Indian Journal of Entomology 86(1): 105-110 (2024) DoI. No.: 10.55446/IJE.2024.1585

EFFECT OF SPERMIDINE SUPPLEMENTATION ON TESTICULAR AND OVARIAN DEVELOPMENT IN SEX-LIMITED AND NON-SEX-LIMITED BIVOLTINE SILK WORM BREEDS OF *BOMBYX MORI* L

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ABSTRACT

Silk worm *Bombyx mori* L is a holometabolous, lepidopteran model for investigating the effect of various molecules. The sex-limited character of cocoon colour is useful for the separation of male and female pupae during commercial seed production. Fecundity is the major limitation of cocoon colour sex-limited breeds. Spermidine is a polyamine, present in all living cells involved in the growth and reproduction of many organisms. The effect of spermidine on testicular and ovarian development in the selected bivoltine sex-limited breed; APS27SL and non-sex-limited hybrid; APS45 x APS12 is studied in the present work. Results showed that foliar feeding of spermidine significantly increased pupal growth, testicular and ovarian growth, number of ovarioles and ovules in the sex-limited breed. Moreover, increased expression of Bm-tektin was observed in the testes of spermidine fed groups. Thus, the study suggests that foliar feeding of spermidine can be used to overcome the limitation of reproductive potential in sex-limited breeds.

Key words: *Bombyx mori*, fecundity, spermidine, sericulture, sex-limited breed, reproduction, polyamine, bivoltine, growth, tektin, cocoon colour, sex linked character, testis, ovarioles

The silk worm *Bombyx mori* L has been intensively studied for a long time because of its economic value. It feeds on mulberry and gives silk, which has various traditional and medical applications. In sericulture, rearing, and egg production are two important factors that contribute immensely to silk production. Fecundity is the most important factor and a major challenge to be considered to improve the economic and mechanical parameters of sex-limited breeds and in the preparation of double hybrids (Dayanand et al., 2011; Subhra et al., 2011). Studies revealed that double hybrids produced by CSRTI (Central Sericulture Research and Training Institute) showed better fecundity compared to single hybrids. Many approaches have been used to boost the reproductive rate. The exposure of gamma radiation to *B. mori* eggs caused an increase in fecundity (Salam and Mahoud, 1995). The suitable rearing conditions, environment, and nutrition during the larval period may lead to higher fecundity by silkworm moths (Miller, 2005; Malik and Reddy, 2007; Hussain et al., 2011). A 1% concentration of ascorbic acid increased the number of eggs in the silkworm (Chauhan and Singh 1992). Further studies showed that fecundity and hatchability were improved by the application of 20-hydroxyecdysone on the larvae in various concentrations (Prasad and Upadhyay, 2012). Mulberry leaves fortified with Zinc increased fecundity when fed to silkworms (Wani et al., 2018). APS27SL is a bivoltine cocoon colour sex-limited breed, male spin yellow cocoons and female spin white cocoons. Spermidine supplementation (Spd) increased the fecundity of this breed in previous studies (Madhavi et al., 2023). APS45 x APS12, a hybrid was developed by Andhra Pradesh State Sericulture Research and Development Institute (APSSRDI), Hindupur, under the collaborative project entitled "Popularization of authorized silkworm hybrids among the farmers of South India", funded by Central Silk Board (CSB), Bangalore. It is characterized by bluish-white plain larvae, white coloured hybrid cocoons with medium grains. The hybrid is quantitatively and qualitatively superior and suitable for rearing under favorable seasons resulting in yielding consistent cocoon crops.

The major constraint of sex-limited breeds was low fecundity (Suresh Kumar et al. 2008). Studies revealed that double hybrids produced by CSRTI showed better fecundity compared to single hybrids (APS45 x APS12),

with respect to growth and reproductive potential. Spd is a biologically important polyamine that serves as a key regulator of processes like DNA stability, protein synthesis, cell proliferation, differentiation, and apoptosis (Eisenberg et al., 2009, Minois et al., 2014, Morselli et al., 2011). Spermine was essential for spermatogenesis in rats (Weiner KX et al., 1992). Infertile men have low spermine and spermidine levels (Calandra RS et al., 1996). Studies show that polyamines increase sperm motility and capacitation in humans (Morales ME et al., 2003; Rodriguez-Paez et al., 2021). Spd concentrations in the testis were reported to be associated closely with the maturation of the testis as well as germ cell differentiation (Maclndoe and Turkington, 1973). In the newt testis, Spd and spermine levels were low in the winter, increased in the spring, and reached a peak in the summer during active spermatogenesis (Matsuzaki et al, 1981). During spermatogenesis in roosters, the ratios of spermine and Spd/DNA were relatively constant throughout spermatogenesis, whereas the ratio of putrescine/DNA increased in elongated spermatids. The cellular contents of putrescine, Spd, and spermine decreased markedly in mature spermatozoa of roosters (Oliva et al., 1982). These data suggest the importance of polyamines in testicular maturation and spermatogenesis. The Micromolar concentrations of polyamines increased the growth and economic parameters of *B. mori* (Lattala et al., 2014). Oral supplementation of Spd in hybrid (CSR2 x CSR4) *B. mori* boosted testicular development and egg production (Mysarla et al., 2016). The current study is focused to check and compare the effect of Spd on testicular and ovarian development in bivoltine cocoon color sex-limited breed (APS27SL) with non-sex-limited bivoltine promising hybrid (APS45 x APS12).

MATERIALS AND METHODS

Standard polyamine Spd free base (RM5438) was purchased from Hi Media Chemicals. The bivoltine *B. mori* cocoon colour sex-limited breed APS27SL (the male and female spin white and yellow cocoons respectively) and non-sex-limited promising bivoltine hybrid APS45 x APS12 were selected. The disease-free layings of both the breeds were released from the schedule and reared under standard rearing conditions (26-28^{o}C, 65- 85% RH), up to the 4th moult with superior cultivar, V_1 mulberry leaves as feed. The 5th instar larvae on day 1 were divided into two groups, one group was fed with Spd (50 µM) swabbed leaves till the day of spinning. Three replications for each breed (25 male and female each) were maintained with batches fed on the mulberry leaves. The matured larvae of both the control and treated groups were allowed to spin cocoons. After harvesting cocoons, the male and female were separated easily based on their colour in the sex-limited breed, and sex separation was carried out manually in the non-sex-limited breed. Testis and egg samples were collected from the male and female pupa at different time points during pupal development in control and Spd-supplemented groups of both sex-limited and non-sex-limited breeds and analyzed. The cocoons were cut open and the weight of the male and female pupa was taken (at the beginning, middle, and at end of the pupal stage; days 13, 16, and 19) till emergence and recorded. Pupae were dissected at different time points of the pupal development (days 13, 16, and 19), testes and ovaries obtained from pupae were weighed. Weighments were taken from randomly selected pupae. Three biological replications were performed and three samples were analyzed from each replication (n = 3). Testes and ovaries were weighed individually and averages with mean error values were recorded.

For tissue sample preparation the testes and ovaries were dissected out from the pupae at different time points during pupal development (days 13, 16, and 19) from treated and control groups using insect Ringer's solution (0.68% NaCl) and transferred into a fresh microfuge tube and stored at -80 ^{o}C till use. The expression of the Bm-tektin and RP49 genes was carried out by RT-PCR (reverse transcription polymerase chain reaction). Total RNA was isolated from 0.4 g of the testes (n=3 pooled) of control and Spd-treated groups of sex-limited and non-sex-limited breeds on day 19 of pupal development by the TRIzol method. The RNA samples (3 µg) were reverse transcribed into cDNA using SRL-RT-PCR Kit (First Strand cDNA Synthesis Kit) 94837-(BOS001). Three replicates of PCR were performed with each sample. In each run of PCR, the housekeeping gene RP49 was used as the reference. 5′- AGCGAATGCTTTCTCTGGAA -3′ and 5′- TGTCGCTCCAATCAAATTCA -3′ were used as forward and reverse primers for the Bm-tektin gene amplification, and 5′- CAGGCGGTTCAAGGGTCAATAC -3′ and 5′- TGCTGGGCTCTTTCCACGA -3′ primers were used for RP49 gene amplification (control). The densitometric analysis of Bm-tektin and RP49 was carried out in the control and Spd-treated APS27SL and APS45 x APS12 using Image Lab software in GelDoc^{XR} by BioRad. Statistical analysis was carried out by taking the measurements of pupal weights, egg weights and testes weights in triplicates and compared

between the control and treated groups (Spd). The experimental data was subjected to statistical analysis by t-test; two samples assuming equal variances (T <= t, one tail; p= 0.05).

RESULTS AND DISCUSSION

Results showed that the effect of Spd was more prominent in the sex-limited *B. mori* breeds when compared to the non-sex-limited breed. This study for the first time showed foliar supplementation of 50 µM Spd during 5th instar larvae enhanced growth and development of pupa, testis and ovary of cocoon colour sex–limited breeds and APS45 x APS12. The weights of the pupae were recorded on days 13, 16, and 19 of the control and Spd treated groups. Spd-treated male pupae showed a significant increase in weights on day 13 and day 16 of APS27SL and on day 13, 16 and 19 of APS45 x APS12 compared to the control group (Fig. 1A). Spd-treated female pupae showed a significant increase in weight on days 13, 16, and 19 of APS27SL compared to the control group, the Spd-treated APS45 XAPS12 showed increase in female pupal weights compared to control (Fig. 1B). These results were in accordance with the earlier studies which showed the role of Spd in the growth and sexual reproduction of *Fusarium graminearum* (Tang Guangfei et. al., 2021). Spd treated mice showed an increment in body weight (Wang et al., 2022). Larvae of strain CSR2 X CSR4 fed with Spd showed increased male and female pupal weights than the control (Mysarla et al., 2016).

The testicular and ovular weights were recorded on days 13, 16 and, 19. Day 19 is considered the final day as moths emerge on day 20. The weight of Spd-treated APS27SL testes showed a significant increase on days 13 and 19 compared to a control group (Fig. 1C). The results obtained matched with the earlier findings of Spd treatment. Testicular weights were shown significantly higher in Spd-treated groups than in the control groups of CSR2×CSR4 cross-breed silkworms (Mysarla et al., 2016). Spd treated mice showed improved testicular

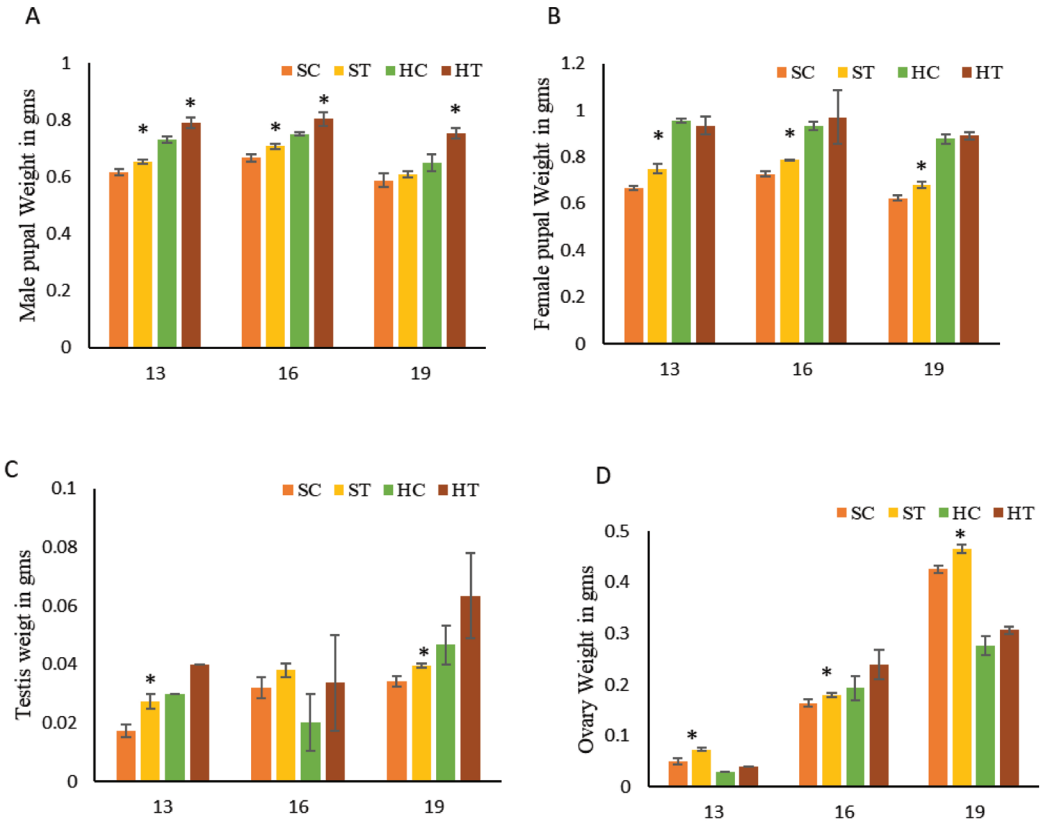


Fig. 1. Effect of Spd on the growth of APS27SL and APS45×12 pupae A. male pupal weights B. Female pupal weights C. Testes weights D. Egg weights, X-axis represents different days of pupal development and Y–axis represents average weight in grams (gms). SC and ST represent the control and Spd treated groups of the sex-limited breed, HC and HT represent the control and Spd-treated groups of the non-sex-limited breed. * represents ($p \leq 0.05$)

function, with an increment in the testis weight (Wang et al., 2022). Spd-treated ovary weights showed a significant increase on days 13, 16 and 19 of APS27SL when compared to the control groups and an increase in ovary weight was observed Spd-treated compared to control groups of APS45 x APS12 (Fig. 1D). In vitro, studies in Kiwi fruit showed the positive effect of Spd on ovary growth by reduction in necrosis (R. Biasi et al., 1997). The increased ovarian weights in Spd supplemented groups could be due to decreased cell death. The Spd group of both sex-limited and non-sex-limited breeds showed 5 ovarioles whereas, 4 ovarioles were observed in control groups (Fig. 2A and B). Spd protected the ovarian function by increasing antioxidants in mice (Jiang D et al., 2023). The size of the testes increased in Spd-treated sex-limited and non-sex-limited breeds compared to the control. The distinct tubular formation can be noticed in Spd-treated sex-limited and non-sex-limited breeds compared to the control (Fig. 2). The exogenous supplementation of Spd enhanced the growth of testis in mice (Qi Zhao et al., 2021). The Spd supplementation enhanced the testicular and egg production in the CSR2×CSR4 breed of *B. mori* (Mysarla et al., 2016).

To understand the molecular levels differences, expression analysis of Bm-tektin on day 19 testicular homogenates was performed with sex-limited and non-sex-limited breeds. Cloning and characterization of testis-specific tektin in *B. mori* was done earlier (Ota et al., 2002). The Bm-tektin gene is responsible for the flagellar activity of sperm which in turn facilitates the motility of the sperm., which plays a major role in reproduction. RP49 gene was used as an internal control to normalize the expression of Bm-Tektin. Results showed elevated expression of Bm-tektin in the Spd-treated group of the sex-limited breeds compared to the control group (Fig. 3A). The fold change in the Spd-treated group was calculated by normalizing the Bm-tektin expression with RP49. Results showed a 0.5 fold increase in the Spd-treated sex-limited breed when compared to the control (Fig. 3B). Tektin 3 and Tektin 4 were shown to be essential for sperm motility in mice (Roy et al., 2009). Tektins found abundance in centrioles and axonemal microtubules (Steffen et al.,1988). The transcriptome analysis of the testis of the silkworm, *B. mori* was carried out and the testis-specific genes were found to be accumulated more on the Z chromosome (Arun Kumar KP et al., 2009). Thus the foliar supplementation of Spd during 5th instar larvae resulted in a significant increase in pupal weight testicular and ovarian growth in the sex-limited breed compared with the non-sex-limited breed. Further

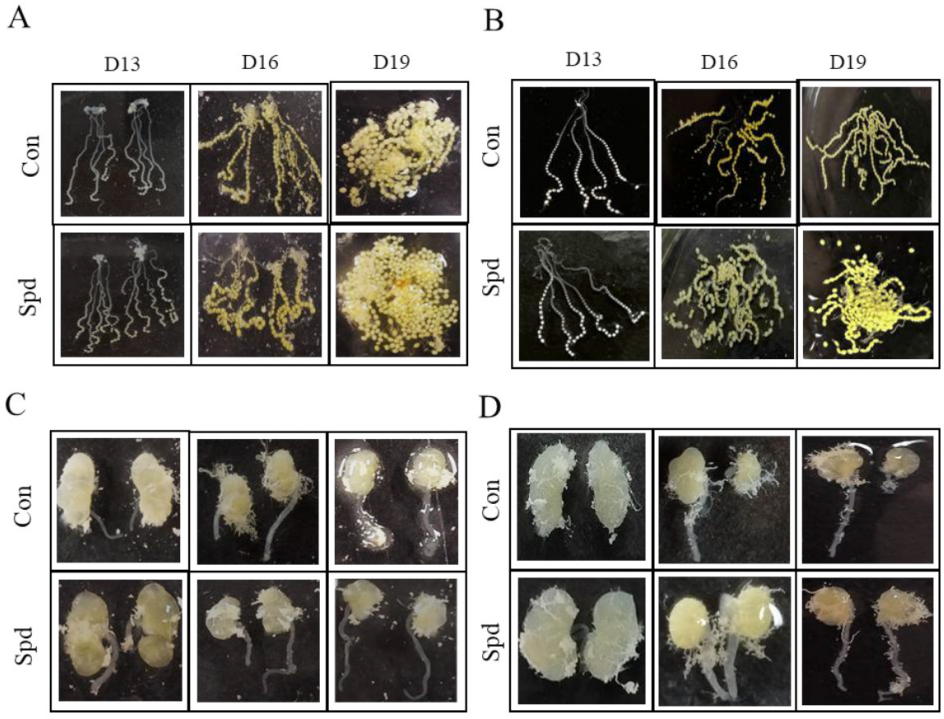
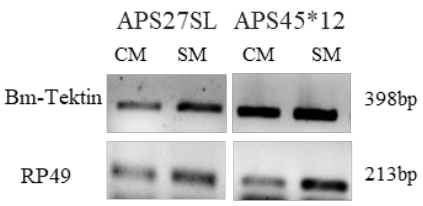


Fig. 2. Effect of Spd on testicular and ovular morphology of sex-limited and non-sex-limited pupae in control and treated groups (A) ovary of (B). ovary of non-sex-limited breed. (C). testis of sex-limited breed (D). testis of non-sex-limited breed.

A



B

	Tektin	RP49	Normalization	Fold change
SC	34,20,402	17,76,717	1643685	1
ST	41,35,658	15,99,868	2535790	1.542747
HC	28,76,317	14,49,635	1426682	1
HT	31,30,925	24,76,776	654149.3	0.458511

Fig. 3. Expression analysis of Bm-tektin. (A) RT-PCR of Bm-tektin and RP49 genes in testicular homogenates of *B. mori*. (B) Relative expression of Bm-tektin was quantified and normalized with RP49 of control and Spd-treated testes of APS27SL and APS45×12 on day 19.

studies are required to understand the role played by Bmtektin in the growth and reproduction of *B. mori*.

ACKNOWLEDGEMENTS

Andhra Pradesh State Sericulture Research and Development Institute (APSSRDI) is acknowledged for providing us with a platform to conduct silkworm rearing, as well as the GITAM University, Visakhapatnam for providing us with lab facilities to conduct this research work. We also thank Ms. Shruthika and Ms. Roshini, B.Sc project students in silkworm rearing.

AUTHOR CONTRIBUTION STATEMENT

All authors equally contributed. MK carried out growth analysis. BGLD carried out RT-PCR analysis. SJ and PJR provided rearing facilities and checked rearing performance experiments. AM conceptualized the idea and supervised the project work.

CONFLICT OF INTEREST

No conflict of interest.

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(Manuscript Received: August, 2023; Revised: November, 2023;
Accepted: December, 2023; Online Published: January, 2024)
Online First in www.entosocindia.org and indianentomology.org Ref. No. e23585