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Original Article

Impact of microsporidian infection on growth and development of silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae)

Sunil Kumar Gupta,[a] Zakir Hossain,[b,*] Madana Mohanan Nanu,[c] Kalidas Mondal[d]

[a] P2 Basic SeedFarm, National Silkworm Seed Organization, Central Silk Board, Purnea 854 301, Bihar, India
[b] Central Sericultural Research & Training Institute, Central Silk Board, Berhampore 742 101, West Bengal, India
[c] Silkworm Seed Production Centre, Central Silk Board, Palakkad 678 551, Kerala, India
[d] Zonal Silkworm Seed Organization, Central Silk Board, Malda 732101, West Bengal, India

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ABSTRACT

Several species and strains of microsporidia have been isolated from infected silkworms among which pebrine caused by *Nosema bombycis* Nageli is the most important. Infection from this disease causes severe economic loss in sericulture. Reduction of larval and pupal development and reduced weights in silkworms due to infection has been reported. In the present study, five microsporidian (*Nosema)* isolates from mulberry silkworm, *Bombyx mori* L. collected from different locations in West Bengal, India were sampled to study the impact of their infection on the growth and development of *B. mori*. The study revealed significant differences among the isolates in their ability to cause a reduction in the larval and pupal development of silkworm. Healthy larvae showed better body and tissue weights which were significantly higher than in infected lots. Among the isolates, M5 registered the maximum reduction in relative growth rate, larval silk gland tissue somatic index, larval male and female gonad tissue somatic index (GTSI) and pupal female GTSI compared to the healthy control. Male and female pupa treated with M5 spores died before emergence, suggesting that the M5 isolate was the most virulent.

Introduction

Microsporidia are obligately intracellular protozoon parasites which infect most invertebrate phyla and vertebrates (Canning and Lom, 1986). Their importance as pathogens is recognized in several spheres—they are harmful and cause significant damage to economically important hosts, including silkworms—and they are ubiquitous in nature including more than 1200 species belonging to about 150 described genera (Patrick and Naomi, 2002). These organisms are well adapted in pathogenicity, transmission, ecology and resistance to the defense mechanism of their hosts (Wittner and Weiss, 1999; Wasson and Peper, 2000; Weiss, 2001), the majority of which are insects and fish (Cali and Takvorian, 2003; Lom and Nilsen, 2003). Nearly all the taxonomic orders of the class Insecta are susceptible to the pathogen (Sprague, 1977), out of which the orders Lepidoptera and Diptera account for over half of these hosts (Tanada and Kaya, 1993). The microsporidium *Nosema bombycis* N., which is the type species of this genus (Sprague, 1981) is well known to cause the transmitted disease, 'pebrine' in silkworm which was responsible for the collapse of silk industry in France during the mid 19th century (Govindan et al., 1997).

Earlier studies have shown that apart from *N. bombycis*, several other microsporidian isolates belonging to the genera *Nosema, Pleistophora* and *Thelohania*, which differ in their spore morphology, sites of infection and virulence, have been isolated from silkworms (Fujiwara, 1980, 1984, 1985; Jolly, 1986; Ananthalakshmi et al., 1994). In the present study, five microsporidian (*Nosema*) isolates collected from different regions of the state of West Bengal, India were taken to study the impact of their infection on the growth and development of *Bombyx mori.*

Materials and methods

Samples collection

Five microsporidia (coded M1, M2, M3, M4 and M5) infecting *B. mori* L. were collected from different locations of West Bengal, India and used in the study. Each of the purified microsporidian isolates was maintained *in vivo* in isolation, through per oral inoculation of healthy silkworm larvae of the breed, Nistari (M). The

* Corresponding author.
E-mail address: zakirayub@yahoo.com (Z. Hossain).

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spores were isolated from 5th instar larva by macerating and suspending them in 0.85% NaCl, followed by filtration through a cheesecloth and centrifugation at 3000 revolutions per minute for 10 min. The spore pellet was purified by Percoll gradient centrifugation as described by Sato and Watanabe (1980).

This study assessed the impact of microsporidian infection on the relative growth rate (RGR) in different stadia (Waldbauer, 1968; Madana Mohanan, 2004), the silk gland tissue somatic index (SGTSI) and the male and female gonad tissue somatic index (GTSI) at various metamorphic stages (Reddy et al. 1991; Ponnuvel et al. 1997). The influence of microsporidian infection on the reproductive potential of *B. mori* was also studied (Rath et al. 2001).

Study of relative growth rate

Newly hatched, healthy and uniform sized larvae (one day 1st instar) were counted using a magnifying lens and fine brush and were separated into four groups of 50 larvae for each of the five isolates. A spore suspension (1×10^6 spores/mL) of each isolate was prepared. Each of the four larval groups for each isolate was given one leaf disc (22.26 cm^2) smeared with microsporidian spore suspension (0.25 mL) separately. The larvae were exposed to the feed for 4–5 h and then all four larval groups of each isolate were pooled together in one batch and reared up to spinning. A control batch was also reared under identical conditions, where the larvae were given leaf discs smeared with Endotoxin free (ETF) water instead of spore suspension, thereby making a total of six batches of silkworm larvae. The spore suspension for the inoculation was purified using Percoll and each microsporidian-treated leaf was prevented from drying during inoculation by placing moist foam pads around the rearing bed.

The initial larval weight (immediately after molt) and the mature (final) larval weight were measured in every instar using a balance (Electronic Monopan; Sartorius AG; Goettingen, Germany). Three larval weights were taken at intervals of 1 h before the molt and the highest among these was selected as the mature (final) larval weight. Initial and mature larval weights were recorded from an average of 50 larvae. The mean larval weight recorded was the average of the initial and final larval weight taken during the feeding period (Soo Hoo and Fraenkel, 1966). The feeding period measured in days was also recorded in every instar for calculating the RGR, using Eq. (1) (Waldbauer, 1968):

$$\mathrm{RGR} = \frac{\text{Mature (final)larval weight (g)} - \text{Initial larval weight (g)}}{\text{Mean larval weight (g)} \times \text{feeding period (d)}} \quad (1)$$

Study on tissue somatic index

Healthy and uniform sized first day, 3rd instar larvae of *B. mori* L. were separated into five batches of 20 larvae for each of the isolates. The larvae were then starved for 3–4 h to induce hunger after which, 0.25 mL of purified spore suspension (1×10^6 spores/mL) of each isolate was smeared on a mulberry leaf disc (22.26 cm^2) and fed to each batch separately. A healthy control batch was also maintained under identical conditions, where the larvae were given a mulberry leaf disc dipped in ETF water. The larvae were exposed to the treated leaves for 4–5 h and then pooled larvae for each isolate and for the control were reared separately on quality mulberry leaves in wooden trays.

Twenty matured (5th day 5th instar) larvae were randomly collected from infected and healthy lots separately. After weighing individually, the silk gland and male and female gonads from each larva were dissected out and kept in chilled insect saline (0.85% NaCl). The weight of paired silk glands and the male and female gonads were recorded after blotting out the excess water from the tissues. Similarly, after taking pupal and adult weights, the male and female gonads from 5th day pupae and one-day adults were also separated and weighed. From the weights, the tissue somatic index (TSI) was calculated using Eq. (2):

$$\mathrm{TSI} = \frac{\text{Weight of tissue (g)}}{\text{Weight of insect (g)}} \quad (2)$$

Reproductive potential of silkworm

Newly hatched, healthy and uniform sized larvae (day 1 of the 1st instar) were counted using a magnifying lens and fine brush and were separated into four groups of 50 larvae for each of the five isolates and control and reared up to second molt. A sample of 250 μL of spore suspension (1×10^6 spores/mL) of each isolate was smeared on a leaf disc (22.26 cm^2) and given separately to each batch of larvae on the day 1 of the 3rd instar. After exposure (4–5 h) to the treated leaves, the larvae were pooled isolate-wise and reared on quality mulberry leaves in wooden trays. Simultaneously, a healthy control lot was also reared, in which the larvae were given a leaf disc dipped in ETF water. After spinning, grainage was conducted to produce eggs and the surface sterilized eggs were incubated for rearing. Data on the fecundity and the percentages of hatching, dead and sterile eggs were recorded from both microsporidian-infected and healthy lots. The experiment was conducted progressively during September, October, November and February.

Results

Determination of relative growth rate

The results revealed that after inoculation of the five isolates of microsporidian in different stages of silkworm development, the RGR deteriorated from the 3rd instar of silkworm onwards (Fig. 1). The RGR reached its maximum in the 2nd instar in both healthy and infected lots and its minimum in the 5th instar. The 2nd instar, healthy larvae showed a higher RGR (0.674) among the isolates, with M2 recording the maximum RGR (0.634) among the treatments, followed by M5 (0.576), M3 (0.554), M4 (0.528) and M1 (0.503). However in the 5th instar, healthy larvae showed a higher RGR (0.237) and among isolates, M1 recorded the maximum RGR (0.230) followed by M2 (0.226), M4 (0.225), M3 (0.216) and M5 (0.195). Significant ($p < 0.05$) differences were observed among the instars (Table 1).

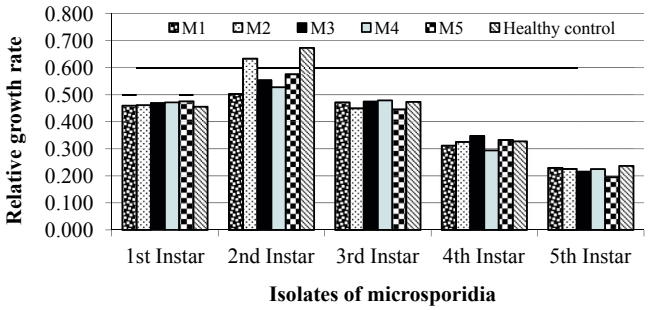

Fig. 1. Instar-wise relative growth rate of *Bombyx mori* L. infected with five isolates of microsporidia.

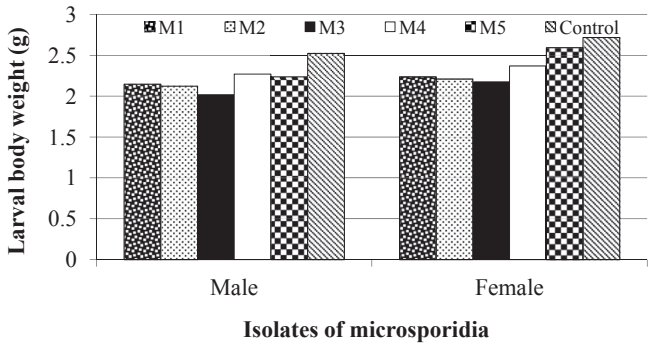

Fig. 2. Mature male and female larval body weight of *Bombyx mori* L. infected with five isolates of microsporidia.

Effect of infection on body weight and tissue somatic index

The five microsporidian isolates were also studied for their effect on the body weight and the TSI of the silkworm larva (silk gland and gonads), pupa and adult. The results revealed changes in the weight of the silkworm larval body, silk gland tissue somatic index (SGTSI) and gonad tissue somatic index (GTSI) during the 5th instar.

The mature larval weight of both healthy and microsporidian-infected silkworms indicated that all microsporidian isolates reduced their mature larval weight compared to the control. Among the microsporidia, the least body weights (2.017 g and 2.174 g for male and female respectively) were found in M3, followed by M2 (2.122 g and 2.210 g) and M1 (2.147 g and 2.236 g), whereas the healthy larva had higher values for both male (2.522 g) and female (2.717 g) as shown in Fig. 2. Significant ($p < 0.05$) differences were found among the isolates (Table 2) with respect to the control.

The TSI values of the larval silk gland ranged from 0.0847 (M5) to 0.1259 (M4) in infected larvae compared to 0.1623 in the healthy control (Fig. 3). The TSI of larval male gonads ranged from 0.0016 (M5 and M1) to 0.0027 (M3) in infected larvae compared to 0.0031 in the control, whereas in case of female larval gonads, the range was from 0.0009 (M5) to 0.0024 (M4) in infected larvae compared to 0.0026 in the control (Fig. 4). Significant ($p < 0.05$) differences were observed among the isolates with respect to the values of the larval male and female GTSI values (Table 3).

Studies on the TSI of silkworm pupal gonads (Figs. 5 and 6) revealed that the TSI values of infected male gonads ranged from 0.0218 (M2) to 0.0270 (M1) compared to 0.0276 in the control, while in infected female gonads, the range was from 0.0727 (M5) to 0.1896 (M4) against compared to 0.2969 in the control. Significant ($p < 0.05$) differences among the isolates were observed (Table 4).

The TSI values with respect to adult males were 0.0391 for M4 and 0.0471 for M2, while the M1, M3 and M5 spores of treated male pupa died before emergence. Adult female TSI values ranged from 0.3842 (M3) to 0.5807 (M1). The female pupae treated with spores of M5 isolate died before emergence (Figs. 7 and 8).

Effect of infection on reproductive potential of silkworms

The reproductive potential of *B. mori* was studied for three seasons, producing a reduction in M1, M2 and M3 whereas in M4 and M5, infected moth pairing did not take place. The results are summarized in Figs. 9–12.

Grainage and fecundity data during September and October revealed that moth emergence ranged from 12.5% (M5) to 57% (M2), whereas in the control it was 93.29%. Moth pairing was 12.5% (M3), 59.25% (M2), and 66.67% (M1) compared to 100% in the control. No pairing was observed in M4 and M5 and the average fecundity was recorded as 146 (M1), 163 (M2) and 218 (M3) compared to 369 in the control.

The results during November revealed that moth emergence ranged from 14.59% (M5) to 79.12% (M4), whereas in the control it was 95.67%. Moth pairing was 60 % (M1), 67.74% (M4), 78.76% (M2) and 79.24% (M3) compared to 99.24% in the control. No pairing was observed in M5. The average fecundity was 155 (M1), 253 (M4), 257 (M3) and 259 (M2) compared to 427 in the control. The average hatching percentage was 43.58% (M1), 58.37% (M3), 59.88% (M2) and 64.85% (M4) compared to 95.94% in the control.

Similarly for the February crop, among the isolates M5 recorded the least moth emergence (17.8%) and M4 the maximum (78.8%), compared to the control (98.6%). Here too, no pairing was observed in M5.

Significant ($p < 0.05$) differences among the isolates with respect to all four parameters (moth emergence, moth pairing, fecundity and hatching) studied were observed. However, the effect of season was only found to be significant ($p < 0.05$) on the emergence of moths (Tables 5–8).

Discussion

Relative growth rate

The RGR was higher in the early rather than in the late instars. The consumption rate, appropriate digestibility and efficiency of conversion of digested food into the body interact to determine the growth rate value of insects (Slansky and Scribe, 1985). In the present study, a general pattern of growth rate with a maximum RGR in the 2nd instar and a minimum in the 5th instar was in agreement with Madana Mohanan (2004). Generally silkworms with the longest larval duration showed the lowest RGR and with

Table 1
ANOVA for instar-wise variation in relative growth rate of *Bombyx mori* L. after microsporidian infection.

Source of variation	Sum of squares	DF	MS	F	*p*-Value	F crit
Instars	0.465367	4	0.116342	115.491382	1.60263E-13	2.866081
Treatments	0.005017	5	0.001003	0.995980	0.445220214	2.710890
Error	0.020147	20	0.001007			
Total	0.490531	29				

DF, degrees of freedom; MS, mean square; F crit, critical value of F.

Table 2
ANOVA for variation in mature male and female larval weight of *Bombyx mori* L. after microsporidian infection.

Source of variation	Sum of squares	DF	MS	F	*p*-Value	F crit
Treatment	0.37438	5	0.074876	13.99551402	0.005799	5.050329
Larval weight	0.080688	1	0.080688	15.08186916	0.0116	6.607891
Error	0.02675	5	0.00535			
Total	0.481818					

DF, degrees of freedom; MS, mean square; F crit, critical value of F.

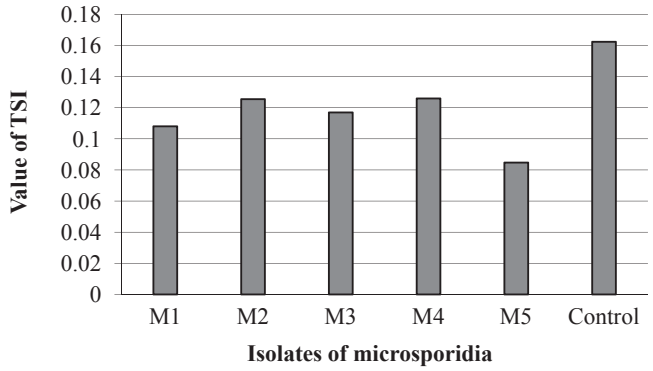

Fig. 3. Larval silk gland tissue somatic index (TSI) of *Bombyx mori* L. infected with five isolates of microsporidia.

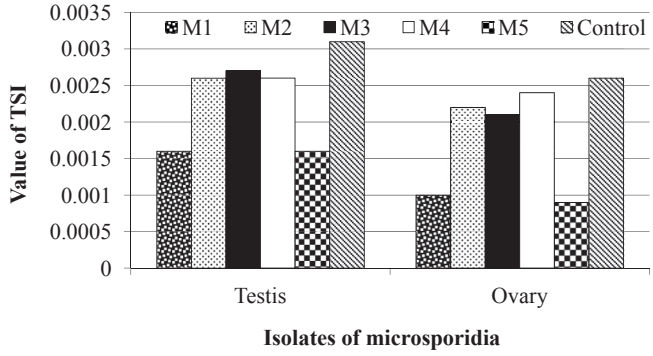

Fig. 4. Larval gonad tissue somatic index (TSI) of *Bombyx mori* L. infected with five isolates of microsporidia.

the shortest larval duration showed the highest RGR (Rema Devi et al. 1991). The average instar-wise feeding period was shortest in the 2nd instar (2.5 d) and longest in the 5th instar (6.25 d). The RGR of the 2nd instar larvae was more than double that of the 5th instar. This indicated a direct influence of the feeding period on the RGR. Earlier studies (Veber and Jasic, 1961; Gaugler and Brooks, 1975) showed that the developmental time in infected larvae was extended due to the depletion of nutritional reserves and the reduced ability to assimilate food efficiently. Krishnan and Chaudhuri (2002) reported that the RGR and the development of the 5th instar larvae of *B. mori* were significantly inhibited during the progression of nuclear polyhedrosis virus (NPV) disease. RGR is important, since it reflects the time needed to attain mature weight. If the RGR is lower than the ideal value, fitness may also be reduced because of an extended period of vulnerability to predators and parasitoids (Price et al., 1980).

M5 infection resulted in the maximum reduction in the RGR followed by M3, M4, M2 and M1. The differences in the RGRs of larvae infected with microsporidian isolates can be correlated to variations in the virulence of the isolates. The differences in the RGR between the healthy and infected larvae in the early instars were less than in the late instars. This may have been due to the increased intensity of infection in the late instars which in turn reduced the growth and development of the insects and prolonged the feeding period.

Larval weight and tissue somatic index

The mature larval body weight decreased in infected lots compared to healthy larvae. Thomson (1958) reported a reduction in the larval and pupal development and reduced weights due to infection of the spruce budworm *Choristoneura fumiferana*, by the microsporidium, *Perezia fumiferanae*. *Per os* inoculations of 4–6 day-old larvae of corn earworm, *Heliothis zea*, with *Nosema acridiophagus* or *Nosema cuneatum* retarded the growth and development of the larvae and the weights of earworm larvae inoculated with *N. acridiophagus* were significantly lower than in the uninfected larvae (Henry et al. 1979). Krishnan et al. (1998) observed a significant reduction in the mature larval weight of NPV-infected *B. mori* due to a reduction in food assimilation ability.

The TSI in modern biology offers great advantages in the understanding and interpretation of the growth of various organs of an organism (Reddy and Benchamin, 1989). Growth of tissues varies markedly with the silk gland growing relatively faster than other tissues (Ueda et al. 1971). This is more pronounced during the final instar. However, under infected conditions, the growth rate of the silk gland decreases compared to the larval body weight mass, which may be attributed to the reduced food uptake and utilization and the higher rate of pathogen multiplication and spore production in the silk gland (Madana Mohanan, 2004).

Observations indicated that microsporidian infection reduced the weight of the silk gland and the SGTSI. The decreased SGTSI after infection suggests a slow rate of silk gland growth against larval body weight due to the reduced intake of food and its utilization. In the present study, the extent of weight loss, however, could not be directly correlated to the reduced food uptake and its

Table 3
ANOVA for variation in larval gonad tissue somatic index (GTSI) of *Bombyx mori* L. after microsporidian infection.

Source of variation	Sum of squares	DF	MS	F	*p*-Value	F crit
Treatment	4.53E-06	5	9.05E-07	56.58333333	0.000212	5.050329
Larval GTSI	7.5E-07	1	7.5E-07	46.875	0.001015	6.607891
Error	8E-08	5	1.6E-08			
Total	5.36E-06	11				

DF, degrees of freedom; MS, mean square; F crit, critical value of F.

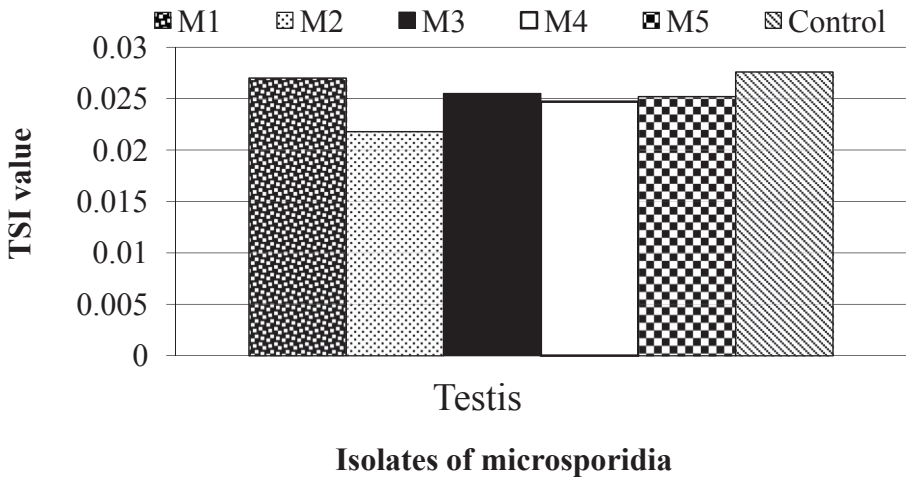

Fig. 5. Male pupa tissue somatic index (TSI) of *Bombyx mori* L. infected with five isolates of microsporidia.

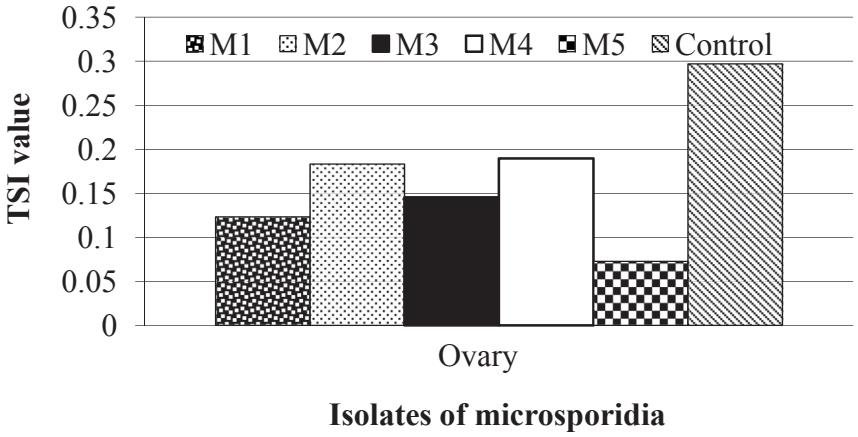

Fig. 6. Female pupa tissue somatic index (TSI) of *Bombyx mori* L. infected with five isolates of microsporidia.

Table 4
ANOVA for variation in pupal gonad tissue somatic index (GTSI) of *Bombyx mori* L. after microsporidian infection.

Source of variation	Sum of squares	DF	MS	F	*p*-Value	F crit
Treatment	0.014622	5	0.002924	1.022229	0.490670	5.050329
Pupal GTSI	0.061547	1	0.061547	21.51406	0.005641	6.607891
Error	0.014304	5	0.002861			
Total	0.090473	11				

DF, degrees of freedom; MS, mean square; F crit, critical value of F.

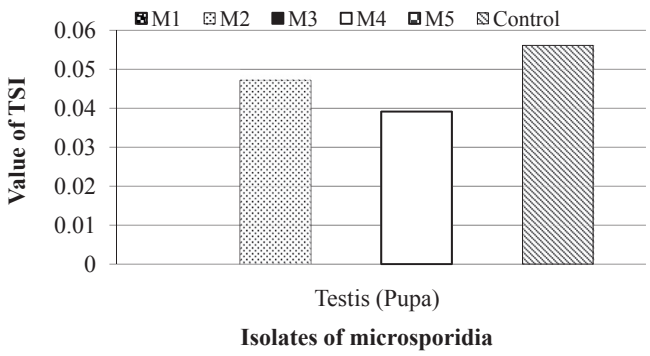

Fig. 7. Adult male tissue somatic index (TSI) of *Bombyx mori* L. infected with five isolates of microsporidia.

conversion efficiency alone, since the exploitation of nutritional resources by the invading pathogen may also be a cause for reduction in weight. Decreased protein, glycogen and cholesterol contents in silk glands (Madana Mohanan, 2004) substantiate this view. Studies on the multiplication of these microsporidian isolates in various tissues of larvae showed that in the silk gland, the pathogen multiplication and spore production occurred at a higher rate, which further emphasizes this point.

The male and female GTSI both reached their maxima in the pupal stage followed by the larval stage. The increases in the protein and nucleic acid contents concomitantly increased the male and female gonadal weights during the pupal period (Chaudhuri and Medda, 1986). These changes are indications of the enhancement of spermatogenesis during the pupal stage of males and of increased synthesis by the follicular cells of the vitellogenic ovary

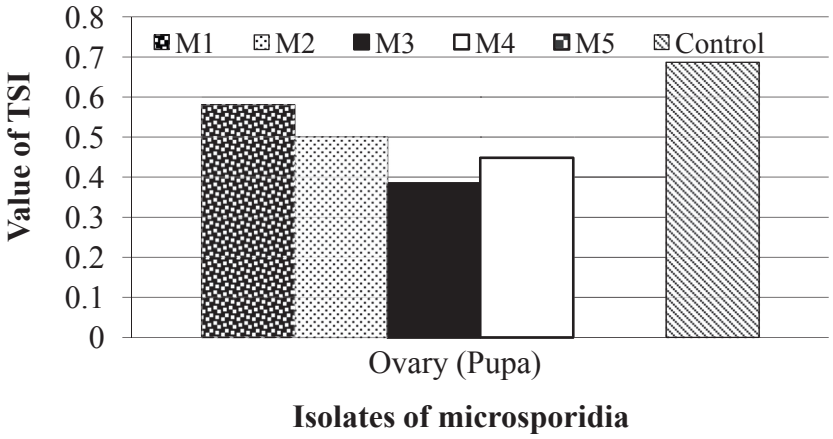

Fig. 8. Adult female tissue somatic index (TSI) of *Bombyx mori* L. infected with five isolates of microsporidia.

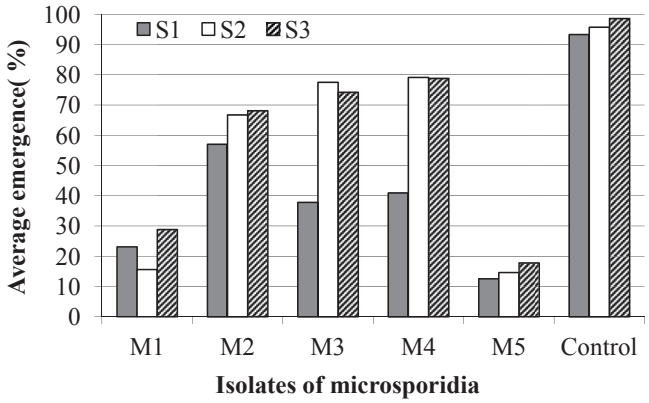

Fig. 9. Seasonal variation in moth emergence of *Bombyx mori* L. infected with five isolates of microsporidia.

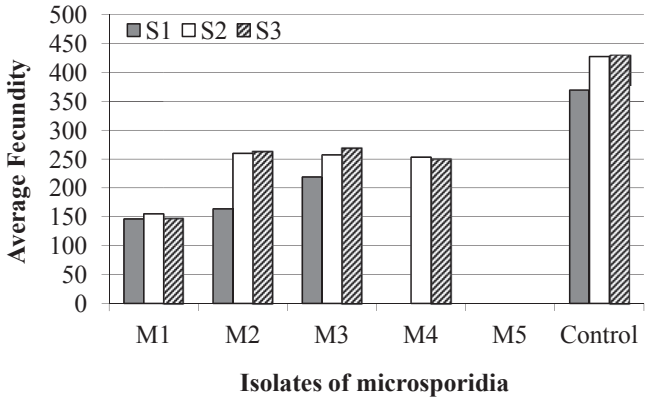

Fig. 11. Seasonal variation in fecundity of eggs of *Bombyx mori* L. infected with five isolates of microsporidia.

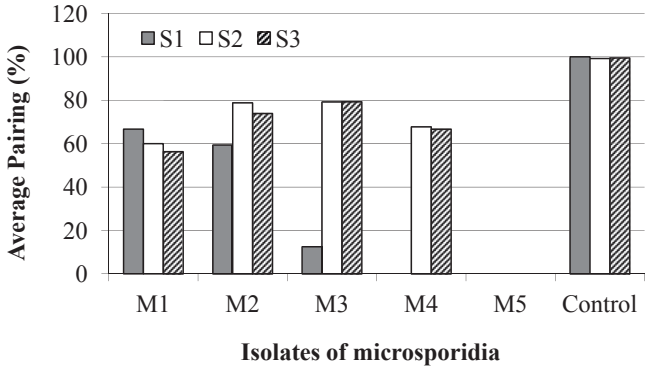

Fig. 10. Seasonal variation in moth pairing of *Bombyx mori* L. infected with five isolates of microsporidia.

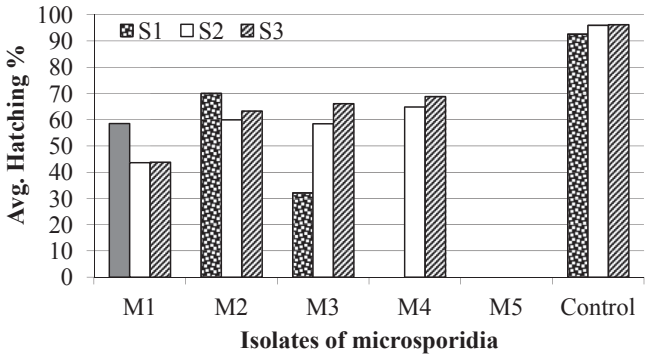

Fig. 12. Seasonal variation in hatching of eggs of *Bombyx mori* L. infected with five isolates of microsporidia.

(Anderson and Telfer, 1969; Bast and Telfer, 1976; Glass and Emmerich, 1981) in females. The reduced GTSI in male-infected lots and female-infected lots could also be correlated to the possible involvement of the host endocrine system. All the major proteins are synthesized by the fat body and secreted into the haemolymph for their temporary storage and are then resequestered into the fat body for deposition in the form of dense proteinaceous granules (Chipendale and Kilby, 1969; Locke, 1981; Tojo et al. 1981). Microsporidian infection in the fat body might have reduced the protein turnover of the host, which ultimately affected the normal growth and development of the gonad. Compared to larval weight, the male and female gonadal weights were much less, which may have been due to the low intensity of infection in the gonads or to no infection during early stages. On the other hand, the significant reduction in the larval weight may be attributed to the higher infection in the gut, silk gland and fat body. Similar trends were observed in adult male and female moths. Since M5 was the most virulent among the isolates, no male or female moth emergence took place. However the M1-infected and M3-infected

Table 5
ANOVA for variation in moth emergence of *Bombyx mori* L. infected with microsporidian isolates.

Source of variation	Sum of squares	DF	MS	F	*p*-Value	F crit
Treatment	13801.87	5	2760.374	24.28831	2.710223E-05	3.325835
Season	988.47	2	494.235	4.348733	0.04376089	4.102821
Error	1136.503	10	113.6503			
Total	15926.85	17				

DF, degrees of freedom; MS, mean square; F crit, critical value of F.

Table 6
ANOVA for variation in moth pairing of *Bombyx mori* L. infected with microsporidian isolates.

Source of variation	Sum of squares	DF	MS	F	*p*-Value	F crit
Treatment	16197.42	5	3239.483	8.094737	0.002732881	3.325835
Season	2241.191	2	1120.596	2.800115	0.108229317	4.102821
Error	4001.962	10	400.1962			
Total	22440.57	17				

DF, degrees of freedom; MS, mean square; F crit, critical value of F.

Table 7
ANOVA for variation in fecundity of eggs of *Bombyx mori* L. infected with microsporidian isolates.

Source of variation	Sum of squares	DF	MS	F	*p*-Value	F crit
Treatment	271036.9	5	54207.38	18.66693	8.77701E-05	3.325835
Season	23394.77	2	11697.38	4.028128	0.052102863	4.102821
Error	29039.26	10	2903.926			
Total	323470.9	17				

DF, degrees of freedom; MS, mean square; F crit, critical value of F.

Table 8
ANOVA for variation in hatching of eggs of *Bombyx mori* L. infected with microsporidian isolates.

Source of variation	Sum of squares	DF	MS	F	*p*-Value	F crit
Treatment	14269	5	2853.8	9.080295	0.001760606	3.325835
Season	682.4844	2	341.2422	1.085774	0.374344173	4.102821
Error	3142.849	10	314.2849			
Total	18094.33	17				

DF, degrees of freedom; MS, mean square; F crit, critical value of F.

male pupae also died before emergence. The differences in the reduced gonadal weight and somatic index in different isolates may have been due to variation in their levels of virulence.

Reproductive potential

Several reports are available on the adverse effects of *N. bombycis* on insect tissues, reproductive potential and fertility (Steinhaus, 1949; Yup-lian, 1995; Bansal et al. 1997). Reduced fecundity and egg hatching in microsporidian-infected silkworm may not only be due to high temperature (>29 °C) but also due to severe damage of the fat body and gonads (Madana Mohanan et al., 2005). Damage to the reproductive tissues and the dissolution of muscular tissues following infection were possible reasons for the reduced fecundity in insects (Hassanein, 1951; Smirnoff and Chu, 1968; Yup-lian 1995) and which also could be contributing factors toward increased sterility and mortality of eggs. It has also reported that microsporidia depleted the nutritive reserves used for reproduction and thereby reduced fecundity (Thomson, 1958; Veber and Jasic, 1961; Smirnoff and Chu, 1968) and fertility (Tanabe and Tamashiro, 1967). In the present study, severely infected male and female adults did not mate properly which could be a reason for sterility in the infected eggs. Embryonic development may have ceased due to embryonic infection causing the death of eggs and an increased number of sterile and dead eggs (Yup-lian 1995).

Microsporidian variation in fecundity, hatching, sterility and mortality of eggs may be due to variation in virulence of the microsporidia. This study helped to explain the adverse effect of microsporidia on reproductive potential of *B. mori*.

All five microsporidian isolates considered were found to be pathogenic but differed in their virulence as revealed by this study with respect to their effect on the silkworm larval RGR and body weight, the TSI of larva (silk gland and gonads), pupae and adults and the reproductive potential. Among the isolates, M5 was found to be the most virulent.

Conflict of interest

There is no conflict of interest.

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