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Embryonic Development Stages in Muga Silkworm, *Antheraea assamensis* Helfer–Suitable Embryonic age for Preservation

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ABSTRACT: Embryonic development in muga starts within a few hours of egg laying. Chronological variations during embryonic development of normal eggs of *Antheraea assamensis* Helfer were recorded from 24 h to till hatching. During 10-16 h of embryonic development, the ventral plate is formed and by 24 h trough shaped embryonic premodium floats in yolk and the protocephalon and protocorn are separated by transverse furrows. After 96 h the embryo became C-shaped. The thoracic region is clearly divisible into three thoracic segments. After 120h, organogenesis in embryo takes place. The antennae bear segments and head region was detachable into three segments. The head capsule formation completed after 144 h and mouth parts got matured. Three segmented antennae with antennal setae, the mandibles and labrum are well developed. After 168 h old, mandible become sclerotised and pigmented at distal ends. Larval eyes appear as six brown spot on either side of head. The spiracles are clearly visible on the sides of the body. Fully developed muga silkworm larva comes out from the egg cell after 8th day of oviposition rupturing the anterior part of egg shell by the mandibles. The suitable age of egg preservation at low temperature is 36h to 48h old embryo and the eggs at this stage can be preserved for 20 days without affecting hatchability. From sericulture industrial point of view, eggs of mix ages (24 h to 72 h old) are considered for preservation since the muga silk moth continue egg laying for 72 h and it has been found that Muga silkworm eggs of mix ages can be preserved up to 15 days at 7°C and 70-75% relative humidity without hampering its hatching in compare to that of control. The result of the bioassay of the preserved eggs showed that preservation of eggs at 7°C did not affect the survivability and cocoon parameters of the muga silkworm.

Key words: *Antheraea assamensis*, embryonic development, organogenesis, preservation, bioassay.

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INTRODUCTION

The muga silkworm, *Antheraea assamensis* Helfer is semi-domesticated and polyphagous sericigenous insect producing the golden lustrous silk. It is multivoltine in nature having 5 to 6 generations in a year.

Although there are about 15 known food plants, the larvae of muga primarily feed on the foliages of Som, *Persea bombycina* Kost and Soalu, *Litsaea monopetala* Juss (Kumar, 2017; Neog *et al*., 2006; Mech *et al.,* 2015; Goswami *et al.,* 2015). Egg is considered as the key factor of sericulture industry.

Only quality eggs can ensure a healthy crop and good harvest. The oviposition and hatching of eggs of muga silkworm are severely affected during the adverse summer months of the year i.e. July to September owing to the prevalence of high temperature coupled with very high humidity . The low moth emergence at initial and last part of seed preparation resulted the wastage of valuable eggs due to lack of technology for silkworm egg preservation which affects the muga silk industry. Postponement of hatching sometimes becomes inevitable for solving problems associated with synchronized brushing, as per suitability of season and availability of good foliage (Pandey *et al.,* 1992). In traditional practice, muga silkworm eggs are preserved at room condition or BOD incubator at 26 ± 2°C for incubation just after the completion of the daily grainages in a piece meal system during all the seasons of rearing. The eggs are collected after 72 hours of laying. This practice creates problems like, synchronizing hatching with leaf sprouting, non uniform hatching due to mixing of eggs of different ages leading to unequal development of worms during rearing and inconvenient in timely distribution of eggs in bulk with uniform hatching for commercial rearing. Therefore, for commercial seed preparation, it becomes necessary to develop appropriate technologies of preservation of eggs in muga culture to postpone hatching for synchronizing hatching with leaf sprouting, to skip unfavourable seasons and timely supply of eggs in bulk with uniform hatching. This will protect the wastage of valuable biological material. For preservation of embryo at low temperature, it is essential to know the chronology of embryonic developmental stages and discern the suitable embryonic stage for preservation at low temperature. Technologies for preservation of multivoltine muga silkworm eggs have not been established thereby facing a big hurdle in the management of seed sector of the industry. The non-diapause eggs mulberry silkworm, *B. mori* can be preserved at low temperature for more than 70 days without affecting the hatching (Yu, 1993; Rajanna *et al*., 2009). In mulberry silkworm, the longest embryonic stage i.e. stage 15 has been identified as the suitable stage of effective preservation for longer duration (Reddy *et al*., 2003). In muga silkworm, the different embryonic developmental and suitable stages of egg preservation have not been studied. In this paper, the chronology of embryonic development stages of muga silkworm, suitable stage and safe duration of preservation of eggs are presented.

MATERIALS AND METHOD

Muga silkworm eggs were collected from the Muga Silkworm Breeding Section, Central Muga Eri Research and Training Institute, Central Silk Board, Jorhat, India. To isolate the different embryonic developmental stages of muga silkworm in different ages, the standard technique developed by Reddy *et al*. (2003) for mulberry silkworm was followed with slight modification as the eggs of muga silkworm has thick chorionic layer covered with thick gummy substances.

The zero age eggs of muga silkworm, *A. assamensis* were collected and incubated at 26 ± 2°C and relative humidity of 75-85 % for different durations from 24 h to till hatching and the stages of embryonic development at the different ages were studied at an interval of 12h. The egg samples of different age groups were boiled in 3–4 % KOH solution for 2-3 minutes and washed in 60°C water. Care is taken so that KOH does not dissolve the embryo. The embryo was then kept in distilled water maintained at room temperature in a transparent glass petridish and kept under the dissecting stereo zoom microscope. Embryos were released by squirting the eggs with water with the help of a Pasteur Pipette. The egg shell was removed from the micropylar end using a sharp surgery blade, with the help of a pointed needle and soft brush, the embryo was freed from the yolk material. The photographs of the embryos were taken for identification of stages of development with respect to particular age.

After identification of the developmental stages of the embryo, muga silkworm eggs of different embryonic ages (24 h, 36 h, 48 h, 60 h, 72 h and mix ages from 24 h to 72 h) were preserved at 5°C, 7°C and 10°C for different durations and observe their hatching performance. The suitable embryonic age, temperature and safe duration of preservation was identified. Bioassay of the preserved eggs was conducted by rearing the hatched larvae on the foliages of Som, *Persea bombycina* Kost in outdoor condition under nylon net cover and the rearing performance was analyzed.

RESULTS AND DISCUSSION

Like most other insects, life of muga silkworm begins as an independent egg. Each egg is manufactured within the female's genital system and is eventually released from her body through an ovipositor, a component of her external genitalia (Fig. 1).

The cell's cytoplasm is usually distributed in a thin band just inside the vitelline membrane and in diffuse strands that run throughout the yolk. The egg cell's haploid nucleus lies within the yolk, usually close to one end of the egg. The egg's anterior/posterior polarity is determined by the relative positions of the nucleus and the oosome. The egg is covered by a protective "shell" called the chorion made of protein secreted before oviposition by accessory glands in the female's reproductive system. The chorion is perforated by microscopic pores called aeropyles that allow respiratory exchange of oxygen and carbon dioxide with relatively little loss of water. The micropyle, a special opening near the anterior end of the chorion, serves as a gateway for entry of sperm during fertilization.

A **B**

Fig. 1. A-mother moth laying eggs, B-eggs(enlarged).

Embryogenesis is a developmental process that usually begins once the egg has been fertilized. After two hours of oviposition, male and female pronuclei unite at a definite position near the anterior pole to form zygote (Takami, 1969). The zygote yields about 5000 daughter nuclei through 12-13 cycles of mitosis without cytokinesis. The cleavage nuclei migrate through the yolk toward the perimeter of the egg. They settle in the band of periplasm where they engineer the construction of membranes to form individual cells and a one-cell-thick layer, the blastoderm is formed. Blastoderm cells on one side of the egg begin to enlarge and multiply. This region, known as the germ band is where the embryo's body will develop. The rest of the cells in the blastoderm become part of a membrane that forms the yolk sac. The cells from serosa grow around the germ band, enclosing the embryo in an amniotic membrane.

At the cellular blastoderm stage, when secondary membrane is formed between blastoderm cells and the yolk system, some cleavage nuclei migrating towards egg periphery are prevented from entering into the periplasm and they remain attached to the secondary yolk membrane. These become vitellophages. Cleavage nuclei remaining in yolk becomes the centers of yolk granules to supply nutrition to the developing embryo. Yolk membrane folds during late germ band stage after which the yolk system divided into masses each enclosing one or several nuclei and yolk organelles. This process is known as yolk segmentation (Miya, 1984).

The embryonic developmental stages in *Bombyx mori* were serially numbered from 1-30 (Takami and Kitazawa, 1960). The embryonic developmental stages in eri silkworm (*Samia ricini*) were studied and identified the suitable stage for cold preservation (Sarkar *et al*., 2012).

Stage 1-3: The fertilization is considered as stage-1, Cleavage is stage -2 and Blastoderm is stage -3. The earliest embryonic stage that can be isolated and removed is from stage 4 *i.e.* germ band onwards.

The chronological variations during embryonic development of normal eggs were recorded from 24 h to till hatching (Fig. 2A- N). Thirteen different embryonic stages were detected and among these stages the longest stage *viz*., Hei - B stage was observed at 68 h to 72 h old embryo.

Stage-4: This stage is called germ band, which develops to an embryo. A group of cells attaches to the inside at a specific region of the germ band. The cytoplasm of these cells appears dense than that of germ band cells. These are primordial germ cells. The germ band is concave on the inner side and the shape is of an oval plate. In muga silkworm, it forms within 24 h of oviposition (Fig. 2A).

Throughout 22-28 h, the germ band becomes slender and elongated and by 28-34 h, a long narrow depression called the primitive groove or streak or median plate is formed along the mid portion of the germ band on upper side. By 24-36 h, the development of embryo was well differentiated into head and trunk region (Fig. 2B).

Stage-5, 6 & 7: The embryo gradually contracts to the shape of Dharuma (Japanese doll). Gradually the head and tail region can be identified. After 48 h, segmentation of the body is clearly visible.

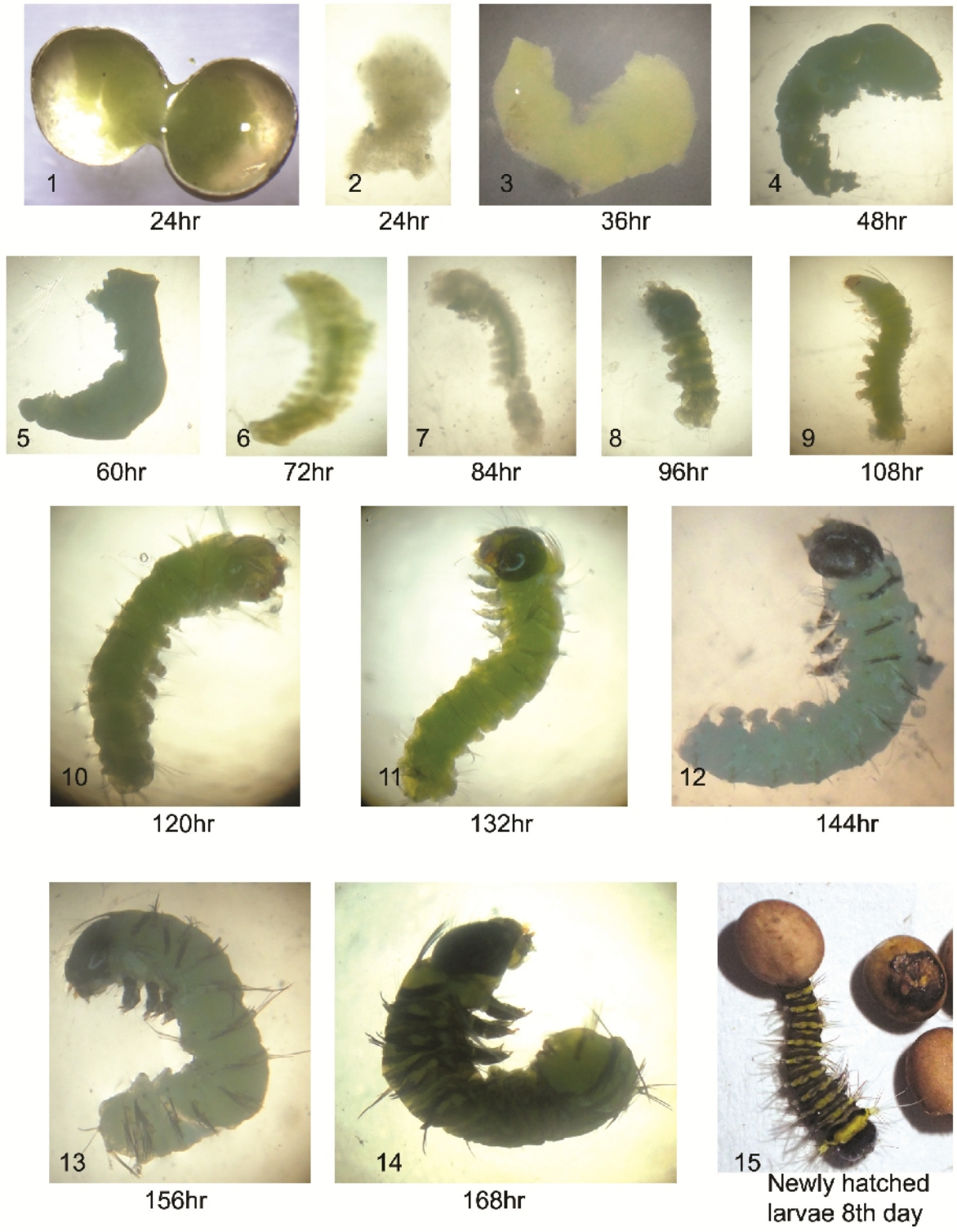

Fig. 2. 1-15. Different developmental stages of embryos of muga silkworm (24 h to till hatching).

The head end is called the protocephalon and the tail end is called caudal lobe. This stage is

Stage-15: In 72 h of age, the embryo reaches this stage wherein the metamerism of mesoderm is completed and mesoderm is arranged segmentally (Fig. 2. 6). In 72 h, embryo showed three well differentiated distinct regions of the body i.e. head, thorax and abdomen. The segmentations with

Stage-16-20: In 84-96 h, rudiments of appendages appear in thoracic region and cephalic region formed by the beginning of stomodaeum (Fig. 2. 7-8).

Stage-21: The process of blastokinesis begins in 108-120 h after oviposition. Embryo starts to move around. Blastokinesis first start in the abdominal region and extend toward heads. Posterior abdominal segments are first turned vertically so that the abdominal region as a whole forms a straight line. The abdominal region then turns towards anterior side and reaches the level of prothorax. The anterior and posterior ectodermal invaginations extend to form the fore gut and hind gut respectively (Fig. 2. 9-10).

Stage-22: The head capsule formation completed in after 132 h and mouthparts got matured (Fig.2. 11). Three segmented antennae with antennal setae, the mandibles and labrum are well developed. Yolk mass inside the eggs serves as a source of nutrients for the developing embryo and also help in holding the embryo on its surface as a necessary foundation.

Stage-23: After 144 h lateral walls complete and tips of labrum and labium become segmented (Fig. 2. 12). Thoracic legs become segmented with claws at distal end. Rudiments of the setae develop on the body surface.

Stage-24: At about 156 h, entire body of embryo is covered by strong setae and embryonic moult occurs in this stage (Fig. 2. 13). The caudal horns also occur in this stage.

continued from 36 h to 60 h in muga silkworm egg (Fig. 2, 1-5).

enough length and the amniotic fold covering the embryo were clear and serosa was completely covered with the yolk. The embryo was slender with a well-defined head and caudal region. The head has a clear cut depression in the middle. Mesoderm segments are clearly visible.

Stage-25-29: After about 168 h, mandible become selerotised and pigmented at distal ends (Fig. 2. 14). Larval eyes (ocelli) appear as six brown spot on either side of head. The spiracles are clearly visible on the sides of the body. Head capsule and mouth appendages are sclerotised and well pigmented. The amnion and serosa disappear by fragmentation. Embryo ingests the embryonic membranes and sensitive for adverse environmental condition. Entire body of embryo becomes scierotised.

Stage-30 (Newly born muga larvae): Fully developed muga silkworm larva comes out from the egg cell on the 8th day of oviposition rupturing the anterior part of egg shell by the mandibles and swallowing the portion of the chorion in the early morning of exposure of light (Fig. 2. 15). Newly born larvae are generally blackish or brownish in colour. Generally healthy larvae are blackish brown in colour with distinct yellow lines at the intersegment region. Head portion is shining black with elongated spot and larval body is yellowish with blackish tubercle.

Effect of Refrigeration of Eggs of Different Ages at Different Durations on the Hatchability:

Muga silkworm eggs of different embryonic ages (24 h, 36 h, 48 h, 60 h, 72 h and mix ages from 24 h to 72 h) were preserved at 5 °C for different durations to observe their hatching performance after long term preservation. The results of hatching performance of different duration of preservation are shown in Table 1.

Table 1: Hatching performance of different aged embryos preserved at 5°C.

Duration of preservation	Embryonic age							CD	
	24h	36h	48h	60h	72h	Mixed	Control	5%	1%
10 days	67.82	71.32	69.37	65.27	61.87	63.27	75.32	4.76	6.45
20 days	65.42	69.78	69.95	65.3	64.72	65.07	75.32	3.54	4.62
30 days	34.47	41.2	31.75	32.87	29.5	32.9	75.32	4.41	5.77
40 days	25.807	31.02	30.40	28.00	25.12	25.25	75.32	3.66	4.78

In 10 days of preservation at 5 °C, embryo of 24 h showed 67.83% hatching against 75.32% of control which was significantly lower than that of control (P≤0.01& P≤0.05). Similarly, in 10 days of preservation at 5°C, embryos of 36h showed 71.32% hatching which was not significantly different to that of control (P≤0.01& P≤0.05). Hatching performance of 69.37% of embryos of 48h after 10 days of preservation was not significantly different than that of control (P≤0.01&P≤0.05). Embryos of 60h, 72 h and mixed ages of 24h to 72 h showed hatching of 65.27%, 61.87% and 63.27% respectively after 10 days of preservation which were significantly lower than that of control (P≤0.01&P≤0.05).

In 20 days of preservation at 5 °C of embryos of 24 h, 36 h, 48 h, 60 h, 72 h and mix ages, hatching were recorded as 65.42, 69.78, 69.95, 65.3, 64.72 and 65.07 respectively which were significantly lower than that of control (P≤0.01 & P≤0.05). However, hatching performances of embryos of 36 h and 48 h after 20 days of preservation were at par to that of control (P≤0.01& P≤0.05).

However, from 30 days of preservation onward at 5°C, hatching performance of embryos of different ages and mixed ages (24h to 72h) were significantly lower than that of control (P≤0.01 & P≤0.05). It was found that embryos were fully developed inside the egg shell but could not emerge out of the egg shell. In view of the industries' need for preserving eggs of mix ages(24h to 72h), the experiment was modified by preserving eggs of mix ages at three different temperature ranges *i.e.* 5°C, 7°C and 10°C for different durations. Muga silkworm eggs are generally harvested after 72h of egg laying.

The result of the hatching performances of the different durations of the preservation of the eggs of mixed ages at three different temperature *viz*., 5°C, 7°C and 10°C are presented in Table 2. As evidenced from the table, at 5°C the eggs of the mixed ages can be preserved up to 5 days without affecting the hatching performance. At 7°C, the eggs can be preserved for a maximum period of 15 days with hatching of 71.85% which is not significantly different from that of control (P≤ 0.05). Similarly at 10°C, the eggs of mixed ages can be preserved for a maximum duration of 6 days with hatching of 70.00% without significant difference from that of control (P≤0.05). It was observed that, while preserving the eggs at 10°C beyond 6 days the embryos developed inside the BOD incubator and some of the embryos hatched from the eggs after about 15 days of preservation. The embryos, though developed fully inside the egg shell at 10°C could not break the egg shell and could not hatch which may be due to weakness of the developed embryo at 10°C.

Table 2: Preservation of muga silkworm eggs (mixed) at different temperature and duration.

Temperature	Duration of preservation (days)																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
5°C	73.25	71.75	71.25	71	70	66.25	60.5	53.5	58.25	53.25	54	52.75	55.75	51.25	52.25	44.5	33.25	31.5	30.75	29.75
7°C	73.75	73.75	72.75	73.25	72.75	73.25	72	72.25	72	72.25	71.5	72.25	71.90	72.0	71.85	69	64.5	63	60.5	57.25
10°C	72.75	71.5	70.5	71	70	70	66.75	65	61.75	57.75	53.5	51.25	48.75	47	45.25	-	-	-	-	-
Control	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0
CD 1%	2.56	3.06	3.89	2.51	2.87	3.68	7.48	4.24	3.95	5.03	4.36	3.55	4.81	4.68	5.81	3.27	5.14	4.27	2.85	3.75
CD 5%	1.85	2.22	2.81	1.81	2.07	2.66	5.41	3.06	2.86	3.64	3.16	2.56	3.48	3.39	4.20	2.32	3.64	3.02	2.02	2.66

Table 3: Bioassay Result of Preserved egg during Chatua Crop, 2015.

Location	No. of dfl		Hatching %		E. R. R. %		Cocoon weight (g)	
	T	C	T	C	T	C	T	C
I	100	100	72.00	75.00	54.60	54.65	5.42	5.45
II	100	100	73.00	74.00	51.60	52.00	5.7	5.65
III	100	100	70.00	71.42	53.97	54.5	5.65	5.55
IV	100	100	72.00	75.00	52.72	52.05	5.55	5.65
Average			71.75	73.86	53.22	53.3	5.58	5.57
CD (P≤0.05)			1.73		0.63		0.16	
CD (P≤ 0.01)			1.83		0.93		0.24	

T: Treatment, C: Control

Another reason for unsuccessful preservation of eggs for longer duration may be due to the low relative humidity (<60%) inside the BOD incubator at low temperature which affected the development of the young embryos. Thus, from the analysis it is found that among the three different temperatures of preservation of the eggs of mixed ages (Table 2), 7°C is the most effective temperature for preservation.

After confirming the suitable temperature and duration of preservation of the eggs of mixed ages, 400 disease free layings (dfls) in four replications were preserved at 7 °C for 15 days and bioassay of the preserved eggs was conducted in four different locations by rearing 100 dfls per location during 'Chatua Crop' 2015 and the rearing performances were compared with that of control (Table 3).

It is observed that hatching percentage in the different locations was not significantly different from that of control (P≤ 0.05) (Table 3). In effective rate of rearing (ERR), significant differences were not observed between the preserved and the control eggs in the different locations (P≤0.05 & P≤ 0.01). Similarly, in cocoon weight also there was no significant difference between the preserved and the control lots. The above result of the bioassay showed that preservation of eggs at 7°C did not affect the survivability and cocoon weight of the muga silkworm. Therefore, it is deduced that muga silkworm eggs of mixed ages (24 h to 72 h) can be effectively preserved at 7°C up to 15 days in BOD incubator.

Present finding indicates that, the embryonic development starts within a few hours of egg laying and it requires proper incubation for healthy development of embryos since proper incubation of eggs plays a vital role in embryonic development of silkworm leading to timely hatching. Any change in temperature can hamper the development, hatching and rearing performance. Embryonic development and hatching were hampered at the stressed temperature and humidity condition because high temperature and low humidity were unfavorable condition for embryonic development (Mech *et al.,* 2015; Goswami *et al.,* 2015; Dinesh *et al*., 2012). Due to global warming, this type of condition prevails during summer season seed crop grain ages of *A. assamensis* there by affecting hatchability of the eggs. Higher temperature and low humidity during embryonic development leads to death of embryo during early age. Temperature stress caused poor egg laying, poor hatching, depression of eggs and death of fully formed larvae inside egg shell.

CONCLUSION

Embryonic development in muga starts within a few hours of egg laying and it requires proper incubation for healthy development of embryos. The suitable age of egg preservation at low temperature is 36h to 48h old embryo and the eggs at this stage can be preserved for 20 days without affecting hatchability. From sericulture industrial point of view, eggs of mix ages (24 h to 72 h old) should be considered for preservation and it has been found that Muga silkworm eggs of mix ages can be preserved up to 15 days at 7°C and 70-75% relative humidity without hampering its hatching in compare to that of control. The result of the bioassay showed that preservation of eggs at 7°C did not affect the survivability and cocoon weight of the muga silkworm. Another advantage of the preservation technology is that eggs when released from preservation of any duration of preservation starting from 1st day to 15th day, hatched after 6 days of releasing which facilitates in large scale supply and convenient effective planning for brushing.

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