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Microsporidian Multiplication and Spore Production in Various Tissues of Pupa and Adult, in Relation to Age and Development of Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae)

Madana Mohanan Nanu*, Sunil Kumar Gupta[1], Beera Saratchandra[2] and Durga Prasad Haldar[3]

Silkworm Seed Production Centre, National Silkworm Seed Organization, Central Silk Board, Palakkad 678 551, Kerala, India
[1]Silkworm Pathology Laboratory, Central Sericultural Research and Training Institute, Berhampore 742 101, West Bengal, India
[2]Central Silk Board, Madivala 560 068, Bangalore, Karnataka, India
[3]Protozoology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741 235, West Bengal, India

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Multiplication and spore production of three microsporidia (*Nosema bombycis*, *Nosema* sp. 1 and *Nosema* sp. 2) in selected tissues of pupa and adult of silkworm, *Bombyx mori* L. were studied in two seasons (SI, SII) with distinct temperature (SI: 20.1±0.8°C and SII: 25.1±0.7°C) regimes. Multiplication of the microsporidia followed a logistic pattern with a lag phase, an exponential phase and a stationary phase. In SII, spore production was significantly (P<0.01) higher in various tissues. Highest spore production was observed 30 days post inoculation (p.i.) in SI and in SII, it was 21~23 days p.i. Spore production was significantly (P<0.01) higher in the gut tissues than other tissues. *Nosema* sp. 2 registered significantly (P<0.01) higher spore production in both the seasons compared to *Nosema bombycis* and *Nosema* sp. 1. Results indicate that the multiplication and spore production of microsporidia are tissue specific and extremely sensitive to the temperature at which the host is reared. Through this study, the precise day that the spore numbers of the microsporidia are maximized can be predicted in both pupa and adult in case the infection is initiated in the first instar.

Key words: Microsporidian, Spore, Multiplication, Tissue, *Bombyx mori*, Temperature

*To whom the correspondence addressed
Silkworm Seed Production Centre, National Silkworm Seed Organization, Central Silk Board, Kodumbu.P.O., Erattayal 678 551, Palakkad (Dist.), Kerala (India). Tel: 0491- 2572699; E-mail: madanamohanann@rediffmail.com

Introduction

Microsporidia are eukaryotic, intracellular, obligatory parasites of all animal phyla and are common in arthropods (Larsson, 1988; Sprague, 1977). They are characterized by infectious spores with unique organelles for their invasion into the host (Undeen, 1997). After entering into the host cell, the pathogen begins development either asexually or sexually leading to the production of new generation of infective spores. The pathogen multiplication and spore yield gives an indication of its development (Becnel and Undeen, 1992). Various workers studied multiplication of microsporidia (Fowler and Reeves, 1975; Lai and Canning, 1980, 1983; Pilley *et al*., 1978). In *Pieris brassicae*, the pathogen yielded maximum spores only when injected into hemocoel (Lai and Canning, 1983). Similarly, the influence of temperature on development of parasite/host system of *Edhazardia aedis* (Microsporidia) and *Aedes aegypti* was studied (Becnel and Undeen, 1992). The rate of spore production of *Nosema bombycis* L. in pupae and adults of *Bombyx mori* L. in relation to development of the host was studied (Sasidharan *et al*., 1994). Microsporidian multiplication and spore production is dependent on age of the host (Lai and Canning, 1983; Whitlock and Brown, 1991), temperature (Fowler and Reeves, 1975), spore dosage (Hostounsky and Weiser, 1972; Lai and Canning 1983) and even development of the host from one stage to another stage (Lai and Canning, 1983).

Pebrine, a protozoan disease of silkworm, *Bombyx mori* L. caused by *Nosema bombycis* Nageli (Dihaplophasia, Nosematidae) of phylum microsporidia. Unlike other silkworm diseases, it shows both horizontal and vertical type

of transmission. The horizontal transmission occurs through contaminated leaf and vertical transmission through infected eggs. Microsporidia infecting tropical tasar silkworm, *Antheraea mylitta* D. and mulberry pest, *Diacrisia obliqua* W. were found to be cross-infective to *Bombyx mori* L. and their infection lead to retarded growth and development in the host (Madana Mohanan, 2004; Madana Mohanan *et al*., 2004). The best method for checking pebrine disease is the systematic examination of mother moth after oviposition and elimination of pebrine infected eggs (Pasteur, 1870). However, some times identification of pebrine infection through individual mother moth examination becomes difficult when infection is late/low. Thus, it was decided to under take a systematic investigation on tissue specific multiplication and spore production of above microsporidia in relation to development of the host. The study would help in the proper selection of tissues for the microscopic examination and makes the examination more easy and accurate.

Materials and methods

Insect and food plant

The multivoltine mulberry silkworm, *Bombyx mori* L. breed Nistari (marked) fed on mulberry (*Morus alba* cv S1) leaves was used for the study.

Microsporidia

Three microsporidia, *Nosema bombycis*, *Nosema* sp.1 and *Nosema* sp. 2 isolated from *Bombyx mori* L., *Antheraea mylitta* D. and *Diacrisia obliqua* W. respectively were selected for the investigation.

Microsporidian inoculation

Spore inoculum was prepared by collecting spores from microsporidian infected adults and purified by iso-density equilibrium centrifugation using percoll i.e., Polyvinyl silica particles (Sato and Watanabe, 1980). Healthy larvae of uniform size (average weight 0.419 g) were separated into 50 groups of 10 after fourth moult (1st day of 5th instar). The larvae were starved for 3~4 h to induce hunger and 250 µl of spore suspension (1×10^6/ml) in Endotoxin Free (ETF) water obtained from Milli Q system was smeared on a fresh mulberry leaf disc (22.26 cm sq). Spore count was enumerated using a Neubauer hemocytometer (Cantwell, 1970). Each larval group was exposed to one leaf disc smeared with microsporidian inoculam. Moist foam pads were placed around the larvae and covered with paraffin paper to prevent the drying of leaves. After complete consumption of the treated leaves, the larvae were pooled and reared in four replications with 125 larvae/replication in wooden trays (60 cm × 40 cm). All the three microsporidia were inoculated in the same method and the larvae reared till cocooning and adult eclosion.

Tissue processing and determination of spore count

From the first day of pupation (0 day pupa), 10 pupae were collected at random from each replication (irrespective of sex) on alternate days. Whole pupa, thorax and abdomen were weighed separately (Sartorius Monopan Electronic balance) and homogenized in a Teflon coated Potter and Elvejam tissue homogenizer (local make) using suitable quantity of 0.6% K_2CO_3 solution. The homogenate was then filtered through a cotton pad of 3~4 layers. The tissue debris collected in the cotton pad was resuspended in fresh K_2CO_3 solution, agitated on a cyclomixer and again filtered to collect any spores entangled in the debris. The filtrate was centrifuged (REMI R4C Model centrifuge with swing out rotor) at 3000 rpm for 3 m to sediment the spores (Rekha *et al*., 1998). After decanting the supernatant, the sediment was suspended in 1ml ETF water. The gut and fat body tissues were dissected out from individual insect in insect saline (0.65% NaCl) and weighed after blotting out excess water. Each pooled tissue was homogenized in 0.6% K_2CO_3 solution and after centrifugation sediment was suspended in 1 ml ETF water. The spore count was assessed in a Neubauer Hemocytometer counting chamber (Cantwell, 1970) and is expressed in per gram tissue wet weight.

Spore concentrations were also determined for whole body, thorax, abdomen, gut and fat body of the emerged adults at 1st, 2nd, 3rd, 4th and 5th day post emergence using the above methods. The studies were undertaken in two different seasons with distinct average temperatures and relative humidity [November- December (SI): Temp. 20.1 ±0.8°C, RH 72.6 ±6.2% and February-March (SII): Temp. 25.1 ±0.7°C, RH 83.5 ±7.0%]. The data were subjected to ANOVA and means were compared for significant difference.

Results

The duration of experiment was 30 days in SI and 23 days in SII. Among the microsporidia, *Nosema* sp. 2 yielded significantly ($P < 0.01$) higher numbers of spore in all the tissues in both the seasons than did the other two microsporidia. In SII, spore yield was significantly ($P < 0.01$) higher than SI. Gut tissues produced the highest number of spores in both the seasons which was significantly ($P < 0.01$) higher than other tissues and abdomen yielded the least. In SI, spore production in most of the tissues of pupa slowly increased until 9 days post pupation

(21 days p.i.) and then sharply increased on the 11th or 13th day. The spore production from tissues for 13-day pupae was significantly ($P<0.01$) higher than that for 9-day pupae. In SI, maximum spore production was observed in 4~5 day adults (29~30 days p.i.). In SII, spore yield reached the maximum in gut and abdomen of 3-day adult (21 days p.i.) whereas it reached highest in thorax and fat body of 4-day adult (22 days p.i) with production highest in the gut and lowest in the abdomen.

In SI, among the microsporidia, in general *Nosema* sp. 2 produced the highest number of spores in various tissues. In this season, *Nosema* sp. 2 produced the highest number of spores in gut tissues (205.24×10^6 g^{-1} tissue) of 5-days aged adult (30 days p.i.) and least in the abdomen (63.44×10^6 g^{-1} tissue), which was 1.7 and 5.3 times more than *Nosema bombycis* and 1.35 and 1.38 times more than *Nosema* sp.1 respectively. Similarly, in SII, *Nosema* sp. 2 produced the highest number of spores in the gut tissues (197.7×10^6 g^{-1} tissue) and lowest in abdomen (64.6×10^6 g^{-1} tissue) both on 21 days p.i., which was 1.8 and 3.4 times higher than *Nosema bombycis* respectively. In the same season, *Nosema* sp.1 produced 1.4 times less spores in gut and 2.8 times less spores in abdomen than *Nosema* sp. 2 (Table 1~4).

In SII, spore yield was higher in various tissues. In SII, various microsporidian species produced 3.7~4.1 times more spores in the gut tissues than SI after 23-days p.i. Similarly, in this season, these microsporidia produced

Table 1. Spore yield of three microsporidia in various tissues of silkworm, *Bombyx mori* L during season-I. Data in parenthesis are logarithmic transformed values

Body part/ Tissue	Microsp-oridia	Spore yield ($\times 10^6$ g^{-1}. wet wt. of tissue) at various days post inoculation											
		13	15	17	19	21	23	25	26	27	28	29	30
		Pupal Stage							Adult Stage				
Whole Body	*Nosema bombycis*	0.18 (0.07)	0.56 (0.19)	0.74 (0.24)	1.22 (0.34)	2.12 (0.49)	2.94 (0.59)	4.09 (0.70)	10.90 (1.07)	17.56 (1.27)	22.17 (1.36)	29.86 (1.49)	23.88 (1.40)
	Nosema sp1	0.28 (0.11)	0.67 (0.22)	1.58 (0.41)	1.76 (0.44)	2.15 (0.50)	2.95 (0.59)	5.15 (0.79)	28.43 (1.47)	79.79 (1.91)	100.41 (2.01)	125.45 (2.10)	105.26 (2.03)
	Nosema sp2	0.33 (0.12)	1.25 (0.35)	1.67 (0.43)	1.95 (0.46)	2.37 (0.53)	2.94 (0.59)	21.67 (1.35)	91.61 (1.97)	94.54 (1.98)	125.46 (2.10)	141.37 (2.15)	138.20 (2.14)
Thorax	*Nosema bombycis*	0.32 (0.12)	0.84 (0.26)	0.94 (0.29)	1.15 (0.33)	1.45 (0.39)	1.97 (0.47)	2.88 (0.58)	16.85 (1.25)	23.20 (1.38)	29.05 (1.48)	24.50 (1.40)	16.50 (1.24)
	Nosema sp. 1	0.83 (0.26)	1.07 (0.31)	1.22 (0.34)	1.57 (0.41)	1.63 (0.42)	2.34 (0.52)	9.18 (1.01)	17.50 (1.26)	27.90 (1.46)	38.90 (1.60)	42.45 (1.64)	34.51 (1.55)
	Nosema sp. 2	1.05 (0.31)	1.17 (0.33)	1.55 (0.40)	1.67 (0.43)	2.40 (0.53)	5.46 (0.81)	12.64 (1.13)	33.24 (1.53)	54.21 (1.74)	138.63 (2.14)	145.56 (2.17)	132.02 (2.12)
Abdomen	*Nosema bombycis*	0.023 (0.01)	0.33 (0.12)	0.43 (0.15)	0.54 (0.19)	0.91 (0.28)	2.11 (0.49)	2.59 (0.55)	2.11 (0.49)	2.79 (0.58)	5.42 (0.80)	7.66 (0.93)	12.05 (1.11)
	Nosema sp. 1	0.018 (0.01)	0.40 (0.15)	0.46 (0.16)	0.60 (0.20)	1.09 (0.31)	5.39 (0.80)	10.47 (1.06)	13.13 (1.15)	22.28 (1.36)	31.88 (1.52)	33.50 (1.54)	45.85 (1.67)
	Nosema sp. 2	0.030 (0.01)	0.46 (0.16)	0.47 (0.17)	0.73 (0.24)	1.37 (0.37)	12.72 (1.13)	13.21 (1.15)	24.26 (1.40)	29.97 (1.49)	42.68 (1.64)	52.34 (1.73)	63.44 (1.81)
Gut	*Nosema bombycis*	1.55 (0.41)	2.05 (0.48)	3.22 (0.62)	3.47 (0.65)	3.80 (0.68)	29.16 (1.48)	34.41 (1.55)	40.64 (1.62)	49.66 (1.70)	69.42 (1.85)	93.94 (1.98)	119.95 (2.08)
	Nosema sp. 1	1.63 (0.42)	2.28 (0.51)	3.84 (0.66)	4.51 (0.74)	5.33 (0.80)	33.61 (1.54)	54.43 (1.74)	65.54 (1.82)	71.48 (1.86)	84.45 (1.93)	161.33 (2.21)	151.73 (2.18)
	Nosema sp. 2	2.17 (0.50)	2.88 (0.59)	4.03 (0.69)	6.73 (0.89)	7.63 (0.93)	40.58 (1.62)	58.83 (1.78)	66.73 (1.83)	76.53 (1.89)	155.02 (2.19)	188.95 (2.28)	205.24 (2.31)
Fat Body	*Nosema bombycis*	0.22 (0.09)	0.25 (0.10)	0.24 (0.09)	0.26 (0.10)	0.36 (0.13)	1.83 (0.44)	2.03 (0.48)	6.10 (0.85)	7.95 (0.95)	10.80 (1.07)	20.75 (1.34)	17.07 (1.25)
	Nosema sp. 1	0.27 (0.10)	0.28 (0.11)	0.30 (0.11)	0.43 (0.15)	0.66 (0.22)	3.64 (0.66)	6.54 (0.87)	19.59 (1.31)	39.17 (1.60)	47.25 (1.68)	55.82 (1.75)	51.81 (1.72)
	Nosema sp. 2	0.28 (0.11)	0.37 (0.14)	0.41 (0.15)	0.48 (0.17)	1.42 (0.38)	3.65 (0.67)	8.31 (0.97)	24.77 (1.41)	44.81 (1.66)	50.96 (1.71)	74.03 (1.87)	67.92 (1.84)

Table 2. ANOVA for spore production of three microsporidia in various tissues of *Bombyx mori* L.in relation to development of the host in season-I.

Source	DF	SS	MS	F	P<0.05
Microsporidia (M)	2	8.02	4.00	1886.30**	0.009
Tissue (T)	4	6.38	1.59	749.87**	0.012
Day (D)	9	6.67	0.74	348.90**	0.017
M × T	8	22.33	2.79	1313.29**	0.020
M × D	18	9.71	0.54	253.75**	0.029
T × D	36	85.57	2.38	1118.22**	0.037
M × T × D	72	149.38	2.08	976.08**	0.064
Error	450	0.96	0.002		
Total	599	289.01			

14.9~43.6, 14.5~25.7, 3.6~7.4 and 16.8~32.6 times more spores than SI in whole body, thorax, abdomen and fat body respectively after 23 days p.i.

It was assumed that all the larvae consumed equal quantities of leaves smeared with spore and hence each larva received a spore load of 1×10^4. From this, rate of multiplication of each microsporidium in various body parts/ tissues was calculated considering the initial number of spores consumed, maximum number of spores produced and time (in days) taken. Rate of multiplication of microsporidia was higher in SII and among the tissues multiplication was maximum in the gut and minimum in the abdomen. In SII, in the gut *Nosema bombycis*, *Nosema* sp.1 and *Nosema* sp. 2 were produced 574, 652 and 941

Table 3. Spore yield of three microsporidia in various tissues of silkworm, *Bombyx mori* L. during season-II. Data in parenthesis are logarithmic transformed values

Body part/ Tissue	Microsp-oridia	Spore yield ($\times 10^6$ g^{-1}. wet wt. of tissue) at various days post inoculation									
		10	12	14	16	18	19	20	21	22	23
		Pupal Stage						Adult Satge			
Whole Body	*Nosema bombycis*	0.71 (0.21)	0.96 (0.28)	3.26 (0.63)	4.31 (0.72)	6.07 (0.85)	12.10 (1.11)	27.02 (1.44)	32.30 (1.52)	41.07 (1.62)	43.88 (1.65)
	Nosema sp1	0.90 (0.28)	2.60 (0.55)	7.67 (0.94)	13.42 (1.16)	17.54 (1.27)	17.75 (1.27)	28.51 (1.47)	38.20 (1.59)	47.91 (1.69)	66.44 (1.83)
	Nosema sp2	1.29 (0.36)	9.73 (1.03)	19.81 (1.31)	21.73 (1.35)	22.63 (1.37)	23.26 (1.38)	53.51 (1.74)	85.49 (1.94)	123.88 (2.10)	128.10 (2.11)
Thorax	*Nosema bombycis*	0.29 (0.10)	1.57 (0.40)	3.15 (0.61)	3.65 (0.66)	7.27 (0.92)	12.58 (1.13)	16.97 (1.25)	19.34 (1.31)	30.63 (1.50)	28.62 (1.47)
	Nosema sp1	0.60 (0.20)	3.83 (0.68)	13.27 (1.15)	22.54 (1.37)	25.18 (1.41)	25.94 (1.43)	28.78 (1.47)	45.77 (1.67)	47.81 (1.69)	47.48 (1.69)
	Nosema sp2	2.26 (0.51)	15.17 (1.21)	35.47 (1.56)	39.01 (1.60)	43.78 (1.65)	45.01 (1.66)	56.32 (1.76)	98.61 (2.00)	149.51 (2.18)	140.57 (2.15)
Abdomen	*Nosema bombycis*	0.02 (0.01)	0.88 (0.27)	2.18 (0.50)	2.59 (0.55)	3.31 (0.63)	5.70 (0.82)	13.67 (1.16)	18.80 (1.30)	19.51 (1.31)	15.51 (1.21)
	Nosema sp1	0.42 (0.15)	1.86 (0.46)	4.95 (0.77)	7.81 (0.94)	11.96 (1.11)	14.72 (1.20)	18.59 (1.29)	23.35 (1.38)	25.76 (1.43)	22.09 (1.36)
	Nosema sp2	1.23 (0.34)	11.11 (1.08)	13.66 (1.16)	15.33 (1.21)	23.26 (1.38)	50.92 (1.72)	60.65 (1.79)	64.60 (1.82)	47.37 (1.68)	45.77 (1.67)
Gut	*Nosema bombycis*	0.29 (0.11)	3.84 (0.68)	4.20 (0.71)	23.79 (1.39)	58.54 (1.77)	79.18 (1.90)	105.48 (2.03)	110.95 (2.05)	126.19 (2.10)	118.08 (2.08)
	Nosema sp1	1.17 (0.34)	8.11 (0.96)	22.46 (1.37)	66.32 (1.83)	94.49 (1.98)	99.25 (2.00)	123.60 (2.10)	137.71 (2.14)	143.54 (2.16)	135.11 (2.13)
	Nosema sp2	3.42 (0.64)	30.20 (1.49)	40.79 (1.62)	124.88 (2.10)	130.20 (2.12)	136.54 (2.14)	178.14 (2.25)	197.70 (2.30)	163.22 (2.21)	150.95 (2.18)
Fat Body	*Nosema bombycis*	0.19 (0.07)	0.50 (0.17)	4.92 (0.76)	15.00 (1.20)	15.99 (1.23)	20.49 (1.33)	26.67 (1.44)	55.96 (1.75)	62.58 (1.80)	55.20 (1.75)
	Nosema sp1	0.54 (0.19)	2.99 (0.60)	10.76 (1.07)	22.07 (1.36)	31.97 (1.52)	33.66 (1.54)	57.73 (1.77)	71.64 (1.86)	63.90 (1.81)	61.06 (1.79)
	Nosema sp2	0.91 (0.28)	19.65 (1.31)	23.78 (1.39)	40.10 (1.61)	45.29 (1.66)	46.34 (1.67)	81.56 (1.92)	98.96 (2.00)	118.26 (2.08)	119.11 (2.08)

Table 4. ANOVA for spore production of three microsporidia in various tissues of *Bombyx mori* L. in relation to development of the host in season-II.

Source	DF	SS	MS	F	P< 0.05
Microsporidia (M)	2	27.90	13.95	5387.78**	0.010
Tissue (T)	4	26.83	6.71	2590.07**	0.013
Day (D)	9	143.95	15.99	6176.79**	0.018
M x T	8	1.18	0.15	57.03**	0.022
M x D	18	3.71	0.21	79.65**	0.032
T x D	36	5.48	0.15	58.74**	0.041
M x T x D	72	2.32	0.032	12.46**	0.071
Error	450	1.17	0.003		
Total	599	212.54			

spores/day, whereas, the same in SI was 400, 556 and 684 spores/day respectively. In SII, in the abdomen *Nosema bombycis*, *Nosema* sp.1 and *Nosema* sp.2 were produced 89, 117 and 308 spores/day respectively in comparison to 40, 153 and 211 spores/day in SI. Among the microsporidia, *Nosema* sp. 2 showed the highest multiplication (308~941 spores/day) followed by *Nosema* sp.1 (117~652 spores/ day) and *Nosema bombycis* (89~574 spores/day) in various tissues in SII. In SI also same trend was followed with highest multiplication in *Nosema* sp. 2 (211~684 spores/day), followed by *Nosema* sp.1 (146~556 spores/ day) and *Nosema bombycis* (40~400 spores/day).

Discussion

Microsporidian development can be influenced by a number of environmental factors among those, which have been commonly evaluated are temperature and host nutritional sources and levels. Because these intracellular parasites are so closely adapted to and dependent on their hosts, it is difficult to determine whether a factor directly influences the parasite or indirectly affects the parasite via host effects. The influence of environmental factors, therefore, is best considered as the influence on the host-parasite system. In our study, multiplication of all the three microsporidia in silkworm pupae and adults followed a logistic pattern, which conforms to the three stages of development, a lag phase, an exponential phase and a stationary phase of *Vairimorpha* (=*Nosema*) *necatrix* in *Trichoplusia ni* and *Heliothis zea* described by Fowler and Reeves (1975). A similar pattern of spore replication has been reported for *Nosema algerae* in *Pieris brassicae* (Lai and Canning, 1983) and in silkworm, *Bombyx mori* (Sasidharan *et al*., 1994). The lag phase suggests that a certain population density of vegetative forms must be attained before development proceeds to spore stage (Maddox, 1968). The lag phase of tested microsporidia in *B. mori* was 5~7 days in SI and 4~5 days in SII. Weiser (1978) explained the stationary phase in infections of *Vairimorpha* (=*Nosema*) *plodiae* and *V.* (=*N*osema) *heterosporum* in *Gulleria mellonella* as the period, when the fat tissue available in the insect had been completely utilized by the parasites, so that there could be no further increase in parasite number.

The ecologically relevant temperature and relative humidity range for the normal development of silkworm, *B. mori* is 25 ± 1°C and 70 ± 5% respectively (Dandin *et al*., 2000). In SII, a steady increase in spore production was observed during the exponential phase in all the tissues irrespective of microsporidia and maximum spore production was obtained after an incubation period of 22 days. But, in SI, from 12 days p.i. to 22 days p.i., very low rate of multiplication occurred. A sharp increase was noticed, however, when the insects were undergoing pupal-adult transformation and the highest spore counts were obtained after an incubation period of 29 ~ 30 days. Similar results were also obtained from *P. brassicae* (Lai and Canning, 1983), where a sudden increase in spore production was observed on the 19th and 20th days p.i., when the pupae were transitioning to imago. It is possible that physiological changes in the host caused the developmental cycle of the parasites to switch from a predominantly vegetative phase to sporogony, resulting in an increase in spore production.

In SII, the exponential replication (>) phase lasted approximately 13 ~ 14 days, whereas this phase was prolonged to 17 ~ 18 days in SI. In this respect, all the three microsporidia behaved in a similar manner. In general, a stationary/ declining phase was observed on day 22 p.i. in SII and on day 29 ~ 30 p.i. in SI with a few exceptions in SI, where the pathogens were still in the exponential phase. Fowler and Reeves (1975) observed that the length of "spore exponential phase" changed with the temperature at which the host was cultured. They found that in cabbage looper, *Trichoplusia ni* and corn earworm, *Heliothis zea,* the exponential phase of pathogen multiplication varied, when the hosts were cultured at different temperatures. They explained that the prolonged "exponential phase" observed at high and low temperature extremes was considered to be the effect of temperature on the metabolic state of the host and parasite. In the present study, "exponential phase" was not tested at high and low temperature extremes and yet a prolonged "exponential phase" was noticed in SI, when average temperature was low.

As observed in pupae, the spore count in adults was highest in gut tissues and lowest in abdomen. However, the highest spore count was observed in SI. The higher final spore numbers in some of the body parts and tissues in SI

adults compared to SII adults may be due to the prolonged larval and pupal stages in SI. In a similar study with *Nosema bombycis* in two silkworm races, one multivoltine (Pure Mysore) and one bivoltine (NB_{18}), Sasidharan *et al.* (1994) reported highest spore production in the wings, followed by gut then fat body in NB_{18} pupae and thereafter showed a declining trend. But, in adults, they found the highest spore production in cephalothorax followed by wings then gut and finally fat body. In adults, a continuous increase in spore production was noticed from 0 h to 96 h post emergence and in this respect results of the present study do not agree. In the present work, the highest spore numbers were obtained from gut (wings were not taken for this study), followed by thorax, with lowest numbers in the abdomen. Unlike infections in Pure Mysore and NB_{18}, in Nistari (m), all the microsporidia showed a continuous increase in spore production in all the tissues of pupae tested. The same trend of increasing numbers was observed in adults up to 3 days post emergence and then decreased or remained same.

Compared to production of *Nosema bombycis* spores in Pure Mysore, production was higher in Nistari (m) even though temperature (SII) was more or less the same. This difference in spore production may be due to differences between the silkworm breeds or spore concentration of the initial inoculum, which was 1×10^7/ml for Pure Mysore and 1×10^6/ml for Nistari (m). Hostounsky and Weiser (1972) obtained similar spore numbers from *Mamestra brassicae* with different dosages (5×10^3, 5×10^4 and 7.2×10^5) of *V. plodiae* spores. They allowed the microsporidium to develop for 16~18 days until the spore numbers had reached the stationary phase. Teetor and Kramer (1976) studied the effect of dosage on the level of parasitism of *Octosporea muscaedomesticae* in *Phormia regina* and found that, when a trial was allowed to continue through several parasite generations, the dose effect became clouded, when the maximum spore carrying capacity of the host was reached. Henry *et al.* (1979) obtained the highest spore numbers in *Melanoplus sanquinipes* inoculated with 10^6 spores of either *Nosema acridiophagus* or *N. cuneatum*, when three dosages 10^4, 10^5 and 10^6 were fed, but efficiency of spore production was best in those insects inoculated with 10^5 spores of either microsporidium. Lai and Canning (1983) found that higher spore doses resulted in higher spore production, but in contrast to total spore yields, lower spore dosages resulted in higher replication rates of *Nosema algerae* in *Pieris brassicae*. Spore production figures from a low dosage are considerably higher than from a high initial dose and investigators explained the difference by the fact that a small number of parasites in a given amount of host tissue can proliferate without restriction, while the proliferation of a large number of parasites in a similar amount of tissue is limited.

In general, fewer spores were recorded in the whole body and abdomen than in gut, thorax and fat body in both the seasons, especially in the pupal stage. The reason for such a result could be that in the whole body and abdomen both infected and uninfected tissues are in the homogenates, but gut, thorax and fat body homogenate was obtained only from infected tissues and when spore count was expressed in per gram wet tissue there is the chance of dilution in the case of whole body and abdomen.

Among the microsporidia, *Nosema* sp. 2 produced more spores, followed by *Nosema* sp.1 and *Nosema bombycis* in most of the body parts and tissues for both the seasons. The spore counts for *Nosema* sp. 2 and *Nosema* sp.1 were significantly higher than those for *Nosema bombycis*. This difference in spore production is a reflection of the host-parasite interaction and possibly in virulence. Results on lethal concentration and host mortality (Madana Mohanan *et al.*, 2004) indicated that *Nosema* sp.2 as the most virulent, followed by *Nosema* sp.1 and *Nosema bombycis*. *Nosema bombycis* used for the investigation was obtained from a laboratory culture maintained through animal passage (*Bombyx mori* L.) in Central Sericultural Research and Training Institute, Berhampore, West Bengal (India). But, the other two microsporidia, *Nosema* sp.2 and *Nosema* sp.1 were isolated from *Diacrisia obliqua* W. and *Antheraea mylitta* D. respectively. Since, *Nosema bombycis* was maintained through animal passage in silkworm, *B. mori* a process of adaptation of the host to the parasite may have occurred, thus rendering the host as less susceptible to this particular microsporidium and hence showed lowest spore production in silkworm compared to *Nosema* sp. 2 and *Nosema* sp. 1. In a preliminary study Kellen and Lindegren (1968, 1969) found that *N. heterosporum* was more virulent than *N. plodiae* in the larvae of Indian meal moth *Plodia interpunctella*, where *N. heterosporum* showed relatively low LC_{50} compared to *N. plodiae*. Henry *et al.* (1979) reported that migratory grasshoppers, *Melanoplus sanguinipes*, inoculated with *N. acridiophagus* produced fewer spores than similarly inoculated corn earworms, but spore production was similar in these insects, when they were inoculated with *N. cuneatum*.

The results indicate that the most susceptible tissues are the gut and thorax and maximum spore yield occurred after 3~4 days of adult emergence. Hence, for pebrine detection, gut and thorax of adult can be tested after 3 days of emergence. The current practice of eliminating pebrine disease in commercial seed production is exclusively based on strict mother moth examination immediately after oviposition. In the tropics, silkworm rearing is a continuous process and therefore, examination of mother

moths cannot be delayed to allow sufficient time for sporulation of microsporidia in multivoltine silkworm. However, in bivoltine sericulture, where large quantities of hibernating eggs are produced and the mother moth examination can be delayed through proper preservation of moths for a few days after oviposition for increasing the efficiency of the test. It also indicates that the tissue specific parasite development is extremely sensitive to the temperature at which the host is reared and the precise day that spore numbers are maximized can be predicted in case the infection is initiated in the first instar.

References

Becnel JJ, Undeen AH (1992) Influence of temperature on developmental parameters of the parasite/host system *Edhazardia aedi* (Microsporida: Amblyosporidae) and *Aedes aegypti* (Diptera: Culicidae). J Invertebr Pathol 60, 299-303.

Cantwell GE (1970) Standard methods for counting *Nosema* spores. Am Bee J 110, 222-223.

Dandin SB, Jayaswal J, Giridhar K (2000) Silkworm rearing; in *Handbook of sericulture technologies*, Central Silk Board, Bangalore, India, pp. 177-198.

Fowler JL, Reeves EL (1975) *In vivo* propagation of a microsporidian pathogenic to insect. J Invertebr Pathol 25, 349-353.

Henry JE, Oma EA, Onsager JA, Oldacre SW (1979) Infection of corn earworm, *Heliothis zea*, with *Nosema acridophagus* and *N. cuneatum* from grasshoppers, relative virulence and production of spores. J Invertebr Pathol 34, 125-132.

Hostounsky Z, Weiser J (1972) Production of spores of *Nosema plodiae* Kellen et Lindegren in *Mamestra brassicae* L. after different infective dosages. Vest Cs Spol Zool 36, 97-100.

Kellen WR, Lindegren JE (1968) Biology of *Nosema plodiae* sp.n., a microsporidian pathogen of the Indian meal moth, *Plodia interpunctella* (Hubner), (Lepidoptera: Phycitidae). J Invertebr Pathol 11, 104-111.

Kellen WR, Lindegren JE (1969) Host-pathogen relationships of two previously undescribed microsporidia from the Indian meal moth, *Plodia interpunctella* (Hubner), (Lepidoptera, Phytitidae). J Invertebr Pathol 14, 328-335.

Lai PF, Canning EU (1980) Infectivity of a microsporidium of mosquitoes (*Nosema algerae*) to larval stages of *Schistosoma mansoni* in *Biomphalaria glabrata*. Int J Parasitol 10, 293-301.

Lai PF, Canning EU (1983) Some factors affecting spore replication of *Nosema algerae* (Microspora, Nosematidae) in *Pieris brassicae* (Lepidoptera). J Invertebr Pathol 41, 20-26.

Larsson JIR (1988) Identification of microsporidian genera (Protozoa, Microspora) - a guide with comments on the taxonomy. Arch Protistenkd 136, 1-37.

Madana Mohanan N (2004) Microsporidian parasites of mulberry pests and their cross-infectivity to silkworm, *Bombyx mori* L. Ph.D. Thesis, University of Kalyani, India.

Madana Mohanan N, Krishnan N, Mitra P, Saratchandra B, Haldar DP (2004) Developmental cycle of a new microsporidium from *Diacrisia obliqua* (Walker) and its cross-infection to mulberry silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae); in *Recent Advances in Animal Science Research*. Ghosal SK, Ray D (eds.) Vol.III, pp. 448-460, Orion Press International, West Bengal, India.

Maddox JV (1968) Generation time of the microsporidian *Nosema necatrix* in the larvae of the armyworm, *Pseudoletia unipuncta*. J Invertebr Pathol 11, 90-96.

Pasteur L (1870) Etudes sur la Maladie des vers soie; in *Pebrine disease of silkworm,* 1971, Overseas Technical Cooperation Agency, Japan.

Pilley BM, Canning EU, Hammond JC (1978) The use of a microinjection procedure for large-scale production of the microsporidian *Nosema eurytremae* in *Pieris brassicae*. J Invertebr Pathol 32, 355-358.

Rekha L, Nataraju B, Sivaprasad V (1998) Studies on group moth inspection method for production of pebrine free layings in commercial seed production centres. Indian J Seric 37, 148-153.

Sasidharan TO, Singh RN, Samson MV, Manjula A, Santha PC, Chandrashekharaiah (1994) Spore replication rate of *Nosema bombycis* (Microsporidia: Nosematidae) in the silkworm, *Bombyx mori* L. in relation to pupal development and age of moths. Insect Sci Appl 15, 427-431.

Sato R, Watanabe H (1980) Purification of mature microsporidian spores by iso-density equilibrium centrifugation. J Seric Sci Jpn 49, 512-516.

Sprague V (1977) Classification and phylogeny of the microsporidia; in *Comparative pathobiology*. Bulla LA, Cheng TC (eds.), Vol.2, pp.1-30, Plenum press, New York.

Teetor GE, Kramer JP (1976) Dosage effect study of microsporidian *Octosporea muscaedomesticae* in the adult black blowfly *Phormia regina*, with reference to the reproductive cycle of the pathogen. J Invertebr Pathol 28, 37- 41.

Undeen AH (1997) Microsporidia (Protozoa) A handbook of Biology and Research Techniques. Southern Cooperative Series Bullettin. No.387.

Weiser J (1978) Transmission of microsporidia to insects via injection. Vestn Cesk Spol Zool 42, 311-317.

Whitlock VH, Brown ST (1991) First record for *Nosema locustae* in the brown locust *Locustana pardalina* in South Africa and the yield of spores in laboratory bioassay. J Invertebr Pathol 58, 164-167.