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Pathogenic Effects of the Microsporidian *Nosema sp.*, on Larval and Post-cocoon Parameters in Tasar Silkworm, *Antheraea mylitta* Drury (Daba TV).

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Abstract

The commercial rearing of polyphagous Indian tasar silkworm, Antheraea mylitta Drury being practiced on naturally grown primary food plants like Terminalia arjuna, (Arjun) Terminalia tomentosa (Asan), and Shorea robusta (Sal) available in the tropical forests of central India , at times, is seriously affected by the disease- Pebrine, caused by Nosema sp., a microsporidian pathogen. The present investigation on comparative larval, silk gland weight and also cocoon parameters in Pebrine-free and Pebrine- infected ecorace of tasar silkworm Antheraea mylitta Drury (Daba TV), illustrates the tasar silkworm larvae infected with pebrine disease causing heavy losses to the economy of the silk industry.

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Introduction

The tropical tasar culture is an important forest based agro-industry of producing vanya silk by rearing a wild silkworm, *Antheraea mylitta* Drury (Lepidoptera:Saturniidae), primarily on *Terminalia arjuna* (arjun), *Terminalia tomentosa* (asan) and *Shorea robusta* (sal) and secondarily on *Lagerstroemia parviflora*, *Zizyphus mauritiana*, *Anogeis suslatifolia*, *Syzigium cumini*, *Careya arborea* and *Hardwickia binata*, *etc*. Tasar Culture is "a forest based industry". It plays a major role in improving socio-economic status of tribal, weaker sections, landless people and women.

The tasar food plant leaf quality in terms of nutrition can influence the health and growth of larvae, effective rate of rearing (ERR) and crop yields as it has correlation with the weights of cocoon, shell and silk ratio and can influence the crop economics. The leaf nutrient status of tasar food plant is fundamental not only for silk productivity, but also for its metamorphosis during life cycle and subsequent parental moth reproductive efficiency. The larval feeding status of any polyphagous commercial insect has impact on food storage and budgeting for biological activities so as to combat the adverse or to excel during favorable conditions (Reddy *et al.*, January 2012).

The diseases in silkworm are the major constraints

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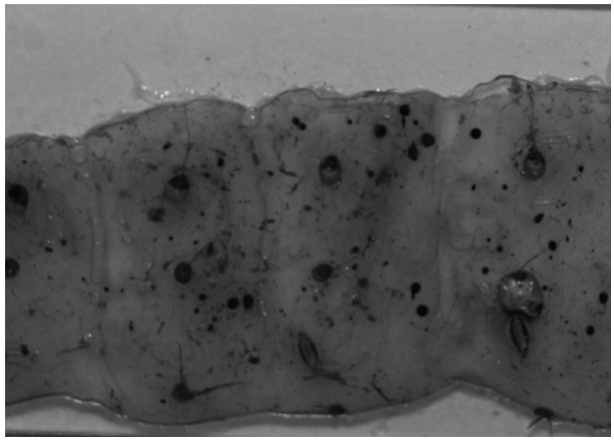

Fig. 1. The integument of Tasar silkworm, *Antheraea mylitta* D(Daba TV) showing black spots.

Fig. 2. ThePebrine infected tasar silkworm, *Antheraea-mylitta.* D (Daba TV).

in tasar culture, which adversely affect the economics of this culture by causing 35-40% crop loss. Among the diseases, pebrine is causing most devastating effect on the rearing of the tasar silkworm accounts for 20-25% yield loss (Sahay *et al.*, 2000). Even though some work has been done on the breeding aspects of Tasar silkworm, not much work has been published so far on pathological aspects of tasar silkworm (Reddy *et al.*, 2010).

Tasar silkworm is often infected with the intracellular parasite of the genus Nosema. Pebrine can be acquired from the mother moth (primary infection) or from the environment through food (secondary infection). Infected larvae show black pepper like spots on the integument. These infected hypodermal cells become enlarged and vacuolated and blackened due to the formation of melanin (Ganga, 2003). Larvae infected with Nosema sp. show extended development period, reduced size and larval weight in comparison to uninfected ones (Rath *et al.*, 2003).

Pebrine is a disease caused in tasar silkworms, by a parasitic microsporidian-*Nosema mylittensis*, belonging to the family Nosematidae, Genus -*Nosema*, Species -*mylettensis* ; affected by which, the larvae show slow growth, undersized body and poor appetite. Diseased larvae reveal pale and flaccid body. Tiny black spots appear on larval integument (Fig. 1). Dead larvae remain rubbery and do not undergo putrefaction shortly after death. The pathogen comes from infected eggs laid by infected mother moths or exists in rearing facilities as spores or come from wild insects naturally infected (Fig. 2) with *Nosema mylittensis*.

This dreaded disease is also known as pepper disease or corpuscle. The name pebrine was given to the disease in 1860 by De Quatrefages because the black spots that appear on the diseased silkworms look like pepper grains. The disease was not properly understood until the researches of Louis Pasteur during the years 1865-70 brought out a method of diagnosis. Pasteur established that the disease invariably manifests itself in the mother moth though sometimes it may not be possible to detect it in the larval and cocoon / pupal stages (Fig. 3 and 4).

There are two stages in the life cycle of this organism, the spore stage and the vegetative stage. The mature spore is oval and measures 3-4 microns by 1.5-2 microns. The spore consists of spore membrane, which encloses the sporoplasm, in the form of girdle across the width of the spore, anterior and posterior vacuoles, two nuclei in the sporoplasm and polar capsule which encloses a spiral polar filament (30 times the length of the spore) which is projected through the small opening. The pathogen comes from infected eggs laid by infected mother moths. It may also exist in rearing facilities or *T. arjuna* garden as spores.

During the vegetative stage the *T. arjuna* leaf acts as a means of transmission, to which the spores stick and enter along with the leaves, which are ingested by the

Fig. 3. Pebrinefree and Pebrine infected Cocoons of *Antheraeamylitta* (Daba TV).

Pebrine free

Pebrine infected

Fig. 4. Pebrine free and Pebrine infected pupae of Tasar silkworm, *Antheraeamylitta* (Daba TV).

silkworm. The spores multiply in number; the digestive juices act on the spores, the polar filament is extruded and soon becomes detached from the spore. Soon these develop into globular planonts, which invade the haemocoel, intestine, gonads, malphigian tubules and silk glands by rapid proliferation. In the various body tissues of the silkworm, they gradually increase in size and become meronts / schizonts by draining the cytoplasmic contents of the host and finally becoming a sporont. The mode of infection and transmission of the spores are mainly through oral, cuticular and ovarial pathways (Kramer, 1976). In the ovarial pathway, the pathogen is incorporated into the egg within the female reproductive tract and the progeny from such females are also infected. The rate of disease transmission and degree of infection in the offspring is directly related to the intensity of infection in the mother moth (Griyaghey and Sengupta, 1989; Talukdar, 1995).

Some of the microsporidia show transovarial transmission and some are not (Fujiware, 1980; Fujiware, 1984; Ananthalakshmi *et al*., 1994; Nageshwara Rao *et al.*, 2004). Most of the species are highly virulent and mortality caused by them also varies. No silkworm race is reported to be completely immune to pebrine. Spores of *Nosema* sp. can be detected at any stage of life cycle in Tasar silkworm (Sharan *et al.*, 1992) and are different in size, shape and pathogenicity (Shabir Ahmad Bhat *et al.*, 2009). The present experiment is an attempt to study in detail the pathogenic effects of Pebrine on tasar silkworm, *Antheraea mylitta* D (Daba TV).

The silk protein fibroin is fibrous in nature, forming the main silk filament content, while sericin is a sticky coating substance between the layers of fibroin. Thus, the quality of cocoons depends both on sericin and fibrin which are controlled by atmospheric conditions (Shamitha and Rao, 2006). The sericin content as being the deciding factor in the quality of the cocoon and raw silk reeled was reported by Singhvi and Bose (1991). However, filament length and quality of the shell are based on the fibroin content. The present studies provides insight into the damaging effects of the Pebrine infection in the larval and cocoon stages resulting in poor quality of silk and identifies the need to designate proper measures to prevent the disease.

Material and Methods

Rearing of Tasar silkworm

The trivoltine tasar silkworm, *Antherae amylitta* (Daba TV) were reared in outdoor under normal condition on a diet of fresh *Terminalia arjuna* plantation, at the experimental garden of Kakatiya University at 26-33°C and 37-50% R.H and 13L+11D photoperiodic condition and, about two dfls were reared during December 2011-February 2012. Another batch of Pebrine free larvae (2 DFLs- disease free layings) of tasar silkworm, *Antheraea mylitta* (Daba TV) were collected from neighbouring forest patches for comparative analysis (Fig. 5).

Fig. 5. V-instar larvae of *Antheraeamylitta*(Daba TV) Pebrine-free larvae.

Symptoms of Pebrine

The incidence of Pebrine disease in the rearing lot was identified by the symptoms in the early larval stages. The appearance of black spots on the body and unequal larval development are the most prominent symptoms of this disease. They exhibit poor appetite resulting into stunted growth and irregular moulting. The larvae detected with Pebrine infection are immediately removed and buried far away from the rearing site. The instar-wise larval mortality is presented in the results (Table 1).The larvae soon become pale and dull with wrinkled cuticle. As the larval stages advance, the appearance of black spots in the integument becomes more prominent, exhibiting melanosis.

Identification of the disease

The studies on silk gland were conducted by their collection from individual fifth instar larvae in a dissection tray containing normal saline. The dissected glands were weighed on Citizen Balance (Table 2) by removing any excess water. The infected silk glands (Fig. 6) along with posterior glands (Fig. 7) were spread on the slide and examined under microscope at 40X magnification for *Nosema* spores. The haemolymph was collected by puncturing the

Table 1. Instar-wise Average Temperature (C), Average Relative Humidity (%) and Mortality due to Pebrine disease of Tasar silkworm, Antheraea mylitta (Daba TV)

Instar	Temparature°C	Humidity (%)	Mortality	(%) Loss	Larval life span	
					Pebrine free	Pebrine infected
I	33.02 ± 0.61	37.6 ± 1.51	-	-	4	5
II	33.8 ±1.47	39 ± 2.30	10	4	4	4
III	33.05 ± 3.64	41.28 ± 2.42	51	20.4	6	7
IV	26.67 ± 0.51	49.55 ± 9.98	62	24.8	7	9
V	29.03 ± 2.50	45.43 ± 6.76	117	93.6	29	43

The values are expressed in terms of Standard Error of the Mean.

Table 2. Larval weight,Silk gland weight and % Decrease in Pebrine- free and Pebrine infected V-instar Antheraea mylitta (Daba TV)

SL.NO	*Anthereamylitta Drury*, (Daba TV)	PEBRINE-free of *Anthereamylitta Drury*, (Daba TV)	PEBRINE-infected *Anthereamylitta Drury*, (Daba TV)	Decrease (%)
1	Larval weight	22.48 ± 1.54	19.93 ± 1.80	11.34
2	Silk gland weight	1.05 ± 0.04	0.40 ± 0.26	61.90

The values are expressed in terms of Standard Error of the Mean.

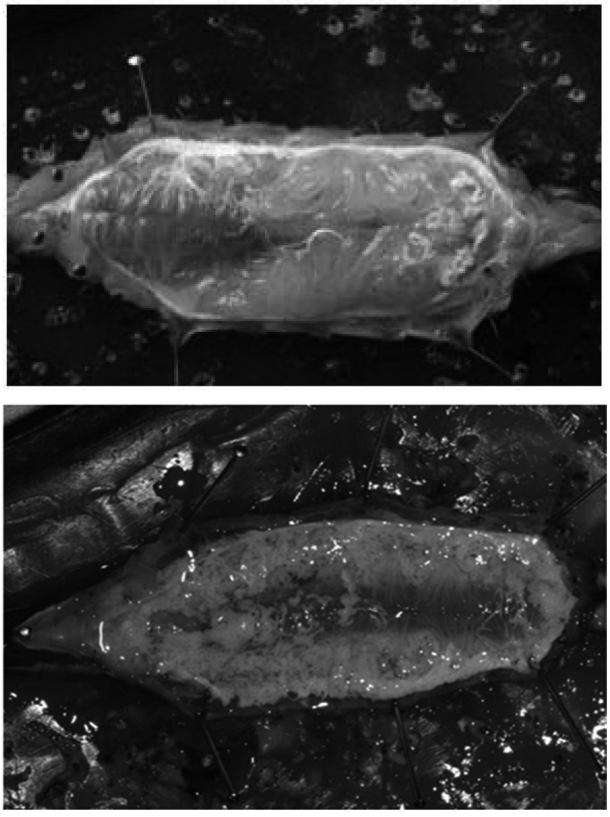

Fig. 6. Silk glands of Pebrine free and Pebrine infected (*Nosemamylittensis*) tasar silkworm. *Antheraeamylitta* (Daba TV).

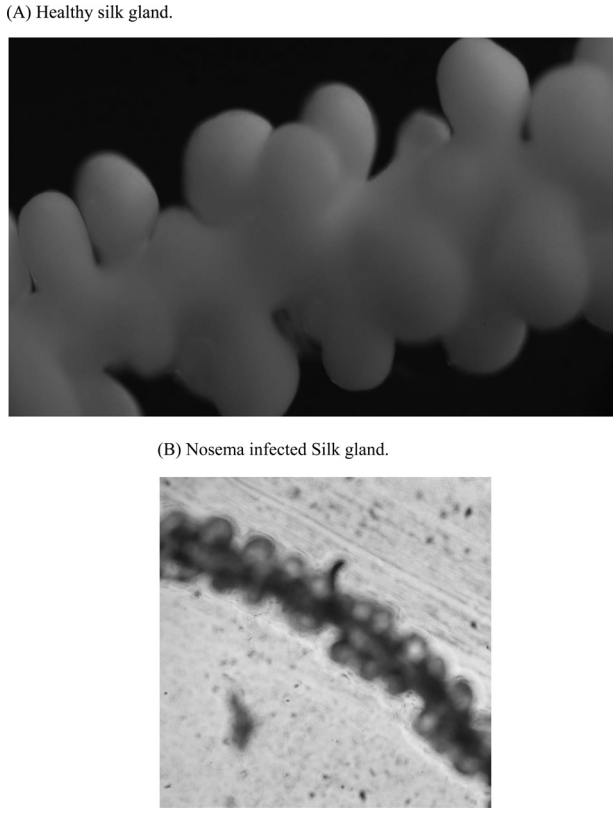

Fig. 7. A comparative diagram of Pebrine free and *Nosemamylittensis* (Pebrine) infected Posterior silk gland of *Antheraeamylitta* (Daba TV).

last pair of legs of fifth instar larvae and smeared on a clean slide (Fig. 8) for examination under microscope at 40X magnification for *Nosema* spores.

A severely infected larva may die before spinning or it may spin a flimsy cocoon or spits and wastes the silk. However, a good number of infected worms have been allowed to spin for studying the post cocoon characters (Table 3). In the pupa, the abdominal area is soft and swollen and dark in colour. Black spots may be seen on the sides near the wing area. Sometimes the infected pupae do not show any symptoms. In order to detect the disease at pupal stage, the pupae were first washed with distilled water for two minutes, and then the lower half of the abdomen (gut) was placed in a

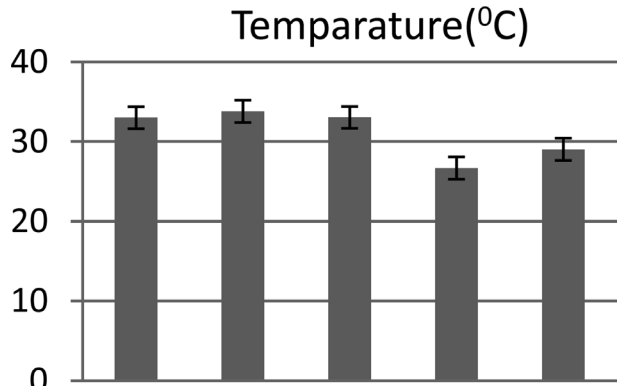

Fig. 8. *Nosemamylittensis*(Pebrine) spores in larval Haemolymph*Antheraeamylitta*(Daba TV).

clean mortar. The tissue was crushed and the smear examined under microscope at 40X magnification for *Nosema spores* (S.K. Sharan, *et al*, 1992).

Evaluation of larval / cocoon parameters

The larval, cocoon and post-cocoon parameters of Pebrine free and infected worms were measured as follows:

Temperature (°C) and relative humidity (%)

The optimum temperature and relative humidity for the tasar silkworm rearing are 25-30°C and 60-70% respectively. In the present study, the temperature and relative humidity were recorded with the help of lab thermometer and hygrometer respectively. The average of lowest and highest values taken and noted them instar-wise.

Mortality

The mortality was calculated by counting the number of worms dead and diseased due to microsporidian pathogen (Pebrine disease) during each instar by observing the symptoms and accordingly the percentage was calculated.

The larval / pupalweight

At the beginning of each instar, 10 healthy worms were selected at random from the rearing lot, which are feeding on the *Terminali aarjuna* plants for measurement of larval weight, taken from the day after hatching during the first instar to fifth instar. Larval weights were measured in an electronic Citizen balance (Model no: CG 203) in grams. The percent difference between Pebrine free and Pebrine infected worms was also calculated. The average weight (g) of 5 randomly selected pupae was also taken.

Table 3. Cocoon parameters of Pebrine free and Pebrine infected tasar silkworm, Antheraea mylitta (Daba TV)

S. no	Silkworm	Cocoon Weight (grams)	Shell Weight (grams)	Shell ratio (%)	Pupal eight (grams)	Reelability (%)	Weight of the filament (grams)	Length of the Filament (meters)	Denier
1	Pebrine-free	9.50 ± 0.20	1.25 ± 0.08	13.19	8.16 ± 0.27	7.9	0.750	744	9.07
2	Pebrine-infected	4.47 ± 0.59	0.27 ± 0.06	6.17	4.12 ± 0.59	3.6	0.164	162	9.11

The Values are expressed in terms of Standard Error of the Mean.

Silk gland weight:

The average silk gland weight dissected from five V fifth instar larvae selected at random feeding on the *T. Arjuna* plants were taken and presented in the results.

Length / weight of the filament

Filament length was measured by using cocoon approvette. It has an axis around which four wooden sticks were arranged at equal distance with the circumference of 9/8 meters. It is rotated with the help of a handle at one end. And to the other hand of the axis is a bell by which number of rotations was known. The number of rotations multiplied by the circumference gives the filament length.

Filament length = 9/8met X No. revolutions

The reeled silk weight measured with the electronic digital balance in grams.

Shell Ratio (%)

The shell ratio is calculated by the following formula

$$\text{Shell ratio}(\%) = \frac{\text{Shell Weight}}{\text{Cocoon Weight}} \times 100$$

Reelability (%)

The reelability of cocoons for economic reeling is the ease with which the cocoons yield the bave in reeling, which is called the reelability of cocoons. This is calculated by the following formula:

$$\text{Reelability}(\%) = \frac{\text{Weight of the silk reeled}}{\text{Weight of the cocoons}} \times 100$$

Denier

The difference in thickness of the size of the bave from beginning to the end is so gradual and minute in tasar cocoon that it does not interfere with the quality of the size of ultimate raw silk reeled. Denier is obtained by the following formula:

$$\text{Denier} = \frac{\text{Weight of the silk reeled}}{\text{Length of the reeled}} \times 9000$$

Results

Temperature and Relative Humidity

The instar wise average temperature and its standard deviation of Tasar silkworm *Antheraea mylitta* (Daba TV) rearing of 2011- 2012 were 33.02 ± 0.61 (S. D), 33.8 ± 1.47 (S. D), 33.05 ± 3.64 (S. D), 26.67 ± 0.51 (S. D) and 29.03 ± 2.50 (S. D) while that of rearing were I, II, III, IV and V instar respectively (Fig. 9)

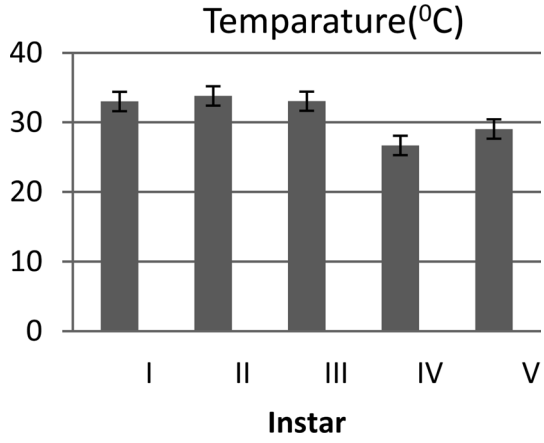

Fig. 9. Instar wise temperature (°C) during the rearing of tasar silkworm, *Antheraea mylitta* (Daba TV).

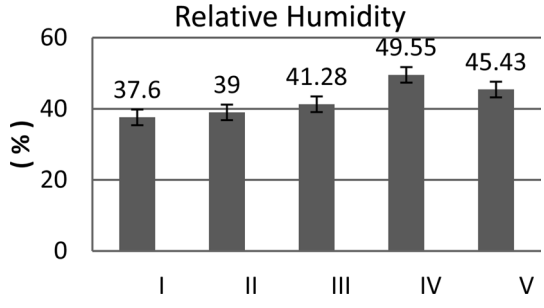

Fig. 10. Instar wise Relative humidity (%) during the rearing of Tasar silkworm, *Antheraea mylitta* (Daba TV).

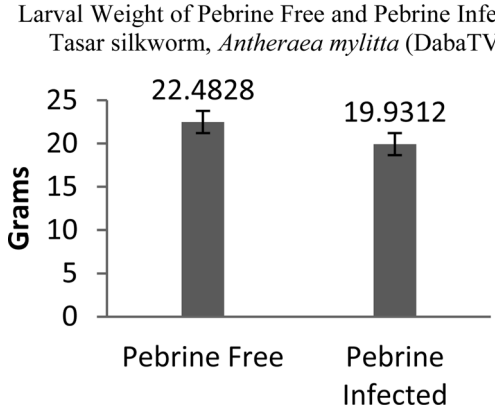

Fig. 11. Larval weight of Pebrine-Free and Pebrine infected Tasar silkworm, *Antheraea mylitta* (Daba TV).

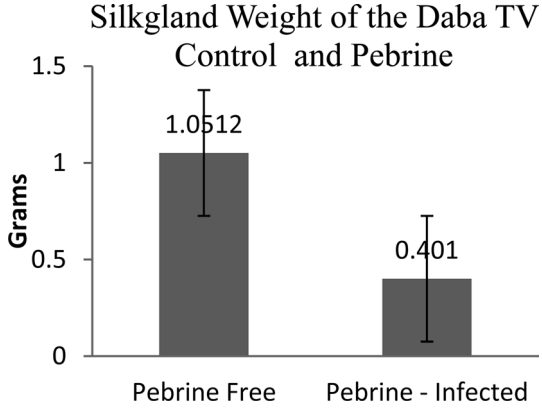

Fig. 12. Silk gland weight of Pebrine free and Pebrine infected Tasar silkworm, *Antheraea mylitta* (Daba TV).

The instar wise average Relative Humidity and its standard deviation of *Antheraea mylitta* (Daba TV) rearing were 37.6 ± 1.51 (S. D), 39 ± 2.30 (S. D), 41.28 ± 2.42 (S. D), 49.55 ± 9.98 (S. D) and 45.43 ± 6.76 (S. D) in the I, II, III, IV and V instars respectively (Fig. 10).

Larval and silk gland weight

The average larval weight and its standard deviation of Pebrine- free tasar silkworm, *Antheraea mylitta* Drury, was 22.48 ± 1.54 (S.D) while that of, Pebrine infected one was 19.93 ± 1.80 (S.D) (Fig. 11).

The average Silk gland weight and its standard

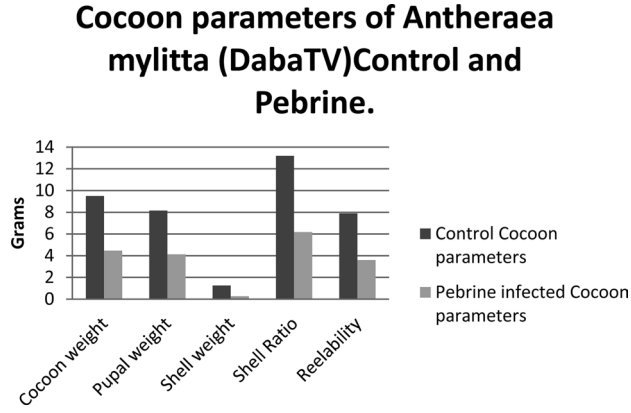

Fig. 13. Cocoon Parameters of Pebrine free and Pebrine infected tasar silkworm, *Antheraea mylitta*(Daba TV).

deviation of Pebrine free tasar silkworm, *Antherae amylitta* Drury, was 1.05 ± 0.04 (S.D) while that of Pebrine infected larva was 0.40 ± 0.26 (S.D) (Fig. 12).

Post cocoon parameters

The post-cocoon characters of Pebrine free and Pebrine infected tasar silkworm, *Antheraea mylitta* Drury (Daba TV), reared during the 2011-2012 include cocoon weight (gr), shell weight (gr), shell ratio (%), Pupal weight, Reelability, weight of the filament, length of the filament and Denier which are: 9.50 ± 0.20 (S.D), 1.25 ± 0.08 (S.D), 13.19%, 8.16 ± 0.27 (S.D), 7.9%, 0.750 grams, 744 meters and 9.07. while than that of Pebrine infected were 4.47 ± 0.59 (S.D), 0.27 ± 0.06 (S.D), 6.17%, 4.12 ± 0.59 (S.D), 3.6%. 0.164 grams, 162 meters and 9.11 respectively (Fig. 13).

Discussion

The etiology of Pebrine (*Nosema bomycis* Naegeli) dates back to mid19th century as an epidemic of silkworm, *Bombyx mori*, when it spread to all sericulture countries of Europe resulting in decline of silk production. From the earlier reports it is understood that in tropics Pebrine is commonly

prevalent in Thailand, Cambodia, and Vietanam *etc*. Later, it has also spread to Indian sub-continent by late 19th century. The disease has been noticed intermittently in silk crops during 1925-30, 1940-47, and 1991-92 as periodic outbreaks in several silk producing parts of our country (Chitra *et al.*, 1975).

A report on Pebrine incidence in Thailand (Somsri, *et al.*, 1991) in two generation of multivoltine race of *Bombyx mori* has shown normal oviposition but decreased hatching and high mortality were observed and subsequently, a decrease in number of egg laying and increase in number of dead eggs were found in the deformed wings moths.

There are also several reports on Nosema bombycis, a highly virulent micro-organism, damaging the silkworm crops in Japan and other Asian countries. A detailed morphology and life cycle of *Nosema assamensis*, pathogenic microsporidian causing pebrine in *Antheraea assamensis*, confined to Assam, India has revealed the ultra structural details of the virulent spore by Scanning Election Microscope (S. Chakrabarti, 2009).

Earlier, tasar mortality due to Pebrine was reported by Jolly in 1968. Studies on larval mortality by instar in varied seasons by Mishra (1992) and Pebrine control measures by feeding of Benomyl, Carbestine, Bngrad in specified dosages from second instar was cited by Sinha (2005). The present study calls for a remedial measure to prevent this epizootic disease which causes alarming loss to tasar culture which has a direct relevance to tribal employment.

From the results it is observed that mortality due to Pebrine was apparent from 2nd instar and gradually increased in 3rd, 4th instars and reached its peak in the fifth instar, which may be due to the manifestation of virulent parasitic microsporidian, Nosema mylittensis, in the late instars. This is in corroboration with the earlier studies that the mortality of A.mylitta larvae accelerated from 3rd instar onwards, with increasing intensity of disease symptoms (Sen *et al.*, 1969).

It is found that there is a significant loss of larval body weight with a corresponding and drastic decrease in the silk gland weight of the Pebrine infected tasar silkworms. It is in good agreement with the view that Dabaecorace recorded highest concentration of silk protein in its silk glands compared to other ecoraces studied (Lokesh *et al.*, 2012). The higher consumption of dietary food and corresponding higher metabolic activities leads to better conversion of leaf proteins in to silk proteins.

The post-cocoon parameters have revealed that the weights of the cocoon, shell and filament have substantially decreased in the Pebrine-infected cocoons. The decrease in the weight and length of the silk filament is extreme. From the literature it is inferred that the quantitative as well as qualitative nutrition is highly essential for sericigenous insects to maintain important physiological status and lack of such nutrition in the tasar silkworm might have extended its larval span (Chapman, 1998; Behmer, 2006; Kumar *et al.*, 2010). This is in agreement with the present studies which show an instar-wise extension of larval life span, more so in the fifth instar (Table 1). The potential fitness and maturity attainment is only possible when the larva obtain adequate amount of nutrients in a required balance, the deficiency of which leads to longer larval duration, lesser ERR and cocoon yield, which are inter-related (Reddy *et al.*, 2012). It is also supported by the view that the larvae can survive better on getting the required nutrition by improving defense system, which also shorten its larval span and lessen the chance of exposure to the diseases, pests and pathogens (Baylis, 1991; Ojala *et al.*, 2005).

From the present studies it is evident that this dreadful disease which may be transmitted through the egg and ingestion of the contaminated food by the larvae / origination of the pathogen from infected eggs laid by infected mother moths / its pre-existence in rearing site as spores / wild insects naturally infected with *Nosema mylittensis* can be prevented by selecting the disease free laying before rearing; burying away of diseased larvae and silkworm waste; disinfection of rearing equipment and dusting 5% bleaching powder with slaked lime (Singh, 2005).

In the present investigation, it is revealed that the

pupal weight in Pebrine infected larva has shown high reduction, which may lead to low fecundity. A recent study of *N. bombycis* on the impact of egg production and disease transmission has revealed that fecundity, hatchability and diseased population are dependent on spores in the female larvae (Jyothi and Patil, 2008). The main characteristic of microsporidian infection is its long lasting and chronic effect to the host. As the fecundity is dependent on the reserves of nutritious substances accumulated in the larva, before pupation and silk moths do not consume any food (Verber and Jassic, 1961), they mainly develop in the fat bodies of the host and the infection causes depletion of nutritive reserves resulting in less fecundity in females (Arm strong and Bass, 1986).

This study suggests that there is an urgent need to develop an efficient method to control the disease. Although medium temperature and relative humidity can stimulate the pathogen (Dasgupta, 1950), some studies have shown that high temperature and relative humidity can cause minimum incidence of Pebrine (Devaiah, 1975). *Nosema mylittensisis* a well known entomopathogen of pebrine and loss incurred due to this disease varies from 35 - 40% (Sahay *et al.*, 2000). The results of the study by Singh G.P. *et al.* (2005) indicated that bleaching powder, slaked lime and formalin were effective in the inactivation of both *Antheraea mylitta* cytoplasmic polyhedrosis virus (AmCPV) and spores of *N. mylittensis*, while a recent report has indicated the indoor rearing performed at lower temperature and disinfected conditions can minimize the Pebrine to some extent (Shiva Kumar and Shamitha, 2009).

The origin of *Nosema* along with other microsporidians which infect a wide range of eukaryotes causing severe diseases in immune-compromised humans and losses to apiaries, fisheries and silk farms has been traced back to fungal relatedness (Patrick *et al.*, 1998). In 2003, Japanese scientists have conducted several identification tests of *Nosema sp*., mainly by Flourescent antibody techniques, ELISA, Latex adhesion tests, analyses of rRNA genes by sequencing and PCR and unveiled the phylogeny of several silkworm microsporidians (Kawarabata, 2003). The position of *Nosema sp* in evolutionary relationships among microsporidian lineages was also depicted by Patrick *et al.*, in 2004. The virulence of *Nosema bombycis* is established by genomic studies (Yoshinori, 2009), however, the molecular studies towards the origin, phylogeny and detailed morphology of Nosema mylittensis is still an unexplored area so that a disease resistant strain of *A.mylitta* with genetic adaptability needs to be evolved using modern biological tools.

The Pebrine infection is found to be one of the major constraints in tasar culture, which causes heavy loss to the crops. Hence, the prevention and management of this disease is a crucial factor for high yield of cocoons and quality silk. As there is a persistent demand for tasar silk in both domestic and international fronts, and on the other hand, the tasar production base is totally tribal community dependent, there is an immediate urge to prevent the disease incidence in the food plant and the silkworms by quick remedial measures and ensure quality production of the silk.

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