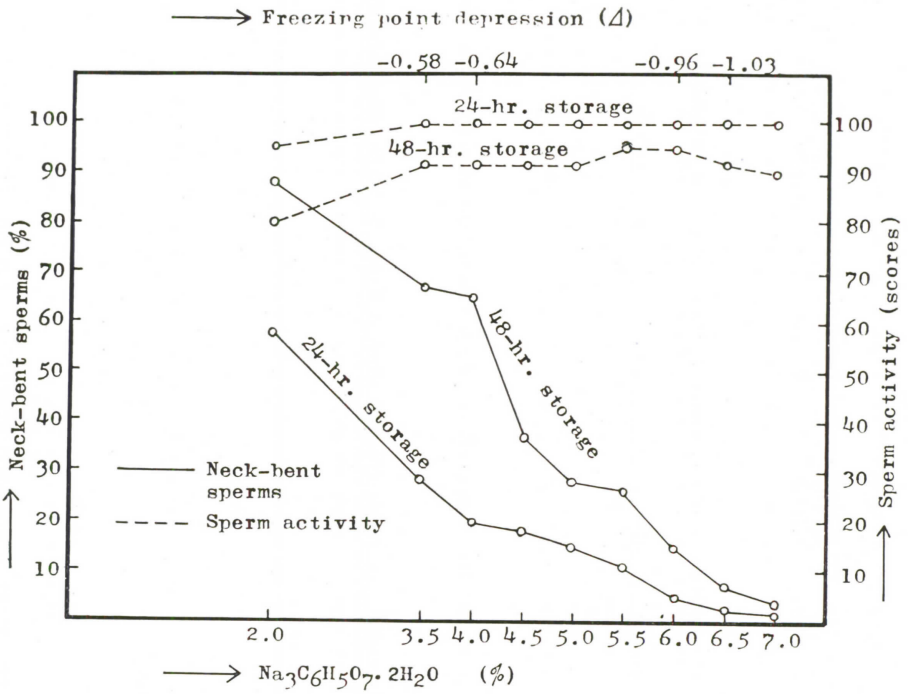


Fig. 16. Relationship between the percentage of neck-bent sperms, sperm activity, and concentration of sodium citrate in the yolk-citrate buffer at 24- and 48- hour storage.

Table IX. Fertility test of fowl semen diluted with an osmotically adjusted yolk-citrate buffer.

No. hens inseminated	No. eggs laid	No. eggs fertile	Average 2-week fertility rate per hen
1—2 hrs. storage			
H_1	9	7	77.7
H_2	12	2	16.6
H_3	12	10	83.3
H_4	6	6	100.0
H_5	4	4	100.0
H_6	10	8	80.0
I_1	5	3	60.0
I_2	5	4	80.0
I_3	9	6	66.6
A_1	8	4	50.0
A_2	8	8	100.0
A_3	9	8	88.8
Flock average	8.1	5.8	72.2
24 hrs. storage			
G_1	8	5	62.5
G_2	14	10	71.4
G_3	11	8	72.7
G_4	9	8	88.8
G_5	11	8	72.7
G_6	12	8	66.6
G_7	6	5	83.3
G_8	6	2	33.3
E_1	8	3	37.5
E_2	12	0	0
E_3	10	3	30.0
E_4	9	3	33.3
E_5	3	3	100.0
Flock average	8.6	5.1	55.5
72 hrs. storage			
C_1	10	7	70.0
C_2	13	13	100.0
C_3	11	8	72.7
C_4	11	0	0
C_5	10	3	30.0
C_6	12	3	25.0
C_7	9	1	11.1
C_8	10	7	70.0
C_9	8	3	37.5
C_{10}	11	0	0
Flock average	10.5	4.5	42.8
96 hrs. storage			
F_1	9	5	55.5
F_2	9	2	22.2
F_3	5	2	40.0
F_4	9	1	11.1
F_5	11	8	72.7
F_6	11	1	9.0
F_7	9	4	44.4
Flock average	9.0	2.6	36.5

the result obtained with 9 to 10% glucose solutions as shown in Fig. 13.

The adjustment of the yolk-citrate buffer to fowl semen should, therefore, be merely an increase of sodium citrate in the buffer until the final composition is settled.

The adjustment of the buffer was done by reducing the concentration of sodium citrate to the half and replacing it with 9.0% glucose solution. In this way, the possibly toxic effect of high dose of sodium citrate will be eliminated without changing the osmotic pressure of the original buffer. The constituents of the final dilutor thus prepared was:

One volume 6.5% $Na_3C_6H_5O_7 \cdot 2H_2O$ solution + one volume egg-yolk + two volumes 9.0% glucose solution, in which the Δ was $-1.03°C$.

The results of artificial insemination with the semen diluted with the osmotically adjusted buffer in the rate of 1 : 4 are given in Table IX.

A comparison of the data in this table with those in the Table VIII clearly shows that the yolk-citrate buffer adjusted to fowl sperms can decidedly promote their fertility. The semen diluted with this buffer exhibited at the storage of 1–2 hours so high fertility rates that no undiluted semen could attain this level, the average two-week fertility being 72.2% per flock. Among 12 hens inseminated, 3 hens maintained 100%, 4 hens more than 80%, and 3 hens more than 60% fertility, though the other 2 hens showed the rate lower than 60% fertility. At longer times of storage, the average fertility rates per flock consisting of 13, 10 and 7 hens, were 55.5, 42.8 and 36.5% for the storage of 24, 72 and 96 hours, respectively. Some selected fertility records of the hens inseminated with semen stored in the adjusted buffer may serve as a proof of the significance of adjustment of the osmotic pressure

Table X. Selected Fertility Records of the White Leghorn Hens during the Period from the Second to the Fifteenth Day following a Single Insemination.

No. hens	Duration of fertility potential of semen in days 2	3	4	5	6	7	8	9	10	11	12	13	14	15	Biweekly fertility per hen %
Inseminated immediately after collection															
H_3	−	+	+	+	+	+	+	+	0	+	+	+	−	?	83.3
A_4	0	+	+	+	0	+	−	+	0	+	+	+	0	?	88.9
Inseminated after 24- hour storage															
G_2	+	+	+	+	+	+	−	+	−	+	+	+	−	−	71.4
G_4	+	+	+	0	0	+	−	0	0	+	+	+	+	0	88.9
Inseminated after 72- hour storage															
C_2	+	+	+	+	+	+	+	+	+	+	+	+	0	+	100.0
C_3	+	+	+	+	0	+	0	+	−	+	+	−	0	−	72.0
Inseminated after 96- hour storage															
F_5	+	+	0	+	+	0	+	+	0	+	+	−	−	−	72.7
F_1	−	0	0	0	0	+	+	+	0	+	+	−	−	−	55.6

Remarks: + fertile, − inferetile, 0 no egg laid, ? not examined.

in thed ilutor through which the neck-bending of the sperms was prevented (Table X).

In this view, the basic principle of devising the dilutor for fowl semen is first to remove the hypotonic effect of its seminal plasma by the promotion of the osmotic pressure of the medium.

7. DISCUSSION

Although SAMPSON and WARREN (1939) observed one case of sterility caused by low sperm concentration associated with a high percentage of defective sperm, they believed that morphological defects of sperms cannot be a serious cause of infertility. On the contrary, LAKE (1954) has pointed out an association between the morphological defect of sperms and infertility.[1] According to this author, semen collected by the massage method of BURROWS and QUINN can consists of one or more of the following components: 1. Spermatozoa and secretions from the seminiferous tubules and epididymal region of the male tract; 2. clear buff-colored fluid; 3. white solid material; 4. highly viscous, colorless fluid; 5. clear watery fluid; 6. faeces. LAKE believes that true semen consists of component 1 and part of component 5 whereas the other components 2, 3, 4, 6 and part of 5 are most certainly derived from the rectum or the ureters during the excitation of the cock. The author was doubtful how far the component 5 can be considered as seminal fluid. If, however, the component 5 partly corresponds to the "transparent fluid" termed by NISHIYAMA (1951), this fluid must be considered as a constant component of fowl semen because, as shown above, it was always present in the ejaculate collected by the "Hiroshima" method in which no squeezing operation was applied.

Although LAKE (1956) has already observed the detrimental effect of the fluids from the lymph folds and vascular bodies on the fertility of sperms, he believes those components to be the products of squeezing at massage. The transparent fluid here considered, however, is no product of squeezing but a constant component of the fowl semen, which acts hypotonically upon the sperms and causes the neck-bending in them.

This apparent contradiction in nature would be easily understood if the role of the transparent fluid from the lymph folds and vascular bodies is considered. It is known that the bird has no special accessory sexual organs homologous to those of mammals which produce at ejaculation some secretions for diluting the thick semen from the *vasa*. Evidently, in the bird also, such secretions are indispensable because the semen stored within the *vasa* is so thick and so little in volume that it is hardly possible to be ejected into the female oviduct without dilution. Under this circumstance, the transparent fluid can play the role of dilutor, firstly enabling the *vasa*-semen to be easily ejected, secondly providing a swimming medium for the sperms. Observations on the pure semen squeezed out from the under-half of both the *vasa* revealed that it was very thick in consistency and dense in sperm concentration, varying from

1) The term "defect" in LAKE's paper seems to have been used rather in a broader sense than "neck-bending".

4,300,000 to 6,500,000 per cu. mm. The sperms thus obtained showed relative slowness of their movement in spite of keeping the high motility potential. In contrast, the sperms in the ejaculate, the concentration being reduced approximately to a half to those in *vasa* (cf. Table I), showed more active forward movements. Even in this case, some sperm agglomerates of varying size were observable, being progressively disintegrated by liberation of free-swimming individual sperms with the laspe of time.

The successful insemination of poultry with stored semen is very little known, though the viability of stored sperms has been manifoldly reported. Excepting the earlier papers published before 1950, JASPER (1950) could obtain only one fertile egg by inseminating with cock semen that had been stored for 20 hours at 4.4°C in human serum, while GARREN and SHAFFNER (1952) secured 38 and 6% fertile eggs with undiluted cock semen stored at 5°C for 3 and 4 hours, respectively. In the similar experiments of SCHINDLER et al. (1955) they found that the fowl semen diluted with whole milk and stored for 24 hours at 4°C gave only 19% resultant fertility though undiluted semen and the semen diluted with either Ringer- or Lock-solution retained a full fertilizing capacity after 4 hours storage at 10°C. The experiments of LAKE, SCHINDLER and WILCOX (1959) transporting fowl semen by air from U.S.A. to Israel and Scotland gave better results. The semen buffered with a phosphate solution containing antibiotics was cooled in an insulated container. The fertility rate of the semen thus transported to Israel was 38% after 38 hours and that arrived in Scotland, 36% after 37 hours, computed from eggs laid within the first week following insemination. In contrast to this, LAKE (1960) communicated the most promising results of 64 and 47% fertility from the semen stored for 24 and 48 hours, respectively, at 0°C in a glutamate-containing saline solution added with fructose. Nevertheless, present results of fertility tests with osmotically adjusted egg-yolk citrate buffer have revealed a remarkable advantage as the semen dilutor compared with those referred above.

While, in mammals, the secretions of the acceessory sexual organs are completely adjusted to the functional activity of the sperm, the transparent fluid of birds appear to be of temporary nature merely for the purpose of ejaculation. Thus, the contradictory phenomenon, the neck-bending of the sperm, can be attributed to the incompleteness of the transparent fluid as a natural semen dilutor, since it is produced by the undifferentiated organs or tissues belonging to the lymph and vascular systems. However, it must be remembered that the neck-bending is only *in-vitro* phenomenon. When the ejected sperms proceed into the female oviduct, they will be quite free from the osmotic effect of the transparent fluid; no neck-bending can take place though its protecting mechanism is still obscure.

8. SUMMARY

In fowl semen, probably avian semen in general, the sperm is not placed in an osmotic equilibrium with the surrounding seminal plasma. Especially the transparent fluid from the lymph folds and vascular bodies exerts hypotonically upon the

midpiece of the sperm though it is a regular component of natural semen, resulting in swell-up or disruption of the midpiece. This hydrating action induces further the sperm to be bent at the damaged midpiece in association with the movement of its tail. It was also found that any medium with the Δ lower than $-0.9°C$, be it body fluid or physiological saline salution or other artificial dilutors available to mammalian sperms, causes the neck-bending in fowl sperms. Hence, the medium with the optimum concentration for the motility of sperm reversely affects its midpiece, resulting in neck-bending, eventually in low fertility. It seems, therefore, to be justified to conclude that the basic principle for making up the dilutor of fowl semen is to inhibit the outbreak of this defectiveness of the sperm by adjusting the osmotic pressure of its medium by promoting the Δ to $-0.90°C$ or $-1.03°C$. The experimental data with egg-yolk citrate-glucose buffer osmotically adjusted showed clearly the promotion of biweekly fertility on eggs produced per bird.

This range of ideas appears to be significant in the routine insemination of the fowl.

CONCLUSION

"Hiroshima" method of artificial insemination of the domestic fowl has aimed to improve the technique along three lines, stress being laid upon the least frequency of insemination with the highest fertility level:

1. Collection of semen without changing its genuine nature when the abdominal massage is applied.
2. Deep insemination into the posterior part of the uterus in order to place sperms directly into their proper environment.
3. Make-up of the dilutor effective for keeping as high fertility potential as in normal matings.

At first sight the technique here presented requires much labor in collecting and introducing semen than that operated by a single person, the so-called "one-man technique".

In birds, differing from mammals, the ovulation is of every day occurrence and sperms can retain their fertility potential in the female oviduct for 16 days or more after a single mating. If, therefore, any dilutor of semen can be devised for keeping the fertility potential of sperms as long as in natural matings, the frequency of insemination will be greatly reduced, in other words, the technique as a whole will save man's labor remarkably.

The data of experiments conducted at this laboratory with this working hypothesis have given sufficient evidence for the possibility of its actualization. In view of this, "a single insemination per hen weekly or more with regular success" would be no far distant goal in the routine insemination of the fowl.

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にわとりの人工授精・広島方式

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要　約

にわとりの人工授精の実用化を目ざして，技術上の改善を行なった．

1. 精液の採取にはマッサージ法を応用したが，従来のものと異なり鶏体保定台と精液受容器の新型を創案し，これを応用した．

2. この方法によると精液を質量共に最も自然に近い状態で，しかも，半無菌状態で回収できる．

3. 精液注入技術に対しては，注入管を約 10cm 延長して，深部注入を可能にし，膣内注入と共に子宮内注入を可能にした．

4. 多数実験の結果，若干の熟練は要するが，子宮内注入が膣内注入よりも，高い受精率を示し，しかも，爾後の産卵率にも悪影響のないことを知った．

5. にわとりの精子は射精後，中片部で屈折して，いわゆる首曲り現象を起こし，これが卵子の受精率を著しく減殺することを確かめたので，その防止策として，稀釈液の改善が必要なことを論じた．

6. 差し当たりブドウ糖・クエン酸ソーダ緩衝液の滲透圧を調節して，$\Delta = -0.90°C \sim -1.03°C$に引揚げることにより．首曲りを抑止し，その結果，精子は1—2週間にわたり高い受精率を保持することを確認した．

7. 広島方式人工授精は，以上のように改善された3技術から成立する．