

Original Research Article

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A Unique Technique for Artificial Insemination in Tasar Silkworm, *Antheraea mylitta* with Fresh and Frozen Semen

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ABSTRACT

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Artificial insemination is a very important tool and has been used for hybrids, mutant lines and producing inbred lines is only possible with a method that ensures controlled mating. During the tasar eggs production, male and female emergence are differ as male moths abundance during early phase where as female later phase of grainage which resulted loss of uncoupled female moths (10 to 19%). To reduce loss of eggs and necessity of hybrid/inbred lines in *Antheraea mylitta*, present study was conducted. A unique and farmers friendly technique of artificial insemination of *A. mylitta* has been developed. A Tris buffer diluent with volume of 100 μ l effective for homogenous mixing of semen collected from single male. The response was decreases on advancement of age of male and female for collection of semen and insemination. The data reveals that there were no significant difference observed among treatment on fecundity, hatching and chawki survival of larvae when female moth inseminated with fresh semen, semen kept at 0°C for 1 day and at -80°C for up to 120 days. It can be concluded that artificial insemination with fresh and frozen is feasible in *A. mylitta* which is very helpful to tasar silk industry.

Introduction

Tropical tasar silkworm, *Antheraea mylitta* Drury (Lepidoptera: Saturniidae) is a polyphagous sericigenous insect, produces tasar silk of high commercial importance. Rearing of DABA ecorace of tasar silkworm conducted two to three times in a year to produce the tasar cocoons. During the tasar silkworm eggs production, male and female moths emerged from its cocoons. The pattern of male and female moth's emergence are differing as male moth's abundance during early phase where as female later phase of

grainage. Therefore, male moth's scarcity during the later phase of emergence and loss of uncoupled female moths was recorded to be 10 to 19%. Some attempts have been to avoid male moths' loss through preserved it in cold condition at 4 to 10°C temperature under refrigerator. But cold preservation of male moths has it limitation due to low longevity (4-9 days).

Daba ecorace is one of the most commercially exploited races among 44 ecoraces of *A. mylitta*. Hybridization studies involving ecoraces and their exploitation could not be taken made in the field for its utilization. In recent

years some attempts were made to characterize ecoraces based on molecular techniques and observations show that the studied ecoraces are genetically divergent. The maintenance of genetic bio-resources is one of the important goals for biological studies. In the case of the silkworm, the importance of commercial strains for effective production of silk and of mutant strains as experimental animals is recognized.

Tasar silkworm ecoraces are one time yielder in its life time. There is hardly any way to keep the genotype for further user. So, to avoid compatibility of males and females and for further use of genetic resources, artificial insemination technique is essential to evaluate feasibility of cross combination of distinct ecoraces.

Artificial insemination techniques have been studied in *Bombyx mori* (Ômura, 1936). Takemura *et al.*, (1996; 1999) developed the most effective method of artificial insemination, by which fertilizes eggs are obtained at normal rate, almost 100%. The artificial insemination in *B. mori* was successfully performed using cryopreserved semen in most strains tested (Takemura and Kanda, 1999; Takemura *et al.*, 2000). This opens the gate to store the resources without rearing the strains every year. Artificial Insemination is a very important tool and has been used for various purposes. Keeping mutant lines, selecting behavioural strains, testing maternal effects and producing inbred lines is only possible with a method that ensures controlled mating.

In the view of above and necessity of tasar silk industry for higher yielding hybrid/ inbred lines, the present study was taken up to develop a simple technique of artificial insemination of *A. mylitta* with utilizing fresh and frozen (cryo) semen.

Materials and Methods

Insect culture

Rearing of tropical tasar silkworm, *A. mylitta* Drury (Lepidoptera: Saturniidae) DABA ecorace was conducted in outdoor conditions of its primary food plants *T. tomentosa* by using slandered rearing protocol (Kumar *et al.*, 2008). Male and female cocoons were kept separately in grainage house under wooden wire mess case after sex determination in pupae i.e. a genital aperture in the form of a minute line in female and a dot in male is present ventrally on 8-9th and 9th segment respectively. Emerged male and female moths of

different age groups were kept separately in wooden wire mess cages for experimental use. The sample size n=30 for each treatment. The experiment was conducted at Silkworm Physiology Laboratory, CTR&TI, Ranchi during 2010-11.

Collection of Semen

For collection of semen, whole internal reproductive systems were dissected from male moth and the organs were lower portion, the seminal vesicle along with accessory gland removed and kept in 1.5ml pre-chilled eppendorf tube containing different volume of buffers i.e. Hey solution (Ruttner, 1975), Tris buffer (Takemura *et al.*, 2000), physiological saline and double distilled water (control). The semen was collected by rupturing of seminal vesicles in 1.5 ml pre-chilled eppendorf tube with buffer. The semen was kept at 0°C and -80°C for insemination use. Cryopreservation and thawing procedure followed the method developed by Takemura *et al.*, (2000). The frozen semen in the eppendorf tube was thawed in water at 37°C for 5 sec before insemination.

Simple technique for artificial insemination

The artificial insemination was carried out according to the method for *B. mori* reported by Omura (1936, 1938); Takemura *et al.*, (1996, 1999) with modification. Data was recorded on age of male and female moths for collection of semen and insemination, response, fecundity, hatching and chawki survival on Tasar Amrit (semi synthetic diet) indoor condition.

Results and Discussion

In *Bombyx mori*, Ômura, 1936; Takemura *et al.*, (1996, 1999) developed the most effective method of artificial insemination, by which fertilizes eggs are obtained at normal rate, almost 100%. In present study, we developed simple and farmers friendly technique of artificial insemination of *A. mylitta*. A simple glass tubule dropper 10 cm long, one end fitted 50 µl micro pipette tip which used for inserting in the female genital opening (bursa copulatrix) for injection of semen and another end fitted with rubber valve which help in airpump (Fig. 1). For insemination, the female moth holds in left hand with help of thumb and middle finger and index finger support dorsally. Female moth excited/warming up by rubbing of genital organs with index finger of right hand (Fig.2). Fresh/ cryo-preserved

10 µl semen injected to the bursa copulatrix with the help glass tubule (modified). Inseminated female moth kept for rest, cut the wings and allows for egg laying in paper box.

Effect of different diluent and volume on response, fecundity and hatching of inseminated female

Artificial insemination of *A. mylitta* silkworm with different diluents buffer and volume for dilution of semen and its impact on response, fecundity and hatching are presented in table 1. The result reveals that the maximum response, fecundity and hatching of eggs were recorded to be in the Tris buffer diluent with 100 µl i. e. 90.00 per cent, 221.50±2.557 eggs and 81.33±0.745 per cent, respectively followed by 83.33 per cent, 197.23±2.303 eggs and 80.60±0.701 per cent, respectively on 50 µl Tris buffer volume. Whereas response, fecundity and hatching were observed to be very less in other buffer diluents as well as volume. Tris buffer diluent with volume of 100 µl effective for homogenous mixing of semen collected from single male of *A. mylitta*. The finding conformity of Moritz (1984) report that a tris buffer diluent containing arginine and lysine proved to be feasible for use in the honey bee queen insemination.

Fitness age of male and female moths for artificial insemination

The adult/moth stage of tropical tasar silkworm is non-feeding and moth longevity varies from 4-9 days. The effect of age of male collection of semen and female for insemination presented in table 2.

The results indicated that the most effective age of male for collection of semen and female for insemination was observed to be up to day 2 in the term of fecundity and hatching i.e. 223.24±1.24 to 198.24±2.02 and 76.35±0.63 to 82.08±0.35, respectively. It can be inferred that the response was decreases on advancement of age of male and female moths. Tobin *et al.*, (2014) observed in *Lymantria dispar* (L.) that increases in male and female age reduce the rate of fertilization, which is furthermore reduced, as males mate multiple times as they age.

To establish possible effects of male age on sperm quality and to evaluate effects of elevated temperatures, food deprivation during sexual maturation, and immune challenges. We found that sperm viability decreases with male age (Stürup *et al.*, 2013). Santhosh and Krishna (2013) suggests that in *D. bipunctata*, male age affects the number of accessory gland cells and the quantity of protein in the accessory gland.

Table.1 Effectiveness of different buffer and volume for artificial insemination in *A. mylitta*.

Treatment	Dilution volume for single SV/AS gland (µl)	Female moth responded (%)	Fecundity (No.) Mean ±SE	Hatching (%) Mean ±SE
Hey buffer	25	6.67	178.03±2.850	10.63±0.488
	50	13.33	177.00±2.787	20.93±1.475
	100	16.67	178.83±2.438	18.23±0.998
	200	3.33	177.20±2.766	10.00±0.534
Tris buffer	25	43.33	189.67±2.294	79.20±0.504
	50	83.33	197.23±2.303	80.60±0.701
	100	90.00	221.50±2.557	81.33±0.745
	200	13.33	182.93±2.726	60.23±0.689
Physiological saline	25	6.67	177.70±2.902	19.57±0.845
	50	13.33	180.57±3.053	28.97±1.359
	100	16.67	177.27±2.859	21.00±1.067
	200	3.33	176.90±2.808	11.63±0.728
Double distilled water	25	6.67	180.20±3.045	12.93±0.314
	50	10.00	181.23±3.014	13.67±0.685
	100	13.67	177.73±2.788	12.93±0.362
	200	3.33	175.53±2.673	9.73±0.403

Table.2 Fitness age of male and female moths for artificial insemination.

Age of male moth collection of semen	Age of female moth for artificial insemination	Fecundity (No.) Mean ±SE	Hatching (%) Mean ±SE
Day 1	Day 1	223.24±1.24	82.08±0.35
	day 2	220.27±1.12	79.87±0.57
	Day 3	219.40±1.68	77.86±0.61
Day 2	Day 1	221.50±1.98	81.30±0.48
	day 2	198.24±2.02	76.35±0.63
	Day 3	178.36±2.17	71.88±0.89
Day 3	Day 1	175.87±1.61	68.53±0.81
	day 2	148.20±2.08	55.30±1.74
	Day 3	113.14±2.56	39.64±2.18
Control (natural coupling) Day 1	Day 1	226.00±1.12	84.35±0.61
	day 2	221.40±1.35	82.98±0.75
	Day 3	181.50±1.56	73.40±0.89

Table.3 Impact of artificial insemination with fresh and frozen semen on fecundity, hatching and survival of *A. mylitta*.

Treatment	Duration	Fecundity (No.)	Hatching (%)	Chawki survival (%) on semi synthetic diet
Fresh semen collected from one day old moth)	1 day old male moth	222.10 ±3.568	80.07±0.851	91.70±0.808
Semen kept in freezer 0°C (collected from 1 day old moth)	1 day	221.50±2.557	79.63±0.736	91.10±0.916
	7 day	180.30±3.075	46.30±1.828	88.63±0.827
	30 days	158.07±1.561	22.80±1.892	84.70±0.925
Semen kept in -80° C (collected from 1 day old moth)	1 day	220.27±2.056	79.87±0.567	91.57±0.789
	7 day	219.40±2.369	79.73±0.614	91.30±0.937
	30 days	220.73±2.637	79.53±0.579	91.27±±0.770
	60 days	220.40±2.344	80.17±0.612	91.57±0.789
	120 days	219.23±2.386	79.90±0.570	91.53±0.527
Natural coupling	1 day	221.60±2.155	84.13±0.449	92.30±0.611

Figure.1 Glass tubules with micro pipette tip



Figure.2 Insemination of female moth



The size and number of main cells in the accessory gland and the size of the accessory gland were related to the production of protein. Effect of delayed mating on the fecundity, fertility and longevity of female of diamondback moth, *Plutella xylostella* was studied by Wang *et al.*, (2011) and found that both males and females delayed mating, the female fertility and fecundity decreased; egg fertility affected marginally.

Impact of artificial insemination with fresh and frozen semen on fecundity, hatching and survival of *A. mylitta*

Freshly emerged female moths inseminated with fresh semen, semen kept at 0°C for one, 7 and 30 days, frozen semen (-80°C) thawed at one to 120 days which was collected from one day old moth and diluted with 100 µl Tris buffer. The results reveals that there were no significant difference observed among treatment on fecundity, hatching and chawki survival of larvae except semen kept at 0°C for 7 and 30 days. It can be concluded that artificial insemination with fresh and frozen is feasible in *A. mylitta*. In *bombyx mori*, artificial insemination with fresh and cryopreserved semen (971 days) was developed and observed that female moths artificially inseminated had no significant difference on fertility, laid eggs and survival of larvae (Omura, 1936; Takemura *et al.*, 1996; Takemura and Kanda, 1999; Takemura *et al.*, 1999, 2000 and Mochida *et al.*, 2007).

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Author Contributions

Dinesh Kumar: Investigation, formal analysis, writing—original draft. B. C. Prasad: Validation, methodology, writing—reviewing. A. K. Sinha:—Formal analysis, writing—review and editing. J. P. Pandey: Investigation, writing—reviewing. P. K. Mishra: Resources, investigation writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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