# ARTIFICIAL INSEMINATION OF *BOMBYX MORI*

By

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[With 3 Text-Figures and 3 Tables]

## Introduction

Artificial insemination (which is by no means the same as artificial fertilization) has played many important rôles in experimental embryology and breeding in some animals such as fishes, amphibians, echinoderms, etc. In fishes and even in some mammals it has now been introduced practically, from the economic point of view.

The method of artificial insemination is simpler in the animals whose eggs and spermatozoa come together outside the body than in those whose ejaculation is carried out inside the body. In the latter case the finer and the more complicated the construction of the sexual organs of both sexes is the more difficult is the carrying out of the artificial insemination. The character of the spermatozoa sometimes influences the method. Therefore there are not many examples of successful artificial insemination in insects.

Since a hundred or more years a method of artificial insemination has been desired eagerly among bee-culturists, for in bees this is the only method for breeding. Recently it was successfully carried out first by L. R. WATSON (1926) after many failures repeated by older investigators. He injected the semen taken out from the male sexual organ into the female organ with a fine injector settled on a micromanipulator. It needed delicate microtechnique at that time, but it has been improved now and demonstrated by some bee-culturists.

In Himenoptera there is one more example which was introduced by H. FEDERLEY (1929), although not published. That is the method tried by A. D. PEACOCK in Tenthredinidae, in which a glass tubule was used. It is notable that the seminal fluid taken out from the male sexual organ was used in both the above methods.

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Only one example in Lepidoptera was introduced by FEDERLEY (1929), which was carried out by J. W. H. HARRISON (1916), who bred a hybrid between *Philosamia cynthia* female and *Philosamia promethea* male. He pushed the penis of *promethea* moth into the ductus bursae of *cynthia* and pressed the hindbody of the male.

For some years the present writer has also tried artificial insemination in *Bombyx mori* and he succeeded finally in 1932 (ÔMURA 1933) with glass tubules and again in 1934 (ÔMURA. 1935)[5] with a method analogous to HARRISON's. Furthermore studies on nearly all necessary conditions for the application of these methods were pursued.

The writer's efforts were first directed to examine the possibility of putting artificial insemination into execution in *Bombyx mori*, and in this direction he succeeded in developing reliable methods. In addition attempts were made to find as many methods as possible, because the final aim was to work out different methods practicable for various biological experiments. Consequently the central feature of the herein reported work is methodology.

This work was carried out under the guidance of Prof. Emer. K. SUDA and Prof. E. KAWAGUCHI. Hearty thanks are offered to both of them for their kind suggestions and encouragement throughout the course of the undertaking.

## Materials

All the representative races of Japanese, Chinese and European silkworm moth cultured in the Sericultural Laboratory of the Hokkaido Imperial University and some from sericulturists in the country were employed at random. There is found scarcely any difference between the races in relative superiority as the materials, yet the larger moths such as found in some race-hybrids are better fitted for the experiment.

## Methods

### I. Glass tubule methods

#### A. Instruments

Not so complex instruments are necessary in these experiments as in the honey bee. A pincette and a pair of scissors for dissecting

and a set of injectors are enough. As the injector two sorts of glass tubules and an airpump were used. One of the glass tubules (Fig. 1: A) was used for the absorption of the seminal fluid, the other (Fig. 1: B) for the injection. Though the total length and the diameter of the basal part of both tubules are at the operator's option, it is recommended that the fine part of tubule "A" be made long enough to be able to include all the semen gathered up by it and that the fine and the thick parts of the tubule "B" both be made as short as possible; it is not very good to make the thick part of the tubule "A" too narrow nor to make the same part of the tubule "B" too wide. There is only one necessary condition about the relative dimentions of the tubules: let the fine part of "A" be so long that its end will reach to the boundary between the fine and thick parts of "B".

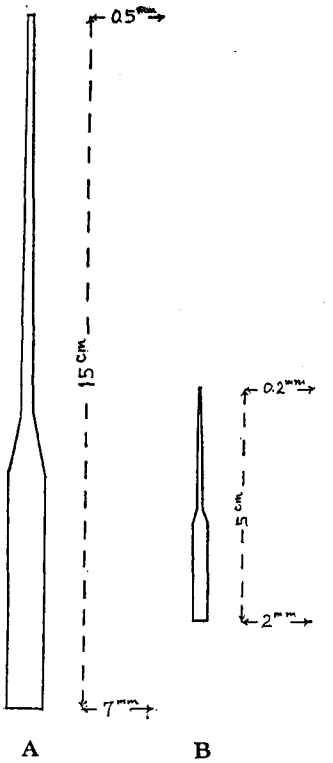


Figure 1. Two sorts of glass tubules. A. absorbing tubule. B. injecting tubule. Dimensions show the lengths and outer diameters roughly

The end of both tubules is so capillarised, so fragile and so readily stopped up by the mucus of the seminal fluid after every use, that many of them must be prepared.

For the airpump, such a one as that held by the foot and pressed by both hands is most suitable, as it is stable and strong in pressure. When the seminal fluid is not viscous, or the inner diameter of the end of the injecting tubule is large, the injection may be done without such a strong airpump but with an air bulb or even with an out-breathing press.

### B. Methods for gathering the seminal fluid

The most important and essential point in the artificial insemination in Lepidoptera is the manner of gathering the seminal fluid.

The spermatozoa of silkworm moth have no activity in the vesicula seminalis or in the ampulla ductus deferentis under normal condition, while those ejaculated out into the bursa copulatrix have violent activity. This activity is brought about by the fluid secreted from the glandula prostatica of the male moth, which is analogous to the prostata of Mammalia (ÔMURA. 1935) (6).[1] Therefore, it was scarcely possible to succeed in the artificial insemination without activating the spermatozoa by mixing them with the prostatic secretion before injection. To gain such a mixture, there are two ways, which will be explained further on.

### 1. The seminal fluid in the bursa copulatrix of the copulated female

In natural copulation, the spermatozoa is usually mixed with the secretion from the glandula prostatica in the course of the ejaculation. Therefore, the complete seminal fluid that contains the spermatozoa which have violent activity as well as the ability of fertilization is found in the bursa copulatrix of the female who has made the copulation.

Under the natural conditions, the male moths continue the copulation with the females many hours and ejaculate several times, but the first ejaculation is completed within some 40 minutes after the beginning of the copulation under the normal temperature 25°C or so. So the most dense semen can be obtained immediately after 40–60 minutes copulation. When the semen is so viscous as not to be suitable for the experiment, it is recommended to keep the female for a while from the male after the copulation, in order to dilute the semen by the transmission of some part of the spermatozoa from the bursa copulatrix to the receptaculum seminis. Yet, to keep the female alone more than 10 hours in normal temperature is to be avoided, for the semen becomes too dilute to contain a sufficient quantity of spermatozoa for fertilization.

In order to gather the seminal fluid from the bursa copulatrix, the following procedure was employed. The female was dissected after the given duration of copulation and the bursa copulatrix was taken out. The spermatophora had been formed up making the membrane of the bursa to expand about its extremity. It has some

1) The details will be found in another paper.

rigidity and attains about half the size of a grain of wheat. So it was easily held by the thumb and forefinger of the left hand. Then the absorbing tubule was taken up in the right hand, and both membranes of the bursa and the spermatophora were penetrated by the point of the tubule. When the tubule was inserted well into the spermatophora the seminal fluid ascended gradually through the tubule and nearly the whole of the fluid could be sucked up into it. When that failed it became necessary to suck the fluid up into the tubule by gentle in-breathing. Then the head of the absorbing tubule which contained the seminal fluid was inserted into the wide part of the injecting tubule deep enough to reach to the entrance of the narrow part of it, and the fluid was transferred to the latter by gentle out-breathing.

Occasional coherence of some of the semen to the wall of the tubules may be partially avoided by soaking them in physiological salt solution before use.

2. The seminal fluid in the male sexual organs

The spermatozoa have no activity in the semen taken from the vesicula seminalis or from the ampulla ductus deferentis, but if a trace of the prostatic secretion is added there they become as vivid as they are usually in the semen which is ejaculated out in the natural copulation, and gain the ability of fertilization.

In order to gather the semen from these organs, an analogous process to that of the first method was repeated. This case is more difficult than in the first method, as the organs are more delicate and smaller than the female one. It was very difficult to hold them by the fingers, hence they were set on the tip of a finger.

After the gathering of the semen, one must add a trace of the prostatic secretion to it. The glandula prostatica is a small duct, which has about the same diameter as the end of the absorbing tubule. Therefore, all of the secretion of this gland could not be sucked up into the tubule, but some of it could be gathered after having, with the point of the tubule, stirred up the gland placed on a finger tip. The prostatic secretion may be sucked up first and then the semen. It was not necessary to mix the secretion previously with the spermatozoa, for they were mingled gradually by the movement of the spermatozoa which had obtained activity in contact

with the secretion. The two sorts of fluid sucked up in the same absorbing tubule were transferred to the other as in the former case.

Using this method, the seminal fluid taken into the tubule is less than in the former method, for it is difficult to gather all of it.

### C. Method of injection

The injecting tubule with the seminal fluid was connected to the gum tube of the injector, or the airpump. Then the end of it was inserted into the ductus bursae of a given female. The airpump was pressed by an assistant. When the semen was dilute enough and the inner diameter of the end of the tubule was prepared not too small and no coherence of the semen on the wall of the tubule occurred, light pressure was enough for the injection. In several trials the writer succeeded only with out-breathing. But in cases when the conditions were not so favourable, strong pressure was necessary.

In practice, it is most difficult to insert the tubule into the ductus bursae. To acquire skill in this technique, it is advisable to practice it with a needle instead of a tubule. It requires skill also to inject all of the semen from the tubule into the bursa copulatrix with the air pressing. Because, if excess pressure was exerted the bursa was broken and the semen was pushed out into the body cavity. Moreover, the insemination was difficult of success unless a part of the semen touched the entrance of the ductus seminalis, otherwise the progression of the spermatozoa to the receptaculum seminis did not occur.

Fig. 2. shows several forms of the bursae copulatrices which have received the semen injected naturally by the ejaculation and artificially by the glass tubule. In the former it is notable that the outlet of the spermatophora is located close to the entrance of the ductus seminalis and the semen touches it. A in Fig. 2. is such a natural form. In the case of artificial injection several forms of the bursae are shown, of which B is the most ideal one and is very like the natural case. But there is not so much semen as in the natural ejaculation, therefore, in practise such forms as C and D are recommendable. D represents a case when a little air is added by a little over-pressure and the semen is returned back so as to touch the entrance of the ductus seminalis. C represents the case

when no air is added as in B, the injection having been stopped just before the finish. E and F are examples of failures. In the former case the pressure is intermediate between C and D, in the latter there is loaded over-pressure. In both cases no spermatozoon can progress to the receptaculm seminis, because no part of the semen whatsoever touches the entrance of the ductus seminalis.

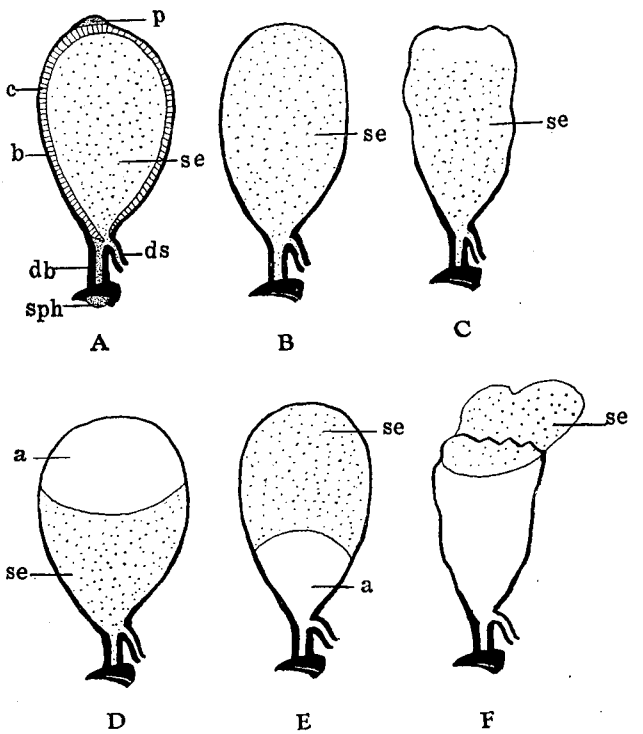


Figure 2. Models of the bursa copulatrix: A.... naturally mated, B-F.... artificially inseminized, B, C, D.... successful cases, E, F.... unsuccessful cases. a. air, b. bursa copulatrix, c. spermatophora, db. ductus bursae, ds. ductus seminalis, p. pearly body of spermatophora, se. semen, sph. spermatophragma.

The techniques described above appear very delicate, but it is not difficult to finish all the treatments, from the dissection to the injection, within five minutes. In 1934, the present writer measured 1.5–5 minutes which was consumed in a trial by the first method.

## II. Artificial mating method

The relation between ejaculation and duration of mating in silkworm moth has been made clear by MACHIDA and WATANABE (1927). In normal temperature, by some 5 minutes mating the male moth is stimulated to ejaculation, which is finished normally even if the pairing is separated. So that one can see the figure of natural ejaculation by drawing out the penis after some 5 minutes mating. In fact, it is thus shown that the ejaculation of spermatozoa begins at 15–20 minutes after the beginning of the mating and lasts about 10–15 minutes.[1)]

This principle has been applied, by the writer, to artificial insemination. The male was separated from the female after some 20 minutes mating, and the penis was inserted into the ductus bursae of a given female and kept in that position during 20 minutes or more. All the procedure was carried out by the fingers. So this method needed no instrument.

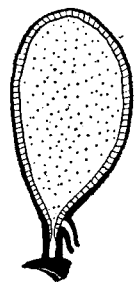

Figure 3. Spermatophora misformed by artificial mating method.

This is a simple method, but is hard to practice. As the penis is very small, short and brittle, it is difficult to insert it into the ductus bursae. Moreover the penis must be put deep enough into the ductus, and the paired individuals be held in such a posture as to receive no unfavourable effect which disturbs the ejaculation. In this experiment, the writer has some examples which had received nearly complete spermatophora but no spermatozoon proceeded to the receptaculum seminis. This may be attributed to the fact the opening of the spermatophora could not reach to the entrance of the ductus seminalis, owing to an insufficient insertion of the penis. Fig. 3 shows a misformed spermatophora.

## Results of the experiments

### I. The rate of success in the experiments

Since 1932, the writer has tried many experiments on artificial insemination. The rate of success in those experiments was not constant, being greatly influenced by many factors.

1) The details will be found in another paper.

TABLE 1.

Percentage of success in artificial insemination.

| Method | Series of experiments | Date | Individuals experimented | Individuals which received semen[1] | Individuals fertilized | % of success | Remarks |
|---|---|---|---|---|---|---|---|
| First glass tubule method | I | Nov. '32 | 13 | 7 | 3 | 23.1 | Sperm were taken from the ♀♀ immediately after 3 hours copulation at 25°C. |
| | II | July '33 | 4 | 4 | 0 | 0 | Sperm were taken from the ♀♀ kept alone 15 hours at 27°C after 1 hour copulation at 25°C. |
| | III | ,, | 2 | 2 | 1 | 50.0 | Sperm were taken from the ♀♀ kept alone 10 hours at 10°C after 1 hour copulation at 25°C. |
| | IV | ,, | 12 | 10 | 6 | 50.0 | Sperm were taken from the ♀♀ immediately after 30 minutes copulation at 25°C. |
| | VII | July '34 | 10 | 10 | 7 | 70.0 | Sperm were taken from the ♀♀ immediately after 30–60 minutes copulation at 25°C. |
| Second glass tubule method | VIII | ,, | 11 | 10 | 8 | 72.7 | Sperm were taken from the vesiculae seminales of non-mated male moths. |
| | IX | ,, | 10 | 8 | 3 | 30.0 | Sperm were taken from the vesiculae seminales of the pupae 2 days before emergence. |
| | XIII | Nov. '34 | 5 | 5 | 4 | 80.0 | Sperm were taken from the ampullae ductuum deferentium of non-mated male moths. |
| Artificial mating method | XX | Oct. '34 | 4 | 2 | 1 | 25.0 | |
| | XXI | ,, | 8 | 2 | 0 | 0 | |
| | XXII | ,, | 7 | 2 | 1 | 14.3 | |

1) Involves the individuals which received the sperm in their bursae copulatrices, disregarding whether the eggs were fertilized or not.

The results are summarised in Table 1. As is shown, some individuals are found into whose bursae copulatrices seminal fluid was injected without any resultant fertilization. Many of them were injected with seminal fluid in such manners as shown in Fig. 2 (E and F) and Fig: 3, while some of them had too little a quantity

TABLE 2.

Total number, fertility and hatchability of eggs per batch.

Materials .... Akajyuku (a Japanese race)

| Series of experiments | No. of batch | Deposited eggs | Fertilized eggs | Hatched eggs | Fertility (%) | Hatchability (%) |
|---|---|---|---|---|---|---|
| VII | 3 | 451 | 334 | 317 | 74.1 | 94.9 |
| | § 6 | 685 | 667 | 582 | 7.4 | 87.3 |
| | 7 | 166 | 40 | 38 | 24.1 | 95.0 |
| | 8 | 606 | 557 | 522 | 91.9 | 93.7 |
| | 10 | 484 | 202 | 181 | 41.7 | 90.0 |
| | § 11 | 274 | 162 | 131 | 59.1 | 80.9 |
| | 12 | 433 | 11 | 4 | 2.5 | 36.4 |
| VIII | 1 | 162 | 14 | 12 | 8.6 | 85.7 |
| | § 2 | 545 | 499 | 300 | 91.5 | 60.1 |
| | 3 | 286 | 203 | 196 | 71.0 | 96.6 |
| | § 4 | 562 | 525 | 514 | 93.4 | 97.9 |
| | 5 | 496 | 47 | 43 | 9.5 | 91.5 |
| | 6 | 395 | 343 | 280 | 86.8 | 81.6 |
| | 8 | 454 | 369 | 337 | 81.3 | 91.3 |
| | 10 | 601 | 560 | 515 | 93.2 | 93.8 |
| IX | § 3 | 402 | 347 | 324 | 86.3 | 93.4 |
| | § 7 | 141 | 120 | 116 | 85.1 | 95.0 |
| | § 8 | 563 | 412 | 374 | 73.2 | 90.8 |
| XX | 4 | 470 | 349 | 322 | 74.3 | 92.3 |
| XXII | 3 | 168 | 74 | 61 | 44.0 | 82.4 |
| Control | § 1 | 740 | 708 | 642 | 95.7 | 90.7 |
| | 2 | 649 | 635 | 592 | 97.8 | 93.2 |
| | § 3 | 677 | 667 | 655 | 98.5 | 98.2 |
| | 4 | 333 | 320 | 214 | 96.1 | 66.9 |

§.... The batch from which hatched out larvae were reared.

of sperm in their receptaculum seminis to fertilize the eggs. The latter were found only when the glass tubule methods were used, and especially in such examples which had been injected with a little quantity of seminal fluid as occasionally seen in the case employing the pupae to gather the semen.

In Table 1, series I is the first successful experiment made with the glass tubule. In series I and VII, the injected semen was taken from the bursae copulatrices of the female moths immediately after 30–180 minutes mating at 25°C. Nevertheless there is found a great difference between the percentage of success in the experiments. This might perhaps depend upon skill in practice. Comparing series II, III and IV, where the semen was taken from the bursae copulatrices as in series I, but the females had been isolated from the males for a different number of hours respectively, bad effects can be found on the rate of success due to the long isolation of the females.

Series VII and VIII show that there is little difference in the rate of success so far as the method of gathering seminal fluid is concerned, though practically the second method was more difficult. The poorer rate of success in series IX than in VIII may be due to the less amount of the seminal fluid in the pupae, and not to the character of the spermatozoa.

The 19 individuals used in series XX, XXI and XXII are all those upon which the artificial mating method was employed. Of these only in two cases did success attend the experiment. It should be noted that in these series four examples were gained with misformed spermatophore as shown in Fig. 3.

The table shows, as a whole, that practice increases the percentage of success and great skill may be expected to approximate it to that of the natural mating, and that it may not be difficult for any one to gain some 50 percent of success with glass tubule methods.

## II. Effect on the number of eggs laid and on fertility

A remarkable difference was found in the number of eggs to a batch and in the fertility but scarcely any difference in the hatchability, between the experimented and controlled series. The former two characters, *i. e.* number and fertility of eggs show larger deviation and less value in the experimented series than in the controlled

TABLE 3.

Some characters of the offspring of artificially inseminized female moths.

| Series of experiments | No. of batch | Reared larvae | Full grown larvae | Ratio full grown to reared (%) | Female[1] | | | Male[1] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Weight of a cocoon (gr.) | Weight of silk (gr.) | Ratio silk to cocoon (%) | Weight of a cocoon (gr.) | Weight of silk (gr.) | Ratio silk to cocoon (%) |
| VII | 6 | 557 | 407 | 73.1 | 1.88 | 0.247 | 13.1 | 1.53 | 0.247 | 16.1 |
| | 11 | 100 | 90 | 90.0 | 2.18 | 0.321 | 14.7 | 1.71 | 0.294 | 17.2 |
| VIII | 2 | 283 | 232 | 82.0 | 2.16 | 0.300 | 13.9 | 1.66 | 0.265 | 16.0 |
| | 4 | 493 | 402 | 81.5 | 2.01 | 0.303 | 15.1 | 1.64 | 0.281 | 17.1 |
| IX | 3 | 298 | 233 | 79.9 | 2.13 | 0.304 | 14.3 | 1.73 | 0.286 | 16.5 |
| | 7 | 110 | 104 | 94.5 | 2.12 | 0.298 | 14.1 | 1.66 | 0.260 | 15.7 |
| | 8 | 303 | 283 | 93.4 | 1.97 | 0.285 | 14.5 | 1.53 | 0.265 | 17.3 |
| Control | 1 | 468 | 422 | 90.2 | 1.84 | 0.258 | 14.1 | 1.50 | 0.248 | 16.5 |
| | 3 | 606 | 438 | 72.3 | 1.91 | 0.280 | 14.7 | 1.53 | 0.262 | 17.1 |

1) Cocoon and silk weight show the average value of 20 individuals which were collected at random.

(Table 2). These results may be attributed to the physiological relation between copulation, spermatozoa, fertilization and oviposition, which may have been unfavourablly effected by the experiments. Details regarding these points will be discussed in another paper.

## III. Effects on the progeny of the treated individuals

In order to study whether the spermatozoa had been influenced by the experiments, the progeny were bred. The result is shown in Table 3. In the table, the ratio of full grown larvae to reared represents viability; cocoon weight indicates indirectly the relative weight of the full grown larva; the ratio of silk weight to cocoon weight expresses roughly the silk producing ability of a larva. The data are not enough to warrant a definitive conclusion, but they enable one to believe that at least no unfavourable effect on the progeny results from the treatments. It is also notable that there is found no difference between the progeny of the spermatozoa taken from the moth and that from the pupa.

## Consideration and conclusion

In 1916, as mentioned above, the first and only one success of artificial insemination in Lepidoptera was gained by HARRISON. His method is analogous to the present writer's artificial mating method. But the possibility of it is questionable, judging from the mechanism of the ejaculation in the silkworm moth. In fact, he made no other success than the one although he made repeated trials (FEDERLEY, 1929). It may not be unreasonable to suggest that the male of *Philosamia promethea,* which carried out the ejaculation in the operator's hands, had accidentally made a mating with a *promethea* female within a few minutes before the experiment and was in the state of imminent ejaculation.

In the silkworm moth, one might think of some other methods than those described above which might be successful: *e. g.*, to gather the spermatozoa either from the testis or from the receptaculum seminis of a mated female; or to inject the spermatozoa directly into the oviduct near the micropyle of the egg. Were these possible, they might contribute many valuable techniques in the

direction of the physiology of the fertilization and genetics, etc. But these methods can scarcely be put into practice. It is hardly possible to gather any sufficient quantity of mature spermatozoa directly from the testis, for those spermatozoa do not stay in the follicles of the testis but progress to the ductus deferens one by one, moreover the character of the spermatozoa in the testis is not the same as of those in the ductus deferens or the vesicula seminalis. It is also very difficult to gather sufficient spermatozoa from the receptaculum seminis to use in the experiment, on account of the minuteness of the organ. Furthermore it may be impossible to fertilize the eggs in the oviduct or ovarial tubes by direct injection of the spermatozoa, because of the delicacy and complexity of the mechanism of the penetration of the spermatozoa into the micropyle (ÔMURA. 1935) (6).

So it will be recognized that no successful method may be found for the artificial imsemination in the silkworm moth other than those described above.

However, it may not be impossible to modify the method. For example, it may be possible to mix the semen with such a solution as physiological salt solution or glucose solution to dilute the seminal fluid in order to make the injection easy, or to substitute some solution for the secretion of the glandula prostatica to activate the spermatozoa in the male sexual organs.

Each of the three methods above described possesses its own characteristics. The first glass tubule method needs healthy moths of both sexes, which have the ability to copulate, in order to gather the spermatozoa from the mated female, and it compels one to kill the female unless he makes the male to ejaculate outside the female body by early separation of the copulation. The second glass tubule method needs no female to get spermatozoa, and further, a feeble male, even the pupa, may be acceptable as the material as far as he can supply mature and sound spermatozoa. But it is the defect of this method that the male has to be killed. The artificial mating method needs healthy sexes and is most difficult in practice, beside consuming much time, yet it requires neither the sacrifice of one of the sexes nor the use of any instrument.

There is, however, a fundamental difference between the glass tubule and artificial mating method. In the latter method spermatozoa can be inseminized exactly as in the natural mating, while

in the former it is impossible; that is to say, in the former any kind of stimulus can be given to the spermatozoa at the operator's will, while in the latter it is impossible.

The operator may select either of the methods according to his circumstances.

In practice, either of the methods results equally without any unfavourable effect on the progeny, though the number of the eggs laid as well as the percentage of the fertilized eggs is reduced more or less. In conclusion, it may be stated that these methods can be applied to various biological experiments.

## Résumé

1. The author has suggested three methods of artificial insemination of the silkworm moth.

2. The first is called the glass tubule method, in which the spermatozoa taken from the bursa copulatrix of a mated female were injected into that of a given female.

3. The second is also a glass tubule one, in which were used the spermatozoa taken from the ampulla ductus deferentis or vesicula seminalis of a male moth or pupa and activated by the secretion of the glandula prostatica.

4. The third is, so to speak, an artificial mating; which depends on the fact that the ejaculation of the male moth is caused by some 5 minutes mating, and not discontinued by early breaking of the copulation.

5. The treatments yielded some unfavourable effects on the total number of eggs laid as well as on their fertility, but not on the physiological characters of the progeny.

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