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Influence of Temperature on Microsporidian Multiplication and Spore Production in Various Tissues of Silkworm (*Bombyx mori* L.) During Larval Development

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Multiplication and spore production of three microsporidia *viz.*, *Nosema bombycis*, *Nosema* sp. 1 and *Nosema* sp. 2 in fifth instar larval tissues of silkworm, *Bombyx mori* L. in two seasons with distinct temperature regimes were studied. *Nosema* sp. 2 produced significantly ($P < 0.01$) higher number of spores in various tissues. Among the tissues, spore production was highest in silk gland, followed by fat body and gut. Spore production was significantly ($P < 0.01$) higher in season-II (Average temperature 29.4 ± 1.1°C). Maximum spore production was observed 25 days post inoculation (p. i.) in season-I (Average temperature 18.9 ± 1.1°C), whereas in season-II, it was 14 days p. i. In season-I, spore production was low up to 21 days p. i., then increased sharply. In season-II, there was a steady increase in spore production. The results indicate that the microsporidian multiplication is tissue specific and extremely sensitive to temperature at which the host is reared. It also reveals that, silk gland, fat body and gut are the most appropriate tissues for microscopic identification of microsporidia in the larval stage.

Key words: Microsporidia, *Bombyx mori*, Multiplication, Spore Production, Temperature

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Introduction

Microsporidia are eukaryotic, intracellular, obligatory parasites of all animal phyla and are common in arthropods (Sprague, 1977; Larsson, 1988). They are characterized by infectious spores with unique organelles for their invasion into the host (Undeen, 1997). Multiplication and spore yield of microsporidia in various host systems are widely studied (Lai and Canning, 1980, 1983) and these are influenced by temperature (Fowler and Reeves, 1975; Becnel and Undeen, 1992), spore dose (Teetor and Kramer, 1976; Henry *et al.*, 1979; Lai and Canning, 1983), age of the host (Lai and Canning, 1983; Whitlock and Brown, 1991) and type of tissue (Sasidharan *et al.*, 1994).

Pebrine caused by *Nosema bombycis* is a unique microsporidium and it exhibits both horizontal and vertical transmission. Different strains and species of microsporidia have been isolated from silkworms, thus disease has become increasingly complex (Fujiwara, 1985). The earliest method developed by Pasteur (1870) for production of pathogen free eggs through examination of mother moths has been successfully used in checking the vertical transmission. However, sample testing is necessary during the larval stage to identify early infection and to check horizontal transmission. As the multiplication of microsporidia is tissue specific (Sasidharan *et al.*, 1994), testing of individual tissues gives more accuracy than the whole larva. However, no report is available on the influence of temperature on tissue specific multiplication of microsporidia in the larvae of silkworm, *Bombyx mori* L. We investigated multiplication and spore produc-

tion of three microsporidia, *Nosema bombycis*, *Nosema* sp. 1 and *Nosema* sp. 2 in various tissues of larvae of *Bombyx mori* L. in relation to development of the host in two distinct temperature regimes.

Materials and Methods

Insect and food plant

A popular multivoltine silkworm, *Bombyx mori* L. race Nistari (marked) fed on mulberry (*Morus alba* cultivar S_1) leaves was selected for the experiment.

Microsporidia

Three microsporidia *viz.*, *Nosema bombycis*, *Nosema* sp. 1 and *Nosema* sp. 2 isolated respectively from mulberry silkworm, *Bombyx mori* L., tropical tasar silkworm, *Antheraea mylitta* D. and a mulberry pest, *Diacrisia obliqua* W. were taken for the study. From infected adults, spores were isolated and purified using standard technique (Rekha *et al.*, 1998; Sato and Watanabe, 1980).

Microsporidian inoculation and tissue preparation

Healthy and uniform sized larvae (0.08 g) of Nistari (marked) immediately after second moult were separated into 25 batches with 20 larvae in each and were starved for 3 – 4 hrs. Spore suspension (1×10^6/ml; 200 µl) in endotoxin free (ETF) water was smeared on a mulberry leaf disc (5.3 cm dia.) and exposed one such leaf disc to each batch of larvae. After complete consumption of microsporidian contaminated leaves, the larvae were pooled equally in four wooden trays (80 cm × 40 cm) with 125 larvae each and were reared on healthy mulberry leaves till spinning following standard technique (Krishnaswamy, 1972). All the three microsporidia were inoculated and maintained in identical condition.

From first day of fifth instar to spinning, 10 larvae were collected at random from each replication on alternate days. Mid gut, silk gland, fat body, malpighian tubules and male and female gonads were dissected out in insect saline (0.65% NaCl). Except male and female gonads, all other tissues were collected from the larvae irrespective of sex. Each weighed tissue was homogenized (Potter and Elvejam teflon coated tissue homogeniser) in 0.6% aqueous K_2CO_3 solution for 2 – 3 m for complete release of spores from the tissues. The homogenate was filtered through 3-layer cheesecloth. The tissue debris collected in the cloth was resuspended in K_2CO_3 solution, agitated over a cyclomixer and again filtered to collect spores entangled in the debris. The filtrate was centrifuged at 3000 rpm for 3 m for complete sedimentation of spores. The sediment was dispersed using a cyclomixer in 1 ml of ETF water. The spore concentration was then determined using a Neubauer hemocytometer (Cantwell, 1970). The same procedure was used to determine spore concentration in different tissues. The spore concentration was expressed in g^{-1} wet tissue. The experiment was conducted in two seasons [season-I (SI) and season-II (SII)] with distinct average temperatures (SI: 18.9 ± 1.1°C; SII: 29.4 ± 1.1°C) and relative humidities (SI: 76.5 ± 6.5% and SII: 85.1 ± 3.2%). In first season, SI, the spore concentration of the tested microsporidia was determined from day 1 to day 11 of 5th instar larvae (larval duration 32 days), whereas the same in the second season, SII was determined from day 1 to day 7 of 5th instar larvae (larval duration 21 days). The data were subjected to ANOVA and means were compared for significant difference.

Results

In general, in SII, all tested microsporidia showed significantly ($P < 0.01$) higher multiplication and spore production in various tissues than SI. Among various tissues, significantly ($P < 0.01$) higher spore production was in silk gland in both the seasons, while minimum in malpighian tubules. *Nosema* sp. 2 produced significantly ($P < 0.01$) higher number of spores in both the seasons (Table 1, 2 and 3). The spore production of tested microsporidia was low up to day 7 of 5th instar larvae (21 days p. i.) and then increased sharply reaching their peak values on day 11 of 5th instar larvae (25 days p. i.) in season I. However, spore production of tested microsporidia showed a steady increase from day 1 to day 7 of 5th instar larvae reaching their maximum values on day 7 of 5th instar larvae (14 days p. i.) in season II (Fig. 1). With regard to multiplication and spore yield in various tissues, significant ($P < 0.01$) interactions were also observed among season, microsporidium and day.

All the tested microsporidia produced more spores in silk gland and lesser in malpighian tubules in day 7 of 5th instar larvae in SII. In this season, *Nosema* sp. 2 produced 1.8 and 1.3 folds more spores than *Nosema bombycis* and *Nosema* sp. 1 in silk gland and 3.2 and 1.8 folds more spores in malpighian tubules respectively. In SI, these microsporidia produced maximum spores in silk gland (*Nosema* sp. 2: 1.8×10^8 g^{-1}; *Nosema* sp. 1: 1.5×10^8 g^{-1} and *Nosema bombycis*: 4.6×10^7 g^{-1}) and minimum in malpighian tubules (*Nosema* sp. 2: 4.0×10^7 g^{-1}, *Nosema* sp. 1: 2.2×10^7 g^{-1} and *Nosema* bombycis: 7.3×10^6 g^{-1}) in 11th day of 5th instar larvae.

Fifth instar larval duration was 11 days in SI, 4 days more than SII and spore yield up to 7th day in both the seasons was statistically analyzed. In SII, the spore yield of

Table 1. Spore production of three microsporidia in various tissues of larvae of *Bombyx mori* L. in relation to development of the host during season I (Temp. 18.9 ± 1.1°C and R.H. 76.5 ± 6.5%). Data represent mean ± S.E. (n = 20).

Tissue	Microsporidia[a]	Number of spores on different days of development (× 10^6 g^{-1}. wet tissue)					
		1	3	5	7	9	11
Gut	N. b	1.29 ± 0.2	2.58 ± 0.3	2.61 ± 0.2	4.12 ± 0.6	5.45 ± 0.6	35.45 ± 1.7
	N. sp. 1	2.22 ± 0.4	6.13 ± 0.9	6.53 ± 0.7	14.72 ± 1.1	18.23 ± 2.2	42.80 ± 2.9
	N. sp. 2	2.13 ± 0.4	3.17 ± 0.6	10.5 ± 1.6	18.39 ± 2.2	33.15 ± 1.8	61.43 ± 3.3
Silk Gland	N. b	0.78 ± 0.1	0.88 ± 0.2	0.91 ± 0.2	1.28 ± 0.2	23.53 ± 2.5	45.78 ± 2.8
	N. sp. 1	0.49 ± 0.1	1.91 ± 0.4	6.25 ± 0.7	13.04 ± 1.3	80.00 ± 7.5	146.8 ± 12.5
	N. sp. 2	0.93 ± 0.2	2.79 ± 0.4	6.75 ± 1.1	18.97 ± 1.3	135.55 ± 6.6	181.03 ± 7.5
Malpighian Tubules	N. b	1.06 ± 0.3	1.88 ± 0.3	2.35 ± 0.2	3.30 ± 0.3	5.20 ± 0.2	7.25 ± 0.3
	N. sp. 1	3.48 ± 0.6	5.36 ± 0.6	9.87 ± 0.7	16.90 ± 3.5	17.92 ± 2.9	22.40 ± 2.1
	N. sp. 2	1.33 ± 0.1	1.47 ± 0.3	3.64 ± 0.6	8.34 ± 1.1	28.13 ± 1.0	40.28 ± 5.5
Fat Body	N. b	0.87 ± 0.2	1.28 ± 0.1	2.93 ± 0.0	3.44 ± 0.2	13.06 ± 1.5	40.25 ± 2.4
	N. sp. 1	1.11 ± 0.2	2.27 ± 0.4	3.79 ± 0.7	6.14 ± 0.4	52.78 ± 6.4	87.45 ± 7.0
	N. sp. 2	2.40 ± 0.4	5.77 ± 0.7	7.39 ± 0.4	9.00 ± 1.1	60.00 ± 5.3	126.1 ± 15.2
Male Gonad	N. b	1.79 ± 0.3	1.86 ± 0.3	1.95 ± 0.3	2.63 ± 0.3	3.73 ± 0.3	8.05 ± 0.5
	N. sp. 1	2.50 ± 0.0	3.13 ± 0.5	3.91 ± 0.7	11.61 ± 1.8	16.25 ± 1.2	30.00 ± 2.9
	N. sp. 2	3.45 ± 0.3	4.17 ± 0.3	5.19 ± 0.9	11.93 ± 1.9	24.95 ± 2.8	41.73 ± 3.8
Female Gonad	N. b	1.89 ± 0.3	2.40 ± 0.2	2.60 ± 0.5	3.18 ± 0.2	6.11 ± 0.6	9.38 ± 1.1
	N. sp. 1	2.92 ± 0.4	3.91 ± 0.7	4.46 ± 0.7	15.63 ± 1.5	19.08 ± 1.9	39.29 ± 3.8
	N. sp. 2	3.52 ± 0.3	4.55 ± 0.8	5.92 ± 0.6	19.44 ± 1.2	41.04 ± 2.1	81.70 ± 10.1

a: *N.b* = *Nosema bombycis*; *N.* sp = *Nosema species*

Table 2. Spore production of three microsporidia in various tissues of larvae of *Bombyx mori* L. in relation to development of the host during season II (Temp. 29.4 ± 1.1°C and R.H. 85.1 ± 3.2 %). Data represent mean ± S.E. (n = 20).

Tissue	Microsporidia[a]	Number of spores on different days of development (× 10^6 g^{-1}. wet tissue)			
		1	3	5	7
Gut	N. b	3.27 ± 0.2	6.46 ± 0.4	41.85 ± 2.1	61.95 ± 4.1
	N. sp. 1	4.77 ± 0.4	8.95 ± 0.9	63.02 ± 3.8	106.05 ± 3.1
	N. sp. 2	9.91 ± 0.8	15.30 ± 1.7	81.25 ± 1.8	136.62 ± 2.6
Silk Gland	N. b	3.23 ± 0.4	5.62 ± 0.2	121.21 ± 13.0	203.45 ± 7.5
	N. sp. 1	5.08 ± 0.3	6.63 ± 0.4	197.13 ± 9.1	296.67 ± 12.8
	N. sp. 2	7.20 ± 0.9	14.06 ± 1.1	333.33 ± 12.5	375.00 ± 11.7
Malpighian Tubules	N. b	3.91 ± 0.4	7.19 ± 0.5	13.44 ± 1.7	16.23 ± 0.9
	N. sp. 1	7.38 ± 0.4	10.75 ± 0.3	14.17 ± 0.9	29.17 ± 2.0
	N. sp. 2	9.09 ± 0.8	13.20 ± 0.8	26.25 ± 1.7	52.21 ± 3.3
Fat Body	N. b	5.56 ± 0.5	7.57 ± 0.8	74.29 ± 2.3	187.50 ± 7.7
	N. sp. 1	6.25 ± 0.8	9.07 ± 0.2	137.00 ± 18.5	210.00 ± 5.5
	N. sp. 2	9.28 ± 1.0	17.83 ± 1.8	153.45 ± 5.5	278.80 ± 5.4
Male Gonad	N. b	3.82 ± 0.3	6.60 ± 0.6	20.84 ± 0.9	29.33 ± 2.7
	N. sp. 1	5.63 ± 0.6	8.22 ± 0.4	33.04 ± 3.0	45.00 ± 3.9
	N. sp. 2	6.62 ± 0.3	14.77 ± 0.6	37.50 ± 1.6	68.24 ± 0.9
Female Gonad	N. b	5.36 ± 0.4	8.21 ± 0.7	29.04 ± 2.5	49.17 ± 2.9
	N. sp. 1	5.81 ± 0.4	8.66 ± 0.5	34.61 ± 1.8	56.52 ± 1.4
	N. sp. 2	7.72 ± 0.6	13.13 ± 1.4	43.13 ± 2.0	95.00 ± 2.5

a: *N.b* = *Nosema bombycis*; *N.* sp = *Nosema species*

Table 3. ANOVA for the multiplication and spore production of three microsporidia in various tissues of 5th instar larvae of silkworm, *Bombyx mori* L. in relation to development of the host

Source	DF	SS	MS	F	P < 0.05
Season (S)	1	377117.7	377117.7	7156.2**	1.2
Microsporidian (M)	2	42917.1	21458.6	407.2**	1.5
Day (D)	3	417081.2	139027.1	2638.2**	1.7
Tissue (T)	5	245491.7	49098.3	931.7**	2.1
S × M	2	26705.3	13352.6	253.4**	2.1
S × D	3	329876.1	109958.7	2086.6**	2.4
S × T	5	246461.1	49292.2	935.4**	2.9
M × D	6	30694.5	5115.8	97.1**	2.9
M × T	10	22115.0	2211.5	42.0**	3.6
D × T	15	272624.4	18175.0	344.9**	4.2
S × M × D	6	20066.7	3344.5	63.5**	4.2
S × M × T	10	20765.9	2076.6	39.4**	5.1
M × D × T	30	26156.7	871.9	16.5**	7.2
S × M × D × T	30	286107.3	9536.9	181.0**	10.2
Error	447	23556.1	52.7		
Total_	575	2387736.6			

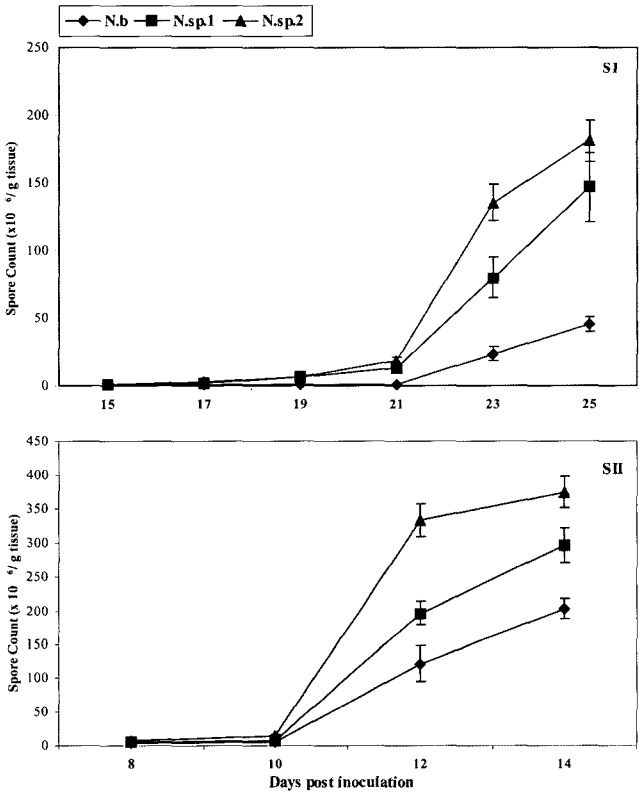

Fig. 1. Spore production of three microsporidia in silk galnd of *Bombyx mori* L. at various d.p.i. in two seasons (SI = Jan - Feb.; SII = June). Vertical bars represent S.E. of Mean (n = 20).

Nosema sp. 2 in silk gland and malpighian tubules of 7th day of 5th instar larvae was 19.8 and 6.3 times higher than that of SI respectively. Similarly, *Nosema* sp. 1 showed 22.8 and 1.7 and *Nosema bombycis* 159.0 and 4.9 times higher spore yield in silk gland and malpighian tubules respectively in SII. *Nosema* sp. 2 showed a minimum of 5.7 (malpighian tubules) and a maximum of 52.1 (silk gland) fold increase in spore production from 1st day of 5th instar to 7th day of 5th instar larvae in SII, whereas the yield increase was 6.3 and 20.4 fold respectively in SI. *Nosema* sp. 1 showed a minimum of 4.0 (malpighian tubules) and a maximum of 58.4 (silk gland) fold spore yield increase in SII, while in SI the same was 4.6 and 26.7 fold respectively. *Nosema bombycis* showed a minimum of 4.2 (malpighian tubules) and maximum of 62.9 (silk gland) fold increase in spore yield in SII and the same in SI was 1.5 (male gonad) and 4.0 (fat body) fold respectively (Fig. 2a, 2b and 2c).

It was assumed that all larvae consumed equal quantities of pathogen smeared leaf and hence received a spore load of 1×10^4. If consider the spore number increase (in fold) from the day of inoculation to the last day larvae (11th day of 5th instar in SI and 7th day of 5th instar in SII), it was highest in silk gland both in SI (*Nosema bombycis*: 458; *Nosema* sp. 1: 1468 and *Nosema* sp. 2: 1810) and SII (*Nosema bombycis*: 2035; *Nosema* sp. 1: 2967 and *Nosema sp.* 2: 3750) and least in malpighian tubules (*Nosema bombycis*: SI-72 and SII-162; *Nosema* sp. 1: SI-224 and SII-292; *Nosema* sp. 2: SI-403 and SII-522) (Fig. 3a, 3b and 3c).

Discussion

The most ideal temperature for the development of late age silkworm larvae is 24 – 26°C (Dandin *et al.*, 2000).

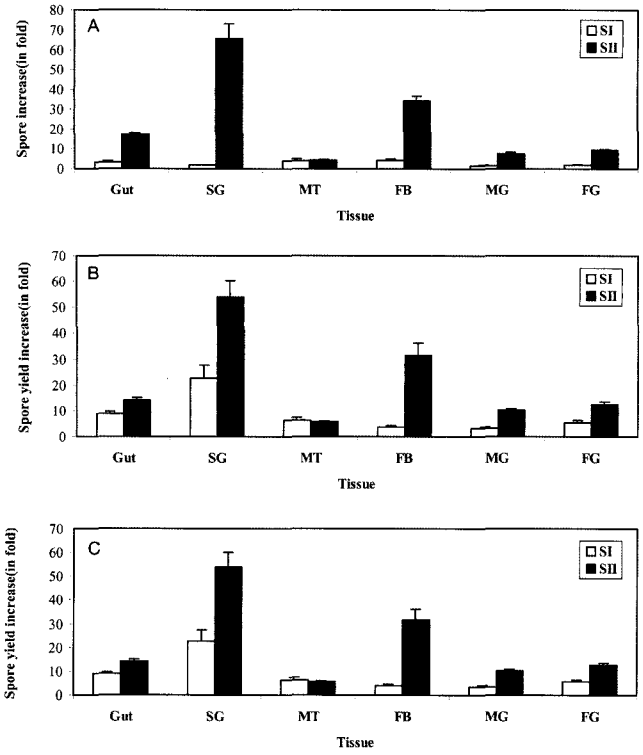

Fig. 2. Spore yield increase in fold of (A) *Nosema bombycis* (B) *Nosema* sp. 1 (C) *Nosema* sp. 2 from 1st day vth instar to 7th day larvae in various tissues of *Bombyx mori* L. in two seasons. Vertical bars represent S.E. of mean (n = 20). G = Gut; SG = Silk Galnd; MT = Malpighian Tubule; FB = Fat Body; MG = Male Gonad; FG = Female Gonad.

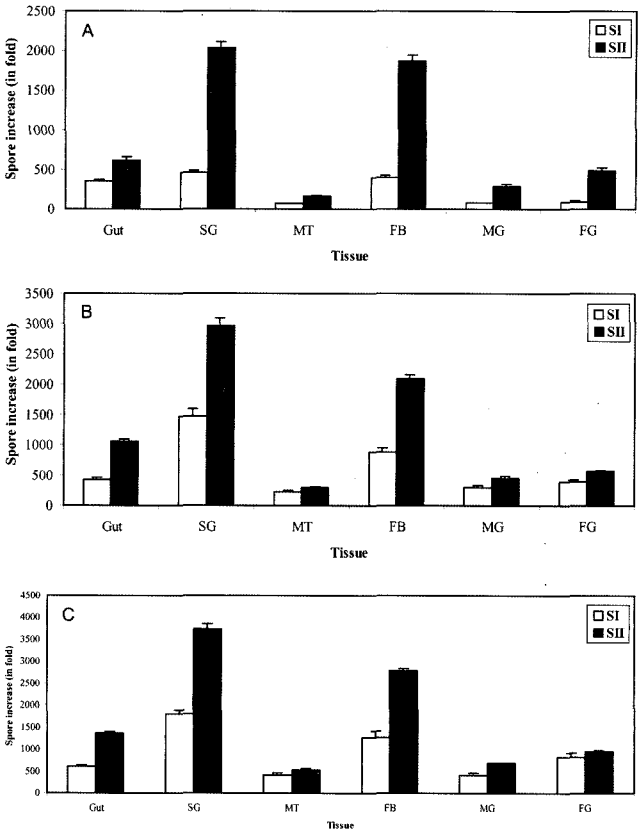

Fig. 3. Spore yield increase in fold of (A) *Nosema bombycis* (B) *Nosema* sp. 1 (C) *Nosema* sp. 2 from the day of inoculation to the last day of vth instar larvae in various tissues of *Bombyx mori* L. in two seasons. Vertical bars represent SE of mean (n = 20). G = Gut; SG = Silk Galnd; MT = Malpighian Tubule; FB = Fat Body; MG = Male Gonad; FG = Female Gonad.

The present study reveals that infectivity and development of microsporidia in insect are dependent on the temperature at which host is being reared. Although, microsporidian spores can tolerate a wide range of temperatures (Weiser, 1963; Maddox, 1973), the infection process is influenced greatly by temperature. In our study, fifth instar larval duration was 11 days in SI and 7 days in SII. This variation was due to difference in temperature during these seasons. The multiplication and spore yield of microsporidia in various tissues of the larvae were significantly higher in SII. Although, spore yield was very low up to 21 days p. i. in SI, a sharp increase was noticed in most of the tissues on 25th day during larval - pupal transformation. The physiological changes in the host possibly regulate the developmental cycle of the parasites and switch over from a predominantly vegetative phase to sporogony, resulting in an increase in spore yield. Similar sudden increase in spore yield was also observed during transformation from pupa to imago in *Pieris brassicae* (Lai and Canning, 1983).

The spore yield of microsporidia in various tissues on day 7 of 5th instar larvae was significantly higher in SII. In SI, even with additional developmental time and resources the microsporidia did not produce more spores. This indicates that while the host and parasite share a sub-optimal temperature for optimum development, the parasite appears to be more sensitive to low temperature than the host. Temperature is one of the determining factors for insect susceptibility and multiplication of pathogens. The optimum temperature for infection of most of the microsporidia lies between 20 – 30°C and no development occurs below 10°C (Maddox, 1973). Infections of grub with *Nosema melolonthae* of *Adelina melolonthae* similarly increased mortality at 20 – 25°C temperature range, compared with chronic long - lasting infections at lower temperatures (Hurpin, 1967). Likewise, temperature optima for *Nosema necatrix* in *Trichoplusia ni* and *Heliothis zea* were observed as 21 – 26°C (Fowler and Reeves, 1975). It was also reported inhibition of vegetative development of *Vavraia culicis* in mosquito, *Culex pipiens fatigans* at a temperature of 34°C (Reynold, 1970).

In *Vairimorpha necatrix*, high and low temperature extremes delayed the microsporidian development resulting in the production of fewer spores (Fowler and Reeves, 1975). Becnel and Undeen (1992) predicted 30.8°C as optimum temperature for maximum spore production of *Edhazardia_aedis* in *Aedes aegypti* by elevating outside the ecologically relevant temperature range of the host.

Among the microsporidia, the highest spore yield in most of the tissues in both the seasons was in *Nosema* sp. 2, followed by *Nosema* sp. 1 and *Nosema bombycis*. This difference in spore yield could be attributed to their virulence (Madana Mohanan, 2004; Madana Mohanan *et al.*, 2005). Difference in virulence of *Nosema heterosporum* and *Nosema plodia* was reported (Kellen and Lindegren, 1969, 1974) in the larvae of Indian Meal moth, *Plodia interpunctella*. Similarly, *Nosema acridophagus* produced fewer spores in migratory grasshoppers, *Melanoplus sanguinipes* than in similarly inoculated corn earworms, while spore production was reported similar in these insects, when they were inoculated with *Nosema cuneatum* (Henry *et al.*, 1979).

The differences in spore yield may be a reflection of the host-parasite interaction, possibly due to differences in virulence of the microsporidia. In this investigation, *Nosema bombycis* was obtained from a laboratory culture maintained through animal passage in silkworm, *Bombyx mori* L., but, *Nosema* sp. 1 and *Nosema* sp. 2 were isolated directly from *Antheraea mylitta* D. and *Diacrisia obliqua* W. respectively. Since, *Nosema bombycis* was maintained through animal passage in silkworm, an adaptation of host to parasite might have occurred, thus rendering the host less susceptible to this microsporidium and hence showed least spore yield.

Multiplication and spore yield were highest in silk gland, then in fat body and gut, which may be due to their tissue specificity. The rate of sporulation of *Nosema bombycis* increased with age of silkworm pupa and moth and cephalothoracic region recorded the highest spore concentration, especially around the wing and wing muscles among the tissues tested (Sasidharan *et al.*, 1994).

The study indicates that: (1) multiplication of microsporidia and spore production depend on their virulence, type of tissue and also the temperature at which the host is reared, (2) Silk gland, fat body and gut are the most appropriate tissues for microscopic examination and detection of microsporidian infection and (3) the precise day that spore numbers are maximized can be predicted in various larval instars when infection initiated in the first instar.

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