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ILLUSTRATION OF HITCHES IN *ANTHERAEA MYLITTA* SEMEN COLLECTION, PRESERVATION AND ITS INSEMINATION

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ABSTRACT

Semen collection, preservation and insemination are extensively used technique in higher organisms but in insects, sperm it is tricky. Owing the wild nature, even in natural condition *A. mylitta* faces coupling and insemination linked problems leading to low coupling percentage, unfertilized egg percentage and low fecundity etc. The insemination is having practical importance in breeding to transfer desired characters. In the present study hitches in semen collection and its insemination has been documented. The collection of semen from male moth of *A. mylitta* and insemination in females was performed. Collected semen was preserved at various temperatures (-20^{0}C & -80^{0}C and Cryo-planner with liquid nitrogen) showed 20-25% viability in sperm in contrast to the freshly collected semen viability 60-80%. Syringe-pipette-tips based probe was firm-up for insemination, although the hatching did not occurred in the inseminated moths but two positive sign was noticed (i) Eggs layed by inseminated moth-took more time to get depress in contrast to eggs layed by virgin female which indicates that insemination occurred in female but due some factors, hatching could not occurred (ii).The same was further verified by dissecting the inseminated females which showed the presence of sperms. To further explore the plausible reason for not getting eggs hatching, the freshly emerged male moth reproductive system was dissected and the sperms presence was checked which showed more sperms in the middle portion of the reproductive system. Based on this leads, the attempt was made to collect the sperm from the middle part of reproductive system and insemination in female moths but success not achieved in hatching. Although protein estimation, SDS-PAGE and microscopy was performed in order to envisage the changes in semen at morphological and protein level but correlation was not established. Present study indicated the unidentified crucial factor which caused hindrance in fertilization and hatching.

Keywords: Tasar silkworm, *Antheraea mylitta,* semen, insemination

Introduction

There are many physio-eco-variants (ecoraces) of tasar silkworm available in nature with marked diversity in morphology, physiology, fecundity, shell weight leading to marked changes in commercial characters. To transfer desired characters through breeding, availability of donar and recipient moths should be there, but this is technically not possible because of difference in emergence pattern of various ecoraces. Therefore, in the present study attempts were made for semen cryopreservation to artificially-inseminate the preserved semen to female moth as per natural emergence schedule. Based on earlier reports, it is clear that the cryo-preserved semen (sperm plus accessory glands secretions) can be used after several years of preservations. The longest reported successful storage is 22 years. Sperm cryopreservation is a widely used and established method in humans, animals including fish and insects. In insects, sperm cryopreservation has played an important role in preservation of genetic species and maintenance of stocks. In wild silkmoths like Tasar, poor grainage performance like low coupling percentage, increased unfertilized egg percentage, erratic emergence of the moths and low fecundity are observed. Semen cryopreservation has a very important contribution in developing breeding techniques such as artificial-insemination (AI) and *in vitro* fertilization (IVF). Cryopreservation of silkworm germplasm in the form of sperm could serve as a useful adjunct to research and commercial usage in sericulture. Although protein estimation, SDS-PAGE and microscopy was performed in order to envisage the changes in semen at morphological and protein level but correlation was not established. Present study indicated the unidentified crucial factor which caused hindrance in fertilization and hatching.

Materials and Methods

As per the standard methodology work conducted with need based modification in protocol of various experiments. Tasar silkworm, *Antheraea mylitta* Drury (Daba ecorace) was reared in the field laboratory as per standard rearing practices. Larvae were fed on fresh leaves of *Terminalia tomentosa* and *Terminalia arjuna.* During the rearing period, care was taken to remove the dead and diseased silkworms and bury them away from the rearing area. Based on requirement of experiment various age groups of *A. mylitta* pupae and adult were used for present study. The semen collection and its storage were performed at the laboratory

condition and semen temperature was maintained at various sets of temperatures in order to know the appropriate conditions for insemination. Humidity and temperature were regulated based on experimental needs. For each set of insemination experiments, parallel control/virgin female lots were maintained in indoor condition for comparison. As per the experimental design of the study semen collection, preservation and insemination was performed as per the other organism's procedure as well as trial and error basis.

Sorting and segregation of good quality male and female cocoons: Subsequent to completion of the rearing and cocoon harvest, the sorting/segregation of good quality male and female cocoons were performed. Finally in this process ready to emerge male and female pupae were separated.

Monitoring of moths in graiange: Male moth emerged from segregated group of cocoons were used for semen collection & cryo-preservation. During the grainage operation male & female moths were maintained separately in different cages. The viability test and temporal changes in sperm was observed using florescent/non-florescent dye using microscopic technique. Semen was inseminated in virgin females.

Collection of Semen: Collection of semen & tube cryo-preservation was performed in the laboratory. Repeated observation on temporal changes on viability of cryo-preserved semen using microscopy was also done.

Semen protein analysis: Collected semen protein concentration analysis was conducted.

The protein content was estimated as per the Bradford (1976) method with slight modifications. The bovine serum albumin was used as standard. Analysis of bio-chemical constituents of preserved semen and control semen was performed.

SDS-PAGE: SDS-PAGE was conducted to visualize the protein in Gel. Comparative study on embryonic development and hatching of cryopreserved-inseminated and control tasar silkworm was performed.

Observation on hatching: Fresh and preserved semen insemination performed and its impact on the hatching was observed. Observation on temporal changes on viability of cryo-preserved semen and analysis of protein constituents was performed. Assessment of temporal impact of cryo-preservation on artificial insemination was observed.

Steps of main experimental activity

- Sorting and segregation of good quality male and female cocoons for its eventual utilization in semen collection & cryo-preservation.
- Grainage operation & collection of male & female moths separately in different cages.
- Separation of ready to emerge male and female pupae.
- Collection of semen & tube cryo-preservation.
- Viability test of sperm using microscopic and florescent dye/ non-florescent dye Semen Protein concentration analysis.
- Observation on temporal changes and SDS-PAGE analysis.
- Fresh and preserved Semen Insemination & Impact on the hatching.
- Observation on embryonic development eggs fertilization and hatching.

Statistical analysis: Results are expressed as mean standard deviation (SD). Difference among the means of control and treatment was analyzed by Student's t-test. Differences were considered statistically significant when $P < 0.05$.

Results & Discussion

Owing the exploratory nature of the study, various fundamental works was conducted on the temporal changed in *A. mylitta* sperm morphology using microscope. In addition, the sorting of BV cocoons was also performed, and sperm collected from the emerged moths. To check the viability of sperms, various available dyes were screened in various concentrations for contrast in live and dead spore. Out of tested, Acridine orange and propidium iodide dyes were found comparatively better for staining with accuracy in staining. The chronological alteration in the earlier stored (-80C) sperms were observed. Male and female reproductive system of moth was dissected out and fertilization process during the postembryonic development was also screened (Fig. 1-2). DABA BV control lot rearing was also conducted to produce large number of cocoons for its eventual utilization in during emergence for study related experiments. These cocoons (male & female) were separated and shell weights was taken for sorting of better cocoons for preservation and its eventual utilization in various experiments. Insemination of fresh semen in the female moths resulted no hatching with inconsistency in perfection. Collected the sperms from the male and preserved in deep freezer at -80C for its impact evaluation in reference to preservation. Reduction of temperature using Cryo-planner was conducted using various programmes in reducing temperature in cyclic manner such as 5C/Min, 10C/Min, 2C/min etc. (Fig. 1-2). After repetition of insemination experiments perfection in modus operandi could not established.

Finding of study

- Method for collection of semen from newly emerged male moth of *A. mylitta* & semen cum activated sperm from newly-mated-females has been attempted.
- Collected semen was preserved at various temperatures including -20^0C & -80^0C showed 20-25% viability in sperm in contrast to the freshly collected semen viability 60-80%. Viability of sperm was checked using dye.
- Syringe-pipette-tips based probe was firm-up for insemination related use.
- Freshly collected semen was inseminated in female moth using probe, although the hatching did not occurred in the inseminated moths but two positive sign was found: (1) Eggs layed by inseminated moth-took more time to get depress in contrast to eggs layed by virgin female which indicates that insemination occurred in female but due some factors, hatching could not occurred. (2). The same was further verified by dissecting the inseminated females which showed the presence of sperms in the inseminated female. Some photographs attached (Fig. 1A-L).
- Although once fewer percentage of hatching was noticed in one inseminated moth but result was not reproducible. After repeated attempted and change in protocol success in egg hatching not observed.
- To further explore the plausible reason for not getting eggs hatching, the freshly emerged male moth

reproductive system was dissected and the sperms presence was cheeked which showed more sperms in the middle portion of the reproductive system.

- Based on aforementioned leads, the attempt was made to collect the sperm from the middle part of reproductive system and insemination in female moths.
- The information generated in study repeated for its consistency but success not achieved in hatching.
- The key equipment Cryo-planner (shifted from CSGRC Hosur 2018-19) also used at CTR&TI Ranchi using liquid nitrogen but success not achieved.
- Insemination in *A. mylitta* and cryopreservation technique is Novel approach. It is observed that, even effective expertise is not available in India. However, very scanty literature available on sericigenous insects at global level in view of above valuable aspects investigators team and Institute have taken core initiatives on this exploratory study by conducting the various plausible experiments but success in eggs hatching could not achieved.

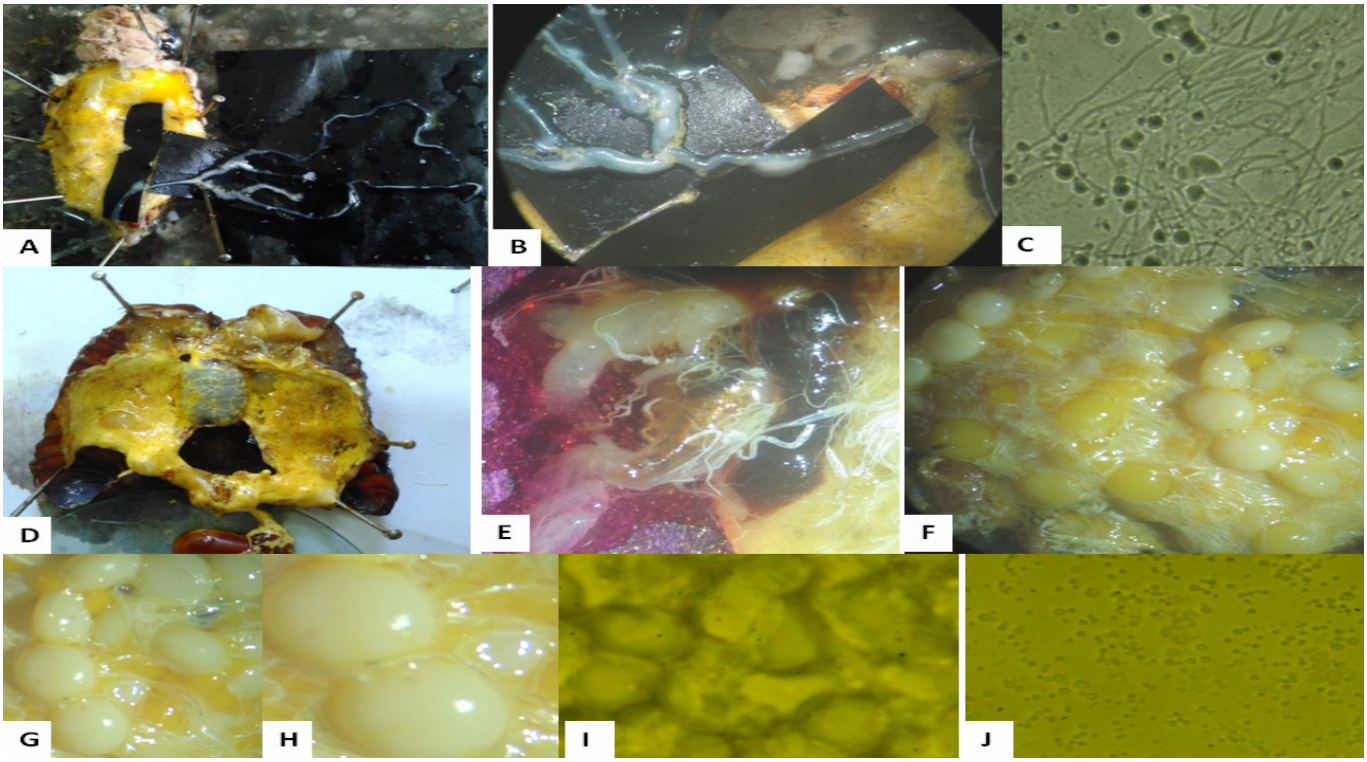

Fig. 1: Showing male and female reproductive organ, sperm and embryo of tasar silkworm *A. mylitta*. (A). Dissected male reproductive organ, (B). Male reproductive organ, (C). Sperm bundles (D). Dissected pupae (E). Female gonad, (F). Eggs in ovarioles (G.) Immature Eggs (H). Enlarged eggs (I) Microscopic observation of eggs (J). Eggs fluid.

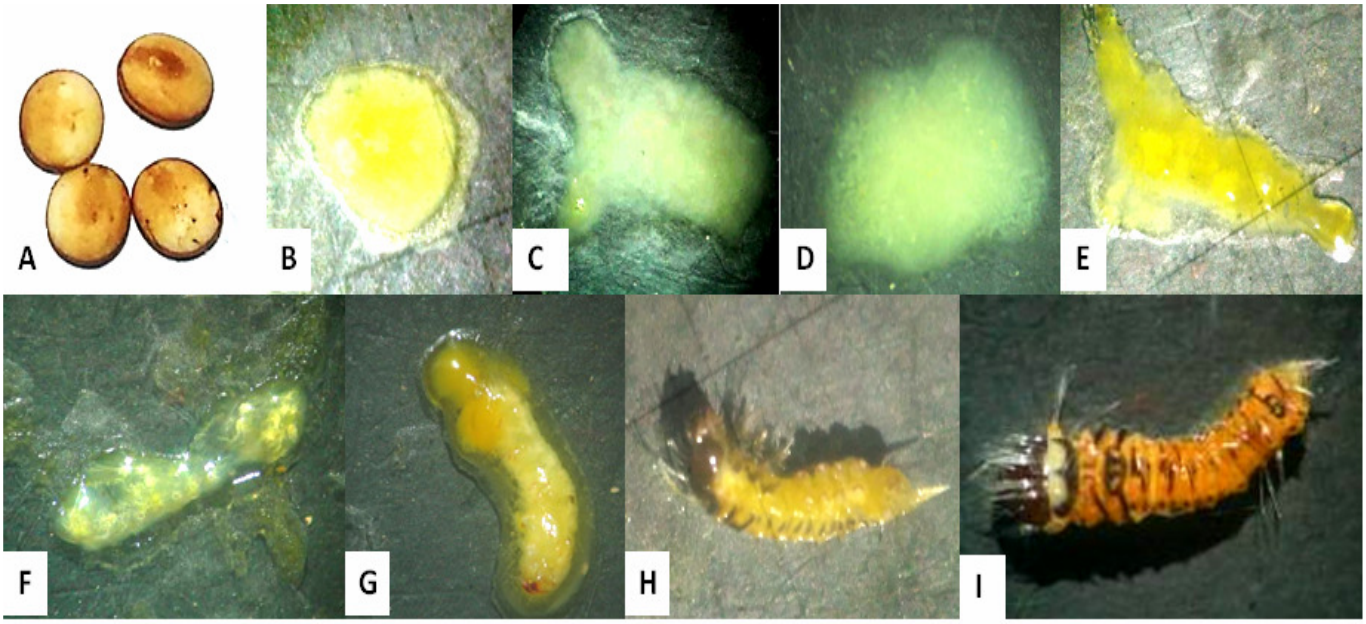

Fig. 2: Showing temporal changed inside the *A. mylitta* eggs during embryonic development: (A) Dissected eggs section, (B). Zero day old eggs, (C.). 1 day old eggs D. 2 day old eggs E. 3 day old eggs, F. 4 day old eggs G. 5 day old eggs (H). 6 day old eggs (I) 7 day old eggs. Note: no postembryonic development noticed in inseminated moths.

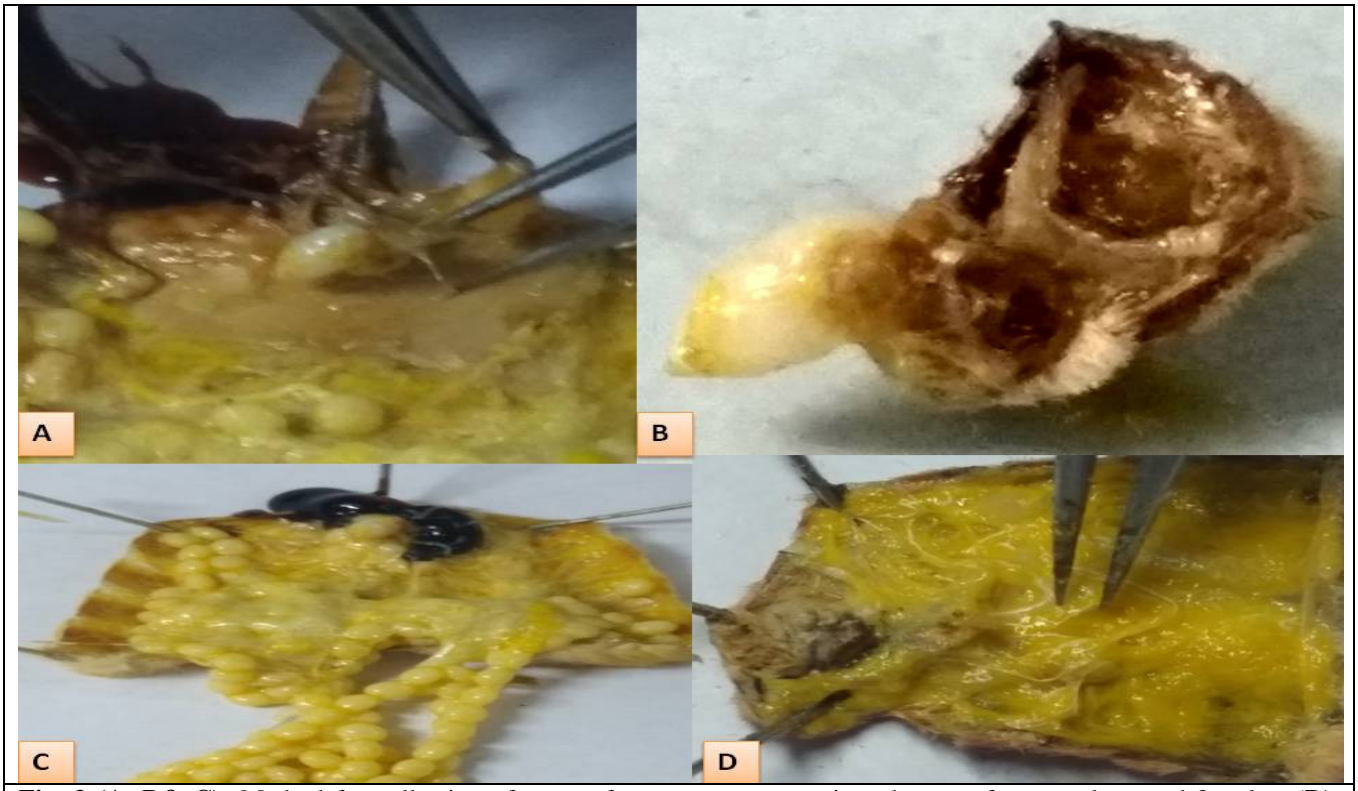

Fig. 3 (A, B& C): Method for collection of semen from semen cum activated sperm from newly-mated-females. **(D).** Method for collection of male reproductive system for semen collection from newly emerged male moth of *A. mylitta*.

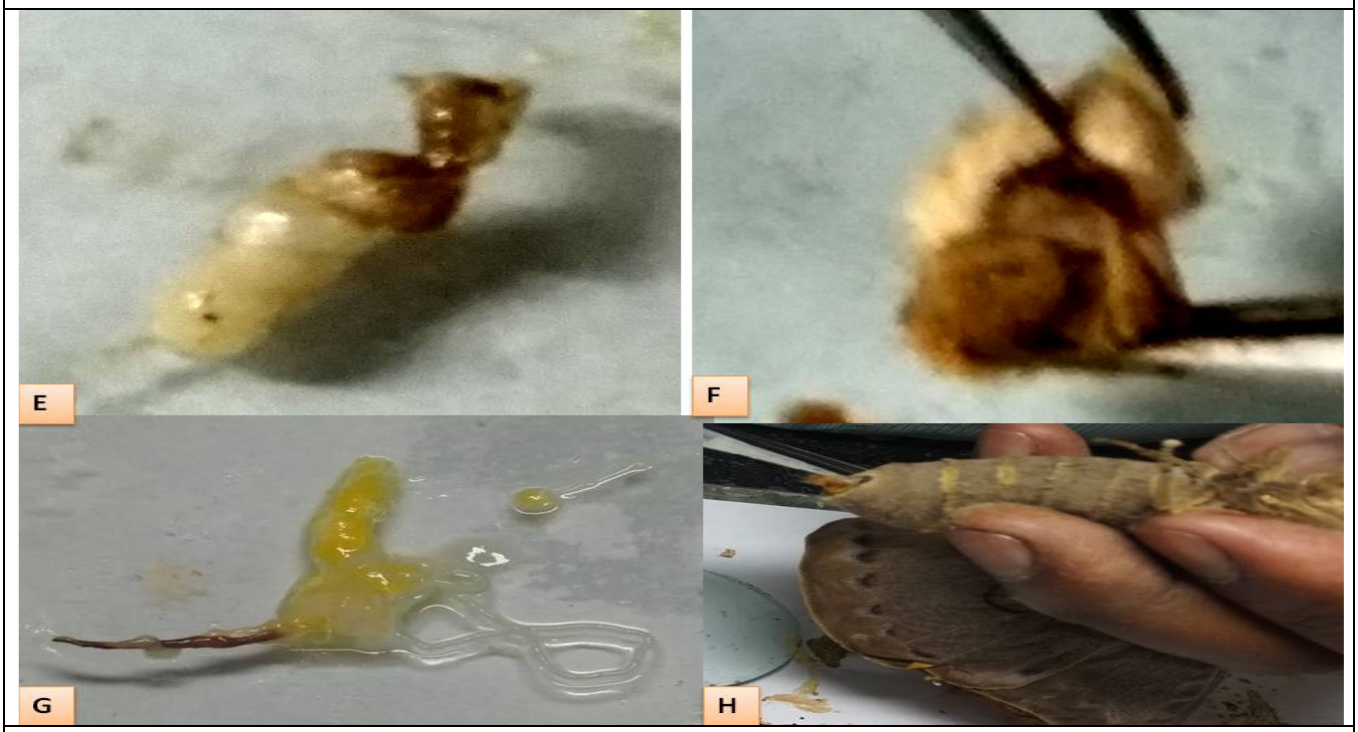

Fig. 3: (E.) Presence of sperm in female reproductive organ of inseminated moths. (F). Scanty female reproductive system of virgin female moth. (G.) Semen collected from the various region of Male reproductive system. H. Insemination of semen in female moth using probe.

Fig. 3 (I&K). Depressed eggs of virgin female. **(J&L)** Eggs of inseminated females

Note: Although the hatching did not occurred in the inseminated moths but two positive sign was found: (1) Eggs layed by inseminated moth-took more time to get depress in contrast to eggs layed by virgin female which indicates that insemination occurred in female but due some factors, hatching could not occurred (please compare Fig. 1 I&J). (2). The same was further verified by dissecting the inseminated females which showed the presence of sperms in the inseminated female (please see Fig. 1 E&F)

Impact of moth incubation at various temperature: Various experiment conducted as mentioned below:

- Incubation of moth at **20**, **25**, **30** and **35**^{0}C was done to identify the better temperature for incubation.
- Identification of sensitive time suitable to respond for insemination based on the natural emergence pattern in grainage house.
- Based on various treatments it is found that 25^{o}C temperature and insemination immediately after emergence is proper to inseminate the sperm.

Correlation of findings: Various experiment indicated that, insemination in *A. mylitta* not established. Various attempt and failure in hatching also indicated the some factors responsible for the proper insemination and it could not be ascertained.

Final observation: Insemination in *A. mylitta* modus operandi is novel approach. It is observed that, even effective expertise is not available in India. However, very scanty literature available on sericigenous insects at global level in view of above valuable aspects investigators team and Institute have taken core initiatives on this exploratory study by conducting the various plausible experiments but success in eggs hatching could not achieved.

Table:-1: Effect of insemination on fecundity and hatching of *A. mylitta.* Denote indicates significant difference between control and treated *P < 0.05 and **P < 0.01.

S. No.	Parameters	Control	Inseminated
1.	Emerged	86±5	84±3
2.	Unmerged	14±3	16±2
3.	Fecundity	196±5	155±5**
4.	Hatching	88±5	00±0**

Note: *Results are expressed as mean standard deviation (SD). Difference among the means of control and treatment was analyzed by Student's t-test. Differences were considered statistically significant when P < 0.05*

Core initiative of study: In the present study semen collection and its cryopreservation has been attempted to artificially-inseminate the preserved semen to female. Core theme was to transfer desired characters through artificial-insemination. Although, semen collected and cryo-preserved but hatching not observed in inseminated moths. Although sperm cryopreservation is a widely used and established

method in humans, animals including fish. But in insects, sperm cryopreservation seems to be very tricky. In tasar silk moths *Antheraea mylitta* many problems exist even in natural grainage (control lots) itself i.e. poor grainage performance, low coupling percentage, unfertilized egg percentage, erratic emergence of the moths and low fecundity etc. Inseminated females layed the eggs but hatching not observed. Although semen cryopreservation is having practical importance in developing breeding techniques such as artificial-insemination (AI) and *in vitro* fertilization (IVF) but in spite of repeated trial success could not achieved. Present study indicated the unidentified crucial factor which resulted un-hatched eggs.

Underpinning idea of study: Semen collection technique in one of the key initiatives of study. Although the information generated in study has no immediate applied value but the initial information in this line will help in future. Cryo-preservation of semen/sperm could not ascertain. The failure in this line is being documented as useful adjunct to further research in sericulture. The semen collection is also useful for semen gene banking. Following are the key issues related to the study:

- In tasar silk moths *Antheraea mylitta* many problems exist even in natural grainage (control lots) itself i.e. poor grainage performance, low coupling percentage, unfertilized egg percentage, erratic emergence of the moths and low fecundity etc.
- Inseminated females layed the eggs but hatching not observed. *Antheraea mylitta* diapause: December-June.
- Management of abiotic factors is necessary to regulate semen preservation.

Inventiveness of findings: Method for collection of semen from newly emerged male moth of *A. mylitta* & semen cum activated sperm from newly-mated-females has been attempted. Collected semen was preserved at various temperatures including -20^{0}C & -80^{0}C showed 20-25% viability in sperm in contrast to the freshly collected semen viability 60-80%. Syringe-pipette-tips based probe was firm-up for insemination related use. Freshly collected semen was inseminated in female moth using probe, although the hatching did not occurred in the inseminated moths but two positive sign was found: (1) Eggs layed by inseminated moth-took more time to get depress in contrast to eggs layed by virgin female which indicates that insemination occurred in female but due some factors, hatching could not occurred. (2). The same was further verified by dissecting the inseminated females which showed the presence of sperms in the inseminated female. After repeated attempted and change in protocol success in egg hatching not observed. To further explore the plausible reason for not getting eggs hatching, the freshly emerged male moth reproductive system was dissected and the sperms presence was cheeked which showed more sperms in the middle portion of the reproductive system. Based on this leads, the attempt was made to collect the sperm from the middle part of reproductive system and insemination in female moths. The information generated in study repeated for its consistency but success not achieved in hatching.

Equipment Cryo-planner (also used at CTR&TI Ranchi using liquid nitrogen but success not achieved. Although protein estimation, SDS-PAGE and microscopy was performed in order to envisage the changes in semen at morphological, physiological/protein content level but its correlation was not established. Changes in protein profile have been also visualized which varied greatly. The marked changes in protein profile were recorded. The promising ground for aforesaid alterations in semen could not be determined.

Prospective utility: Semen collection technique in one of the key initiatives of study. Although the information generated in study has no immediate applied value but the initial information in this line will help in future to proceed in this direction. Cryo-preservation of semen/sperm could not ascertain. The failure in this line is being documented as useful adjunct to further research in sericulture.

Physical target and work done: Detail mentioned in methodology and result part of the study.

Salient findings and initiatives:

- Information generated in line of semen collection technique.
- Method for collection of semen from newly emerged male moth of *A. mylitta* & semen cum activated sperm from newly-mated-females has been firmup.
- Collected semen was preserved at various temperatures including -20^{0}C & -80^{0}C showed 20-25% viability in sperm in contrast to the freshly collected semen viability 60-80%.
- Syringe-pipette-tips based probe was firm-up for insemination related use. Freshly collected semen was inseminated in female moth using probe, although the hatching did not occurred in the inseminated moths but two positive sign was found: (1) Eggs layed by inseminated moth-took more time to get depress in contrast to eggs layed by virgin female which indicates that insemination occurred in female but due some factors, hatching could not occurred. (2). The same was further verified by dissecting the inseminated females which showed the presence of sperms in the inseminated female.
- After repeated attempted and change in protocol success in egg hatching not observed.
- To further explore the plausible reason for not getting eggs hatching, the freshly emerged male moth reproductive system was dissected and the sperms presence was cheeked which showed more sperms in the middle portion of the reproductive system.
- Based on this leads, the attempt was made to collect the sperm from the middle part of reproductive system and insemination in female moths.
- The information generated in study repeated for its consistency but success not achieved in hatching.

A. Virgin Female eggs

B. Inseminated females eggs

Fig.4: Showing eggs of virgin females and eggs of inseminated moths.

Conclusion: In the present study various experiments conducted which revealed following conclusions:

- Method for collection of semen from newly emerged male moth of *A. mylitta* & semen cum activated sperm from newly-mated-females has been firm up. Collected semen was preserved at various temperatures including -20^0C & -80^0C showed 20-25% viability in sperm in contrast to the freshly collected semen viability 60-80%.
- Syringe-pipette-tips based probe was firm-up for insemination related use. Freshly collected semen was inseminated in female moth using probe, although the hatching did not occurred in the inseminated moths but two positive sign was found: (1) Eggs layed by inseminated moth-took more time to get depress in contrast to eggs layed by virgin female which indicates that insemination occurred in female but due some factors, hatching could not occurred. (2). The same was further verified by dissecting the inseminated females which showed the presence of sperms in the inseminated female. After repeated attempted and change in protocol success in egg hatching not observed.
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