

Studies on the Microsporidian Disease (Pebrine) of Silkworm, *Bombyx mori* L.

Sehreen Rasool
(2016-689-D)



Division of Silk Product Science
College of Temperate Sericulture
**Sher-e-Kashmir University of Agricultural Sciences &
Technology of Kashmir**

2023

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Thesis

Submitted to

College of Temperate Sericulture

Sher-e-Kashmir

University of Agricultural Sciences & Technology of Kashmir
In partial fulfillment of requirements for the award of the degree of

Doctor of Philosophy in Sericulture

2023

Someone who hold us in arms as we enter this world
Someone who always encourage us with their loving words of
praise
Someone who constantly support us
Someone who listens patiently to all our worries and stresses
Someone who's eyes shine with pride and happiness at our every
little achievement

Dedicated To
My Mother
Kounsar Akhter

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Division of Silk Product Science,
College of Temperate Sericulture, Mirgund, Baramulla

Certificate – I

This is to certify that the thesis entitled “**Studies on the Microsporidian Disease (Pebrine) of Silkworm, *Bombyx mori* L.**” submitted in partial fulfilment of the requirements for the award of **Doctor of Philosophy in Sericulture**, to the College of Temperate Sericulture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, is a record of bonafide research work carried out by **Ms. Sehreen Rasool (Regd. No. 2016-689-D)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

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ABSTRACT

Silkworm, *Bombyx mori* is categorized as Lepidopteran insect, mainly exploited for the production of natural silk fiber. Globally, silk has the distinction of the elegant light weight and durable textile, enthralling sheen, soft touch durable product possessing strong affinity to dyes. It is rightly known as ‘queen of textiles’ and its production is perceived as an art and science. The science and art of rearing silkworm to produce is called sericulture. In sericulture industry, the output depends on the growth and harvesting of the cocoon crops. However, the problem is the incidence and emergence of diseases, which has the propensity to lower down the cocoon crop production. Microsporidiosis or pebrine disease of the silkworm, caused by microsporidian *Nosema bombycis* (Nageli), is one of the most serious maladies, which determines the success or failure of sericulture industry. The pathogen is infectious and transmitted both vertically and horizontally. The present study was conducted to characterize the morphology of microsporidian spore by means of microscopy (Light and scanning electron microscopy) as well as micrometry. Also the impact of microsporidian infection on survival and economic parameters of six races of silkworm *B.mori*, viz: SK₁, SK₆, SK₂₈, SH₆, NB₄D₂ & CSR₂ was studied. Furthermore, the site (tissue) and intensity of microsporidian infection was ascertained which was then correlated with various biochemical parameters (protein, carbohydrate, lipid) in different tissues of silkworm *B.mori*.

The study revealed that the spore (which was designated as N₁) present in the purified microsporidian isolate differed morphologically from typical *N. bombycis* spores. The N₁ spores were oval in shape measuring $3.33 \pm 0.17 \mu\text{m}$ in length and $1.84 \pm 0.09 \mu\text{m}$ in width whose spore length–width ratio (eccentricity

index) was 1.80:1. The ultra-microscopic studies (SEM) of the microsporidian spores revealed that the external surface of N₁ spores was rough with a depression in the middle of the spores. The study revealed that the microsporidian infection affected the survival and commercial characteristics of all the silkworm races under study, however, different races showed varied responses towards the microsporidian infection. Among the silkworm races under study, SH₆ was least affected by the microsporidian infection and SK₂₈ was most affected in terms of economic and commercial parameters under study viz. Fifth Age Larval Duration, Total larval period, Weight of the matured larvae, Single cocoon weight, Single shell weight, Shell ratio (%), Cocoon yield (by No.), Cocoon yield (by weight), Larval mortality (%), Survivability (%), Pupation rate, Moth emergence, Fecundity, Average filament length, Raw silk percentage and Denier. The microscopic examination of different tissues at larval stage viz. midgut, silk gland, malpighian tubules and at moth stage viz. gonads (ovaries) of six races of silkworm inoculated with microsporidian infection accentuated a high intensity of infection (more than 30 spores/field) in the gonads (ovaries) at moth stage. However, at the larval stage, moderate intensity of infection (5- 30 spores/field) on the 3rd day and 5th day of 5th instar was observed in all the larval tissues of all inoculated silkworm races. The intensity of the infection in the larval tissues was observed in the sequence: midgut > silk gland > malpighian tubules. The sequence of the susceptibility of the races to microsporidian infection based on the intensity of infection in different tissues under study was SK₂₈>CSR₂>SK₆>SK₁>NB₄D₂>SH₆. Gonadal (ovarian) infection was also observed suggesting transovarial transmission in the infected silkworms.

The biochemical findings of the study correlated with the findings of site and intensity. The results of the present study accentuates a general negative effect of the microsporidian infection on all the biochemical parameters (protein, carbohydrate, lipid) under study in all the tissues of infected silkworm larvae in comparison to their healthy control. Based on the results of the present study, the changes in the concentration of bio-molecules can be used as markers to analyze the health status of silkworm, *B.mori* infected with pebrine disease. The study would, however, promote further investigations like TEM, molecular characterization, biochemical analysis of spore (microsporidian) proteins, artificial germination of spore and phylogenetic relationship studies based on molecular markers for confirmation of newer strains of microsporidia for development of appropriate mitigation and management strategies of this disease.

Keywords: *Bombyx mori*, microsporidia, tissue, pebrine, SEM, cocoon

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ACKNOWLEDGEMENT

A formal presentation of mere words is scarcely indicative of my venerable gratitude and indebtedness to my Chairman, **Dr. Khursheed Ahmad Sahaf**, Professor & Head, Division of silk product science, TSRI, Mirgund for his highly inspiring, enthusiastic guidance with never dyeing spirit, sound counseling, meticulous suggestions, enduring encouragement and constructive criticism throughout the entire course of this investigation as well as benevolent help in the preparation of the manuscript. I am also indebted to **Dr. Nisar Ahmad Ganie**, Assistant Professor, Co-chairman of my advisory committee for his counsel, constant monitoring, patient reviewing and punctilious criticism during the course of my research work. I take this auspicious opportunity to express my sincere gratitude and profound reverence to **Dr. Khursheed Ahmad Dar**, **Dr. Zaffar Ahmad Buhroo**, **Dr. Aabid Khaliq**, **Dr. Masarat Bashir**, **Dr. Irfan Khan**, **Dr. Firdous Ahmad**, **Dr. Shabir Ah. Wani**, **Dr. Shakeel Ah Mir** and **Dr. Zia ul Haq Ruffaie** who helped me in every possible way in conducting my research work through their valuable guidance, comments and constructive suggestions for which I shall always remain grateful.

I am immensely thankful to **Dr. Shabir Ahmad Bhat**, **Dr. Mushtaq Rasool Mir**, **Dr. Showkat Maqbool**, **Prof. Shaheen Gul** and **Dr. Imtiyaz Murtaza**, for their highly motivating, unconditional and timely help during the research work. I am extremely thankful to the staff and workers of College of Temperate Sericulture, Mirgund for their assistance and encouragement during the period of study.

I, thankfully acknowledge the help and encouragement received from my sisters **Dr. Sheezan Rasool**, **Shayan Rasool**, **Sana Rasool** and my aunts **Sabina Khan** and **Shafeeqa Akhter**. I sincerely acknowledge the help received from **Dr. Asiya**, **Mr. Shabir Ah. Bhat** (Addl. Director SDD), **Dr. Rajesh Kumar** for rendering valuable support during the entire course of study especially for the compilation of the work.

At last, I would like to appreciate the support rendered by my friends **Shaista Mehraj**, **Mehreen Manzoor**, **Nayeema Jan** and **Raghvendra** that actually made it to happen.

Innumerable numbers of persons have provided their help in course of this study.

Everyone is not mentioned, but no one is forgotten...

Sehreen Rasool

Place: Mirgund

Dated: _____

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Chapter 1

INTRODUCTION

Silkworm, *Bombyx mori* is a Lepidopteran insect which is mainly exploited for the production of natural silk fiber. Globally, silk has the distinction of the elegant light weight, enthralling sheen, soft touch durable textile product possessing strong affinity to dyes. It is rightly known as ‘queen of textiles’ and its production is perceived as an art and science. Over the years, China has emerged as the largest producer of silk in the world and produces 53,359 MT of raw silk followed by India producing 34,923 MT of raw silk (Anonymous, 2022a). India has a unique distinction of being the only country in the world which produces four commercial varieties of silk viz., mulberry, tasar, eri and muga, however, the bulk of the silk produced is from mulberry silkworms, *B.mori*. Among the four varieties of silk produced in 2020-21, mulberry silk production accounts for 25,853 MT, Eri -7395MT, Tasar -1456 MT and Muga-255 MT of total production of 34, 923 MT (Anonymous, 2022b). Sericulture in India is predominantly practiced in tropical region such as Karnataka, Tamil Nadu, Andhra Pradesh and West Bengal and also in temperate regions like Jammu & Kashmir. Jammu and Kashmir is one of the traditional sericulture states of India and sericulture is practiced in 20 districts of union territory of Jammu and Kashmir. The major silk producing districts are Udhampur, Rajouri, Reasi, Kathua, Anantnag, Kupwara, Pulwama, Baramulla and Ganderbal, Kashmir silk is of high quality as per the international quality standards. Presently, in J&K during about 27,000 rural families are directly or indirectly associated with the silk industry and the cocoon production during the year 2021-2022 was 694 MT (Anonymous, 2021).

The success of the enterprise depends on the availability and quality of the raw materials. In sericulture industry, the output depends on the growth and harvesting of the cocoon crop. Environment has a significant impact on silkworm growth and development. There is widespread perception that ambient temperature, quality of mulberry leaf, rearing season and the genetic make-up of

the silkworm strains have an impact on both biological and cocoon-related characteristics. Seasonal variations in the environmental factors have a significant impact on the behaviour of silkworms and the genotypes express variations in phenotypic traits of cocoon weight, shell weight and cocoon shell ratio. However, the main problem in sericulture is the incidence and emergence of diseases, which has the propensity to lower down the cocoon crop production. The silkworm crop loss differs from region to region, season to season and farmer to farmer due to different practices adopted in cultivation and severity of the disease. The crop loss to sericulture industry due to various diseases has been reported to vary from 30-40% (Hanumappa *et al.*, 1986), 15-20% (Baig *et al.*, 1988), 40% (Veerana, 1999), 20-30% (Selvalumar *et al.*, 2002) and 20-35% (Nataraju *et al.*, 2005). In fact, one recent study suggested an average silkworm crop loss of 15-47% at national level due to the incidence of diseases (Tayal and Chauhan 2017). The diseases that are inflicting heavy loss to the sericulture industry include Muscardine (fungal disease), Grasserie (viral diseases), Flacherie (bacterial diseases) and Pebrine (protozoan disease). Individually, the extent of the crop loss in India due to fungal diseases is about 5-20%, viral diseases is 15-20%, bacterial diseases 10-15% and microsporidian infection 5-10% (Bebitha *et al.*, 2016). Out of these diseases, microsporidiosis or pebrine disease of the silkworm, caused by microsporidian *Nosema bombycis* (Nageli), is one of the most serious maladies, which determines the success or failure of sericulture industry as the pathogen is infectious and transmitted both vertically and horizontally (Govindan *et al.*, 1998; Bhat *et al.*, 2009a).

Historically, the emergence and outbreak of pebrine or pepper disease in Italy and France in 1845 diverted the attention of the world scientific community towards this pathogen due to widespread destruction and collapse of the silkworm industry in Europe (Govindan *et al.*, 1998). The data suggested that annual production in France reduced from 26,000 MT in 1853 to 4000 MT in 1865 and the disease spread to Spain, Syria and Romania (Steinhaus, 1949). It was only in

1857 that the first etiological agent of the pebrine was identified and recognized as *N.bombycis* by Nageli. Over the years, it has emerged as the main force responsible for the devastation and destruction with heavy economic losses to the silkworm rearing industries all over the globe. In India, the first report of the occurrence of the disease was in 1866 from Mysore and in Madras provinces in 1890-1900. An epidemic level outbreak of the disease also took place in Kashmir in 1878 (Govindan *et al.*, 1998). The prevalence of the disease in India was about 15% in 1983 that declined considerably during 1984 and 1985 (Baig, 1994; Nataraju and Dandin, 2006). An outbreak of pebrine disease was again witnessed in southern sericultural states of India during 1991-1992 causing a loss of over Rs.200 crores (Quadri and Khatri, 2005).

Most invertebrate phyla and vertebrates are infected by microsporidia, which are parasitic, obligatory intracellular protozoans (Canning and Lom, 1986). Their significance as pathogens is acknowledged in a variety of contexts; they are dangerous and seriously harm economically significant hosts, such as silkworms; and they are widespread in nature, with more than 1200 species and roughly 150 known taxa (Patrick and Naomi, 2002). These species, with a major share of insects and fish, are well suited to express in pathogenicity, transmission, ecology and resistance to the defence mechanism of their hosts (Wasson and Peper, 2000; Weiss, 2001). At the end of 19th century, only *N. bombycis* was known as the causative agent of pebrine in silkworm but at present several microsporidians are known to cause the disease in silkworm (Ananthalakshmi *et al.*, 1994; Kishore *et al.*, 1994; Samson *et al.*, 1999; Singh and Saratchandra, 2003; Nageswara rao *et al.*, 2004; Selvakumar *et al.*, 2005; Bhat and Nataraju., 2007a). They differ in their morphology, serology, site of infection and virulence. Among them, the most common one are the strain of *Nosema bombycis* (NIS-001), *Nosema spp.* (NIS-M11, NIS-M14 and NIK-2r and NIK-3h.), Species of *Vairimorpha* (NIS-M12 and NIK-4m) and *Microsporidium* (NIS-M25), *Pleistophora* (NIS-M27), *Thelohania* (NIS-M32) and *Leptomonas* (Singh and Saratchandra, 2003). *N. bombycis*, NIS-

001, *Vairimorpha* sp. NIS-M12 and *Pleistophora* sp. NISM27 are highly pathogenic with high infection rate and mortality in silkworm larvae. *Nosema* sp. NIS-M14 and *Microsporidium* sp. NIS-M25 are moderately pathogenic while *Thelohania* sp. NIS-M32 is low in infectivity. The other microsporidia with moderate and low infectivity viz., *Nosema* sp. NIS-M11, *Nosema* sp. NIS-M14, *Microsporidium* sp. NIS-M25 and *Thelohania* sp. NIS-M32 cause low level of mortality in the larval stage (Kawarabata, 2003; Singh and Saratchandra, 2003). A microsporidian sp. NIK- 5hm isolated from silkworm in India forms spores within haemocytes (Selvakumar *et al.*, 2005). A new microsporidian associated with Lamerin breed of silkworm has been characterized on the basis of morphology, serological affinity, infectivity etc. and is different from *N. bombycis* (Bhat, 2006).

Most of the *Nosema* species are parasitic invertebrates (Sprague, 1982). Earlier *N. bombycis* was reported as being the only causative agent of pebrine in silkworm, however, at present several microsporidians are documented to be the reason for the emergence of disease in silkworm (Ananthalakshmi *et al.*, 1994; Kishore *et al.*, 1994; Samson *et al.*, 1999; Singh and Saratchandra, 2003; Nageswararao *et al.*, 2004; Selvakumar *et al.*, 2005; Bhat and Nataraju., 2007a). Different silkworm breeds differ in their susceptibility to the microbial infection including the *Nosema* infection. Such differences are genetically determined and large differences exist among various breeds in their susceptibility to infection by *N. bombycis* (Chinnaswamy and Devaiah, 1984). Young silkworms, newly moulted and starving larvae are more susceptible and show high mortality. Tolerance to the microsporidian disease is greater in Chinese breeds, lesser in Japanese and least in European breeds (Govindan *et al.*, 1998). The multivoltine breeds are relatively more resistant than bivoltines (Patil and Geethabai, 1989). The disease resistance appears to depend on the genetic constituents of a particular breed, factors such as pathogen load, inadequate nutrition and the environment in which the insects are reared (Bhat *et al.*, 2009a). Silkworm races

such as Pure Mysore, Nistari and Chotanichi have better surviving ability than other silkworm races (Devaiah and Krishnaswamy, 1975; Devaiah, 1973; Patil and Geethabai, 1989). The high survival of Pure Mysore breed has been attributed to better regenerative capacity of the midgut to recover from the infection (Fujiwara, 1993). Another, silkworm breed namely Lamerin is reported to be comparatively tolerant to the microsporidian diseases as compared to other bivoltine and multivoltine breeds of the silkworm (Bhat and Nataraju, 2007a). Further, Selvakumar *et al.* (2008) observed that, silkworm races such as Mysore Princes, C-Nichi, Kollegal Jawan and Nistari in Multivoltine and 5N, CSR19, 61N and B225 in bivoltine have shown high survival ability than other silkworm races. A microsporidian sp. NIK- 5hm isolated from silkworm in India forms spores within haemocytes (Selvakumar *et al.*, 2005). A new microsporidian associated with Lamerin breed of silkworm has been characterized on the basis of morphology, serological affinity, infectivity etc. and is different from *N. Bombycis* (Bhat, 2006). Normally the occurrence of microsporidiosis in India is of low intensity during summer and high in winter season in tropics due to increase in the lepidopteran insects during winter and rainy seasons. In temperate sericulture region like Jammu and Kashmir, the incidence of microsporidiosis is higher in summer and lower in spring (Bhat *et al.*, 2009a).

N.bombycis belongs to eukaryotes with primitive nuclear division characterized by the absence of mitochondria, ribosomes and RNA (Vossbrinck *et al.*, 1987; Undeen and Cockbum, 1989). The life cycle of *N.bombycis* has three prominent states viz.spore, planont and meront. The mature spores are heavier than water, oval or ovo-cylindrical in shapewith three layered bluish white membrane exhibiting ‘Brownian movement’. The transmission of the pebrine pathogen occurs through transovarial transmission and oral transmission (contamination of food *i.e.*, mulberry leaf). When the spore is ingested by the silkworm through the contaminated food, it germinates under alkaline conditions inside the gut of the host and produces a long polar filament, at the end of which

spores grow as sporoplasm (Peter *et al.*, 1999). The polar filaments anchor firmly with the gut wall by penetrating and rupturing the epithelial cells of the alimentary canal and invades the cytoplasm of the host. The two nuclei of the sporoplasm unite to form a uninucleate planont, about 0.5-1.5 μm matured in 1-2 days. The planont performs amoeboid movement and reproduces by binary fission. The planont which initially infects the gut later passes through the gut wall and invades other tissues. Once the planont penetrates the host cell, it transforms into a sedentary form and becomes localized. The stage is known as meront. Meront is an intracellular stage and has a definite cell wall which absorbs nutrients from host cell. The meront is formed in 2-3 days after infection. It reproduces by binary fission and multiple budding. When the cytoplasm of the host cell is exhausted, meronts get arranged in parallel rows. The meront after massive proliferation fills up the host cells and gets depleted as the nutrients are exhausted and sporulation takes place. The entire cycle of the spore is completed within four days.

N. bombycis completes its relatively simple life cycle with two sporulation sequences both with diplokaryotic sporont and disporoblastic sporogony (Kawarabatta and Ishihara 1984). The primary (early) sporulation sequence produces a thin-walled binucleate spore called “primary spore, internal spore or FC spore” (few coils of polar tube) with 4 coils of short polar filament and a large posterior vacuole. The primary spore can germinate quickly after formation in the infected cells and serve to disseminate infection within *B. mori* (auto infection) and they are responsible for transovarial transmission because of the infection of the gonads. The second sporulation sequence produces a binucleate spore with thicker spore wall and 10 to 13 coils of long polar filament in one row arrangement (Iwano *et al.*, 1981). These external or environmental spores are involved in the infestation of new hosts and function to infect the new host in horizontal transmission.

The pathogen infects all ages, stages, breeds and hybrids of the silkworm. Larvae suffering from pebrine do not show any external symptoms until the

disease is far advanced. At advanced stage, larvae become sluggish and show symptoms like poor appetite, retarded growth and development and irregular moulting. (Jolly, 1986; Singh and Saratchandra, 2003). Infected pupae are flabby and swollen with lusterless, blackish and softened abdomen and black spots occasionally appear on this region (Ishihara and Fujiwara, 1963). Due to the chronic nature of the disease, the infected larvae do not die immediately and continue to survive for some time (Bhat *et al.*, 2009a). Excessive mortality may be the first indication of the microsporidiosis in an insect colony. The silkworm larvae infected during early stages of first and second instar die upto fifth instar, but if infection occurs during fourth and fifth instars, larvae manages to survive and form cocoons but the silk from the cocoon of infected larvae is usually much inferior. When a host cell is fully occupied by mature spores, the cell bursts, releasing the mature spores into the surrounding environment. A significant number of spores are produced from a single infection due to the multiplication during merogony and sporogony, highlighting the enormous reproductive potential of these organisms.

Pathological status of the diseased organism can be evaluated in terms of symptoms resulting from the interactions between host and pathogen, such symptoms are manifested in terms of morphological, physiological and biochemical variations in the host body. The growth of silkworm is correlated with the synthesis of carbohydrates, proteins, amino acids and various enzymes (Sivaprasad *et al.*, 2003). The infections cause many biochemical changes in the silkworm larvae with a change in metabolic pathways to defend against pathogen invasion (Gao *et al.*, 2006). Biochemical compounds of silkworm under stress conditions can be used as appropriate markers, as studied by Laskshmi and Benarjee (2014). In their study, the biochemical components like amino acids were increased in pebrinised fifth instar larvae whereas proteins got reduced. The activity of the enzymes like alanine amino-transferases and aspartate amino-transferases was increased in the pebrinised larvae.

Microsporidiosis has become increasingly complex today because of different strains of microsporidia being isolated from silkworm and some mulberry insect pests making it almost necessary to take up the comprehensive study on these newer strains for the development of better and more appropriate mitigation and management strategies. Although, some work has been done in India, on the characterization of microsporidians infecting silkworm, *B.mori*, but nearly no work has been done on this aspect under the temperate conditions of Kashmir. The present study was aimed to characterize and to find out the strain/species of *Nosema* as well as screen the silkworm races under study for their tolerance/susceptibility towards microsporidiosis, prevailing in the silkworm population of Kashmir. So, the present study was taken up with the following objectives:

1. Morphological characterization of the microsporidian spore from the diseased silkworms.
2. To study impact of microsporidian infection on survival and economic parameters of silkworm, *Bombyx mori* L.
3. To ascertain the site and intensity of the microsporidian multiplication in the infected worms.
4. To study the impact of microsporidian infection on some biochemical constituents of selected silkworm races.

Chapter -2

REVIEW OF LITERATURE

A comprehensive review of literature provides a pathway to identify the research gaps and frame the strategies to overcome the research barriers. It helps to visualize the problem from the different perspective by highlighting the work already carried out and the focus on the push required to carry the momentum forward. The investigation and assessment depict the problems of the previous work and provides an opportunity to think for future developments. This chapter is devoted to analyse the work carried in the area of effect of microsporidian infection on cocoon parameters, spore characteristics and biochemical constituents in different tissues of silkworm at different developmental stages. The available review related to the present study has been presented and organized as:

Ananthlaskhmi *et al.* (1994) isolated three different microsporidians – NIK-2r, NIK-4m, NIK-3h from silkworm *B.mori* from genus *Nosema*. The results revealed that all the three isolates differed in spore size and shape and reacted differently to the different antibody sensitized latex particles in immunological tests.

Kishore *et al.* (1994) collected adults of 26 species of lepidoptera from the outskirts of the mulberry field in the state of Andhra Pradesh and Karnataka, India. They observed microsporidian infections in *Catopsilia pyranthe*, *Catopsilia pomona* and *Catopsilia crocale* (*Catopsilia pomona form crocale*). They isolated that *N. bombycis* and *Nosema spp.* and which were observed to be cross infective to *B. mori*.

Tembhare and Barsagade (2003) studied the effect of microbial infection (microsporidiosis, bacteriosis and virosis) on the posterior silk gland in Tropical tassar silkworm, *A. mylitta*. The results of their study revealed that the infection caused significant reduction in total protein concentration of posterior silk gland.

Nageswararao *et al.* (2004) studied the pathogenicity, mode of transmission, tissue specificity and the small subunit rRNA (SSU-rRNA) gene sequences of the three microsporidian isolates from the silkworm *B.mori*. Out of the three, NIK-2r revealed life cycle features and SSU-rRNA gene sequence similar to *N. bombycis*, suggesting that it is *N. bombycis*. The other two, NIK-4m and NIK-3h, differed from each other as well as from *N. bombycis*. The results of their study revealed that NIK-4m was highly pathogenic and did not show any vertical transmission, in accordance with the apparent lack of gonadal infection, whereas NIK-3h was less pathogenic and vertical transmission was not detected. Also, Phylogenetic analysis based on SSU-rRNA gene sequence placed NIK-3h and NIK-4m in a distinct clade that included almost all the *Vairimorpha* species and *Nosema* species that infect lepidopteran and nonlepidopteran hosts, while NIK-2r was included in a clade containing almost all the *Nosema* isolates that infect only lepidopteran hosts

Bhat and Nataraju (2005) studied the transmission methods of the microsporidian, *Lb_{ms}* and its effect on the fertility and hatching of silkworm *B. mori*. The results of the study highlighted that there was no significant reduction in fecundity and hatchability; however, the infection could have been transmitted in horizontal and vertical direction.

Selvakumar *et al.* (2005) isolated a microsporidian strain NIK-5km spore from the haemocytes of silkworm *B. mori*. It was observed that the isolated NIK-5km was highly pathogenic to the silkworm.

Bhat and Nataraju (2006) conducted comparative investigation of a microsporidian parasite *Lb_{ms}* infecting the Lamerin breed of silkworm, *B.mori*. They observed that *Lb_{ms}* transmits transovarially as well as perorally at low level. Also larvae developed from the eggs of infected moths were as normal as laid by the normal un-infected females. In the infected tissues developmental stages and mature spores were observed but no hypertrophy of the cells was also observed.

Bhat and Nataraju (2007a) isolated a microsporidian spore from the Lamerin breed of silkworm, *B.mori*. The ultrastructure observations and SDS PAGE results revealed well structured endospore, exospore and nuclei. It also showed 11 polar filament coils and posterior vacoules in the new microsporidian *Lb_{ms}*, which were different from *N.bombycis*.

Bhat and Nataraju (2007b) studied the location and severity of infection in various tissues of silkworm, *B.mori* with and the occurrence of two microsporidias (*Lb_{ms}* and *N. bombycis*). The study concluded with the assertion that the intensity of infection due to *Lb_{ms}* was lower in comparison to *N. bombycis* in all the examined tissues.

Etabari *et al.* (2007) studied the effect of *BmNPV* infection on protein metabolism in larvae of silkworm *B.mori*. The results of the study revealed that the amount of all biochemical components except urea were statistically different in both the groups. A decreased total proteins concentration was observed in the infected larvae. However, it was observed that the activity level of two amino-transfereases increased significantly over the entire period.

Madhusudhan *et al.* (2007) studied the biochemical changes during tropical Tasar silkworm-pebrine interaction. The changes in protein concentration, peroxidase and catalase, amylase, acid phosphatase and hydrogen peroxide in Tasar silkworm at various stages of pebrine infection were assesed. It was found that there was an increase in protein concentration with subsequent increase in peroxidase, phosphatase, hydrogen peroxide and amylase enzymes activity in the infected batch. However, a decrease in the concentration of catalase activity was also recorded.

Chakrabarti and Manna (2008) studied the effect of Microsporidian infection on reproductive potentiality on mulberry Silkworm, *B.mori* L. in different seasons. The results of the study revealed that the microsporidian infection decreased the overall egg production, fecundity, hatching percentage and

increased sterile eggs in the infected mulberry silk-moths. They also observed that the all the parameters under study except number of sterile eggs decreased gradually in the successive years.

Selvakumar *et al.* (2008) studied the pathogenicity of newly isolated microsporidian species NIK-5hm in silkworm, *B. mori*. The study revealed that, the isolated species viz., NIK-Is, NIK-5hm, NIK-2r, NIK-3h, NIK-4m, MCpyB1, BCPoB1 and MCcrB1 collected from butterflies of mulberry field were found to infect silkworm, *B. mori* but did not transmit to next progeny. However, NIK-5hm was found to be highly pathogenic and more virulent to silkworm, *B. mori* than *N. bombycis*. The infection rate or level of NIK-5hm was less in the tissues of trachea, gonad and malphigian tubules compared to *N. bombycis*.

Bhat *et al.* (2009b) studied the haemolymph and midgut lipid profiles in 5th instar microsporidian infected Lamerin breed silkworm *B.mori*. They reported the lower lipid profile in midgut tissues of infected larvae as compared to the healthy batches. However, the lipid content didn't show significant changes in case of haemolymph.

Madann *et al.* (2009) studied the microsporidian multiplication and spore production in various tissues of pupa and adult in relation to age and development of silkworm *B.mori*. The results of the study revealed that multiplication of microsporidian spores in the host tissues follows a logistic pattern: lag phase, exponential phase and stationery phase. The highest spore production was observed 30 days post inoculation and highest concentration of spores in the gut tissues as compared to other observed tissues. The results highlighted the multiplication and spore production of microsporidia to be tissue specific and extremely sensitive to host rearing temperature.

Satadal and Buddhaheb (2009) studied and compared the morphological characteristics of *Nosema assamensis* and *Anthereae assamensis*. They concluded

that *A. assamensis* is considerably different from *N.bombycis* in terms of its morphological characters.

Bhat and Nataraju (2010a) compared the infectivity of *Lb_{ms}* with *N. bombycis* and the study revealed that *Lb_{ms}* is less infectivity as compared to *N. bombycis* at all the stages of development. Furthermore, *Lb_{ms}* was found to be morphologically different from *N.bombycis*.

Bhat and Nataraju (2010b) studied and compared pathogenicity of *Lb_{ms}* and *N.bombycis* in different breeds of silkworm, *B. mori*. The per-oral inoculation of *Lb_{ms}* resulted in lower mortality in all the three breeds as compared to *N. bombycis* and concluded that *Lb_{ms}* is low pathogenic/infective to different breeds of silkworm than *N. bombycis*.

Remadevi *et al.* (2010) studied pathological effects and transmission potential of a microsporidian isolate (*Nosema* sp.) from the teak defoliator *Hyblaea puera* (Lepidoptera: Hyblaeidae). The results of their study revealed that the microsporidian spores were ovo-cylindrical and had a mean size of 5.1x 2.8 μ m. The midgut and fat body were the primary organs infected by the microsporidium. Subsequently, infection was observed in Malpighian tubules, tracheal epithelium and gonads. The sequence of infection in different tissues observed was: midgut – fat body – tracheal membrane – Malpighian tubule – gonad. Infection of this microsporidium produced a marked negative effect on the growth and development of larvae.

Madhusudhan *et al.* (2011) studied the variation in larval parameters, concentration of protein and haemocyte level in tassar silkworm *A. mylitta* due to *N.mylitta* infection. The results revealed a significant decrease in the larval parameters and protein concentration in the infected worms, however, an increase was recorded in the haemocyte count.

Jyothi and Patil (2011) studied the growth of *N.bombycis* in the midgut of silkworm *B.mori* and the impact of this infection on the performance/growth

parameters at the larval stage. The autopsy of larvae showed the presence of white pustules on the midgut of *B. mori* five days post infection(pi). At the later stage, a decrease in the growth of midgut, gonads and silk gland with white pustules was observed. The multiplication of the spores was higher at the young larval stage. The parameters like larval and cocoon weight, cocoon shell ratio showed a decrease with an increase in infection period. The transverse section analysis of the midgut showed that *N. bombycis* infection was limited to few columnar cells at 3-5 days of infection. At 7days pi, cell volume increased and cells turned into swollen and elongated fibres. At 8-9 days pi, the spores leaked into the lumen either freely or enclosed within the globules of host cytoplasm. The study concluded that the development of *N. bombycis* can transcend longitudinally and in circular muscles at the late stage of infection.

Bashir *et al.* (2012) investigated the site and intensity of infection in different tissues of silkworm *B.mori* by three different microsporidians. The results of their study revealed varying degrees of intensity of three microsporidian in the different tissues of silkworm. They further concluded that site and intensity of infection is an important parameter for characterization of microsporidian infecting silkworms and the presence of microsporidian spores in the ovaries is an indication of transovarial transmission in the host.

Bhat *et al.* (2012a) conducted histopathological studies on the infected microsporidian tissues of lamerin breed of silkworm, *B.mori* and reported structural disorganization in cells, but no cellular hypertrophy. Also, different developmental stages of the spores *i.e.* meronts and sporonts were observed in direct contact with cell cytoplasm.

Bhat *et al.* (2012b) studied the characteristic features of two microsporidians isolated from silkworm, *B. mori* and the study revealed that the two isolated microsporidians differed considerably from each other as well as from *N. bombycis* in terms of spore structure and number of coils of the polar filament.

Kasmaei and Mahesha (2012) studied the correlation between haemolymph and midgut proteins with commercial characters in four silkworm races of *B. mori*. The study revealed positive correlation of haemolymph protein with commercial characters except larval duration and renditta. Also, the midgut protein was positively correlated except with fecundity, larval weight, shell ratio and renditta.

Madhusudhan *et al.* (2012) studied the effect of pebrine infection in Tropical Tasar silkworm (*A. Mylitta* D.) in relation to catalase activity. The results witnessed a decrease in the catalase activity in pebrinised larval samples in comparison to control samples. The study concluded that a variation in catalase activity can help to identify the pebrine infection in tropical tasar silkworm larvae.

Chakrabarty *et al.* (2012a) studied effect of pathogen (*Nosema ricini*) of pebrine disease on silkworm with the help of light and electron microscopy. The results of the study concluded that the mature spores of *N. Ricini* were elliptical in shape: bulging at the centre and blunt or tapering at the corners. The mature spores were $3.8 \pm 0.06 \mu\text{m}$ in length, $2.6 \pm 0.03 \mu\text{m}$ breadth and $13.7 \mu\text{m}^3$ volume with hard spore wall and slight concavity. The spores possessed smooth external morphology as observed through SEM. The main ingredients of the mature spores like sporoplasm, polar filament, polar cap, anterior and posterior vacuole and spore shell were analysed with the help of TEM. It was observed that the polar filament had 10-12 number of coils which run obliquely backward from its base at the centre of the polar sac. The study concluded that the life cycle of *N. Ricini* has two distinct phases-spore and vegetative and it is species centric.

Chakrabarty *et al.* (2012b) conducted comparative studies on the effect of different types of *Nosema* on mulberry and Vanya silkworms. The results of their study revealed that the mean mortality in *B. mori* due to *N. bombycis* was quite high and varied in different seasons. Also the mortality was high in case of *A. mylitta* infected by *N. mylitta*. In case of *P. ricini* the mortality was comparatively low.

Borphukan and Bhola (2013) studied the haemolymph protein and carbohydrate alteration during larval development in Eri silkworm, *Philosamia ricini* infected with *Nosema*. The results of their study indicated that haemolymph proteins and carbohydrates decreased steeply in the inoculated 5th instar larva and attained its peak on the last day of larval life as compared to control wherein the protein and carbohydrate levels increase in the 5th instar larvae upto the spinning stage.

Chakrabarty *et al.* (2013a) worked on the secondary contamination for spread of pebrine disease in *B. mori*. The results of their study revealed that *B.mori* larvae infected with *N. bombycis* collected from the natural environment (field) were alive till 3rd generation although the concentration of *N. bombycis* spores was very high ($2.4-3.0 \times 10^8$ spores/mL). However, when artificially inoculated with the same dose of *N.bombycis* all larvae died at 3rd instar of first generation.

Chakrabarty *et al.*,(2013b) studied the gender influenced spore dimorphism in *Nosema bombycis* causing pebrine in *B.mori*. The Scanning Electron Microscopy(SEM) and Transmission Electron Microscopy(TEM) of the spores revealed differences in the primary spore which contained short polar tube(ST) with thin walls and environmental spore with long polar tube(LT) and thick wall. It was further observed that the yield of long tube (LT) was highest in female moths and yield of short tube (ST) was more in male moths.

KiranKumar *et al.* (2013) studied the rate of horizontal and vertical/transovarial transmission of *N.mylittansis* in tasar silkworm as well as its impact on cocoon characters. They observed that *N. mylittansis* transmits the infection both horizontally and vertically. The rate of transmission of *N.mylittansis* recorded was 100%. Furthermore, the infection by *N.mylittansis* was also found to affect the cocoon characters of silkworm and significantly reduce the cocoon weight, shell weight and silk ratio.

Mahesha *et al.* (2013) studied the haemolymph glucose level in Pure Mysore and NB₄D₂ breeds of silkworm *B.mori* inoculated per *os* by two doses (1.562×10^6 and 3.125×10^6) of cytoplasmic polyhedrosis virus. The haemolymph samples from the inoculated silkworm larvae were assessed for the changes in carbohydrate (glucose) level and compared with the control batches. The results of their study revealed that the diseased larvae witnessed reduction in the blood glucose level as the infection progressed in comparison to the controlled batch. However, the glucose levels in the controlled batch increased with the advancement of larval age.

Nadia *et al.* (2013) conducted studies to investigate the effect of *N.bombycis* infection in Diamond Black Moth larvae. They inoculated second-instar larvae by different spore concentrations of *N.bombycis*. The results of the study indicated that all the spore concentrations had a significant negative effect on larval survival. Histological observations showed that *Nosema* preferentially infected the adipose tissue and epithelial cells of the midgut, resulting in marked vacuolization of the cytoplasm which suggest that *Nosema* damaged the midgut epithelial cells and proliferated profusely in the midgut. They finally concluded that *N.bombycis* had a direct adverse effect on Diamond Black Moth and could be utilized as an important biopesticide alternative to chemical insecticides in IPM.

Renuka and Shamitha (2013) studied the pathogenic effects of microsporidian *Nosema spp.* on the larval and post cocoon parameters of Tassar silkworm *A.mylitta*, Drury. The results of the study elucidated that both the larval and post cocoon parameters got deteriorated significantly in the inoculated batches as compared to the control (uninoculated) batches.

Sharma *et al.* (2014) studied the morphology, pathogenicity and transmission of a microsporidian isolated from silkworm, *B. mori*. They observed that the isolate of the microsporidia, differed considerably in morphology from *N. bombycis*. Also the results of their study revealed that that the new microsporidian causes low larval and pupal mortality but high infection percentage at moth stage,

however, the infection in the mother moth does not affect the larval health and cocoon parameters of the next generation.

Lakshmi and Benarjee (2014) studied the metabolic alterations in pebrinised 5th instar larvae of tassar silkworm, *A. myelitta* and revealed that the amino acid and protein content gradually decreases in the infected worms whileas the activity of the enzyme aspartate aminotransferase increased post inoculation.

Bajare *et al.* (2015) studied the cumulative effect of assorted bacterial infections on the mortality and protein concentration of *P. ricini* (Donovan) larvae. The study concluded with the assertion that the biological stress induced due to the combined effect of *Escherichia coli*-NCIM2137 and *Staphylococcus aureus* -NCIM2672 resulted in mortality rate of 25.5% and variations in the protein concentration of haemolymph. The protein concentration of the haemolymph decreased from $15.6 \pm 1.2 \text{ mg ml}^{-1}$ in healthy larvae to $9.8 \pm 0.64 \text{ mg ml}^{-1}$ in infected larvae.

Madhusudhan *et al.* (2015) studied the pathogenicity of Pebrine spores isolated from Ichneumon Fly (*Xanthopimpla pedator*) infesting Tropical Tassar Silkworm, *A. mylitta*. The pebrine spores from Ichneumon fly infesting tassar silkworm were isolated and purified. The purified spores were artificially inoculated to healthy Tassar silkworm larvae per orally. The results of the study indicated that inoculated larvae showed the presence of pebrine spores 16 days post inoculation (dpi). The inoculated larvae showed typical symptoms of pebrine disease in tropical tassar silkworm *i.e.*, appearance of black pepper spots on the larval integument which were distributed sparsely. The results of their study confirmed that, pebrine disease can be transmitted by the Ichneumon fly carrying the pebrine spores. This also confirms that, Ichneumon fly can be secondary source of pebrine contamination in the Tassar rearing fields.

Nirupama (2015) conducted studies on the changes in total protein, carbohydrates & lipid contents in the haemolymph and whole larval tissues in two

rices (300H and CSR6) of silkworm, *B.mori* infected by fungi, *Baeuveria bassiana*. The results of the study revealed that there was a progressive hike in the concentration of these bio-molecules in the control batches, however, a reduced level of the same bio-molecules was observed in the haemolymph & larval tissues under study in the inoculated batches.

Gundeti *et al.* (2015) conducted experiment to study biochemical changes in the haemolymph of Eri silkworm infected with two different bacteria viz- *Escherichia coli* (MTCC 106; gram positive) and *Micrococcus luteus* (MTCC 1687; gram negative). The results of their study showed that the proteins and amino acids levels were significantly elevated and 18 individual free amino acids were found that quantitatively varied in the haemolymph post infection with gram positive and gram negative bacteria when compared with control and sterile silkworm haemolymph. They concluded that the changes in insect haemolymph post this infection may lead to fatal consequences.

Gupta *et al.* (2016) studied the impact of five different microsporidian isolates from mulberry on silkworm, *B. mori* and found that the microsporidians differed significantly in causing the reduction in larval and pupal growth. It was observed that healthy larvae possessed better growth and higher tissue weight than infected ones. The analysis of the data revealed that one microsporidian designated as (M5) caused more damage and in terms of reduction in growth and larval tissue somatic index for both male and females.

Lakshmi and Benarjee (2016) studied the effective concentration of benzimidazole for controlling microsporidiasis and improving cocoon characters in *A. mylitta* Drury and hemocyte count in the infected fifth instar silkworm larvae. The results of their study revealed a significant increase in shell weight, cocoon weight, filament length, reelability, reeled silk weight, denier as well as haemocyte count in B1 batch cocoons (0.005% Benzimidazole Treatment) than other treatments- 0.04%, 0.02% and 0.01% Benzimidazole, in comparison with

un-infected control. They further concluded that 0.05% Benzimidazole is efficient in controlling microsporidiasis in *A. mylitta* Drury (Daba TV).

Nuraeni *et al.* (2016) studied the infectivity of two isolates of *N. bombycis* to silkworm, *B. mori* and reported that the isolates are more infective at 3rd and 4th instar and their infection extended larval duration for more than 10 days as compared to the uninfected batches.

Mahusudan *et al.* (2016) conducted studies on the transmission of pebrine disease (*N. mylitta*) to healthy Tropical Tassar silkworm *A. mylitta* through secondary sources of conatimination. The results of their study elucidated that pebrine is transmiited through all the secondary sources of infection viz; soil, leaves, bark and excreta. However, the rate of transmission is highest through soil contaminated plots as compared to other sources of infection.

Borphukan and Bhola (2018) studied the impact of *Nosema* on total hemocyte count of Eri silkworm, *P. ricini* larvae at 5th instar stage. The results of the study revealed that significant changes were induced in the total haemocyte count in the infected larvae due to the attack of the pathogens. They observed that the number of hemocyte cells/mm³ increased successively with an advancement in the larval age at different seasons, both in normal and infected groups. Comparatively, it was also observed that the hemocyte count was significantly ($p<0.05$) higher in infected groups.

Mahusudan *et al.* (2018) studied the biochemical changes during Tropical Tassar silkworm-pebrine interaction. They observed up-regulation in protein concentration, per-oxidase acid phosphatase, amylase enzyme activities, endogeneous hydrogen peroxidase concentration and down-regulation in catalase activities.

Taha *et al.* (2018) studied the metabolic profiles in healthy, flacherie (inoculated with *Bacillus sp.*) and grasserie (inoculated with Nuclear Polyhedrosis Virus (*BmNPV*) - diseased *B. mori* larvae. Results of their study revealed distinct

differences in the amounts of eighteen individual amino acids in healthy and diseased silkworm 5th larval instar. The highest amino acid concentration was recorded in healthy larvae (224.05 mg/g tissue), while the lowest concentration was found in Grasserie-diseased larvae (177.77 mg/g tissue). Flacherie-diseased larvae inoculated with *Bacillus* sp. showed elevation in certain amino acids compared to Gr-diseased larvae inoculated with of Nuclear Polyhedrosis Virus (NPV) occluded bodies. Their study revealed that a decrease in amino acids in fifth-larval stage implies an increase in Nitrogen and Phosphorus percentages in both bacterial and viral infected larvae. They further concluded that the determination of biochemical responses in silkworm against infections may be considered as bio-analytical markers, which are useful for early disease diagnosis and developing disease resistant breeds.

Venkatesh *et al.* (2018) adopted a comprehensive study to analyse the proteomic profiling of *B. mori*'s hemolymph infected with *N. bombycis*. The comparative assessment was made by exploring the Silkworms infected with *N. bombycis* and analysing the changes in infected and un-infected larval haemolymph using inoculation method with a spore load of 1×10^8 spores per os on the 1st day of 5th instar. Latest methods like Nano-drop spectrophotometry and electrophoresis with different staining methods were adopted for the identification and expression of protein sketching. The results revealed that post- inoculation, haemolymph protein concentrations were up-regulated to 25 kDa protein band, targeted in haemolymph after 5th dpi. The targeted protein was systematically analysed through the MALDI-TOF-MS to obtain the sequence of amino acids, which correlated with 38% of homology with Neprilysin-3. The Neprilysin-3 is a Metallo-endoprotease protein associated with *Drosophila melanogaster*.

Rashmi and Raja (2019) analyzed the biochemical changes in midgut tissue of silkworm *B. mori* due to grasserie infection. Quantitative changes in the biochemical components of midgut tissues like, total proteins, carbohydrates, cholesterol, free amino acids, urea and uric acid were studied on the 2nd and 5th

day of 5th instar of silkworm, *B.mori*, infected with grasserie. The results of their study revealed that all the biochemical parameters under study decreased considerably in the midgut tissues of infected silkworm larvae, as compared to the control healthy non-infected silkworms of the same age.

Subramanyam *et al.* (2019) studied molecular identification and characterization of pathogen associated with pebrine disease in *Anthereae assamensis*. The results of their study revealed that the microsporidian infection in Muga silkworm, *A. assamensis* Helfer significantly reduced cocoon weight, pupal weight, shell weight and silk ratios. They further designed a new set of PCR primers suitable for amplification of small subunit ribosomal RNA (SSU-rRNA) of microsporidia infecting *A. assamensis*. They also concluded that microsporidian pathogen of wild silk moth *A. Assamensis* (*Nosema sp.* AA1) has a close evolutionary relationship with microsporidian pathogens of other wild silkmoths. Also, the arrangement and organization of the rRNA genes inferred that *Nosema sp.* AA1 belongs to true *Nosema* group.

Yadav (2019) studied the effect of pebrine disease on the productivity of mulberry silkworm. He concluded that the pebrine disease seriously affects the productivity as well as quality of the cocoons and silk yarn produced.

Dalip and Joshi (2020) worked extensively on the biochemical changes in the haemolymph, Silk Gland & Fat-body of the tiger band infected larvae of Oak Tassar silkworm, *Antherae proyelei*, Jolly. The main objective was to analyse the variations in the biochemical parameters like protein, carbohydrates and amino acids in different body tissues of the silkworm. The results revealed that there was a significant decline in the concentrations of proteins, carbohydrates and amino acids in all the body tissues under study due to the viral disease (Tiger Band disease). Further, among the tissues under study, maximum decline in the biochemical constituents was observed in the silk glands of the infected/ diseased silkworms

Moharrami *et al.* (2022) characterized *N. Bombycis* using SEM and TEM techniques. The results of the SEM analysis indicated that the mean $\pm$ standard deviation length and width of spores were 1.99 ± 0.25 and 2.81 ± 0.32 mm. The size of spores was smaller than the classic *N. bombycis* spores. The TEM analysis showed that these spores were native strain of *N. bombycis* specific to Iran.

Chapter 3

MATERIAL AND METHODS

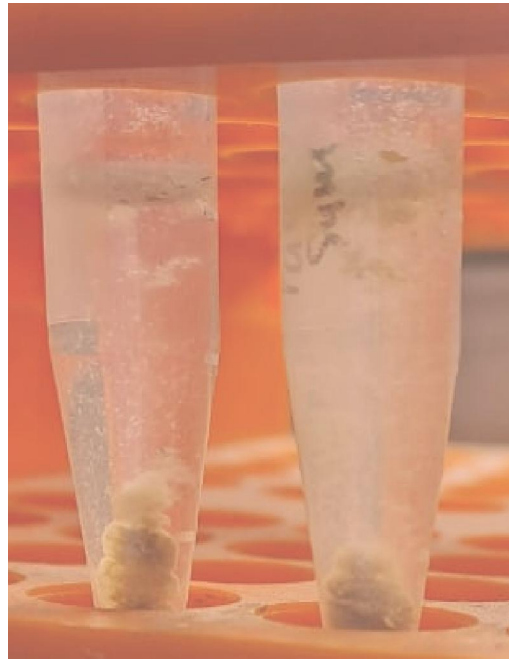
The methodology adopted to reach the broad goal of ‘Studies on the Microsporidian Disease (Pebrine) of Silkworm, *Bombyxmori* L.’ was undertaken as follows:

3.1 Collection, isolation, and purification of microsporidian spores:

The microsporidian infected silkworm samples were procured from Division of Cocoon Crop Production of College of Temperate Sericulture, Mirgund. The silkworm samples were macerated using an autoclaved mortar and pestle. The smear was observed under compound microscope at 600X (MLX-B plus semiplan, Magnus) to confirm the presence of microsporidian. The microscopically examined samples with confirmed microsporidian infection were selected for spore isolation and purification process. The homogenate was filtered through multiple layers of muslin cloth in order to separate the tissue debris from the spore suspension (Plate 1a). The filtrate was transferred to tubes and subjected to centrifugation at 3000 rpm for 10 minutes. The pellet of the initial centrifugation step was separated from the supernatant and re-suspended thoroughly in sterile double distilled water using vigorous vortexing. The suspension containing insect tissue sediments and microsporidian were processed for ultra-purification. The spore pellet was purified based on the density gradient centrifugation using Percoll (Undeen and Alger, 1971). The percoll gradient was prepared by centrifuging 80% percoll in sterile distilled water at 3000 rpm for 20 minutes. The spore suspension in sterile water was gently overlaid through the sides of the centrifuge tube over the percoll gradient without disturbing the integrity of the base gradient. These percoll fractions with top spore suspension layer were first centrifuged at low speed of 3000 rpm for 20 minutes. A sharp white band of mature spores was observed, which was carefully pipetted out in a fresh sterile tube. This fraction containing the Percoll in addition to spores was



a



b

**Plate 1: a) Smear of Pebrne diseased silkworm larvae.
b) Purified microsporidian spores suspended in PBS**

repeatedly centrifuged in order to remove the Percoll particle from the spore suspension by gradually increasing the speed of centrifugation. The spore pellet was thoroughly re-suspended repeatedly in sterile distilled water. After 3 to 4 centrifugation steps, the white pellet containing exclusively the microsporidia was resuspended in PBS (Phosphate Buffer Solution) Plate 1b. The step was used for the preparation of primary stock inoculum and preserved at 5°C.

3.2 Morphological characterization of the microsporadin spore:

The morphological studies at the spore stage are essential to identify, distinguish and compare different strains of microsporadians causing pebrine.

3.2.1 Light microscopy:

The standard method of Fujiwara (1984) was followed for light microscopy of microsporidian spores. The spores isolated from silkworm samples under study were purified by the method described in section 3.1. The smears from each sample were placed on a glass slide and topped with a coverslip and visualized under compound microscope / light microscope microscope (MLX-B plus semiplan, Magnus) at 600X (15 x 40X) and images were captured.

3.2.2 Morphological Characterization of the microsporidian spore using scanning electron microscope (SEM) :

The spores isolated from silkworm samples under study were purified and scanned by the standard scanning electron method (Keohane *et al.*, 1996) to distinguish them on the basis of their external morphology including size, shape and outer spore textures. The samples of the purified spores were air dried at room temperature for electron microscope studies at USIC, University of Kashmir. The microsporidian spores were transferred into double stick cellophane tape pasted on copper stubs for mounting the specimen on scanning electron microscope. The mounted stubs were coated by gold in sputter coater (EMS-550) and scanned in HITACHI S-3000H Scanning Electron microscope at 15 kV voltage at 10,000X.

The spores were observed exhaustively for their shape and texture and images were captured. The observations of spore shape and texture were recorded.

3.2.3 Micrometric parameters of microsporidian isolate:

The size of the spores was observed at 600X using the ocular micrometer (Model-Micron, Optik-CM/L9128879). The micrometry procedure involved placing a 5 µl drop of the pure spore suspension over the already overlaid drop of mineral oil on a slide, which is essential to immobilize the spores for better viewing. The size corresponding to the length and width of the spore is measured with the help of a simple ruled grid of the ocular micrometer which is fixed in the microscope's ocular and calibrated using a stage micrometer. A minimum of one hundred spores were measured for their length and width following the standard procedure for micrometry (Fujiwara, 1980). The stage micrometer has marking divisions and each division represents 10µm. The calibration factor with 15X and 40X eyepieces for the particular microscope and its objective lens was calculated. The unit of each division for 15X and 40X (i.e. 600X magnification) in ocular micrometer was calculated using the following formula:

$$\frac{s}{o} \times 10,$$

Where,

s is the mean value of coinciding divisions of stage micrometer,

o is the mean value of the coinciding divisions of ocular micrometer.

B. Susceptibility of silkworm races to microsporidian infection:

Disease-free layings (DFL) of six mulberry silkworm races *viz*; SK1, SK6, SK28, CSR2, SH6 and NB4D2 were procured from the Germplasm Bank of College of Temperate Sericulture, Mirgund. The layings were surface sterilized with 2% foramlane for 2-3 minutes, incubated, brushed and reared upto 3rd instar as per the standard package of practices (Anonymous 2003). Immediately after 3rd moult, the silkworms from all the races were counted and for every silkworm

race four replications, each consisting of 150 worms was maintained. The silkworms after 3rd moult were fed on mulberry leaves smeared with microsporidian spores. A uniform spore load of 1×10^6 spores/ml was prepared using a haemocytometer. Accurate numbers of spores were calculated in terms of spores in five mm², dilution and depth factor of the chamber (Cantwell, 1970)

$$\frac{\text{spores in five mm}^2 \times \text{dilution} \times \text{depth factor of the chamber}}{\text{number of squares counted}}$$

The inoculated silkworms were allowed to feed on the spore smeared leaves, for 24 hours to ensure complete consumption of the leaf. Thereafter, silkworms were fed with the normal fresh leaves till they settled for spinning. Mortality of larvae was recorded in all the races after every 24 hrs up to spinning stage (Plate 2). One control batch was maintained for each race. In case of control batch, the larvae were fed on mulberry leaves smeared with distilled water which was maintained. The survival and commercial parameters were accordingly recorded. Statistical design of CRD was followed for the experiment.

Table 1: Treatment details of race combinations

Treatment	Combinations
Treatment 1 (T ₁)	SK1 Inoculated
Treatment 2 (T ₂)	SK1 Un-Inoculated/ Control
Treatment 3 (T ₃)	SK6 Inoculated
Treatment 4 (T ₄)	SK6 Un-Inoculated/ Control
Treatment 5 (T ₅)	SK28 Inoculated
Treatment 6 (T ₆)	SK28 Un-Inoculated / Control
Treatment 7 (T ₇)	SH6 Inoculated
Treatment 8 (T ₈)	SH6 Un-Inoculated/ Control
Treatment 9 (T ₉)	NB4D2 Inoculated
Treatment 10 (T ₁₀)	NB4D2 Un-Inoculated/ Control
Treatment 11 (T ₁₁)	CSR2 Inoculated
Treatment 12 (T ₁₂)	CSR2 Un-Inoculated/ Control



a



b

Plate 2: a) Late age silkworm rearing
b) Seriposition of silkworms

Measurement of survival and economic parameters: The survival and economic parameters were measured in terms of larval period (total and fifth age), weight of matured larvae, shell weight, shell ratio percentage, cocoon yield per 10,000 larvae (number and weight), larval mortality, survivability, pupation rate, moth emergence, fecundity, hatching percentage, filament length, denier and raw silk percentage.

1. 5th age larval duration (hr)

It was calculated as hours taken from the 1st day of the fifth instar upto mounting of the ripe worms.

2. Total larval period

Total larval duration was calculated by recording the total days taken from brushing to the mounting.

3. Weight of the matured larvae (g larvae⁻¹⁰)

Ten mature larvae were selected randomly from each replicate of each treatment and weighed.

4. Single cocoon weight (g)

Ten male and ten female cocoons were randomly taken from each replicate of each treatment and weighed to determine the average single cocoon weight.

5. Single shell weight (g)

From each replication, ten cocoons were cut, pupae and cast skin was separated and the cocoon shell weight was recorded and average was calculated to get the mean single shell weight.

6. Shell ratio (%):

It was calculated as the ratio of single shell weight to the single cocoon weight.

$$\text{Shell ratio, \%} = \frac{\text{Single shell weight}}{\text{Single cocoon weight}} \times 100$$

7. Cocoon yield/10,000 larvae:

It was be calculated using the following formulae:

a) By No.

$$\begin{aligned} \text{Cocoon yield per 10,000 larvae} \\ = \frac{\text{No. of cocoons harvested}}{\text{No. of worms retained after 3}^{\text{rd}} \text{ moult}} \times 10,000 \end{aligned}$$

b) By weight (kg)

$$\begin{aligned} \text{Cocoon yield per 10,000 larvae} \\ = \frac{\text{Wt. of cocoons harvested}}{\text{No. of worms retained after 3}^{\text{rd}} \text{ moult}} \times 10,000 \end{aligned}$$

8. Larval mortality (%) :

It was calculated based on the number of dead larvae to the total number of larvae retained after third moult.

$$\text{Larval mortality} = \frac{\text{Total no. of dead larvae}}{\text{Total number of larvae retained after 3}^{\text{rd}} \text{ moult}} \times 100$$

9. Survivability%:

It was calculated by using the below mentioned formula

$$\text{Survival rate} = \frac{\text{No. of live cocoons harvested}}{\text{Total no. of worms retained after 3}^{\text{rd}} \text{ moult}} \times 100$$

10. Pupation rate (%) :

$$\text{Pupation rate, \%} = \frac{\text{Total no. of live pupae obtained}}{\text{Total no. of cocoons harvested}} \times 100$$

11. Moth emergence

Moth emergence for each replication of each treatment was observed and calculated by the following formula:

$$\text{Moth emergence} = \frac{\text{No. of live moths emerged}}{\text{Total no. of cocoons in the tray}} \times 100$$

12. **Hatching percentage:** It was calculated as:

$$\text{Hatching percentage} = \frac{\text{No. of eggs hatched}}{\text{Total no. of eggs in the laying}} \times 100$$

13. **Fecundity:**

10 layings of each replicate of each treatment were selected randomly and no. of eggs will be counted to determine no. of eggs laid per moth.

14. **Average Filament length (m)**

Ten randomly selected cocoons from each replicate of each treatment were reeled to determine the average filament length.

15. **Denier:** It will be calculated as:

$$\text{Denier} = \frac{\text{Weight of the silk filament (g)}}{\text{Length of the filament (m)}} \times 100$$

16. **Raw Silk (%) :**

It refers to the weight of the reeled silk to the cocoon weight on percentage basis.

$$\text{Raw silk, \%} = \frac{\text{Weigh of reeled silk}}{\text{Cocoon weight}} \times 100$$

3.3 Site and intensity of microsporidian multiplication in the infected silkworms:

The site of infection was assessed by dissecting out the different tissues viz. midgut, malpighian tubules, silk gland from the larval stages of silkworm and gonads from adult female silkworm. Ten silkworms from each replicate on 3rd day and 5th day of 5th instar post inoculation were randomly taken from every replicate excluding the controls. The collected tissues were dissected out and washed with distilled water. The tissues were kept in PBS (Phosphate Buffer

Solution) after excision upto examination. Each dissected tissue was individually weighed and 1mg of the tissues was taken and homogenised in 1ml of distilled water (Plate 3a). Smear from each sample was taken on a glass slide topped up by a cover slip. These slides were then examined under microscope (MLX-B plus semiplan, Magnus) at a magnification of 600X. The observations were recorded on the intensity of infection which was recorded as-nil (-) for no infection (+) for low-level infection and (++) for moderate-level infection and (+++) high-level infection. The term nil, moderate, high and very high for spore number is quantitative, nil (-) indicates no spore is present, low level (+) indicated 1-5 spores/microscopic field, moderate level (++) indicated 5-30 spores and high level (+++) indicated 30 or more spores/microscopic field. (Bhat and Nataraju, 2007b)

3.4 Biochemical Analysis:

For the biochemical studies, samples were collected on 3rd and 5th day of fifth instar. Samples of tissues – midgut, silk gland and fat body were collected through dissection except hemolymph. The gonads were collected by dissecting the female moths (a day after emergence).

- i. **Haemolymph:** The haemolymph of the infected silkworms was collected on the 3rd and 5th day of the fifth instar, in pre-cooled eppendorf tubes containing a few crystals of phenyl thiourea @1mg per sample by puncturing the abdominal legs with sterilized scissor (Plate 3b). The samples were centrifuged at 3000 rpm for 5-10 min and supernatant was collected and used as sample for further quantitative estimation (Kasmaei and Mahesha, 2012)
- ii. **Midgut, Fatbody, Silk glands and gonads:** Ten silkworm larvae were randomly collected from every replication of each race, daily on the 3rd and 5th day of 5th instar. Midgut, fatbody and silk glands were collected by dissection from these larvae. The gonads were excised by dissecting ten moths' silkworm female moths (from every replication) on the next day of



a



b

- Plate 3: a) Dissection of silkworms to extract different tissues of silkworms for biochemical studies**
- b) Collection of haemolymph from silkworms**

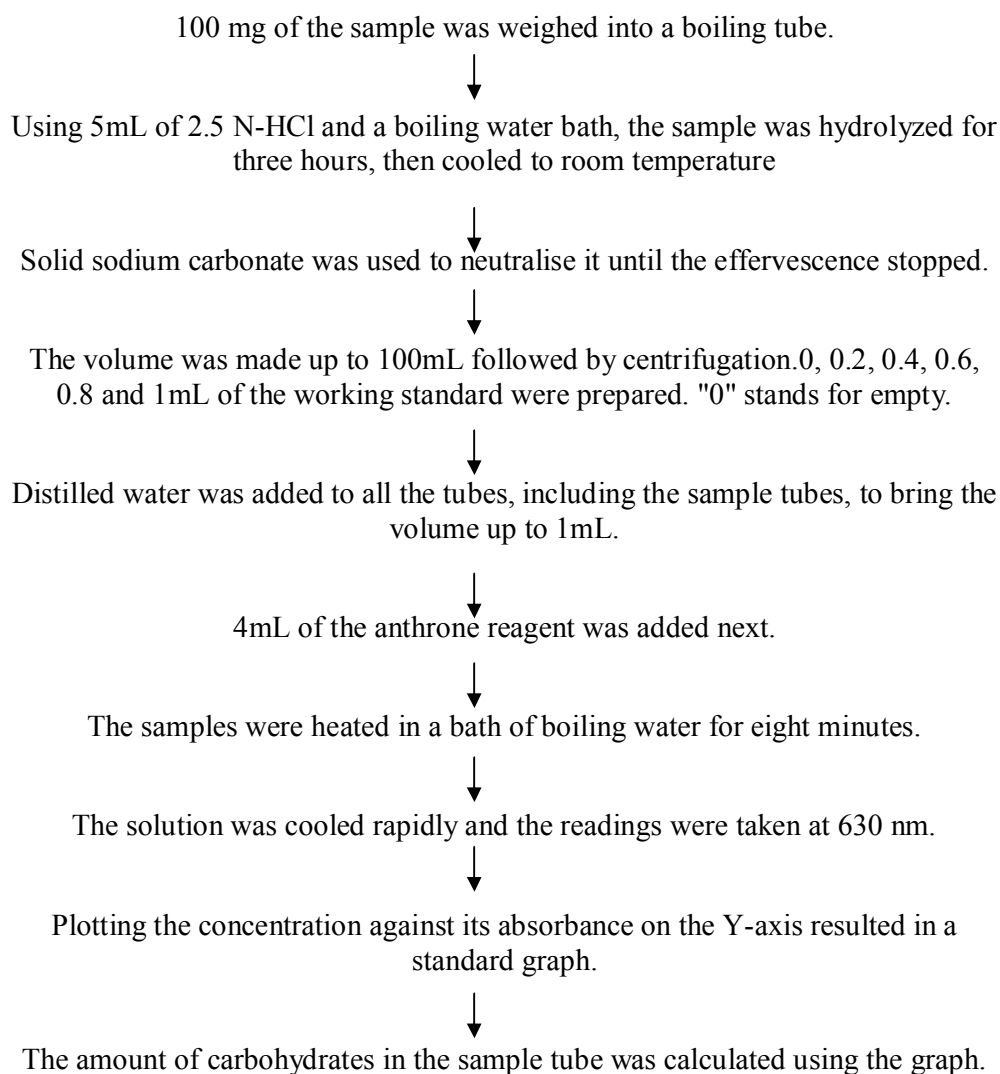
emergence. The dissected tissues were weighed and 10 mg of the sample was transferred to pre-chilled eppendorff tubes. These tissues isolates were then homogenised in 100 ml of distilled water to prepare 10%W/V (weight/volume) homogenate of the tissues using a tissue homogeniser (Potter and Elvehjam tissue homogeniser). The homogenate was centrifuged at 3000 rpm for 10 min, supernatant was collected, diluted, and used as sample for protein estimation (Kasmaeie & Mahesha, 2012; Laskhmi and Banerjee, 2014).

Estimation of biochemical parameters:

The following biochemical parameters for haemolymph and tissue homogenates were measured by standard procedures as:

- **Total protein content:** The most popular colorimetric method for determining protein concentration is the Bradford assay. The fundamental idea is that protein binding causes the maximum wavelength of Coomassie Brilliant Blue G-250 in an acidic solution to shift from 465 to 595 nm. The rise in absorbance at this wavelength is directly proportional to the amount (concentration) of protein present in the sample since the bound form of the dye has an absorption maximum held at 595 nm. Using an ELIZA reader, the protein quantification was carried out in a microtitre plate. Known BSA concentrations were utilised as a benchmark for this estimation. Working BSA concentrations of 5, 2.5, 1.25, 0.625 and 0.313 g were prepared and the final volume was 100ul per well (in duplicates). The microsporidial total protein samples were serially diluted (1:1) and 100 µl of unknown sample and 100 µl of working Bradford reagent were added to each well. Aluminium foil was placed over the plate, which was then incubated at 37°C for 15 minutes. On a spectrophotometer, the absorbance was measured at 592 nm. With the aid of a BSA protein standard curve, the concentration of an unknown sample was estimated.

- **Total Carbohydrate content:** It was measured with the help of Anthrone method (Dubios *et al.*, 1956). The anthrone reagent was prepared in accordance with Trevelyan and Harrison's (1952) instructions by dissolving 0.2g of anthrone in 100 ml of H_2SO_4 , which was created by mixing 500 ml of concentrated acid with 200 ml of water. Until it was completely clear, the reagent was left to stand for 30 to 40 minutes while being occasionally shaken. Each day, the reagent was freshly made and utilised within 12 hours.
- **Procedure:**



- **Total lipid content:** It was measured using the procedure of Folch *et al.*, 1951. The insect tissues (haemolymph, midgut, fatbody and silk glands) from ten random silkworm larvae and gonads from silkworm female moth were taken and were crushed (homogenised). 1mg of each tissue homogenate *viz*: midgut, fatbody, silk glands and gonads was mixed thoroughly with 1ml of chloroform-methanol mixture (2:1,V/W), however in case of haemolymph 1ml in 1ml of chloroform-methanol mixture (2:1,V/V) was taken. It was left for 30- 40 mins in a dark location for lipid quantification. The pooled chloroform-lipid mixture was air dried and the weight of the sample was recorded until constant final weight was ascertained. The difference between the initial and the final weights of the vial gave the total lipid content of the sample. The lipid quantity was expressed in either mg/ml of haemolymph or mg/g weight of tissue.
- **SDS PAGE:** Using SDS-PAGE analysis by following the standard Laemmli (1970) procedure, the total protein extracts in the haemolymph of the test isolates were profiled. The following procedure describes the preparation of small gels that are approximately 10x 10 cm.
- **Preparation of gels:**
 - i) **12% separating gel preparation:**
Using 3.3 ml of double-distilled water, 4 ml of 30 per cent acrylamide mix (acrylamide and bis-acrylamide in a 29:1 ratio), 2.5 ml of 1.5 M tris (pH 8.8) and 0.1 ml of 10 per cent SDS, a separating gel of 12 per cent was formed. To this 10ul of 10 per cent ammonium persulphate and 4ul of TEMED were added for polymerization, Table 2.
 - ii) **5% stacking gel:**
2.1 ml of double-distilled water, 0.5 ml of the 30 per cent acrylamide stock mix, 0.38 ml of 1.0 M tris (pH 6.8), 30 µl of 10 per cent SDS, 30 µl of 10 per cent ammonium persulfate and 2 µl of TEMED were combined to create the stacking gel solution.

Table 2: Reagents used in the preparation of 12% and 3% gel solution

Reagents	12% (10mL)	3% (3mL)
ddH ₂ O	3.3 Ml	2.1 mL
Acrylamide	4.0 mL	0.5 mL
Tris pH 8.8	2.5 mL	0.38 mL (Tris pH 6.8)
10% SDS	0.1 mL	0.03 mL
10% APS	0.1 mL	0.03 mL
TEMED	0.004 mL	0.002 ml

The spacers were used to assemble the glass plates, which were then clamped to the frame. A little amount of thick, molten agarose was poured over the three sides to seal them and the area was then left alone for a while to allow the sealing to take place. In order to later cover the top with stacking gel, the 12 per cent separating gel solution was poured into the spaces between the sealed glass plates. After the separating gel had polymerized, the stacking gel was added and a well-comb was positioned on top. The proteins were loaded onto the gel after being dissolved in a solution containing 12.5M tris HCl (pH 6.8), 2 per cent SDS, 15 per cent glycerol, 5 percent-mercaptoethanol and 0.001 per cent bromophenol blue. The standard protein mixture (molecular weight marker, Puregene) was added to the loading buffer.

Tris-glycine buffer, consisting of 3.0 g of tris and 14.4 g of glycine in 1000 ml of distilled water (pH 8.3) and 0.1 per cent SDS, was used for the electrophoresis. The stacking gel was initially run at 60 V until the protein passed through it. The voltage was raised to 100 V once the dye front entered the separating gel and maintained there until the tracking dye, bromophenol blue, was 1 cm from the gel's bottom. Protein bands were fixed in 10 per cent TCA for an hour at room temperature, stained with coomassie brilliant blue R-250 (CBB)

solution overnight and then de-stained by soaking the gel in a de-stainer solution until the gel turns translucent and the protein bands are visible.

Composition of solutions used:

- CBB stain solution-0.1% Coomassie R-250, 10% acetic acid, 40% methanol.
- Destaining solution-20% methanol, 10% acetic acid in DDW.

Chapter-4

EXPERIMENTAL FINDINGS

The collection and analysis of the recorded data forms an essential component to reach out to scientific and logical conclusion. The study was directed to study the morphological structure of microsporidian spores and its impact on the survival and economic parameters of silkworm *B.mori*. Moreover, a microscopic investigation was carried out to check the site of multiplication of spores as well as the intensity of spores in different larval tissues of silkworm. The study was further extended to record the biochemical parameters in different tissues of silkworm *B.mori*, mainly to assess the impact of microsporidian infection on larval growth and survivability.

4.1 Purification and isolation of microsporidian spores:

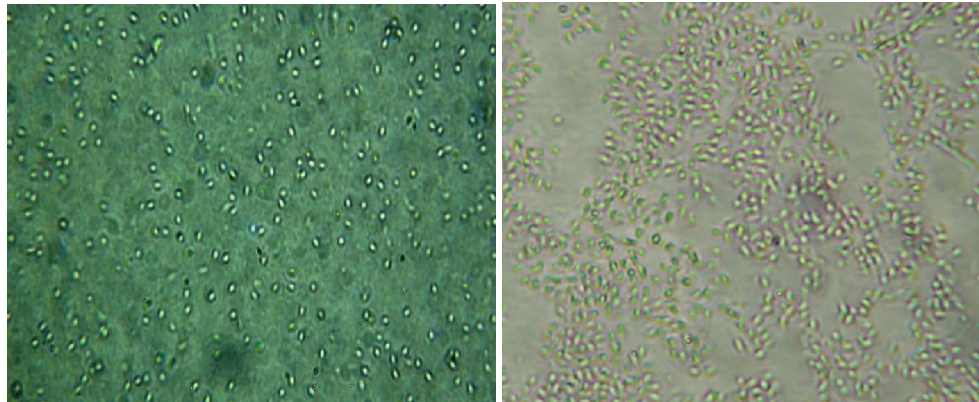
The infected silkworm samples showed presence of spores. Spores of various dimensions were observed, after further purification more clear observations of the microsporidian spores were observed and recorded. (Plate 4a&b).

4.2 Light microscopy and micrometry:

The wet mount of the observed spores exhibited characteristic Brownian's movement and high refractive index which is a typical feature exhibited by spores of *N.bombycis* and other microsporidian spores. The spores observed were designated as N₁ spores and were oval in shape, measuring $3.33 \pm 0.17 \mu\text{m}$ in length and $1.84 \pm 0.09 \mu\text{m}$ in width. The spore length –width ratio (eccentricity index) was 1.80:1. These spores differed distinctly from the standard strain *N. bombycis* spores measuring $3.08 \pm 0.21 \mu\text{m}$ in length and $2.01 \pm 0.05 \mu\text{m}$ in width. The spore length –width ratio was 1.53:1. (Bhat and Nataraju, 2007a)

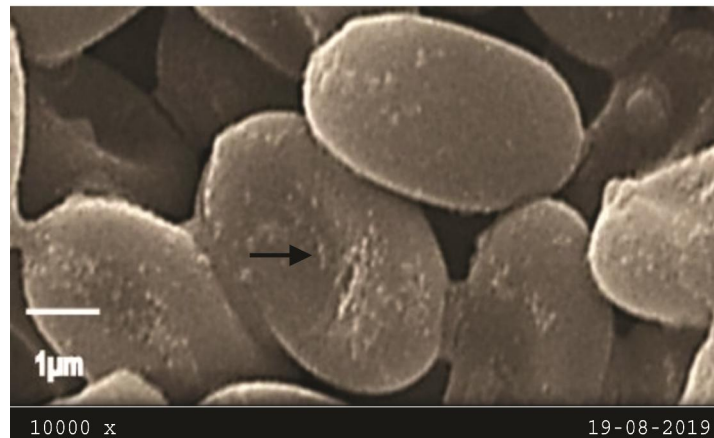
4.2.1 Scanning Electron Microscopy:

Scanning Electron Microscopy (SEM) showed prominent morphological and structural details of the microsporidian spores (Plate 4c). The ultra-

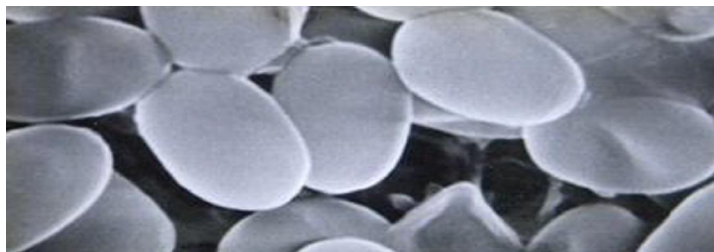


a

b



c



d

Plate 4: a and b) Microphotographs of purified (N1) microsporidian spore.

c) Scanning Electron Microphotographs of isolated (N1) microsporidian spores

*Arrow heads show depression in the spores

d) Scanning Electron Microphotograph of *N. bombycis* spores (Chakrabarty *et al.*, 2013)

microscopic studies of the microsporidian spores revealed that the external surface of N1 spores was rough with a depression in the middle of the spores. These spores distinctly differed from *N.bombycis* spores (Plate 4d) where the exospore or external surface is smooth with no depression. (Bhat and Nataraju, 2007a).

Table 3: Comparison of morphological parameters of isolated microsporidian spore (N1) and standard strain spores of *N. bombycis*:

Microsporidian sample	Shape	Spore size (µm)		Eccentricity index
		Length (Mean±SD)	Width (Mean± SD)	
<i>Microsporidian</i> Spp (N ₁)	Oval	3.33 ±0.17	1.84 ±0.09	1.80:1
<i>N. bombycis</i> *	Oval	3.08 ±0.21	2.01 ±0.05	1.53:1

*(Bhat and Nataraju, 2007a)

4.3 Susceptibility of various silkworm races towards the isolated microsporidian infection:

The experiment was conducted during two consecutive years and the data of two years was later pooled for the results. The parameters of silkworms which were recorded during pre-cocoon and post- cocoon phases is presented as under:

4.3.1 5th age larval duration (hour) :

The results of the experiment showed that the inoculation of different races of silkworm *B. mori* with same spore load of inoculum of *microsporidian* spores caused varied response in terms of fifth age larval duration in all the six races of

silkworm under study. The un-inoculated or control batches of all races showed lesser or shorter larval duration whereas the inoculated races showed prolonged larval durations. Among the inoculated batches, the shortest larval duration of 191.99 hrs was observed in T₇ (SH6), followed by T₉ (NB4D2) wherein the larval duration was 193.33 hours. The longest larval duration of 218.82hrs was observed in T₅ (SK28) followed by T₁ (SK1) -211.55hrs. The 5th age larval duration in the control batches of these races was T₂ (SK1-control) -211.55hours, T₄ (SK6-control) - 183 hrs, T₆ (SK28-control) - 184 hrs, T₈ (SH6) -177hrs, T₁₀ (NB4D2) - 175.5 hours and T₁₂ (CSR2 Control) -174.66 hours (Table 4).

4.3.2 Total larval period (in hours):

The 5th age larval duration and total larval duration parameters are directly proportional to each other; longer the 5th age larval duration, longer will be the total larval duration of silkworms. Significant data was recorded in case of total larval duration in all the races. The un-inoculated or control batches of all races showed lesser or shorter total larval duration whereas the inoculated races showed prolonged larval durations. Among the inoculated batches, the shortest total larval duration was observed in T₉ (NB4D2) - 710.83 hrs followed by T₇ (SH6) - 711.00 hours. The longest total larval duration of 753.16 hours was observed in T₅ (SK28) followed by T₃ (SK6) - 733.16hrs. The total larval duration recorded by other treatment combinations include T₁ (SK1) - 732.50 and T₁₁ (CSR2) - 714.16 (Table 4). The total larval duration observed in the control batches of these races was T₂ (SK1 control) -702 hrs, T₄ (SK6 Control) -699 hrs, T₈ (SH6- Control) - 681.16 and T₁₀ (NB4D2 Control) - 689.5hrs and T₁₂ (CSR2 Control) - 682.5hrs.

4.3.3 Weight of the matured larvae (g larvae⁻¹⁰):

The results of the study exhibit that there is a significant difference in the weight of 10 mature larvae in all the inoculated races of silkworm when compared with the un-inoculated races as well with other races under study. The inoculated races showed lower larval weight as compared to the un-inoculated (control)

batches. The highest larval weight of 38.63g among the inoculated races was observed in T₇ (SH6) followed by T₉ (NB4D2) whose larval weight was 38.18g (Table 4). The lowest larval weight was observed in T₅ (SK28) - 31.81g followed by T₁₁ (CSR2) -34.36g. The larval weights recorded treatment combinations are T₁ (SK1) -34.55g T₂ (SK1 Control) -46.08g, T₃ (SK6) - 33.93g, T₄ (SK6 Control) -45.01g, T₆ (SK28 control) - 45.49g, T₈ (SH6 Control) - 41.58g, T₁₀ (NB4D2 control) - 42.04g and T₁₂ (CSR2 control) - 42.14g respectively.

Table 4: Effect of Microsporidian Infection on Larval Parameters of Silkworm, *Bombyx mori* L

Treatment	Sample	Fifth Age Larval Duration (hours)			Total larval period (hours)			Weight of the matured larvae (g larvae ⁻¹⁰) :		
		Year1 (Spring)	Year2 (Spring)	Pooled Data	Year1 (Spring)	Year2 (Spring)	Pooled Data	Year1 (Spring)	Year2 (Spring)	Pooled Data
T ₁	SK1: Inoculated	212.66	210.45	211.55	729.66	735.33	732.49	33.31	35.8	34.55
T ₂	SK1: Un-Inoculated	185	182	183.5	702	703	702.5	45.5	46.67	46.08
T ₃	SK6: Inoculated	203.33	210	206.66	727	739.33	733.16	40	32.88	36.44
T ₄	SK6: Un-Inoculated	181	185	183	698	700	699	45.44	44.58	45.01
T ₅	SK28: Inoculated	220	217.66	218.83	743	763.33	753.16	31.61	32.02	31.81
T ₆	SK28: Un-Inoculated	182	186	184	70	705	387.5	43.41	47.57	45.49
T ₇	SH6: Inoculated	190.66	193.33	191.99	701.33	713.66	707.49	38.25	39	38.62
T ₈	SH6: Un-Inoculated	178	176	177	680	682.33	681.16	40.6	42.42	41.51
T ₉	NB4D2: Inoculated	194.33	192.33	193.33	712.66	709	710.83	36.54	37.82	37.18
T ₁₀	NB4D2: Un-Inoculated	175	176	175.5	687	692	689.5	40.4	43.68	42.04
T ₁₁	CSR2: Inoculated	200.33	203.33	201.83	712.66	715.66	714.16	33.8	34.92	34.36
T ₁₂	CSR2: Un-Inoculated	171.33	178	174.66	680	685	682.5	42.48	41.8	42.14
	CD (p≤0.05)	4.31	4.87	3.16	6.96	7.60	5.01	3.44	3.74	2.47

4.3.4 Single cocoon weight (g) :

A significant decrease in the single cocoon weight in the inoculated batches as compared to control as well as among the races under study was observed from the results of the study. Among all the inoculated races, lowest single cocoon weight was observed in T₁₁ (CSR2) -1.34g followed by T₅ (SK28) - 1.37g. The highest single cocoon weight among the inoculated races was observed in T₇ (SH6) -1.49g followed by T₉ (NB4D2) - 1.48g, Table 5. The single cocoon weight recorded by other treatment combinations is T₁ (SK1) -1.45g, T₂ (SK1 Control) - 1.89g, T₃ (SK6) -1.46g, T₄ (SK6 Control) -1.92 g, T₆ (SK28 Control) - 1.90g, T₈ (SH6 Control) - 1.73g and T₁₀ (NB4D2- Control) - 1.76 g and T₁₂ (CSR2- Control) -1.78g respectively(Table 5).

4.3.5 Single shell weight (g) :

This parameter also differed significantly among all the treatment combinations. Among the inoculated batches, the highest shell ratio was observed in two treatments T₇ (SH6) -0.23g and T₉ (NB4D2) -0.23g which were statistically at par with each other. The lowest shell ratio was recorded in T₁₁ (CSR2) -0.19g and T₅ (SK28) -0.20g. The shell ratio recorded by other treatment combinations is- and T₁ (SK1) -0.22g, T₂ (SK1) -0.39g, T₃ (SK6) -0.22g, T₄ (SK6 control) -0.39g, T₆ (SK28 Control) -0.40g, T₈ (SH6 Control) -0.35g, T₁₀ (NB4D2 Control) -0.34g and T₁₂ (CSR2 Control) -0.35g (Table 5).

4.3.6 Shell ratio (%) :

The parameter shell ratio is dependent on two parameters- single cocoon weight and shell weight. This parameter also differed significantly among all the treatment combinations. The highest shell ratio among the inoculated batches was exhibited by T₉ (NB4D2) -15.59% followed by T₇ (SH6) - 15.49% (Table 5). The lowest shell ratio was recorded in T₁₁ (CSR2) -13.87% and T₅ (SK28) -14.23%. The shell ratio observed by other treatment combinations is T₁ (SK1) - 14.84%, T₂ (SK1 Control) -20.68%, T₃ (SK6) -15.06%, T₄ (SK6 Control) - 20.97%, T₆ (SK28 Control) - 21.04%, T₈ (SH6-Control) -19.28%, T₁₀ (NB4D2 Control) - 18.82% and T₁₂ (CSR2 Control) -18.87% respectively.

Table 5: Effect of Microsporidian Infection on Cocoon Parameters of Silkworm, *Bombyx mori* L.

Treatment	Single cocoon weight (g)			Single shell weight (g)			Shell ratio (%)		
	Year1 (Spring)	Year2 (Spring)	Pooled Data	Year1 (Spring)	Year2 (Spring)	Pooled Data	Year1 (Spring)	Year2 (Spring)	Pooled Data
T ₁	1.43	1.47	1.45	0.22	0.21	0.22	15.38 (3.92)	14.29 (3.79)	14.84 (3.81)
T ₂	1.82	1.95	1.89	0.37	0.41	0.39	20.33 (5.13)	21.03 (5.54)	20.68 (5.32)
T ₃	1.44	1.48	1.46	0.21	0.23	0.22	14.58 (3.29)	15.54 (3.98)	15.06 (3.58)
T ₄	1.8	1.92	1.86	0.38	0.40	0.39	21.11 (4.49)	20.83 (5.59)	20.97 (5.77)
T ₅	1.35	1.39	1.37	0.19	0.2	0.20	14.07 (3.08)	14.39 (4.03)	14.23 (3.79)
T ₆	1.84	1.96	1.90	0.38	0.42	0.40	20.65 (5.17)	21.43 (6.34)	21.04 (6.03)
T ₇	1.48	1.49	1.49	0.23	0.23	0.23	15.54 (3.99)	15.44 (3.34)	15.49 (3.39)
T ₈	1.78	1.85	1.82	0.34	0.36	0.35	19.1 (4.69)	19.46 (4.86)	19.28 (4.53)
T ₉	1.47	1.48	1.48	0.23	0.23	0.23	15.65 (3.56)	15.54 (3.98)	15.59 (3.97)
T ₁₀	1.74	1.82	1.78	0.33	0.34	0.34	18.97 (4.32)	18.68 (4.16)	18.82 (4.37)
T ₁₁	1.32	1.35	1.34	0.19	0.18	0.19	14.39 (4.02)	13.33 (3.60)	13.86 (3.81)
T ₁₂	1.76	1.79	1.78	0.32	0.35	0.34	18.18 (4.06)	19.55 (4.98)	18.87 (4.97)
CD (p≤0.05)	0.291	0.318	0.590	0.26	0.21	0.16	0.957	1.40	0.825

4.3.7 Cocoon yield/10,000 larva:

- a) **By number:** This parameter reflects the effective rate of rearing and in the present study all the treatment combinations differed significantly from each other and their respective controls. The highest cocoon yield by no. among the inoculated batches was observed in T₇ (SH6) -6764 cocoons and T₉ (NB4D2) - 6434 cocoons, Table 6. The lowest cocoon yield by number was recorded by T₅ (SK28) - 5285 cocoons. Cocoon yield by number observed in other treatment combinations was T₁ (SK1) -6047 cocoons, T₂ (SK1 control) - 9503 cocoons, T₃ (SK6) -5621 cocoons, T₄ (SK6 control) - 9589 cocoons, T₆ (SK28-Control) 9493 cocoons, T₈ (SH6 Control) -9618 cocoons, T₁₀ (NB4D2 Control) - 9588 cocoons -T₁₂ (CSR2 Control) -9483 cocoons respectively (Table 6).
- b) **By weight:** The results of the present study show that cocoon yield by weight was significantly different in all the races under study, also the races differed statistically from their control batches. The highest cocoon yield by weight among the inoculated batches was observed in T₇ (SH6) - 10.04Kg cocoons, followed by T₉ (NB4D2) - 9.49Kg. The lowest cocoon yield by weight was recorded by T₅ (SK28) - 7.17Kg. Cocoon yield by weight observed in other treatment combinations was T₁ (SK1) -8.77 Kg and T₂ (SK1 control) -17.91kg, T₃ (SK6) - 8.21 kg, T₄ (SK6 control) - 17.83kg, T₆ (SK28) -18.04 Kg, T₈ (SH6 control) - 17.46 kg, T₁₀ (NB4D2 control) - 17.06 kg, T₁₁ (CSR2) - 8.21 Kg and T₁₂ (CSR2) - 16.83 kg respectively (Table 6).

4.3.8 Larval mortality:

The results of the study revealed that the inoculation of different races of silkworm *B. mori* with the microsporidian spores caused mortality in all the inoculated races, however the percentage varied in all the races. Statistical analysis revealed that mortality percentage in T₅ (SK28) -46.77% was statistically

the highest among all other treatment combinations (Table 6). The lowest larval mortality was observed in T₇ (SH6) - 31.42% followed by T₉ (NB4D2) -34.85%. The larval mortality observed by other treatment combinations is- T₁ (SK1) - 38.94%, T₂ (SK1 control) -4.15%, T₃ (SK6) -42.91%, T₄ (SK6- control) - 3.48%, T₆ (SK28 Control) -3.75%, T₈ (SH6 control) -3.25%, T₁₀ (NB4D2 Control) - 2.96%, T₁₁ (CSR2) - 37.80 and T₁₂ (CSR2 Control) -4.16% respectively.

4.3.9 Survivability:

Statistical analysis revealed that survivability percentage in T₅ (SK28) - 52.33% was statistically the lowest among all other inoculated treatment combinations. The highest larval survivability was observed in T₇ (SH6) - 67.65% followed by T₉ (NB4D2) -64.34% (Table 6). The larval survivability observed by other treatment combinations is- T₁ (SK1) -59.87%, T₂ (SK1 control) - 95.03%, T₃ (SK6) -56.22%, T₄ (SK6 control) - 95.89%, T₆ (SK28 control) - 94.93%, T₈ (SH6 Control) - 96.19%, T₁₀ (NB4D2 Control) - 95.89%, T₁₁ (CSR2) -61.49% and T₁₂ (CSR2 control) - 94.83% respectively.

4.3.10 Pupation rate (%):

The results of the study revealed that the inoculation of different races of silkworm *B.mori* with the microsporidian spores affected the pupation rate in all the inoculated races, however, the percentage varied in all the races. Statistical analysis revealed that pupation rate in T₅ (SK28) - 53.61% was statistically the lowest among all other treatment combinations followed by T₁₁ (CSR2) which recorded a pupation rate of 55.17%. The highest pupation rate was observed in T₇ (SH6) - 59.87% followed by T₉ (NB4D2) - 59.32% (Table 7). The pupation rate observed by other treatment combinations is- T₁ (SK1) -57.87%, T₂ (SK1 control) -92.41%, T₃ (SK6) -58.31%, T₄ (SK6- control) - 92.52%, T₆ (SK28 Control) - 93.45%, T₈ (SH6 control) - 94.28%, T₁₀ (NB4D2 Control) - 94.40% and T₁₂ (CSR2-control) -93.74% respectively.

Table 6: Effect of Microsporidian Infection on single Cocoon Yield , Larval Mortality and Survivability of Silkworm, *Bombyx mori* L.

Treatment	Cocoon yield (by No.)			Cocoon yield (by weight)			Larval mortality (%)			Survivability (%)		
	Year1 (Spring)	Year2 (Spring)	Pooled Data	Year1 (Spring)	Year2 (Spring)	Pooled Data	Year1 (Spring)	Year2 (Spring)	Pooled Data	Year1 (Spring)	Year2 (Spring)	Pooled Data
T ₁	6150	5945	6047.5	8.79	8.74	8.77	37.87 (5.89)	39.95 (6.14)	38.91 (6.39)	61.5 (7.96)	59.45 (7.61)	59.97 (8.01)
T ₂	9536	9470	9503	17.36	18.47	17.91	3.87 (1.12)	4.43 (1.47)	4.15 (1.97)	95.36 (11.63)	94.7 (11.46)	95.03 (10.65)
T ₃	5356	5887	5621.5	7.71	8.71	8.21	45.44 (6.86)	40.38 (6.09)	42.91 (6.34)	53.56 (7.48)	58.87 (7.92)	56.215 (7.34)
T ₄	9648	9530	9589	17.37	18.30	17.83	2.84 (0.93)	4.11 (1.24)	3.475 (1.33)	96.48 (12.04)	95.3 (11.27)	95.89 (11.91)
T ₅	5443	5023	5233	7.35	6.98	7.17	44.58 (5.73)	48.95 (7.03)	46.765 (6.63)	54.43 (7.89)	50.23 (7.13)	52.33 (7.40)
T ₆	9484	9502	9493	17.45	18.62	18.04	3.21 (1.03)	4.31 (1.92)	3.76 (1.77)	94.84 (11.54)	95.02 (10.67)	94.93 (10.14)
T ₇	6903	6626	6764.5	10.22	9.87	10.04	30.03 (5.11)	32.81 (6.34)	31.42 (5.97)	69.03 (8.98)	66.26 (8.91)	67.645 (8.97)
T ₈	9663	9574	9618.5	17.20	17.71	17.46	2.87 (0.95)	3.64 (1.49)	3.255 (1.28)	96.63 (12.01)	95.74 (11.23)	96.185 (11.97)
T ₉	6634	6234	6434	9.75	9.23	9.49	32.65 (5.53)	37.04 (6.33)	34.845 (6.03)	66.34 (8.12)	62.34 (8.08)	64.34 (8.92)
T ₁₀	9674	9503	9588.5	16.83	17.30	17.06	2.18 (0.86)	3.74 (1.29)	2.96 (1.07)	96.74 (12.19)	95.03 (10.41)	95.885 (11.89)
T ₁₁	6233	6064	6148.5	8.23	8.19	8.21	36.77 (5.41)	38.84 (6.14)	37.805 (6.11)	62.33 (8.10)	60.64 (7.39)	61.485 (7.85)
T ₁₂	9586	9380	9483	16.87	16.79	16.83	3.13 (1.09)	5.19 (2.12)	4.16 (1.82)	95.86 (11.89)	93.8 (10.1)	94.83 (10.05)
CD (p≤0.05)	378.73	448.73	285.91	2.65	0.982	1.377	0.342	0.500	0.294	0.350	1.37	0.690

4.3.11 Moth Emergence Percentage:

The results of the present study show that Moth Emergence Percentage was significantly different in all the races under study, also the races differed statistically from their control batches. The highest moth emergence percentage among the inoculated batches was observed in T₇ (SH6) - 59.15% cocoons followed by T₉ (NB4D2) - 57.45%. The lowest moth emergence percentage was recorded by T₅ (SK28) - 52.94% and T₁₁ (CSR2) - 54.60%. Cocoon yield by weight observed in other treatment combinations was T₁ (SK1) -55.42%, T₂ (SK1 control) -92.52%, T₃ (SK6) - 56.25%, T₄ (SK6 control) -93.45%, T₆ (SK28) - 93.48%, T₈ (SH6 control) -95.31%, T₁₀ (NB4D2 control) - 94.64% and T₁₂ (CSR2) - 95.20%. respectively (Table 7).

4.3.12 Fecundity:

A significant decrease of fecundity in the inoculated batches as compared to control as well as among the races under study was observed from the results of the present study. Among all the inoculated races, lowest fecundity was observed in T₅ (SK28) -349 eggs followed by T₁₁ (CSR2) - 360 eggs (Table 7). The highest fecundity among the inoculated races was observed in T₇ (SH6) -391 eggs followed by T₉ (NB4D2) - 383 eggs. The fecundity recorded by other treatment combinations is T₁ (SK1) -370 eggs, T₂ (SK1 Control) - 517 eggs, T₃ (SK6) -378 eggs, T₄ (SK6 Control) -523 eggs, T₆ (SK28 control) 520 eggs, T₈ (SH6 control) - 515 eggs, T₁₀ (NB4D2 control) - 519 eggs and T₁₂ (CSR2 control) -518 eggs respectively.

Table 7: Effect of Microsporidian Infection on Pupation rate, Moth emergence, Fecundity of Silkworm, *Bombyx mori* L.

Treatment	Pupation rate			Moth emergence			Fecundity		
	Year1 (Spring)	Year2 (Spring)	Pooled Data	Year1 (Spring)	Year2 (Spring)	Pooled Data	Year1 (Spring)	Year2 (Spring)	Pooled Data
T ₁	55.74 (7.03)	59.85 (7.17)	57.87 (7.21)	52.45 (6.90)	58.38 (6.99)	55.20 (7.03)	372	368	370
T ₂	89.45 (8.97)	95.37 (9.18)	92.43 (9.22)	89.58 (9.14)	95.45 (9.22)	92.54 (9.25)	527	507	517
T ₃	55.16 (7.12)	61.46 (7.28)	58.60 (7.33)	53.76 (6.98)	58.74 (7.01)	56.26 (7.13)	385	371	378
T ₄	88.36 (8.99)	96.67 (9.22)	92.55 (9.25)	90.32 (9.10)	96.58 (9.17)	93.45 (9.35)	511	535	523
T ₅	51.56 (6.79)	55.58 (6.96)	53.61 (7.02)	50.73 (7.32)	55.14 (7.75)	52.91 (7.93)	357	341	349
T ₆	91.56 (9.13)	95.34 (9.27)	93.40 (9.34)	90.34 (9.16)	96.62 (9.37)	94.55 (9.48)	511	529	520
T ₇	57.17 (7.17)	62.56 (7.38)	59.84 (7.49)	55.18 (7.10)	63.11 (7.23)	59.18 (7.33)	385	397	391
T ₈	91.67 (9.23)	96.89 (9.36)	94.37 (9.42)	92.72 (9.34)	97.89 (9.49)	95.38 (9.60)	520	510	515
T ₉	55.78 (7.10)	62.85 (7.22)	59.30 (7.38)	52.34 (7.11)	62.56 (7.19)	57.59 (7.25)	378	388	383
T ₁₀	91.45 (9.39)	97.34 (9.64)	95.88 (9.76)	92.56 (9.19)	96.72 (9.35)	94.58 (9.48)	513	525	519
T ₁₁	52.25 (6.77)	58.12 (6.98)	55.17 (7.06)	51.78 (7.47)	57.42 (7.68)	54.59 (7.98)	348	372	360
T ₁₂	91.56 (9.13)	95.67 (9.29)	93.74 (9.39)	92.56 (9.33)	97.84 (9.49)	95.20 (9.54)	525	511	518
CD (p≤0.05)	0.265	0.249	0.177	0.136	0.259	0.142	18.40	12.32	10.78

4.3.13 Average Filament Length:

Significant data was recorded in case of Average Filament Length in all the races. The un-inoculated or control batches of all races showed longer Average Filament Length whereas the inoculated races recorded shorter Average Filament Length. Among the inoculated batches, the shortest Average Filament Length was observed in T₉ (NB4D2) - 512.64 m followed by T₁₁ (CSR2) - 520.09m, Table 8. The longest Average Filament Length of 728m was observed in T₃ (SK6) followed by T₁ (SK1) - 695.14 m. The Average Filament Length recorded by other treatment combinations include T₂ (SK1 Control) - 1028m, T₄ (SK6 Control) - 1063m, T₅ (SK28) -674.74m, T₆ (SK28 control) -1038.51m, T₇ (SH6) - 532.58m and T₈ (SH6 Control) - 745.96m, T₁₀ (NB4D2 Control) - 733.34m and T₁₂ (CSR2 Control) - 778.06m respectively.

The present study also revealed that the inoculation of different races under study caused the highest per cent reduction over control in T₅ (SK28) where the per cent reduction over control was 35.02% followed by T₁₁ (CSR2). The lowest per cent reduction over control was observed in T₇ (SH6) - 28.60% followed by T₉ (NB4D2) -30.09%. The per cent reduction over control observed in other races is T₁ (SK1) 32.44% and T₆ (SK6) -31.46 % (Table 8).

4.3.14 Raw silk percentage:

This parameter also differed significantly among all the treatment combinations. Among the inoculated batches, the highest Raw silk percentage was observed in two treatments T₇ (SH6) -28.86% and T₉ (NB4D2) -28.36g which were statistically at par with each other and were significantly different from their respective controls whose shell weight was 30.07% (T₈-SH6 Control) and 30.89% (T₁₀NB4D2 control) respectively. The lowest Raw silk percentage was recorded in T₅ (SK28) -25.68% followed by T₁₁ (CSR2) -27.11%. The Raw silk percentage recorded by other treatment combinations is-T₃ (SK6) -28.17 T₄ (SK6 control) - 31.98g, and T₁ (SK1) -27.66%g and T₂ (SK1 control) -31.85% (Table 8).

4.3.15 Denier:

Significant data was recorded in case of denier in all the races. The uninoculated or control batches of all races showed lesser denier, whereas the inoculated races recorded higher deniers. Among the inoculated batches, the least denier was observed in T₇ (SH6) - 2.27 followed by T₉ (NB4D2) - 2.50 whereas the denier observed in the control batches of these races was T₈ (SH6- Control) - 2.17 and T₁₀ (NB4D2 Control) - 2.40. The highest denier was observed in case T₃ (SK6) - 3.04 which was statistically at par with T₅ (SK28) - 3.01. The denier observed in control batches of these races was T₄ (SK6-Control) - 2.91 and T₆ (SK28- Control) - 2.78. The denier recorded by other treatment combinations include T₁ (SK1) - 2.91 and T₂ (SK1 control) - 2.80 (Table 8).

4.3.16 Site and intensity of microsporidian multiplication in the infected silkworms:

The results of the microscopic examination of different tissues of six races of silkworm inoculated with microsporidian infection are presented in (Table 9). The results showed that all the six silkworm races were susceptible to the microsporidian infection, however, the degree of susceptibility varied between the races. It was further observed that the grade of infection/ intensity increased on 5th day of 5th instar in comparison to 3rd day of fifth instar. Comparatively, SK28 and CSR2 were most susceptible to the microsporidian infection followed by SK1, SK6, NB4D2 and SH6. Among the tissues, the highest intensity of spores was observed in the gonads (ovaries-female) at the moth stage suggesting transovarial transmission in the infected silkworm. At the larval stage the highest intensity of spores was observed in the midgut of the infected larvae followed by silk glands and malpighian tubule.

Table 8: Effect of Microsporidian Infection on Post Cocoon Parameters of Silkworm, *Bombyx mori* L.

Treatment	Average filament length			% Reduction over control	Raw silk percentage			Denier		
	Year1 (Spring)	Year2 (Spring)	Pooled Data		Year1 (Spring)	Year2 (Spring)	Pooled Data	Year1 (Spring)	Year2 (Spring)	Pooled Data
T ₁	702.36	687.93	695.14	32.44	27.06 (5.02)	28.26 (5.18)	27.66 (5.26)	2.93	2.88	2.91
T ₂	1034.33	1023.56	1028.94		31.17 (5.37)	32.54 (5.51)	31.85 (5.60)	2.85	2.74	2.8
T ₃	720.4	737.26	728.83	31.46	27.67 (5.12)	28.68 (5.21)	28.17 (5.30)	3.12	2.95	3.04
T ₄	1081.33	1045.46	1063.39		32.36 (5.21)	31.6 (5.48)	31.98 (5.67)	2.92	2.89	2.91
T ₅	663.73	685.76	674.74	35.02	26.28 (4.79)	25.08 (4.98)	25.68 (5.09)	2.97	3.05	3.01
T ₆	1033.43	1043.6	1038.51		31.45 (5.27)	31.44 (5.41)	31.44 (5.59)	2.72	2.83	2.78
T ₇	508.73	556.43	532.58	28.60	29.1 (5.11)	28.63 (5.26)	28.86 (5.36)	2.31	2.23	2.27
T ₈	735.36	756.56	745.96		31.8 (5.22)	29.6 (5.47)	30.70 (5.53)	2.22	2.12	2.17
T ₉	519.73	505.56	512.64	30.09	27.87 (4.97)	28.85 (5.19)	28.36 (5.25)	2.47	2.53	2.5
T ₁₀	747.43	719.26	733.34		30.57 (5.29)	31.22 (5.46)	30.89 (5.55)	2.38	2.41	2.4
T ₁₁	518.83	521.36	520.09	33.15	27.51 (5.10)	26.71 (5.16)	27.11 (5.23)	2.62	2.54	2.58
T ₁₂	768.5	787.63	778.06		31.58 (5.16)	30.45 (5.38)	31.01 (5.57)	2.39	2.32	2.36
CD (p≤0.05)	21.81	31.42	18.62		2.97	3.00	3.05	2.08	0.478	0.522

Table 9: Intensity of the infection for different races of silkworm *Bombyx mori*

S.No.	Silkworm Race	Host Tissues observed for spore intensity	Intensity of the Infection (no. os spores observed per field)		
			Day 3 of 5 th instar	Day 5 th of 5 th instar	At moth stage
1.	SK1	Midgut	++	++	-
		Silkgland	++	++	-
		Malphigian tubule	++	++	-
		Gonads	-	-	+++
2.	SK6	Midgut	++	++	-
		Silkgland	++	++	-
		Malphigian tubule	++	++	-
		Gonads	-	-	+++
3.	SK28	Midgut	++	++	-
		Silkgland	++	++	-
		Malphigian tubule	++	++	-
		Gonads	-	-	+++
4.	SH6	Midgut	++	++	-
		Silkgland	++	++	-
		Malphigian tubule	++	++	-
		Gonads	-	-	+++
5.	NB4D2	Midgut	++	++	-
		Silkgland	++	++	-
		Malphigian tubule	++	++	-
		Gonads	-	-	+++
6.	CSR2	Midgut	++	++	-
		Silkgland	++	++	-
		Malphigian tubule	++	++	-
		Gonads	-	-	+++

Note:-

Nil (-) indicates no spore is present,

Low-level (+) indicates 1-5 spores/microscopic field

Moderate level (++) indicates 5-30 spores/microscopic field and

High level (+++) indicates 30 or more spores/microscopic field (Bhat and Nataraju, 2007b).

4.4 Biochemical studies

The data was collected for two consecutive years and later on pooled for better interpretation. The experimental findings of the biochemical parameters under study are described below:

4.4.1 Protein estimation in different tissues of silkworm, *Bombyx mori*:

a) Haemolymph:

The haemolymph was collected on two alternate days viz; 3rd day of 5th instar and 5th day of 5th instar. Significant data was recorded in case of protein concentration in haemolymph in all the races. However, in the inoculated batches the protein concentration was on the higher side as compared to the un-inoculated or control batches. The protein concentration in the midgut of inoculated larvae on the 3rd day of 5th instar was highest in T₉ (NB4D2) -34.44 mg/ml in comparison to its control whose protein concentration was T₁₀ (NB4D2- control) 39.64 mg/ml. The lowest protein concentration on the 3rd day of 5th instar was observed in T₁₁ (CSR2) -27.15mg/ml whose control batch T₁₂ (CSR2) - recorded a protein concentration of 33.98 mg/ml, Table 10. On the 5th day of 5th instar the highest protein concentration among the inoculated batches in haemolymph in comparison to their control / un-inoculated batches was observed in T₉ (NB4D2) - 38.37mg/ml followed by T₃ (SK6) - 37.35 mg/ml whose control batches had a protein concentration of T₉ (NB4D2-Control) - 44.34mg/ml & T₄ (SK6- Control) - 44.65 mg/ml respectively (Table 10). The lowest protein concentration on the 5th day of 5th instar was observed in T₁₁ (CSR2) - 31.45mg/ml followed by T₅ (SK28) - 33.38mg/ml. The protein concentration observed in the control batches of these two races is T₁₂ (CSR2- control) -38.74 mg/ml & T₆ (Sk28 Control) -44.12 mg/ml (Fig. 4.1).

The present study also revealed that the inoculation of different races under study by microsporidian infection caused the highest per cent reduction of protein in haemolymph over control in T₅ (SK28) where the per cent reduction

over control was 21.89% followed by T₁₁ (CSR2) -20.10%. The lowest per cent reduction over control was observed in T₉ (NB4D2) -13.11%. which was statistically at par with T₇ (SH6) -13.97%. The per cent reduction over control observed in other races is T₁ (SK1) - 17.50% and T₃ (SK6) -15.99% (Fig. 4.2).

b) Midgut:

Significant data was recorded in case of protein concentration in midgut in all the races. However, in the inoculated batches the protein concentration was on the higher side as compared to the un-inoculated or control batches on both 3rd and 5th day. The pooled protein concentration in the midgut of inoculated larvae was highest in (T₁-SK1) - 28.42 which was statistically at par with T₉ (NB4D2) - 28.17 mg/g and T₇ (SH6) -27-08 mg/g in comparison to their respective controls whose protein concentrations were: T₂ (SK1-control) -35.43mg/g, T₁₀ (NB4D2-Control) -34.60 mg/g and T₈ (SH6-Control) - 33.59 mg/g. The lowest pooled protein concentration was observed in T₁₁ (CSR2) -25.93mg/g whose control batch T₁₂ (CSR2-Control) recorded a protein concentration of 33.85mg/g which was statistically at par with T₅ (SK28) -26.68 mg/g, whose control T₆ (SK28-Control) observed a protein concentration of 35.47 mg/g (Table 10).

The present study also revealed that the inoculation of different races under study by microsporidian infection caused the highest per cent reduction of protein in midgut over control in T₅ (SK28) where the per cent reduction over control was 24.80% followed by T₁₁ (CSR2) -23.40%. The lowest per cent reduction over control among the races under study was observed in T₇ (SH6) - 19.38% followed by T₉ (NB4D2) -18.57%. The per cent reduction over control observed in other races is T₁ (SK1) - 19.80% and T₃ (SK6) -19.78 %.

c) Fat- body:

The results of the present study show that protein concentration in the fat body was significantly different in all the races under study, also the races differed statistically from their control batches. A significant decrease in the protein

concentration was observed in all the inoculated batches in comparison to their control / un-inoculated batches on both the days of observation viz; 3rd and 5th day of 5th instar. The pooled protein concentration in the fatbody of inoculated larvae was highest in T₉ (NB4D2) -44.19 mg/g followed by T₇ (SH6) - 42.37 mg/g in comparison to their respective controls whose protein concentration was T₁₀ (NB4D2- Control) -53.43mg/g and T₈ (SH6- Control) -51.50mg/g. The lowest pooled protein concentration was observed in T₁₁ (CSR2) -37.95mg/g followed by T₅ (SK28) - 39.30 mg/g whose control batches recorded protein concentration of T₁₂ (CSR2- Control) -46.02mg/g and T₆ (SK28- Control) 51.60mg/g (Table 10).

Among the six different races under study microsporidian infection caused the highest per cent reduction over control of protein in fatbody in T₅ (SK28) where the per cent reduction over control was 23.83% followed by T₁₁ (CSR2) - 21.33%. The lowest per cent reduction over control among the races under study was observed in T₉ (NB4D2) -17.29% followed by T₇ (SH6) -17.73%. The per cent reduction over control observed in other races is T₁ (SK1) - 20.39% and T₃ (SK6) -20.22%.

d) Silkgland:

Statistical analysis revealed that protein concentration in the silkgland of inoculated silkworm races was lower than their respective controls on both the recorded days. The pooled protein concentration was statistically the lowest in T₁₁ (CSR2) - 53.44mg/g among all other inoculated treatment combinations and was statistically at par with T₅ (SK28) - 55.02 mg/g. The control batches of these races recorded a protein concentration of 66.09 mg/g and 69.50 mg/g respectively. The pooled protein concentration was statistically the highest in T₃ (SK6) - 57.73mg/g and was statistically at par with T₇ (SH6) - 57.53 mg/g and T₉ (NB4D2) - 57.32 mg/g among all other inoculated treatment combinations. The control batches of these races recorded a protein concentration of 70.23 mg/ml, 68.78 mg/g and 68.69 mg/g respectively (Table 11). The pooled protein concentration in other races under study are: T₁ (SK1) -55.25 mg/g, T₂ (SK2-Control) - 67.87 mg/g.

Among the six different races under study microsporidian infection caused the highest per cent reduction over control of protein in silk glands of silkworm in T₅ (SK28) where the per cent reduction over control was 20.83% followed by T₁₁ (CSR2) -19.13%. The lowest per cent reduction over control among the races under study was observed in T₇ (SH6) -16.36% followed by T₉ (NB4D2) -16.55%. The per cent reduction over control observed in other races is T₁ (SK1) -18.59% and T₃ (SK6) -17.79 %.

e) Gonads:

Among the six different races under study microsporidian infection caused the highest reduction of proteins in gonads of silkworm in T₁₁ (CSR2) -13.93 mg/g which was statically at par with T₅ (SK28) -14.71mg/g. The lowest reduction in protein concentration among the inoculated races was observed in T₃ (SK6) - 15.30mg/g which was statistically at par with T₁ (SK1) - 20.16mg/ml 16.68 mg/g. The protein concentration observed in other races was T₉ (NB4D2) -16.90 mg/g and T₇ (SH6) -16.90 mg/g. The highest per cent reduction over control of proteins in gonads of inoculated silkworms was observed in T₅ (SK28) where the per cent reduction over control was 29.47% followed by T₁₁ (CSR2) -27.66%. The lowest per cent reduction over control among the races under study was observed in T₇ (SH6) -21.65% followed by T₉ (NB4D2) -14.39%. The per cent reduction over control observed in other races was T₁ (SK1) - 25.71% and T₃ (SK6) -25.57% (Table 11).

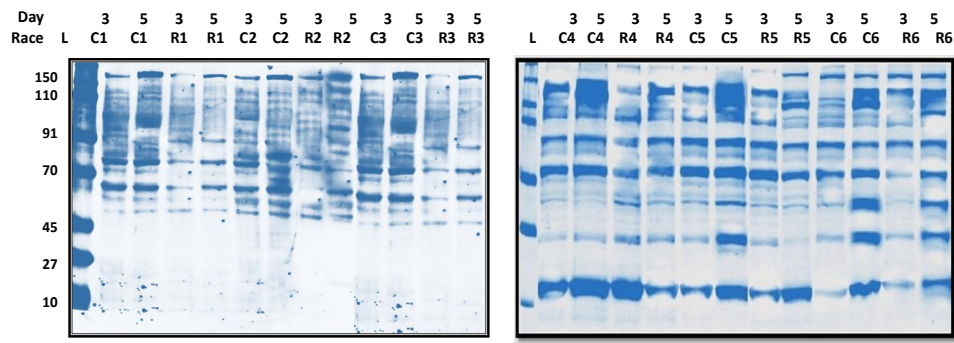
SDS PAGE analysis: The qualitative analysis of proteins (SDS-PAGE) indicated changes in the expression of proteins as follows:

- The intensity of the protein bands decreased in the inoculated silkworm tissues in the range of 27-45kDa as compared to their controls, respectively.(Fig 4.1a)

- Some of the proteins bands are absent in the inoculated silkworm races in the range of 70-91kDa (Fig4.1c) in comparison to their respective controls.
- Some of the new protein band are seen only in few inoculated silkworm tissues which may be due to the synthesis of some biotic stress related proteins (due to *N.bombycis* infection in inoculated silkworms) in the range of 45-70 kDa to meet the physiological need of inoculated silkworms. It may help us to identify some marker proteins for biotic stress in silkworms.(Fig 4.1b)
- Less intensity and absence of proteins bands indicates either, the non-production or utilization or degradation of proteins

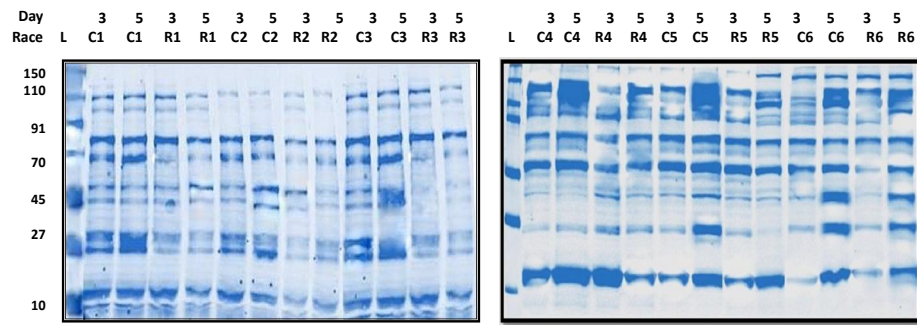
The pronounced effect was seen in race SK28 followed by CSR2.

(A) Haemolymph



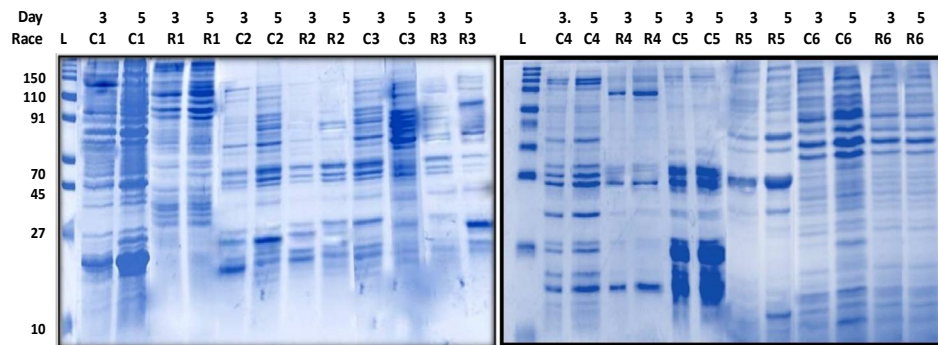
12¹ PAGE showing proteins isolated from haemolymph of different races on day 3 and day 5 on inoculation with respective controls. The races stand were named as R1- SK1, R2-SK6, R3-SK28, R4-SH6, R5-NB4D2, R6-CSR2.

(B) Midgut



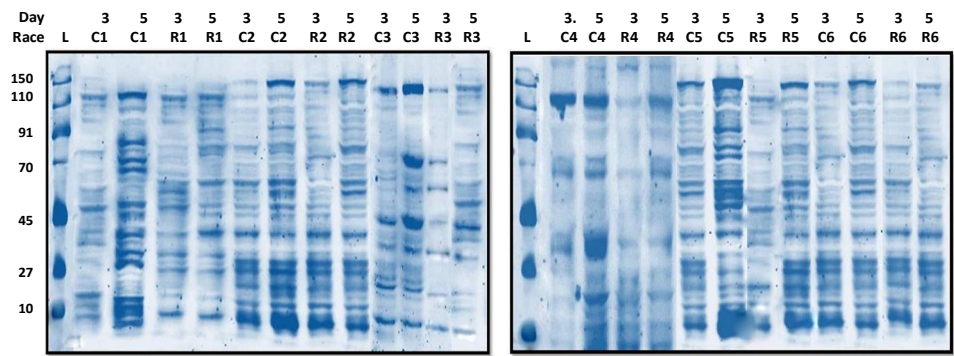
12² PAGE showing proteins isolated from mid-gut of different races on day 3 and day 5 on inoculation with respective controls. The races stand were named as R1- SK1, R2-SK6, R3-SK28, R4-SH6, R5-NB4D2, R6-CSR2.

(C) Fat Body



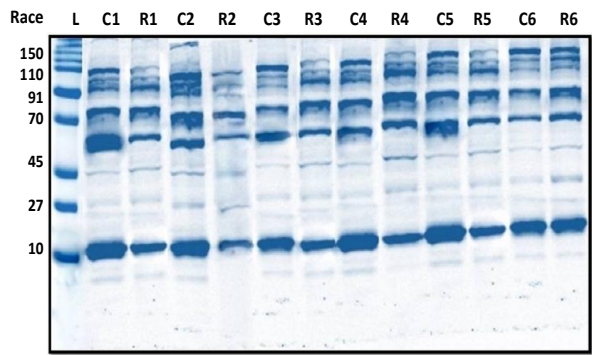
12³ PAGE showing proteins isolated from fat body of different races on day 3 and day 5 on inoculation with respective controls. The races stand were named as R1- SK1, R2-SK6, R3-SK28, R4-SH6, R5-NB4D2, R6-CSR2.

(D) Silk Gland



12 PAGE showing proteins isolated from silk glands of different races on day 3 and day 5 on inoculation with respective controls. The races stand were named as R1- SK1, R2-SK6, R3-SK28, R4-SH6, R5-NB4D2, R6-CSR2.

(E) Gonads



12 PAGE showing proteins isolated from gonads of different races on day 3 and day 5 on inoculation with respective controls. The races stand were named as R1- SK1, R2-SK6, R3-SK28, R4-SH6, R5-NB4D2, R6-CSR2.

Fig. 4.1: SDS page analysis of different tissues of silkworms infected by microsporodian infection

Table 10: Effect of microsporidian infection on Protein concentration in Haemolymph (mg/ml), midgut (mg/g) and Fatbody (mg/g) of silkworm *Bombyx mori* L.

Treatment	Haemolymph				Midgut				Fat body			
	Day 3	Day 5	Pooled data	% reducti on over control	Day 3	Day 5	Pooled data	% reducti on over control	Day 3	Day 5	Pooled data	% reducti on over control
T ₁	28.11	35.55	31.83	17.50	26.27	30.56	28.42	19.80	38.33	45.73	42.03	20.39
T ₂	33.82	43.34	38.58		32.02	38.84	35.43		47.43	58.16	52.80	
T ₃	29.71	37.35	33.53	15.99	25.91	29.52	27.72	19.79	36.63	46.96	41.80	20.22
T ₄	35.17	44.65	39.91		31.24	37.87	34.56		45.5	59.28	52.39	
T ₅	28.12	33.38	30.75	21.89	24.83	28.52	26.68	24.80	36.18	42.42	39.30	23.83
T ₆	34.62	44.12	39.37		32.18	38.76	35.47		46.07	57.12	51.60	
T ₇	29.87	37.07	33.47	13.97	24.89	29.26	27.08	19.38	39.69	45.04	42.37	17.73
T ₈	34.19	43.62	38.91		30.14	37.03	33.59		47.14	55.85	51.50	
T ₉	30.51	38.37	34.44	13.11	25.76	30.58	28.17	18.57	39.77	48.61	44.19	17.29
T ₁₀	34.93	44.34	39.64		31.41	37.78	34.60		48.11	58.74	53.43	
T ₁₁	22.84	31.45	27.15	20.10	24.18	27.68	25.93	23.40	33.46	42.43	37.95	21.33
T ₁₂	29.21	38.74	33.98		30.53	37.17	33.85		42.02	54.45	48.24	
CD (p≤0.05)	Days (D)	: (0.564)			Days (D)	: (0.637)			Days (D)	: 0.815		
	Races (R)	: (1.383)			Races (R)	: (2.340)			Races (R)	: 2.00		
	DxR	: NS			DxR	: NS			DXR	: NS		

Table 11: Effect of microsporidian infection on Protein concentration in Silk glands (mg/g) and Gonad (Ovaries) (mg/g) of silkworm, *Bombyx mori*

Treatment	Silk gland				Gonads			
	Day 3	Day 5	Pooled data	% reduction over control	Year 1	Year 2	Pooled data	% reduction over control
T ₁	49.68	60.82	55.25	18.59	16.23	17.12	16.68	25.71
T ₂	60.16	75.57	67.87		21.13	23.76	22.45	
T ₃	50.41	65.05	57.73	17.79	15.75	14.84	15.30	25.57
T ₄	62.63	77.82	70.23		19.94	21.16	20.55	
T ₅	49.74	60.30	55.02	20.83	14.17	15.24	14.71	29.47
T ₆	61.82	77.17	69.50		21.24	20.46	20.85	
T ₇	51.70	63.35	57.53	16.36	15.62	18.18	16.90	21.65
T ₈	60.92	76.63	68.78		19.81	23.33	21.57	
T ₉	51.02	63.61	57.32	16.55	16.65	17.14	16.90	22.45
T ₁₀	61.03	76.34	68.69		20.71	22.86	21.79	
T ₁₁	46.62	60.26	53.44	19.13	12.17	15.69	13.93	27.66
T ₁₂	58.30	73.87	66.09		18.47	20.04	19.26	
CD (p≤0.05)	Days (D) : (0.663) Races (R) : (1.417) DxR : NS				1.90	1.91	1.31	

SDS page results of various tissues of silkworm:

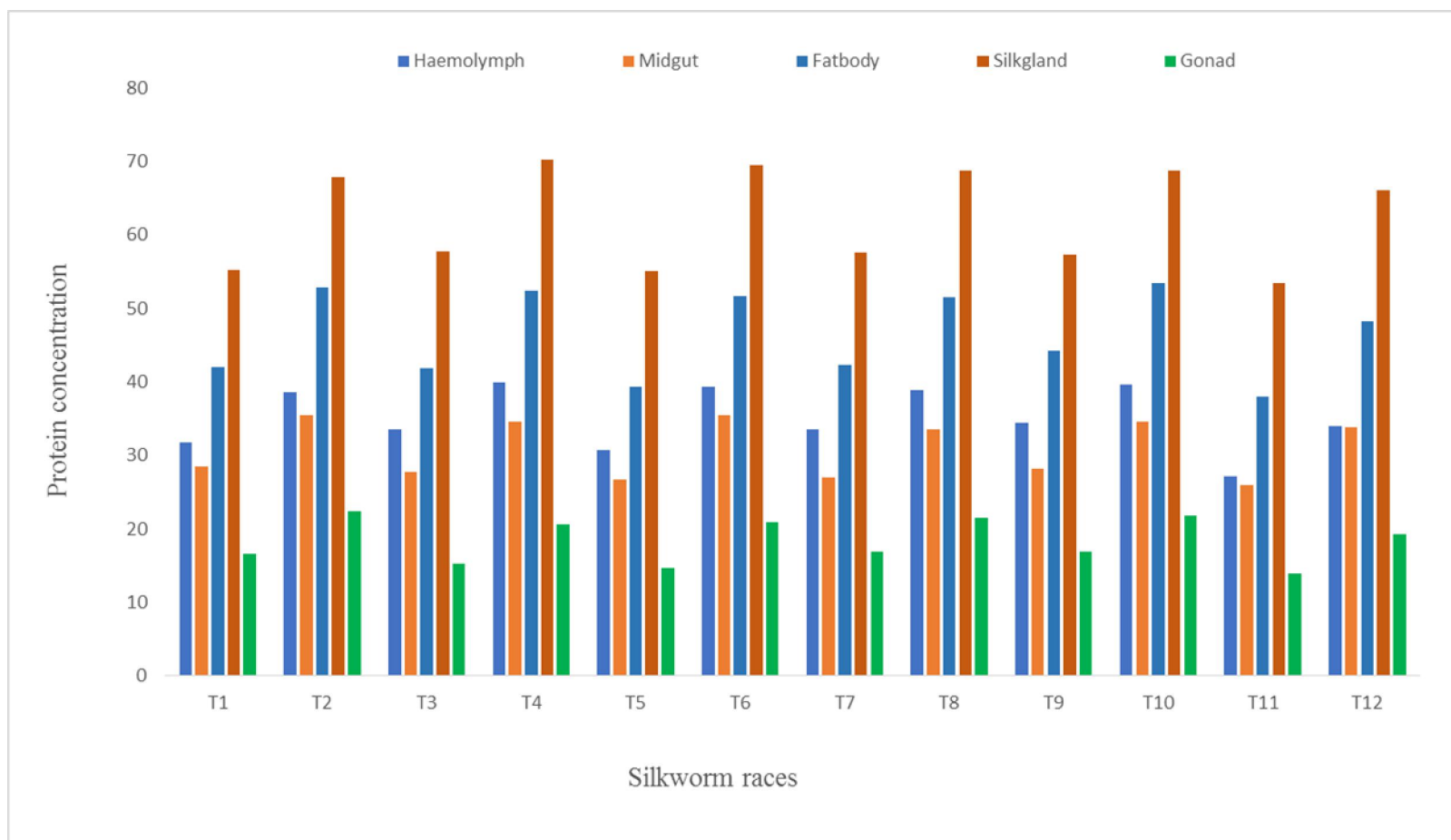


Fig. 4.2: Effect of microsporidian infection on protein concentration in different tissues of six races of silkworm

4.4.2 Carbohydrate estimation in different tissues of silkworm *Bombyx mori*:

a) Haemolymph:

The results of the experiment showed that the inoculation of different races of silkworm *Bombyx mori* with same spore load of *microsporidia* caused varied response in terms of carbohydrate concentration (mg/ml) in the haemolymph of all the six races of silkworm under study. In general, the un-inoculated or control batches of all races showed more carbohydrate concentration (mg/ml) in the haemolymph whereas the inoculated races showed lesser protein concentrations. Significant data was recorded in case of carbohydrate concentration in haemolymph of all the races. Among the inoculated batches highest pooled carbohydrate concentration in haemolymph was observed in T₁ (SK1) - 23.55 mg/ml which was statistically at par with T₇ (SH6) - 23.35 mg/ml. The control batches of these races had a protein concentration of 28.95 mg/ml & 27.92mg/ml respectively. The lowest pooled protein concentration was observed in T₅ (SK28) - 21.58mg/ml which was at par with T₁₁ (CSR2) - 20.45mg/ml. The protein concentration observed in the control batches of these two races is T₆ (Sk28 Control) - 27.08mg/ml and T₁₂ (CSR2- control) - 25.39 mg/ml (Table 12).

The present study also revealed that the inoculation of different races under study by microsporidian infection caused the highest per cent reduction of carbohydrate in haemolymph over control in T₅ (SK28) where the per cent reduction over control was 20.33% followed by T₁₁ (CSR2) - 19.44%. The lowest per cent reduction over control was observed in T₇ (SH6) - 16.37% followed by T₉ (NB4D2) - 17.60%. The per cent reduction over control observed in other races is T₁ (SK1) - 18.67% and T₆ (SK6) - 17.00% (Fig. 4.3).

b) Midgut:

Significant data was recorded in case of carbohydrate concentration in midgut in all the races. However, in the inoculated batches the carbohydrate

concentration was on the lower side as compared to the un-inoculated or control batches on both the observation days. The pooled carbohydrate concentration in the midgut of inoculated larvae was highest in T₇ (SH6) - 19.70 mg/g which was at par with T₉ (NB4D2) - 19.63mg/g in comparison to their respective controls whose carbohydrate concentration was 23.44 mg/g and 23.47 mg/g respectively. The lowest pooled carbohydrate concentration on was observed in T₁₁ (CSR2) - 15.93mg/g whose control batch recorded a carbohydrate concentration of 19.94mg/g. the carbohydrate concentration in the midgut of other races was: T₁- (SK1) - 17.30 mg/g, T₂- (SK1-Control) -21.30 mg/g, T₃- (SK6) - 18.35 mg/g, T₄ (SK6-Control) -22.74 mg/g, T₅ (SK28) 18.08 mg/g and T₆ (SK28-Control) 23.23mg/g (Table 12).

The present study revealed that the inoculation of different races under study by microsporidian infection caused the highest per cent reduction of carbohydrate in midgut over control in T₅ (SK28) where the per cent reduction over control was 22.19% followed by T₁₁ (CSR2) - 20.12%. The lowest per cent reduction over control among the races under study was observed in T₇ (SH6) - 15.98% followed by T₉ (NB4D2) - 16.34%. The per cent reduction over control observed in other races is T₁ (SK1) - 18.78% and T₃ (SK6) -19.32%.

c) Fat- body:

The results of the present study show that carbohydrate concentration in the fat body was significantly different in all the races under study, also the races differed statistically from their control batches. A significant decrease in the carbohydrate concentration was observed in all the inoculated batches in comparison to their control / un-inoculated batches on both the days of observation viz; 3rd and 5th day of 5th instar. The pooled carbohydrate concentration in the fatbody of inoculated larvae was highest in T₃ (SK6) - 18.62mg/g followed by T₁ (SK1) - 17.88 mg/g in comparison to their respective controls whose carbohydrate concentration was 22.82mg/g and 22.22 mg/g, Table 12. The lowest pooled carbohydrate concentration was observed in T₁₁ (CSR2) -

14.08mg/g whose control batch recorded a carbohydrate concentration of 17.68mg/g.

Among the six different races under study, microsporidian infection caused the highest per cent reduction over control of carbohydrate in fatbody in T₅ (SK28) where the per cent reduction over control was 21.75% followed by T₁₁ (CSR2) - 20.37%. The lowest per cent reduction over control among the races under study was observed in T₇ (SH6) - 16.89% followed by T₉ (NB4D2) - 17.27%. The per cent reduction over control observed in other races is T₁ (SK1) - 19.51% and T₃ (SK6) - 18.43 %.

d) Silk gland:

Statistical analysis revealed that protein concentration in the silk gland of inoculated silkworm races was lower than their respective controls on both the observation days. The pooled protein concentration was statistically the lowest in T₁₁ (CSR2) - 25.63mg/g among all other inoculated treatment combinations followed by T₅ (SK28) - 26.47 mg/g. The control batches of these races recorded a protein concentration of T₁₂ (CSR2- Control) - 31.43mg/g and T₆ (SK28- Control) - 32.94mg/g respectively. The pooled protein concentration was statistically the highest in T₃ (SK6) - 28.96mg/g followed by T₉ (NB4D2) - 28.44 mg/g, T₁ (SK1) - 28.49 mg/g and T₇ (SH6) - 27.46mg/g. The control batches of these races recorded a protein concentration of 33.95mg/ml, 34.80mg/g and 32.75mg/g respectively (Table 13).

Among the six different races under study, the microsporidian infection caused the highest per cent reduction over control of protein in silk glands of silkworm in T₅ (SK28) where the per cent reduction over control was 19.64% followed by T₁₁ (CSR2) - 18.45%. The lowest per cent reduction over control among the races under study was observed in T₇ (SH6) - 16.07% followed by T₉ (NB4D2) - 16.22% The per cent reduction over control observed in other races is T₁ (SK1) - 18.13% and T₃ (SK6) - 17.48 %.

e) Gonads:

Among the six different races under study microsporidian infection caused the highest reduction of carbohydrates in gonads of silkworm in T₁₁ (CSR2) - 11.33 mg/g which was statically at par with T₅ (SK28) -11.81 mg/g. The lowest reduction in carbohydrate concentration among the inoculated races was observed in T₇ (SH6) -16.85 mg/g which was statistically at par with T₉ (NB4D2) - 15.96mg/g. The carbohydrate concentration observed in other inoculated races was T₁ (SK1) - 13.44mg/g and T₃ (SK6) -14.96 mg/g (Table 13).

The highest per cent reduction over control of lipids in gonads of silkworm was observed in T₅ (SK28) where the per cent reduction over control was 24.73% followed by T₁₁ (CSR2) -22.56%. The lowest per cent reduction over control among the races under study was observed in T₇ (SH6) -15.96% followed by T₉ (NB4D2) -17.17%. The per cent reduction over control observed in other races was T₁ (SK1) - 19.08% and T₃ (SK6) -18.17%.

Table 12: Effect of microsporidian infection on carbohydrate concentration in Haemolymph (mg/ml), midgut (mg/g) and Fatbody (mg/g) of silkworm *Bombyx mori*

Treatment	Haemolymph				Midgut				Fat body			
	Day 3	Day 5	Pooled data	% reducti on over control	Day 3	Day 5	Pooled data	% reducti on over control	Day 3	Day 5	Pooled data	% reducti on over control
T ₁	20.43	26.66	23.55	18.67	15.84	18.76	17.30	18.78	16.12	19.64	17.88	19.51
T ₂	26.19	31.71	28.95		18.89	23.71	21.30		19.26	25.17	22.22	
T ₃	19.33	25.89	22.61	17.00	16.313	20.38	18.35	19.32	17.12	20.11	18.62	18.43
T ₄	24.07	30.41	27.24		20.07	25.41	22.74		20.46	25.18	22.82	
T ₅	19.63	23.52	21.58	20.33	17.03	19.12	18.08	22.19	15.15	17.04	16.10	21.75
T ₆	24.31	29.85	27.08		21.21	25.25	23.23		18.11	23.03	20.57	
T ₇	19.15	27.54	23.35	16.37	18.15	21.24	19.70	15.98	14.55	18.67	16.61	16.89
T ₈	25.77	30.06	27.92		21.77	25.11	23.44		17.71	22.26	19.99	
T ₉	19.05	24.81	21.93	17.60	18.05	21.21	19.63	16.34	15.12	19.18	17.15	17.27
T ₁₀	23.47	29.76	26.62		20.17	26.76	23.47		18.43	23.03	20.73	
T ₁₁	17.74	23.16	20.45	19.44	14.19	17.66	15.93	20.12	12.42	15.73	14.08	20.37
T ₁₂	22.73	28.04	25.39		17.73	22.14	19.94		15.82	19.53	17.68	
CD (p≤0.05)	Days (D) : (0.691) Races (R) : (1.693) DxR : NS				Days (D) : (0.374) Races (R) : (0.917) DxR : NS				Days (D) : (0.258) Races (R) : (0.633) DxR : NS			

Table 13: Effect of microsporidian infection on carbohydrate concentration in Silk glands (mg/g) and Gonad (Ovaries) (mg/g) of silkworm, *Bombyx mori*

Treatment	Silk gland				Gonads			
	Day 3	Day 5	Pooled data	% reduction over control	Day 3	Day 5	Pooled data	% reduction over control
T ₁	26.85	30.13	28.49	18.13	13.72	13.16	13.44	19.08
T ₂	31.95	37.65	34.80		15.74	17.48	16.61	
T ₃	28.76	29.17	28.96	17.48	12.46	17.45	14.96	18.17
T ₄	32.08	38.12	35.10		17.13	19.42	18.28	
T ₅	24.12	28.83	26.47	19.64	12.75	10.87	11.81	24.73
T ₆	30.11	35.78	32.94		16.66	14.72	15.69	
T ₇	25.76	29.17	27.46	16.07	13.78	14.53	14.16	15.97
T ₈	30.02	35.43	32.72		15.21	18.48	16.85	
T ₉	25.15	31.74	28.44	16.22	12.18	14.26	13.22	17.17
T ₁₀	31.03	36.87	33.95		14.81	17.11	15.96	
T ₁₁	23.15	28.11	25.63	18.45	9.28	13.37	11.33	22.56
T ₁₂	29.14	33.72	31.43		12.54	16.71	14.63	
CD (p≤0.05)	Days (D) : (0.338) Races (R) : (0.828) DxR : NS				1.38	1.47	1.01	

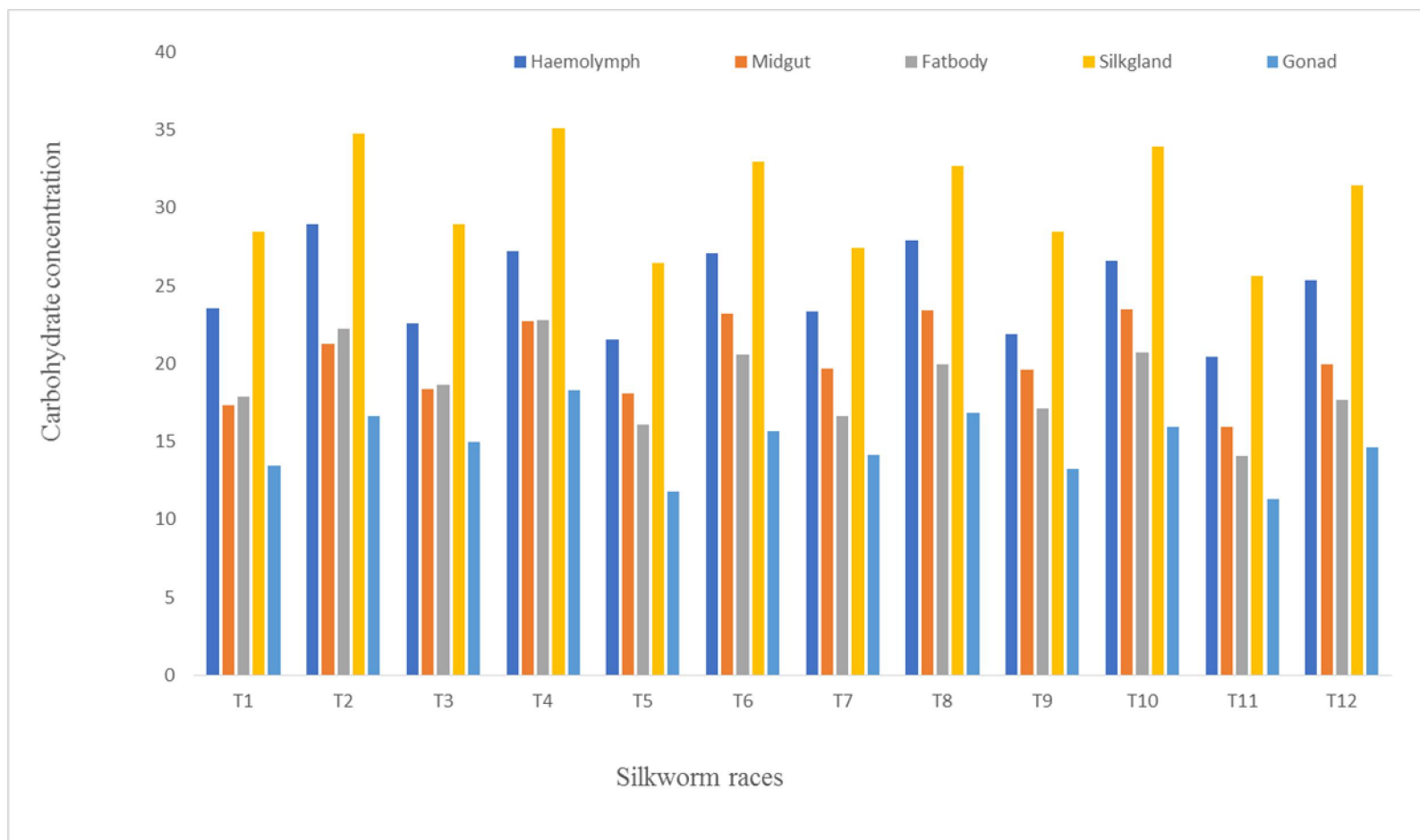


Fig. 4.3: Effect of microsporidian infection on carbohydrate concentration in different tissues of six races of silkworm

4.4.3 Lipid estimation in different tissues of silkworm *Bombyx mori*:

a) Haemolymph:

Significant data was recorded in case of lipid concentration in haemolymph in all the races. However, in the inoculated batches the lipid concentration was on the lower side as compared to the un-inoculated or control batches. The lipid concentration in the haemolymph of inoculated batches was lower on both 3rd day as well as 5th day of 5th instar in comparison to their healthy controls. The pooled lipid concentration among the inoculated races was highest in T₇ (SH6) - 27.99mg/ml in comparison to its control whose lipid concentration was 33.74mg/ml. The lowest pooled lipid concentration was observed in T₁₁ (CSR2) - 23.40mg/ml whose control batch recorded a lipid concentration of 27.61mg/ml. The lipid concentration observed in the other treatment combinations was T₉ (NB4D2) - 25.82mg/ml, T₁₀ (NB4D2 Control) - 34.80mg/ml, T₁ (SK1) - 25.86mg/ml, T₃ (SK6) - 24.55mg/ml and T₂ (SK1 Control) - 32.03mg/ml.

The present study revealed that the inoculation of different races under study by microsporidian infection caused the highest per cent reduction over control of lipid in haemolymph in T₅ (SK28) where the per cent reduction over control was 21.24% followed by T₁₁ (CSR2) - 20.39%, Table 14. The lowest per cent reduction over control among the races under study was observed in T₇ (SH6) - 17.06% followed by T₉ (NB4D2) - 18.80%. The per cent reduction over control observed in other races is T₁ (SK1) - 19.27% and T₃ (SK6) -24.55% (Fig. 4.4).

b) Midgut:

Significant data was recorded in case of lipid concentration in midgut in all the races. However, in the inoculated batches the lipid concentration was on the lower side as compared to the un-inoculated or control batches. The pooled lipid concentration in the midgut of inoculated larvae was highest in T₉ (NB4D2) - 31.02mg/g in comparison to its control whose lipid concentration was 36.95mg/g.

The lowest lipid concentration on the 3rd day of 5th instar was observed in T₁₁ (CSR2) - 24.08 mg/g whose control batch recorded a lipid concentration of 31.30mg/g which was significantly at par with T₇ (SH6) - 24.54mg/g. The lipid concentration observed in the control batches of these two races is T₁₂ (CSR2-control) - 31.30mg/mg & T₆ (Sk28 Control) - 32.49mg/g. The lipid concentration observed in the other treatment combination is T₃ (SK6) - 26.51mg/g, T₄ (SK6 Control) - 33.55mg/g, T₁ (SK1) - 27.95mg/g and T₂ (SK1 Control) - 35.44mg/g (Table 14).

The present study revealed that the inoculation of different races under study by microsporidian infection caused the highest per cent reduction of lipid in midgut over control in T₅ (SK28) where the per cent reduction over control was 24.28% followed by T₁₁ (CSR2) - 23.05%. The lowest per cent reduction over control among the races under study was observed in T₉ (NB4D2) - 16.06% followed by T₇ (SH6) - 17.68%. The per cent reduction over control observed in other races is T₁ (SK1) - 21.29% and T₃ (SK6) -20.97%.

c) **Fatbody:**

The results of the present study show that lipid concentration in the fat body was significantly different in all the races under study, also the races differed statistically from their control batches. A significant decrease in the lipid concentration was observed in all the inoculated batches in comparison to their control / un-inoculated batches on both the days of observation viz; 3rd and 5th day of 5th instar (Table 14). The pooled lipid concentration in the fatbody of inoculated larvae was highest in T₇ (SH6) - 37.01 mg/g in comparison to its control whose lipid concentration was 45.49mg/g. The lowest lipid concentration was observed in T₁₁ (CSR2) - 32.25 mg/g whose control batch recorded a lipid concentration of 41.69mg/g.

Among the six different races under study microsporidian infection caused the highest per cent reduction over control of lipid in fatbody in T₅ (SK28) where

the per cent reduction over control was 23.14% followed by T₁₁ (CSR2) - 22.63%. The lowest per cent reduction over control among the races under study was observed in T₉ (NB4D2) - 18.48% followed by T₇ (SH6) - 18.65%. The per cent reduction over control observed in other races is T₁ (SK1) - 20.44% and T₃ (SK6) -19.28 %.

d) **Silk glands:**

Statistical analysis revealed that lipid concentration in the silk gland of inoculated silkworm races was lower than their respective controls on both the observation days. The pooled lipid concentration was statistically the lowest in T₁₁ (CSR2) - 29.91mg/g among all other inoculated treatment combinations and was statistically at par with T₅ (SK28) - 30.45 mg/g. The control batches of these races recorded a protein concentration of 37.11 mg/g and 38.12mg/g respectively. The pooled protein concentration was statistically the highest in T₇ (SH6) - 34.15mg/g followed by T₉ (NB4D2) - 33.35 mg/g among all other inoculated treatment combinations. The control batches of these races recorded a lipid concentration of 40.90 mg/ml and 39.80 mg/g respectively. The pooled lipid concentration in other races under study are: T₁ (SK1) -30.87mg/g, T₂ (SK2-Control) - 37.94 mg/g., T₃ (SK6) - 33.85 mg/g and T₄ (SK6 Control) - 41.22 mg/g, Table 15.

Among the six different races under study, microsporidian infection caused the highest per cent reduction over control of lipids in silk glands of silkworm in T₅ (SK28) where the per cent reduction over control was 20.12% followed by T₁₁ (CSR2) -19.43%. The lowest per cent reduction over control among the races under study was observed in T₉ (NB4D2) -16.20% followed by T₇ (SH6) -16.52%. The per cent reduction over control observed in other races is T₁ (SK1) - 18.62% and T₃ (SK6) -17.87 %.

e) **Gonads:**

Among the six different races under study, microsporidian infection caused the highest reduction of lipids in gonads of silkworm in T₅ (SK28) - 18.53 mg/g

followed by T₁₁ (CSR2) -24.13 mg/g. The lowest reduction in lipid concentration among the inoculated races was observed in followed by T₉ (NB4D2) -21.56 mg/g which was statistically at par with T₃ (SK6) -20.42 mg/g and T₇ (SH6) -20.29 mg/g. The lipid concentration observed in other races was T₁ (SK1) - 20.16mg/g (Table 15).

The highest per cent reduction over control of lipids in gonads of silkworm was observed in T₅ (SK28) where the per cent reduction over control was 26.34% followed by T₁₁ (CSR2) -24.25%. The lowest per cent reduction over control among the races under study was observed in T₇ (SH6) -20.42% followed by T₉ (NB4D2) -121.57% followed by The per cent reduction over control observed in other races was T₁ (SK1) - 22.76% and T₃ (SK6) -23.73%.

Table 14: Effect of microsporidian infection on Lipid concentration in Haemolymph (mg/ml), midgut (mg/g) and Fatbody (mg/g) of silkworm *Bombyx mori*

Treatment	Haemolymph				Midgut				Fat body			
	Day 3	Day 5	Pooled data	% reducti on over control	Day 3	Day 5	Pooled data	% reducti on over control	Day 3	Day 5	Pooled data	% reducti on over control
T ₁	24.17	27.54	25.86	19.27	26.61	29.28	27.95	21.14	32.35	36.81	34.58	20.44
T ₂	29.82	34.23	32.03		32.12	38.75	35.44		40.72	46.21	43.47	
T ₃	23.32	25.78	24.55	19.43	25.23	27.79	26.51	20.97	33.24	37.67	35.46	19.28
T ₄	28.13	32.81	30.47		30.27	36.82	33.55		41.31	46.54	43.93	
T ₅	24.71	27.82	26.27	21.24	22.26	26.81	24.54	24.48	32.64	35.13	33.89	23.14
T ₆	30.03	36.67	33.35		29.31	35.67	32.49		41.32	46.85	44.09	
T ₇	26.14	29.83	27.99	17.06	25.07	29.84	27.46	17.68	35.22	38.79	37.01	18.65
T ₈	31.44	36.04	33.74		30.47	36.23	33.35		42.75	48.23	45.49	
T ₉	23.81	27.82	25.82	18.81	29.38	32.65	31.02	16.06	34.75	37.96	36.36	18.48
T ₁₀	29.46	34.13	31.80		34.38	39.52	36.95		41.82	47.37	44.60	
T ₁₁	21.68	25.12	23.40	20.39	21.69	26.47	24.08	23.05	30.39	34.11	32.25	22.63
T ₁₂	26.61	32.18	29.40		28.75	33.84	31.30		38.66	44.71	41.69	
CD (p≤0.05)	Days (D)	:	(0.363)		Days (D)	:	(0.316)		Days (D)	:	(0.218)	
	Races (R)	:	(0.891)		Races (R)	:	(0.776)		Races (R)	:	(0.534)	
	DxR	:	NS		DxR	:	NS		DxR	:	NS	

Table 15: Effect of microsporidian infection on Lipid concentration in Silk glands (mg/g) and Gonad (Ovaries) (mg/g) of silkworm, *Bombyx mori*

Treatment	Silk gland				Gonads			
	Day 3	Day 5	Pooled data	% reduction over control	Day 3	Day 5	Pooled data	% reduction over control
T ₁	27.19	34.55	30.87	18.62	19.71	20.6	20.16	22.76
T ₂	34.73	41.14	37.94		24.17	25.38	24.78	
T ₃	31.59	36.11	33.85	17.87	18.54	22.3	20.42	23.73
T ₄	38.11	44.32	41.22		23.14	26.16	24.65	
T ₅	27.61	33.28	30.45	20.12	19.45	17.6	18.53	26.34
T ₆	35.04	41.19	38.12		25.23	23.1	24.17	
T ₇	32.17	36.12	34.15	16.52	19.18	21.4	20.29	20.42
T ₈	37.77	44.03	40.90		24.25	24.54	24.4	
T ₉	31.44	35.26	33.35	16.20	20.52	22.6	21.56	21.57
T ₁₀	36.77	42.82	39.80		24.06	26.31	25.19	
T ₁₁	26.96	32.85	29.91	19.40	17.68	19.87	18.78	24.25
T ₁₂	33.82	40.39	37.11		23.41	24.84	24.13	
CD (p≤0.05)	Days (D) : (0.283) Races (R) : (0.745) DxR : NS				1.87	1.78	1.26	

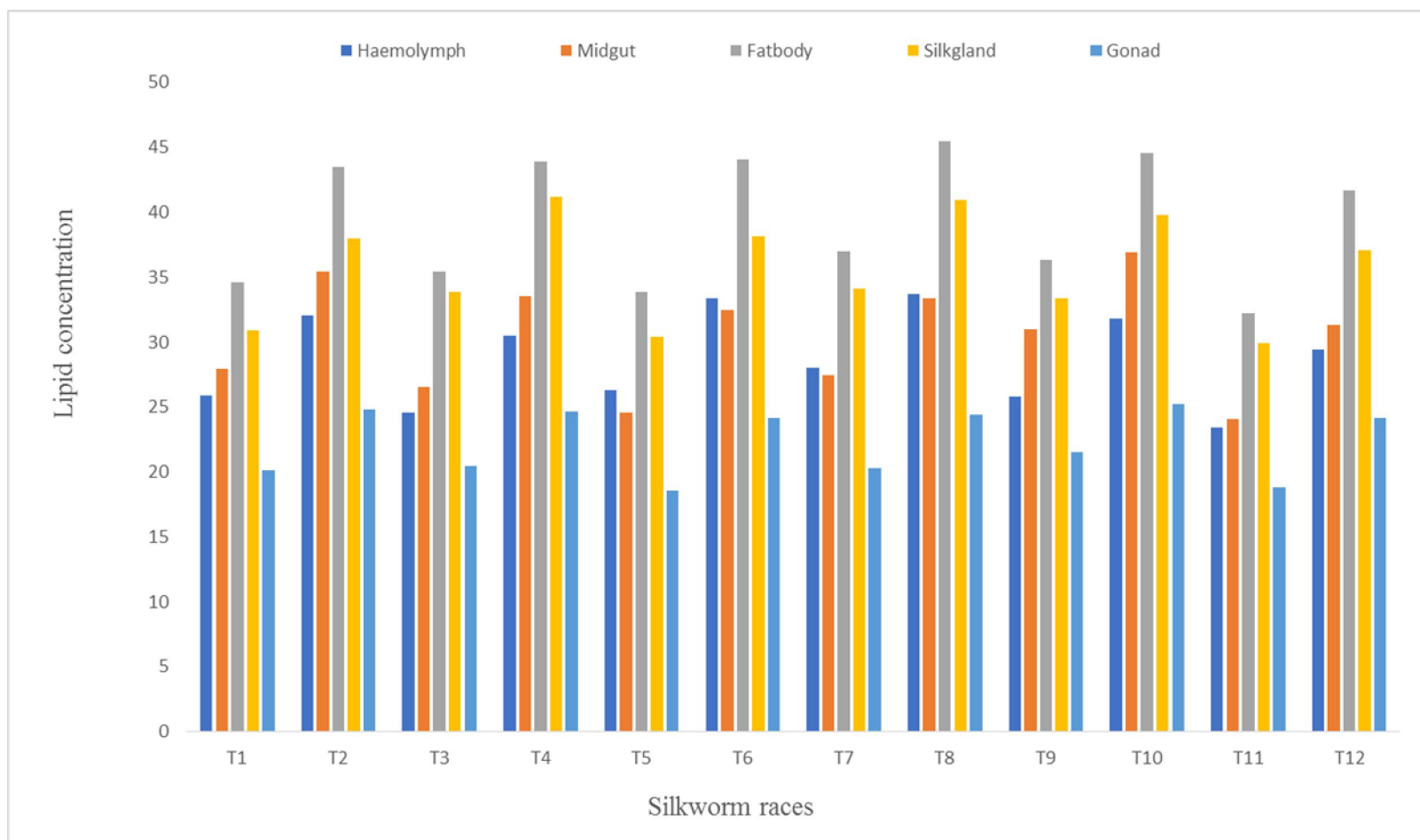


Fig. 4.4: Effect of microsporidian infection on lipids concentration in different tissues of six races of silk worm

Chapter 5

DISCUSSION

5.1 Morphological characterization of the microsporidian spore from the diseased silkworms

The microsporidians are obligate intracellular pathogens of the silkworm *B. mori*, causing the epidemic Pebrine disease and extensive economic losses in sericulture. Most of the silkworm breeds are reported to be susceptible to the microsporidian infection (Rao *et al.*, 2004). Due to the economic importance of sericulture the detection and classification of different microsporidia infecting silkworm is extremely important for proper management of pebrine disease. Microsporidians form spores with rigid spore walls that protect against various environmental pressures, ingested spores, however, germinate immediately under the extremely alkaline host gut condition (Lepidoptera gut pH > 10.5), which is a key developmental turning point from dormant state to infected state (Undeen and Avery, 1984). Because of its durability and resistance to external conditions the microsporidian spore is of prime importance to pathologists and very often the spores are the only material available for taxonomic determination and for this reason a large part of microsporidian taxonomy is based on the shape and size of the spore (Vavra, 1980, Larsson, 1999). The dimensions of the spore have been shown to be remarkably constant for any given species (Thomson, 1960). The microsporidian spores are small, being usually only several micrometers in length and width. In addition to being small, some spore types also look similar under light microscope. The microsporidia lack enough characters at light microscopic level to be useful for classification and the use of ultrastructure studies like Scanning Electron Microscopy (SEM) in the study of microsporidian spores is a logical extension to the preliminary light microscopic observations (Vavra, 1980). The present study revealed that the isolated N₁ spores differed morphologically (in terms of spore size and shape) from the standard spores of *N.bombycis*. Similar results were observed by Selvakumar *et al.* (2008) who reported a

microsporidian NIK-5hm in the haemocytes of silkworm *B.mori*. The microsporidian was oval in shape measuring 4.55 μm in length and 2.10 μm in width and differed in size, form and shape from *N. bombycis* (Standard strain). Bhat (2006) also reported a microsporidian infecting the Lamerin breed of silkworm which differed morphologically from the typical *Nosema* spores in terms of size, form and shape. The measurements of this microsporidia were 4.36 μm x 2.14 μm which were distinctly different from the measurements of standard strain of *N.bombycis* Chakraborty *et al.* (2012) also reported a microsporidian infecting *P. ricini* which differed morphometrically from *N.bombycis* as it was elliptical in shape, having blunt or tapering ends having measurements-length (3.784 $\mu\text{m} \pm 0.056$), breadth (2.630 $\mu\text{m} \pm 0.027$) and volume (13.695 μm^3). The results are also in line with the study of Sharma *et al.* (2014) who reported a microsporidian infecting silkworm, *B.mori* which differed from the typical spores of *N. bombycis* morphometrically. The spores they isolated were ovo-cylindrical in shape measuring 4.03 $\pm$ 0.24 μm in length and 2.30 $\pm$ 0.14 μm in width with 1:57 length: width ratio. These results are also in line with the findings of Moharammi *et al.* (2022) who observed a new strain of *Nosema* infecting silkworms in Iran. SEM studies of this species revealed that its measurements-length: 1.99 $\pm$ 0.25 and breadth- 2.81 $\pm$ 0.32 μm were lesser than normal *Nosema* spores. They further concluded that this strain was a native strain of *Nosema* specific to Iran.

5.2 Impact of microsporidian infection on survival and economic parameters of silkworm, *Bombyx mori* L.

5.2.1 5th age larval duration and Total larval period:

In the present study, a decrease in the 5th age larval duration as well as total larval duration of all the silkworm batches infected with *Nosema* has been recorded over the control /healthy batches. This is in agreement with the findings of Kumar *et al.* (2011) who reported that quantitative as well as qualitative nutrition is highly essential for sericigenous insects to maintain important physiological status and lack of such nutrition in the silkworm under stress

conditions extends its larval span. These observations are also in line with the study of Madhusudan *et al.* (2011), where the pebrine (*Nosema*) infected larvae showed increased larval duration in comparison to the healthy ones in *A. mylitta* and they concluded that the developmental time in the infected larvae usually gets delayed due to depletion of nutritional reserves as well as their reduced ability to assimilate food efficiently. The results of the present study are also in accordance with the findings of Renuka and Shamitha (2013), who observed similar results of instar-wise extension of larval life span, more so in the fifth instar while studying the pathogenic effects of the microsporidian *Nosema sp.*, on larval and post-cocoon parameters in Tasar Silkworm, *A.mylitta* Drury. These results are also supported by the view that the larvae can survive better on getting the required nutrition by improving defense system, which shortens its larval span and lessen the chance of exposure to the diseases, pests and pathogens and vice –versa (Baylis and Pierce, 1991; Ojala *et al.*, 2005). Infection prolongs the developmental time due to depleted nutritional reserves, increases the chances of exposure to pathogens and reduces ability to assimilate food efficiently (Veber and Jasie, 1961). The results are duly supported by the findings of Fisher and Sanborn (1964) who demonstrated that *Nosema apis* is capable of influencing the normal growth and development of the host by producing a substance with activity identical to juvenile hormone, thereby extending the larval period and in the present study, prolonged larval duration may be due to activity of such substances in the infected larvae.

5.2.2 Weight of the matured larvae (g larvae⁻¹⁰) :

The present study highlights a negative impact of microsporidiosis on the weight of matured silkworm larvae in all the infected batches of silkworms over their control batches. These findings are in accordance with the findings of Rajitha and Savithri (2014) who found that there is a significant loss of larval body weight due to infection of the Pebrine in silkworm *A. mylitta* and observed 13% decrease in the body weight of silkworm infected with pebrine over its

control batches. They attributed this to the reduced feeding by the diseased larvae as well as reduced metabolic activities leading to lesser conversion of leaf proteins into essential nutrients. Rath *et al.* (2003) also attributed the reduction in the larval weight of silkworms infected with pebrine to the fact that there may be decrease in the food consumption, digestion, relative consumption rate, efficiency of conversion of ingested food in fifth instar of silkworms' larvae due to the invading pathogen. Absorption of food constituents from digested leaves is mostly depending on midgut of larvae. However, in pebrine infected silkworms the midgut epithelial cells get severely disrupted which reduces the absorption and subsequent assimilation of the ingested food thereby causing a reduction in the mature larval weight (Ali and Sharma, 2014).

5.2.3 Single cocoon weight (g), Single shell weight (g) and Shell ratio (%)

In the present study a declining trend can be seen in case of single cocoon, single shell weight of the infected silkworm batches as compared to their healthy uninfected control batches. This is in confirmation with the findings of Kumar *et al.* (2011) and Reddy *et al.* (2012) who reported that the potential fitness and maturity attainment in silkworm larvae is only possible when the larva obtain adequate amount of nutrients in a required balance and the deficiency of these nutrients due to any biotic or abiotic stress leads to longer larval duration, lesser ERR and cocoon yield. Vellide and Rao (2011) observed similar results in Pebrine infected silkworm and these results corroborate with the decreased protein content in the silk glands of inoculated silkworms. Yogananda *et al.* (2014) suggested that *N. bombycis* interferes with the normal physiology of the silkworm and affects protein content of the silk gland possibly by reducing the assimilation of proteins. They also observed that Silk gland weight and shell weight significantly declined following *Nosema* infection in comparison to uninfected control silkworm larvae. These results are well supported by the findings of (Rath *et al.*, 2003) who observed the weight of the silk gland will reduce in *A. mylitta* larvae infected with *Nosema spp.* which finally reduces the silk production. Velide *et al.* (2013) also

reported that the healthy growth and development of the silkworm is directly related to economical cocoon traits. She also noticed poor cocoon characters from pebrine infected Tropical Tasar silkworm *A. mylitta*.

5.2.4 Cocoon yield/10,000 larva

The present study signifies a decrease in the ERR% (both by number and weight) in all the inoculated batches of silkworm as compared to their healthy controls. This is in conformation with the findings of Velide *et al.* (2013) who observed that effective rate of rearing is directly proportional to the healthy growth and development of the silkworm. She also reported poor economic and commercial cocoon characters from pebrine infected Tropical Tasar silkworm *A. mylitta*. 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). Yadav (2019) also observed a decline in ERR% of pebrine infected silkworm, *Bombyx mori* L. These results are also in line with the findings of Kirankumar *et al.* (2013) observed that the infection by *N. mylittansis* affects the cocoon characters of Tropical Tassar silkworm, *A. mylitta* and significantly reduces the cocoon weight, shell weight, silk ratio and ERR% when compared to control batch.

5.2.5 Larval mortality and Survivability

The present study highlights a significant increase in the larval mortality of the silkworms inoculated with Nosema spores as compared to its control healthy batches. An inverse relationship has been observed in the larval mortality and survivability in the present study. Corresponding to an increase in larval mortality a significant decrease in the survivability of the inoculated batches of silkworms has been observed over the healthy un-inoculated controls. These results are in accordance with the results of Velide and Bhagavanulu (2012) who observed a significant increase in mortality percentage of the Tropical Tasar

silkworm *A. mylitta* infected by microsporidiosis (Pebrine). These results are also in accordance with the findings of Bajare *et al.* (2015) who observed mortality in silkworms inoculated by bacterial infection (*Staphylococcus aureus*). Mishra *et al.* (1992) observed mortality in all instars of silkworm larvae infected by Pebrine. These results are also in accordance with the results of Chakrabarti and Buddhadeb (2008) who while studying the infection of *N.bombycis* and cross-infection of *N. mylitta* on silkworm *B.mori* observed 36.66 to 57.58 % mortality. These results are in line with the results of Khurad *et al.* (2005) who observed 17.5, 20 and 23% mortality in *B.mori* silkworm when infected by a dose of 1300, 1800 and 2000 OBs/larva of viral polyhedral occlusion bodies (OBs) respectively. They suggested that infection of microorganism has profound effect on mortality due to loss of hunger, dysentery, vomiting, dehydration and depletion of essential nutrients. Fujiwara (1984) suggested that silkworm infected with pebrine does not succumb to death by any toxin, but the normal functions of the infected silkworm either stops or is disturbed by mechanical disruption of the host tissues and by deprivation of nutrients by the invading pathogen.

5.2.6 Pupation rate

The present study showed a remarkable decrease in the pupation rate (%) of all inoculated silkworm batches in comparison to their healthy controls. These results are in corroboration with the findings of Vellide and Rao (2011) who observed a reduction in all the commercial and economic parameters including pupation rate of *A. mylitta* infected with pebrine, *N. mylettensis*. Mishra *et al.* (1992) also observed a decrease in the pupation rate of in silkworm *B.mori* infected by *Nosema spp.* These results are also in conformity with the results of Lakshmi and Benarjee (2016) who observed reduction in the commercial and economic parameters of silkworm *A. mylitta* infected with pebrine. All the major proteins are synthesized by the fat body and secreted into hemolymph for their temporary storage and are then resequenced into the fat body for deposition in the form of dense proteinaceous granules (Tojo *et al.*, 1981; Chen, 1978).

Bhorphukan and Bhola (2013) suggested that microsporidian parasitisation of the fat body might have reduced the protein turnover of the host silkworm, which ultimately affected the normal growth and development and decreased the pupation rate in pebrine infected silkworms.

5.2.7 Moth emergence

The present study showed a significant decrease in the moth emergence of silk moths from infected batches as compared to their respective healthy controls. Similar results were also observed by Bhat (2006) in the *Nosema* infected silkworm batches. These results are also in conformity with the studies of Baig (1994) who observed a reduced moth emergence in silkworm *B.mori* post pebrine infection. He observed that during moth stage, the infection with the microsporidian increased many fold and attributed this high infection rate at moth stage to longer time availability to the pathogen for its multiplication in the host.

5.2.8 Filament length (m), Raw Silk (%) and Denier:

The present study accentuates the negative impact of pebrine infection on the reeling parameters of cocoons viz; filament length, raw silk percentage and denier of all the pebrine infected silkworm batches under study over their respective controls. Vellide and Rao (2011) observed similar results in pebrine infected *A.mylitta*. They also observed that the reeled silk weight from single cocoon reduced a lot in case of infection compared to the healthy cocoons. Rath *et al.* (2003) have reported a decrease in silk gland weight in *A.mylitta* larvae infected with pebrine, which finally reduces the silk production, thereby decreasing the shell ratio, silk filament length and raw silk percentage. These results are also in accordance with findings of Bhat *et al.* (2009a) who reported that silk from the pebrine infected cocoons is usually of inferior quality. Also, the findings of Renuka and Shamita (2013) revealed that there is a substantial decrease in the post cocoon parameters of pebrine infected *A.mylitta*. Rath and Sinha (2005) while studying the parasitisation of fifth instar larvae of

A.mylitta by Uzifly have reported a decrease in cocoon weight, shell weight and filament length in the infected larvae. Lakshmi (2003) observed similar reduction of post cocoon parameters due to *B.bassiana* infection and attributed the decline in the synthesis of silk proteins to direct or indirect effect by *B. bassiana* on the growth and development of silk gland of the silkworm *B.mori*. More number of breakages and higher denier were also noticed in experimental cocoons in comparison to healthy ones. They further suggested that the physiological and biochemical stress induced by a fungal pathogen causes to exude uneven amounts of silk fluid in lumps. Ganga (2003), have reported a significant reduction in the reeling parameters of *Nosema* infected cocoons of *A. mylitta* comparative to control pebrine- free cocoons.

5.2.9 Fecundity

The present study highlights the decrease in the fecundity of pebrine infected silkworm batches as compared to their respective healthy controls. These results are in accordance with the results of Bhat and Nataraju (2005) who observed a reduced fecundity in the *Nosema* infected silkworms as compared to *Lb_{ms}* infected silkworms. The fecundity is dependent on the reserves of nutritious substances accumulated in the larva, before pupation, because silk moths do not consume any food (Veber and Jassie, 1961). The microsporidians develop in the fat bodies of the host and the infection causes depletion of nutritive reserves resulting in less fecundity in females (Armstrong and Bass, 1986). Chakrabarti and Manna (2008) also observed a reduction in the number of eggs in pebrine infected silkworms and attributed it to the effect of the parasite on the host protein turnover of silkworms. Protein synthesis in the fat body outside the ovary plays an important role during egg maturation. They further concluded that the degeneration of fat body due to the microsporidian infection affects the protein synthesis during egg maturation which might be one of the reasons for the reduction in the number of eggs produced by the diseased females. A decline in ovary weight, fecundity and fertility in *A.mylitta* larvae infected with *Nosema sp.*

was also reported by Rath *et al.* (2005). These results are also in line with the results of Bansal *et al.* (1997) who reported that the high spore concentration of *Nosema* in the gonads of *A.mylitta*, *A.assamensis* and *B. mori* affects their reproductive potential and fertility.

5.2.10 Hatching percentage:

The decreased hatching percentage of pebrine infected silkworm batches as compared to their respective healthy controls in the present study are in conformity with the findings of Rath *et al* (2005), who observed the number of underdeveloped non-chononated eggs was more than that of their disease-free counterparts in pebrinised adults of *A.mylitta*. A decrease in egg laying capacity of infected adults of *A.mylitta* and other insects was also reported. These results are also in accordance with the results of Jolly and Sen (1972), who observed that microsporidiosis in *A.mylitta* caused atrophy of fat cells due to which yolk could not be deposited for proper egg development. They also reported that infected eggs contain more spores and are less likely to hatch due to a depletion of embryonic food reserves by Microspondian. These results are also in agreement with the findings of Bhat and Nataraju (2005) who observed a decreased hatchability in *Nosema* infected silkworm races compared to Lb_{ms} infected silkworms.

5.3 The site and intensity of the microsporidian multiplication in the infected worms

The present study accentuates a high intensity of infection (more than 30 spores/field) in the gonads (ovaries) at moth stage. At the larval stage, moderate intensity of infection (5- 30 spores/field) on the 3rd day and 5th day of 5th instar is observed in all the larval tissues of all inoculated silkworm races.. The intensity of the infection in the larval tissues is observed in the sequence: midgut> silkgland> malphigian tubules. The silkworm races under study showed varying degree of intensity of the infection in the tissues examined. The sequence of the suceptibility

of the races to *Nosema* infection depending on the intensity of infection in different tissues under study is SK₂₈>CSR₂>SK₆>SK₁>NB₄D₂ and SH₆. These observations are in accordance with the findings of Remadevi *et al.* (2010) who while studying some pathological effects and transmission potential of a microsporidian isolate (*Nosema sp.*) from the teak defoliator *Hyblaeapuera* observed that the midgut and fat body were the primary organs infected by the microsporidium. Subsequently, infection was observed in malpighian tubules, tracheal epithelium and gonads. The sequence of infection observed was: midgut – fat body – tracheal membrane – Malpighian tubule – gonad. They further suggested that infection of this microsporidium produced a marked negative effect on the growth and development of larvae due to depletion of the nutritive reserves of host by pathogen. Kawarbata (2003) and Bhat (2006) also observed that the microsporidian spores germinate in the midgut under alkaline conditions which is the primary site of infection of microsporidian spores. After invading and depleting the nutrition in target primary tissue the spores then proliferate other tissues like fatbody, malpighian tubules, trachea, haemolymph and gonads. Sasidharan *et al.*, 1994 reported microsporidian development to be closely related to the host body nutritional state and suggested that the sporulation of *N. bombycis* increased with the age of the silkworm larvae and higher infection levels were observed in silkworm pupae and moths. Among all the tissues tested the highest spore concentration was observed in the cephalothoracic region especially around the wing and the wing muscles. Madann *et al.* (2005) reported that the difference in spore yield in different tissues is a reflection of the host parasite interaction. Their results indicated that the most susceptible tissues are the gut and thorax and maximum spore yield occurred after 3 - 4 days of adult emergence. These results are also in conformity with the study of Bashir *et al.* (2012) who studied the site and intensity of infection in different tissues of silkworm by three different microsporidia. The results of their study indicated spore formation takes place in midgut which then proliferates to other tissues like fatbody, malpighian tubule, trachea, silk glands, haemolymph and gonads. The highest intensity was observed

in midgut followed by fat bodies and comparatively low infection was observed in other tissues in case of all three microsporidians under study indicating difference in the preference of tissues for multiplication and sporulation by the microsporidia. Bhat (2006) observed that *N. bombycis* derives nourishment from the host cells but do not disrupt the basal membrane and microvilli immediately. Thus, the infected host survives for longer durations possibly for the perpetuation of the pathogen. This shows that the parasite lives in close association with the host during the early stages of its development and extensive destruction of the host cell organelle takes place only in the later stages of the development of parasite resulting in severe depletion of nutrients, retarded growth and mortality.

5.4 Impact of microsporidian infection on some biochemical constituents of selected silkworm races

The bio-molecules like proteins, carbohydrates and lipids play an important role in the biochemical processes underlying growth and development of insects (Ito and Horie, 1959). Increasing attention has been given by number of researchers to study the metabolism and accumulation of the bio-molecules in insect tissues during metamorphosis and also under stress conditions (Bhattacharya and Kaliwal, 2005). In the present study, biochemical changes after inoculation of microsporidian spores showed major changes in the total protein, carbohydrate and lipid content in the haemolymph and whole larval tissues (midgut, fatbody, silk gland and gonads) during the course of microsporidian infection. The physiological anomalies and infection by pathogen are responsible for altered metabolism in any organism. The decreased protein, carbohydrate and lipid levels in haemolymph and tissues can be attributed to the excessive utilization of biomolecules to meet the demand of energy by invading pathogen. In the present study a decrease of protein, carbohydrate and lipids content level under infection condition was observed and an increased level of the same biomolecules was observed simultaneously in the control conditions, where in the worms were healthy. In the present study the six races of silkworm under study

after inoculation showed a decreasing trend in the levels of all the biomolecules in all the larval tissues examined over the control healthy batches. The higher and lower level of these biomolecules in infected silkworm races may be due to the susceptible and resistant nature of races which is a polygenic character; resistant race feeding activity is slightly normal as compared to susceptible races after infection. (Ito and Horie, 1959).

5.4.1 Protein Estimation in different tissues of silkworm races under study

1) Haemolymph:

Proteins are the derivatives of high molecular weight polypeptides. They play a vital role in the formation of structures in organisms and are considered as the building blocks of insects. They are the chief organic constituents of the cell. These macromolecules are concerned with the regulation of all biochemical events in the organism. High protein concentration is usually an indication of greater metabolic activity. The survival ability of an insect to stress majorly depends on its protein synthesis potential. Any stress (biotic or abiotic) on an insect invokes compensatory metabolic adjustments in its tissues through modifications or modulations of proteins (Bano *et al.*, 1981). Silkworms have an open circulatory system with haemolymph consisting of blood and tissue fluids. The Haemolymph plays a dual role of transportation and storage. It not only transports the body fluid but is also a depository of nutrients, energy and metabolic intermediates for all organs and cells. It stores and transports about 241 to 298 proteins involved in metamorphosis, ecdysis, chitin and haemocyte formation, growth of silk gland and reproductive organs (Chai *et al.*, 2008; Nakahara *et al.*, 2009). It is conceivable that the metabolite composition (metabonome) of silkworm haemolymph varies with the silkworm development and growth (Li *et al.*, 2012).

In the present study a significant decline in the haemolymph protein concentration is observed in all the inoculated races compared to their controls.

This is in line with the findings of Madhusudhan *et al.* (2011) who also observed a decreasing protein concentration in the haemolymph of tropical tassar silkworm infected by pebrine. The total protein profile of a tissue may be taken as a diagnostic tool in assessing the physiological status of it or the organism as a whole (Nelson and Cox, 2001). Pathogens are reported to induce several biochemical and physiological alterations in insect tissues (Bergold 1963; Benz and Borusiewicz, 1963). Watnabe *et al.* (1968) also observed hypoproteinemia in the hemolymph of silkworm inoculated with *B.mori* Nucleo-Polyhedrosis virus (BmNPV). The present studies are in corroboration with the studies of Davadas (1991) who reported a significant reduction in hemolymph and gut proteins content after inoculation of *Serratia marcescens* and attributed it to reduced feeding by infected larva as well as altered metabolism of protein and amino acid of haemolymph by developing pathogen. Madhusudan *et al.* (2011) studied the effect on *Nosema* infection on various parameters of silkworm *A. mylitta* including protein concentration and reported a decrease in haemolymph protein concentration. It was suggested that decrease was due to drastic degradation of structural proteins. But decrease in protein content failed to coincide with increase in amino acid concentration suggesting that degraded proteins might be utilized by invading pathogen for rapid development. The findings of the present investigation are also in line with the findings of Sarma *et al.* (1994) observed that significant decrease of total protein content in the haemolymph of BmNPV infected silkworm larvae. These results are also in line with the results of Bhorphukan and Bhola (2013) who observed a decrease in the hemolymph protein concentration of silkworm, *S. ricini* post *Nosema* inoculation. They attributed this to the reduced feeding by infected larva as well as due to metabolism of protein and amino acid of hemolymph by developing pathogen. Low protein content may also be due to reduced digestion/absorption from the midgut since the spores of *Nosema* have been found invading the midgut epithelial cells.

2) Midgut :

Although *N. bombycis* forms spores with rigid spore walls that protect them against various environmental pressures however when ingested, these spores germinate immediately under the extremely alkaline host gut condition (Lepidopteran gut pH > 10.5) which is the first step in host cell invasion and is a key developmental turning point for *Nosema* spores from dormant state to infected state (Liu *et al.*, 2016). Accumulation of biomolecules is mostly dependant on leaf quality, quantity and rate of consumption by silkworm larvae. Absorption of food constituents *viz.* proteins and amino acids from digested leaves is mostly depending on midgut of larvae. In the present study a decrease in the total protein content of midgut is evident in all the inoculated batches over the control batches. These results are in line with the findings of Watanabe (1970) who also observed a decrease in the midgut protein metabolism of silkworms infected with cytoplasmic polyhedrosis virus and attributed this decrease to the active synthesis of polyhedron proteins of virus rather than the midgut proteins of silkworm. Kawase and Hayashi,(1971) observed hypoproteinemia in silkworm larva infected with Cytoplasmic Polyhedrosis Virus (CPV) and suggested that low protein content may be due to reduced digestion/absorption from the midgut since the spores of *Nosema* have been found invading the midgut epithelial cells. They concluded that hypoproteinemia was due to reduction of protein content caused by formation of polyhedral bodies and starvation due to dysfunctioning of midgut. Studies have shown the amounts of amino acids and proteins in both midgut and haemolymph of highly diseased larvae significantly decreased (Kawase, 1965). The results of the present study are also in accordance with the findings of Ali and Sharma (2014) who observed a reduced midgut protein content in the Tropical Tasar Silkworm, *A. mylitta* D during pathogen infected conditions. Govindan *et al.* (1998) noticed a reduction of protein content in *BmNPV* infected midgut epithelial cells of silkworm, *B.mori*. These results are in accordance with the results of Bhat *et al.* (2009 b) who observed a decrease in the midgut protein

content of microsporidian infected silkworm. Similar results were also observed by Joshi and Raja (2019) who observed a reduction in the protein content, of mid gut tissue of silkworm *B.mori* infected with grasserie as compared to the control healthy non-infected silkworms of the same age.

3) Fatbody:

The insect fat body represents the chief site of protein synthesis and amino acid metabolism. Functionally, it is analogous to the liver and adipose tissue of higher vertebrates. In silkworm, the fatbody synthesizes as well as stores over 177 proteins implicated in larval growth and development and responds to the fluctuation of the metabolites in the haemolymph fairly quickly (Hak *et al.*, 2005; Hou *et al.*, 2007; Tojo *et al.*, 1981). When insect larva cease to feed (under any stress) proteins are selectively removed from haemolymph and stored as intracellular granules in fatbody for use at the time of metamorphosis (Chen, 1978). The present study highlights the negative impact of microsporidian infection on fatbody protein of microsporidian infected silkworm races over their healthy control batches. This is in accordance with the observations of Anitha *et al.*, 2010 who reported a decrease in the biochemical constituents under stress conditions (abiotic or biotic stress) to serve the growing energy demands of the body under stress and also observed a considerable decrease in the protein content of fatbody of silkworm under cold stress. Ambika (1990) also observed a decreased protein content in fat body of the infected silkworms during fungal infection by *B. bassiana*. Similar results were observed by Dalip and Joshi (2020) in tigerband infected Oak Tassar silkworms wherein the protein content of the fatbody of infected silkworms decreased significantly. Bansal *et al.* (1997) reported a relatively high spore concentrations in fat body as compared to other tissues (midgut, silk gland and gonads) which corroborates with the reduced protein concentrations in the fat body of infected larvae. Vellide (2012) observed a significant reduction in the protein of fatbody of Tropical Tassar silkworm under cold stress. In another study, Watnabe (1968) observed hypoproteinemia in the

hemolymph of silkworm inoculated with *B. mori* Nucleo-polyhedrosis virus (*BmNPV*). They attributed this to fat body protein depletion as it is the most susceptible tissue to infections like Nucleo polyhedrosis virus (NPV).

4) Silk glands:

The silk gland is the major site of silk proteins synthesis. Apart from two silk proteins (fibroin and sericin), it synthesizes and stores 91 other proteins involved in metabolism, immunity, heat-shock mechanism, cytoskeleton formation, protease inhibition, transport and transcription (Jin *et al.*, 2004; Takasu *et al.*, 2005 ;Kyung *et al.*, 2006; Zhang *et al.*, 2006; Hou *et al.*, 2007). Silk gland assimilates the mulberry protein in the course of larval development and grows considerably in size and weight to provide protein material during the spinning of the cocoon. The process of protein assimilation and growth of silk gland depends mainly on the physiological conditions of the silkworm during larval growth. The present study accentuates a decrease in protein content of the silk gland in the infected larval batches when compared with control group. Similar results were observed by Rajasekhar and Pathak (1994) who reported significant reduction of total protein concentration in the silk glands of flacherie infected larvae, the decrease was suggested to be due to a decrease in the level of protein and aminoacids in the haemolymph of diseased worms which affects the formation of silk protein in the glands. These results are also in line with the results of Thirupathama and Savithri (2014) who observed a decreased protein content in silk gland of mulberry silkworm larvae due to *B. bassiana*. They suggested that the infection interferes with the normal physiology of the silkworm and affects protein content of the silk gland possibly by reducing the assimilation of proteins. They also observed that silk gland weight and shell weight significantly declined following fungal infection in comparison to uninfected silkworm larvae. Mamatha and Balavenkatasubbaiah (2014) observed a decrease in the silk gland of *BmIFV* infected silkworms and suggested that the decrease in protein content indicated decreased metabolic activities and enhancement of proteolysis of silk

gland *Philosamia ricini* larvae under cold stress conditions have shown the decrease in protein content in the fat body and silk gland (Anitha *et al.*, 2010). Earlier works of Sathish (1998) also suggested that, haemolymph protein in sericigenous insects is responsible for the formation of silk proteins in silk glands. Shimura (1978) also reported that, haemolymph acts as an amino acid reservoir between midgut and silk gland, supplied amino acids to silk gland for silk synthesis. This corroborates with the results of the present study wherein there is a decrease in the haemolymph proteins and subsequently in the silk glands has been observed due to *microsporidian* infection.

5) Gonads:

The concentration of the biomolecules *viz.* protein, carbohydrates and lipids showed a maximum decreasing trend in the gonads of the inoculated silkworms when compared with the healthy control batches. Reports have shown that these biomolecules play an important role in the biochemical processes underlying growth and development of insects (Ito and Horie, 1959). Bansal *et al.* (1997) reported highest spore concentration in gonads among other tissues (fat body, midgut and malpighian tubules) under study in *A. mylitta*, *A. assamensis* and *B. mori* which corroborates with the decrease of essential molecules in the gonads in the present study. Also, Madann *et al.* (2005) observed that the intensity of microsporidian infection has been found to be more in female gonads than male gonads which are in support of the results of this study. The microsporidian infected silkworms exhibit severe damage of fat body tissue and gonads which could be the possible reason for the reduced fecundity in insects. Reports have shown that microsporidia prevents cell differentiation in gonads as the nutrients are utilized for pathogen development (Syme and Green, 1972, Tanabe and Tamashiro, 1967). Microsporidia uses nutritive reserve of gonads which is otherwise utilized for reproduction of silkworms and thereby resulting in reduced fecundity and fertility in the infected silkworms (Thomson, 1958; Veber and Jasie, 1961 and Smirnoff and Chu, 1968). Studies have shown that infection

in ovaries affects the process of oogenesis resulting in sterile eggs even after successful copulation due to depletion of nutritive reserves by the invading pathogen in infected silkworms. (Mercer and Wigley, 1987).

SDS page analysis of different tissues of silkworm: As already discussed in biochemical studies, after inoculation of microsporidian infection, all the inoculated silkworm races showed major changes (mostly reduced levels) in the total protein content in the haemolymph and whole larval tissues (midgut, fatbody, silk gland and gonads) during the course of microsporidian infection. The decreased protein levels in haemolymph and whole larval tissues can be attributed to the excessive utilization of biomolecules to meet the demand of energy by invading pathogen (Kadwey, 2009). After inoculation of microsporidian infection, observations were taken on 3rd and 5th instar stage which were compared with the respective controls. And these observations on the protein fractions in third and fifth instar revealed that the expression of proteins showed a drastic decrease compared to the controls. This decline in the concentration of the essential macronutrients i.e. proteins in different body tissues results in retarded growth and development of the larvae, leading to increased mortality rate and ultimately crop loss and also invokes compensatory metabolic adjustments in its tissues through modifications or modulations of proteins (Bano *et al.*, 1981).

The qualitative analysis of proteins (SDS-PAGE) indicated changes in the expression of proteins as the intensity of the protein bands decreased between 27-45 kDa in the inoculated silkworm tissues as compared to their respective controls (especially in R3- SK₂₈). Also, some of the protein bands were absent in the inoculated silkworm races in comparison to their respective controls. These results are in line with the findings of Chavan *et. al* (2021), wherein SDS-PAGE map revealed a variation (down-regulation) not only in protein bands and but also the variation in staining intensity of protein bands in the hemolymph, midgut, fatbody and silk gland samples of silkworm, *B. mori* infected by *BmNPV*, ranging between 32 kDa to 75 kDa in comparison to their respective controls. Also, some

of the new protein bands were seen only in few inoculated silkworm tissues, which may be due to the synthesis of some biotic stress related proteins to meet the physiological need of inoculated silkworms. In present study more no. of protein bands were observed in silk gland of inoculated silkworms between 10-27 KD and 91 to 110 KD compared to their respective controls and midgut of infected silkworm (R4-SH6 and R5-NB4D2) as compared to their controls, which is in line with the findings of Venkatesh *et. al* (2019), wherein SDS-PAGE map revealed a greater number of protein bands in the hemolymph of silkworm, *B.mori* infected by *N.bomycis* infection, ranging between 15 kDa to 80 kDa in comparison to its respective control.

The present study revealed that no. of protein bands in fat body of infected silkworms decreased between 10-27KD compared to their controls, respectively. It could be correlated with the biochemical studies wherein the concentration of protein in infected silkworm decreases in comparison to their controls. Borphukan and Bhola (2013) observed changes in the haemolymph protein during the larval development in *P. ricini* infected with *Nosema* and found that the content of protein continuously reduced with the progression of the infection. Dalip and Joshi (2020), revealed a significant decline in the concentration of protein, carbohydrates and amino acids in the haemolymph, silk gland and fat body of the tiger band infected larvae.

Our study depicted that the intensity of protein bands decreased at 10KD in gonads of infected worms as compared to their controls which could be correlated with less protein concentration in gonads of infected silkworms. This correlates with our biochemical study wherein the protein concentration in gonads of infected silkworms ranges from 13.93-16.90 mg/g while in their respective controls protein concentration ranges from 19.26-22.454 mg/g which justifies SDS-PAGE results as well.

5.4.2 Carbohydrate estimation in different tissues of silkworms under study

1) Haemolymph:

In the present study a significant decrease in the haemolymph carbohydrate concentration levels is evident in all the inoculated races in comparison to their control batches. Carbohydrates serve as a main source of energy to a number of insect spp. (Thomas 1998). This biochemical constituent forms a predominant carbon source of chitin, a participant in energy metabolism and the substrate for protein and lipid synthesis insects. The decrease in the carbohydrate contents in haemolymph may be due to the reduced feeding by infected larvae as well as due to increased utilization of carbohydrates of haemolymph by the developing pathogen (Reddy, 2004). The results of the present study also corroborated with the findings of Mamatha and Balavenkatasubbaiah (2014) who reported a decrease in the carbohydrate levels of haemolymph of race CSR2 due to *BmIFV* infection. They attributed this to the decreased level of carbohydrate in the haemolymph to the excessive utilization of carbohydrates to meet the increased demand of energy by *BmIFV*. It has been reported that high concentration of carbohydrates in hemolymph are maintained during larval development as energy reserve to be utilized later during metamorphosis, pupal and adult stage (Simex and Kodrik, 1986). Besides being a transport tissue for transporting the nutrients to and fro from different tissues haemolymph also acts as a reservoir of many materials essential for the insect growth and metabolism (Satish, 1998). The decrease in carbohydrate content of haemolymph may be due to compensation of deficiency of energy caused by the biological stress. This decline in turn results in retarded growth and development of the larvae, leading to increased mortality rate and ultimately crop loss (Bhorphukan and Bhola 2013). The decrease of carbohydrates (glucose) at the end of larval instar may be due to the decrement of trehalase activity in silkworm haemolymph caused due to NPV interference (Mahesha *et al.*, 2013).

2) Midgut :

The results of the present study indicate a maximum decline in the carbohydrate contents of midgut in the infected silkworm batches when compared with their control batches. Studies have confirmed midgut as a favourable habitat for the multiplication of microsporidians thereby corresponding to the decline in carbohydrate content which may be utilized by the invading pathogen for its metabolic activities. (Lakshmi and Benarjee 2014 , Lakshmi and Rao 2011). These findings are also in line with the studies of Gururaj *et al.* (1996) who recorded a significant decline in the carbohydrate content of midgut of silkworms infected with *BmNPV*. Yaginuma *et al.* (1990) reported decline in the concentration of carbohydrates in the midgut epithelium of the silkworm affected with CPV. The glycogen content in the tissues of *B. mori* was utilized for viral multiplication along with serving as fuel reserve for the growing energy demand of the host for combating virus infection (Kodaya *et al.*, 1984).

3) Fat body:

Fat body is an active organ important for mobilizing major metabolic constituents and their exchange from cell to cell consequent to the coordination of growth, metamorphosis and reproduction in silkworms and is quite susceptible to infections as the spore concentrations in fat body is quite high (Bansal *et al.*, 1997). A vivid decrease in the carbohydrate content of fat body of the infected silkworms in comparison to their un-inoculated control batches can be observed in the present study. This is in line with the observations of (Anitha *et al.*, 2010) who reported that under any stress condition, whether that may be biological or physiological, the biochemical constituents usually get decreased to serve the growing energy demands of the body and also observed a considerable decrease in the carbohydrate content of fatbody of silkworm under cold stress. Similar results were observed by Dalip and Joshi,(2020) in tigerband infected Oak Tassar silkworms wherein the carbohydrate content of the fatbody of infected silkworms decreased significantly. Ambika (1990) also observed the decreased carbohydrate

(glycogen) content in fat body of the infected silkworms during fungal infection by *B.bassiana*. Kochi and Kaliwal (2006) reported a decrease in the available haemolymph carbohydrates and also a decreased conversion in fatbody, with the progress of the age in fifth instar, due to potassium bromide toxicity. In insects, the sugars (carbohydrates) are supplied from carbohydrate-rich tissues such as fat body and midgut into haemolymph for transport to different tissues. During the development of infection these organs get infected which decreases carbohydrate content as the same is utilized by the pathogen for its metabolic and growth requirements (MaryFlora 1991).

4) Silkglands:

A considerable decrease in the carbohydrate levels of silkgland of infected silkworms over control batches has been observed in the present study. This is in line with the findings of Reddy and Venkatappa (2016) who observed significant reduction in the carbohydrate content of Silk gland following bacterial *S.aureus* infection over uninfected larvae. He also observed a decline in the silkgland weight and cocoon shell weight in the infected larvae and suggested that this may be due to the reason that *S. aureus* interferes with the normal physiology of the silkworm and utilizes the nutrients of silkworm for its growth and development. Snehal *et al.*, 2021 observed a decrease in carbohydrate levels of silkglands after exposure of silkworms to magnetic field. They suggested that the depletion in carbohydrate content in haemolymph and silk gland of silkworm larvae under stress may be due to excess utilization of carbohydrate molecules to meet the high demand for energy under influence of the magnetic field (stress conditions). These observations are also in accordance with the findings of Vellide (2012), who reported a decrease in the carbohydrate concentration of silkglands under cold stress. Dalip and Joshi (2020) also reported a decreasing trend in the carbohydrate levels of silkglands of oak Tassar silkworm infected with Tiger band virus. This may be attributed to the fact that whenever a pathogen invades a particular tissue of host it depletes its all the nutrients which the pathogen utilizes for its benefit.

5.4.3 Lipids estimation in different tissues of silkworms under study:

1) Haemolymph:

The haemolymph is the only extracellular fluid in insects. Among its primary functions are the storage and translocation of metabolic products throughout the body. It is well known that the physiological changes occur in the haemolymph during the course of NPV infection. (Reddy *et al.*, 2012)

The lipid concentration of the inoculated silkworm batches showed a reducing trend when compared to the healthy un-inoculated silkworm batches. This is in corroboration with the findings of MallikArjuna *et al.* (2002) who observed a decline in lipid concentration in fungal infected silkworms and attributed it to the depletion of nutritive reserves of silkworms by the invading pathogen which utilizes the same as a source of energy for development and multiplication. Rajitha and Savithri (2014) also reported that, inoculation of fungal pathogen in silkworms resulted in gradual reduction of proteins, lipids and carbohydrates contents in the haemolymph during the progress of *B. bassiana*. Studies have shown that during viral infection, the host cellular metabolism, including the homeostasis of nucleotides, amino acid and lipids are altered which can limit the host cell growth and proliferation as it is hijacked by the infecting virus (Liu *et al.*, 2019; Ghazal *et al.*, 2000). There are reports that lipid metabolism has been observed to be significantly reduced by the differentially expressed cirRNAs in the haemolymph and midgut of silkworms infected by *BmCPV* (Hu *et al.*, 2018).

2) Midgut:

The lipid contents of midgut have shown a drastic decrease in the inoculated silkworm batches as compared to the uninoculated batches. These findings are in line with the findings of Joshi and Raja,(2019) who observed significant a decrease in lipid content of midgut tissues of grasserie infected silkworms. The decrease in the lipid content as reported during Grasserie infection

had been attributed to the altered host lipid metabolism to defend the infections (Komano *et al.*, 1966; Boctor 1981).

Reddy and Venkatappa (2016) also observed a decreased lipid concentration in the midgut profiles of *S. aureus* infected silkworms. Dubovskiy *et al.* (2008) observed that formation of lytic pores in the plasmatic membrane of midgut due to bacterial infection and the disconnection of oxidative phosphorylation processes may result in the overproduction of reactive oxygen species (ROS) which causes lipid peroxidation and hence decreased lipid concentrations in *Galleria mellonera* (Lepidopteran) larvae.

3) Fatbody :

The fat body is an organ unique to insects as it plays an essential role in energy storage and utilization. It is the central storage depot for excess nutrients. Lipid is the main fat body component and more than 90% of the lipid stored is triglyceride (Bailey 1975). The amount of fatty acid or acetate incorporated by the fat body is dependent on the developmental stage and feeding status of the insect (Lorenz 2001; Pontez *et al.*, 2008). In the present study a steep decline can be observed in the lipid concentration of all the inoculated larvae as compared to their controls. These results are in line with the results of Babu *et al.* (2009) who also observed a decline in the fatbody lipid content in viral infected (Grasserie) silkworm larvae. MaryFlora (1991) studied histopathology of NPV infected silkworms and reported appearance of polyhedra in the nuclei of fat bodies and suggested that the decrease in the lipid content of fat body during Grasserie infection can be attributed to reduced host lipid metabolism to meet the increasing energy demands of growing pathogen.

4) Silk glands:

The lipid contents of silk glands have shown a drastic decrease in the inoculated silkworm batches as compared to the uninoculated batches. This is in line with the findings of Reddy and Venkatappa (2016) who observed significant

reduction in the lipid content of Silk gland following bacterial *S. aureus* infection over uninfected larvae. Studies by Thirupathama and Savithri (2014) also showed a decreased in lipid content of silk gland of mulberry silkworm larvae due to Infectious Flacherie Virus (BmIFV) infection. They suggested that the fungus interferes with the normal physiology of the silkworm and affects lipid content of the silk gland possibly by interfering the normal physiology and functioning of fat body. A decrease in lipid content of silk glands has also been observed by Mamatha and Balavenkatasubbaiah (2014) in the silk gland of *BmIFV* infected silkworms.

Chapter-6

SUMMARY AND CONCLUSION

The important findings of the present investigation titled, “Studies on the Microsporidian Disease (Pebrine) of Silkworm, *Bombyx mori* L.” are summarized below:

The present study revealed that the isolated N₁ spore was different from the typical *N. bombycis* spore in terms of size and measured $3.33 \pm 0.17 \mu\text{m}$ in length and $1.84 \pm 0.09 \mu\text{m}$ in width with spore length –width ratio (eccentricity index) of 1.80:1. The ultra-microscopic (SEM) studies of the microsporidian spore also revealed that the external surface of N₁ spores was rough with a depression in the middle of the spores.

The results of the present study revealed that the microsporidian infection affected the survival and commercial characteristics of all the silkworm races under study, however, different races showed varied responses towards the infection. Among the silkworm races, SH6 was least affected by the microsporidian infection and SK28 was most affected in terms of economic and commercial parameters viz; Fifth Age Larval Duration, Total larval period, Weight of the matured larvae, Single cocoon weight, Single shell weight, Shell ratio (%), Cocoon yield (by No.), Cocoon yield (by weight), Larval mortality (%), Survivability (%), Pupation rate, Moth emergence, Fecundity, Average filament length, Raw silk percentage and Denier.

The microscopic examination of different tissues viz; midgut, silk gland, malpighian tubules and gonads of six races of silkworm inoculated with microsporidian infection accentuated a high intensity of infection (more than 30 spores/field) in the gonads (ovaries) at moth stage. However, at the larval stage, moderate intensity of infection (5- 30 spores/field) on the 3rd day and 5th day of 5th instar or 8th day post inoculation was observed in all the larval tissues of all

inoculated silkworm races. The intensity of the infection in the tissues was observed in the sequence: midgut > silk gland > malpighian tubules. The sequence of the susceptibility of the races to microsporidian infection depending on the intensity of infection in different tissues under study was SK₂₈>CSR₂>SK₆>SK₁>NB₄D₂>SH₆. Gonadal (ovarian) infection was also observed suggesting transovarial transmission in the infected silkworms

Protein, carbohydrate and lipid concentrations were estimated in various larval tissues *viz*: haemolymph, midgut, silk gland, fatbody and gonads (ovaries of silkworms) infected with microsporidian infection and compared with their respective healthy controls. Furthermore, SDS PAGE analysis was carried out for qualitative protein analysis in different silkworm tissues. Protein concentration was most affected in the gonads of the silkworm moths in comparison to the other tissues under study. At the larval stage protein concentration was most affected in the midgut followed by fatbody, haemolymph and silk glands. The protein deterioration due to microsporidian infection in different races was in the sequence: SK₂₈>CSR₂>SK₆>SK₁>NB₄D₂>SH₆. Carbohydrate concentration in the different silkworm tissues infected by microsporidian infection was affected in the sequence: Gonads (ovaries) > midgut > fatbody > haemolymph > silk glands. The carbohydrate deterioration due to microsporidian infection in different races was in the sequence: SK₂₈>CSR₂>SK₆>SK₁>NB₄D₂>SH₆. Lipid concentration also underwent dramatic changes (decrease) due to the microsporidian infection, however, different races showed varied responses to the same load of microsporidian infection. The lipid concentration decreased the most in the gonads of race SK₂₈ followed by midgut, fatbody, haemolymph and silk glands. The lipid concentration were least affected in the silk glands of race SH₆. The sequence of the tissues as per deterioration in the lipid concentration was Gonads > midgut > fatbody > haemolymph > silk glands. The sequence of the races in the decreasing order of lipid concentration was: SK₂₈>CSR₂>SK₆>SK₁>NB₄D₂>SH₆.

SDS PAGE mapping revealed that the intensity of the protein bands decreased (down-regulated) in the inoculated silkworm tissues as compared to their controls. Also some of the proteins bands were absent in the inoculated silkworm races in comparison to their respective controls. The pronounced effect was seen in race SK₂₈ followed by CSR₂.

It is therefore concluded that the isolated microsporidian spore (N1) is different from the Nosema spores isolated in India and other sericulture practising countries of the world. However, further studies like Transmission electron microscopy (TEM), artificial germination, phylogenetic studies using molecular markers etc are needed to classify the spore at genus level.

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner **Dr Mir nisar Ahmad**, Former Joint Director/Scientist Central Silk Board (CSB) during Viva-Voce examination held on **21-07-2023** have been incorporated in the manuscript entitled **“Studies on the Microsporidian Disease (Pebrine) of Silkworm, *Bombyx mori* L.”** submitted by **Ms. Sehreen Rasool (Regd. No. 2016-689-D)**

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