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Effect of Polyamines on Fecundity (egg laying capacity) trait of Tasar Silkworm, *Antheraea mylitta* (Daba BV)

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ABSTRACT

Antheraea mylitta, a wild sericigenous insect is a species widely distributed in India, from West Bengal in the in the East to Karnataka in the South with its natural inhabitation in the forest areas of Bihar, Orissa, Madhya Pradesh, Maharashtra, Andhra Pradesh and Telangana. It is a polyphagous insect feeding on a number of food plants primarily on *Terminalia arjuna* and *T. tomentosa* and a host of secondary food plants. The wide range of distribution of the species has encountered diverse geographic and climatic variations of the distinct areas, leading to marked differences in not only phonotypical and physiological traits but also in the commercial and technological aspects. In this study, considering the immense effect of polyamines *(Spermidine, Spermine and Putrescine)*, as food supplements was studied, *A. mylitta*. The silkworms in Vth instar larval stage were fed with plain (control) and polyamines sprayed *Terminalia arjuna* leaves in different concentrations. The results explored as, the *spd* and *spm* by *50 µM, 100 µM* increased the fecundity (egg laying capacity) than larvae treated by other drugs and even control. Considerably, the hatching percentage calculations revealed that, the females of *spd* (♀) *50 µM, 100 µM* mates with *spm* (♂) *50 µM, 100 µM* males were predominant, respectively, in hatching percentage into young larvae that was conclude the progressive effect of *spm* 100 on spermatogenesis and the *spd* on oogenesis which could be explored at molecular level.

Keywords: *Antheraea mylitta,* Polyphagous Insect, polyamines, Spermidine, Spermine Putrescine and Fecundity.

INTRODUCTION

A.mylitta D, Daba BV (Bi-Voltine- produce two crops in a year) ecorace is a sericigenous lepidopteran that belongs to the family Saturniidae feed on *Terminalia arjuna* trees, synthesize the silk with two main proteins i.e. sericin (20-30%) and fibroin (70-80%) in the paired silk glands (the modified salivary glands) at the end of the V instar larval period. This silk oozes out via the mouth and harden when exposed to air. The life cycle is completed within 38- 45 days (partially depending on the season), normally, July to September (I crop) and September to November (II crop).

Silkworm growth and development during the larval stage is important due to the rest of the stages depend on it. In our previous study, addition of *polyamines* diet in the silkworm *A. myliita* enhanced the fat body content, silk gland growth in V- instar larval organs (Uppula & Gangupanthula, 2021a) and found increased levels of protein content in fat body, silk gland and gut epithelium (Uppula & Gangupanthula, 2021). Glycogenesis and lipogenesis are the two vital functions of the fat body in silkworms to synthesis carbohydrates and lipids, respectively, in *B. mori* (Inagaki & Yamashita, 1986).*PAs* indulge in fat body synthesis and mediate in the storage of proteins, carbohydrates, and lipids along with other nutrients for the pupal, moth development and fecundity as well. PAs as additional feed to the silkworm during the larval stage contribute the best performance in the next life cycle. Pupal mass determined the adult development and fecundity in *Aedes aegypti* (Armbruster & Hutchinson, 2002).

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Polyamines, cationic molecules, intermediate metabolic derivatives, inside the cells during the catabolism of the amino acids and involve in vital cellular functions. *Polyamines (PAs)* such as *Putrescine (Put), Spermidine (Spd), and Spermine (Spm)* are essential in various tissue development during the larval stage such as silk glad, fat body, gut epithelium and musculature etc. This development contributes the fecundity enhancement during the moth.

Qualitative and quantitative egg production determines crop productivity. Fecundity, the egg laying capacity, determined physical parameters such as humidity, temperature, and duration of light in non-mulberry silkworm *A. mylitta* (Prabhakar et al., 2019), (Reddy et al., 2008). Suitable photoperiodic conditions contribute to fecundity and hatching performances in the eri-silkworm, *S. ricini* (Bhatia & Yousuf, 2014). Supplementation of *PA* rich nutrients increases the noticeable development of silkworms in all stages of its life cycle, which stands the sericulture in a large scale industry (Legay, 1958).*PAs* promoted the testicular development and growth of silk glands in *B. mori* (Mysarla et al., 2016).Heredity; the genetic factors influence the silk production and reproductive ability in *B. mori*. and in *S.cynthia ricini* (Vijayan et al., 2006). Quality food prepared with raw materials from soya along with the pulverized mulberry leaves enhance silk production and fecundity in silkworm *Bombyx mori* (AVRAMOVA et al., 2020). Polyamines promote sperm maturation in testes (Calandra et al., 1996), spermatogenesis, sperm cell motility in testes and follicular development in ovary of vertebrates (Lefèvre et al., 2011). Addition of *DFMO* increased certain egg vitellogenin and trypsin for protein metabolism (Kogan & Hagedorn, 2000). *PAs* promote oogenesis in *Xenopus laevis* by enhancing meiotic cell division (Osborne et al., 1989) and mosquito, *Aedes aegypti* (Kogan, 1991). Polyamines, *diamino propane*, *cadaverine*, and *putrescine* enhance spermatogenesis in crickets and cockroaches (Hamana et al., 1989).

Reproductive organs development and gametogenesis determine fruitful sperm and egg production. During the adult stage, the matured male and female silk moths participate in coupling for 24 to 48 hours. The female lays eggs in 2 to 3 stages. Eggs are hatched and instar I larvae emerge after completing the diapauses period.

ANOVA single factor used to know the significance *P-value* at $a \leq 0.05$ and *F ratio (F value >F Crit.)* in between the Pupal weight to fecundity. Regression analysis used to determine the Co-relation between the variables and explained the statistical development. In our preceding study, *PAs* of *Spd 50 µM, 100 µM and Spm 50 µM, 100 µM* exhibited significant relationship, the regression and correlation in between larval and silk gland weight, cocoon and shell weight prospected close proximity in growth and development (Swamy and Shamitha), which could determine the structure such as organogenesis and maturation and their functionality in the proceeding life cycle. The potential development in the pupae determines the fate of next ongoing stages such as metamorphosis into adult moth, sexual maturation and production of offsprings. Significant relationship *(P<0.01)* was noticed in between the larvae and silk gland growth in *B. mori* (Gündüz and Şahan 2019), (Chandrakanth et al. 2016) and (Seidavi 2010).

This study on tropical Tasar silk moths, *A. mylitta* D, Daba BV ecorace on both, the male and female moths aimed to explore the prominent effect of polyamines i.e., *Spm, Spd* and *Put* at different concentrations on the sexual fitness, gametogenic vigour and egg laying capacity along with the production of fertile progeny.

This investigation also includes morphological development of emerged moths, mating behavior and duration, the egg weight and especially, the estimation of selective cross-fertilization outcomes of adult moths developed from the provision of polyamine rich diet during its V- instar of larval period. This study could also be the platform to take measures on increasing the leaf quality for the sustainable development of wild tasar culture.

MATERIALS AND METHODS

Experimental Design

PAs treatment to larvae

Tasar silkworm, *A. mylitta*, DABA BV ecorace reared in Kakatiya University, supplemented with PAs as additional food additives by applying them to the leaves of feeder plant *T. arjuna*., during the V instar larval period. *The tetraamine Spermine (Spm), triamine Spermidine (Spd)* and *diamine Putrescine (Put)*, purchased from Himedia Laboratories Pvt Ltd., Hyderabad, India. Diluted the drugs to (1mg/1ml) as of manufacturer's instructions and applied to the matured leaves of the feeder plant during the first crop rearing from July last week to September second week, 2019.A large nylon nets were placed half a meter above the trees to protect the growing larvae from predatory birds, rodents, grazing mammals and prevent the entry of pathogenic invaders into the culturing unit (fig. ia). Average temperature was in between 28 °C and the relative humidity was in between the (75 %- 83%) during the crop periods. Ten (10) groups of V- instar larvae (fig. id), on day-4, made batches into each group with 30 individuals (9 for *PAs* treatment and 1 for control) were grown on separate trees applied with different polyamines (concentrations at *Spd 50 µM, 100 µM & 150 µM Spm 50 µM,100 µM &150 µM and Put 50 µM,100 µM & 150 µM)* (fig. ic). After a day of grazing, at the end of day-5, they were transferred to separate trees for normal feeding of leaves. This treatment was repeated on day 8 and day 12 to boost the provision of nutritious diet. On

a-Culturing field b- I instar larva c- Separately labeled plantation

d- V instar larva e- Cacoon d- Labeled cacoons

Figure-1. PAs treatment to larvae

completion of treatment from day- 13 onwards the larvae were allowed to graze on normal trees till spinning into cocoons (Figure-1a-g).

Grainage Activity

Cocoons harvested from the field at the end of the crop separately in group wise (Figure-1f), and allowed to dry at RT for two days in laboratory, marked and segregated into 10 groups, made them into garlands, hanged on wires and admitted for grainage activity (from the moth emergence till egg laying and hatching into I-instar larvae). Mosquito nets were arranged around the setup to avoid the entry of predators such as lizards and rats etc (Figure-2).

Figure-2. Grainage activity

Wiped out the excreted liquids frequently and maintained proper sanitation. Dead moths were picked up from the platform and burnt outside the laboratory. Moths exhibited poor movements and underdevelopment were isolated and quarantined in separate plastic tubs.

Percentage of Emerged moths

Moths emerged from the cocoons (Figure-4) were kept separately according to their experimental groups in different cartons which contain holes for passage of light and air. Daily, 5-6 times, all the chambers of grainage activity were keenly observed to any emergence of moths. The emerged moths were segregated and counted on time, periodically during the entire grainage activity. Mean and standard deviation calculated at the end and noted.

Morphological observations of emerged moths:

Moths emerged from the *spd 100 µM, spm 100 µM, spm 50 µM, spd 50 µM, and 150 µM, spd 150 µM, put 50 µM, put 100 µM and put 150 µM* treated were periodically observed the growth and development of wings, antennae, abdomen and noted (Figure-4c).

Percentage of moth involved in the coupling:

In all the experimental groups, the male and female moths exhibited distinct morphological features and involved in copulation after 2-3 days of emergence. The wings of both males and females were cut off to avoid improper movements during copulation. Few of the moths (Figure-4e) (both male and female) which did not participate in coupling were eliminated from the remaining crowed of moths. Percentage of mating moths counted separately and mean calculated along with the STDV at the end of the reproductive period.

a-Cacoon after the emergence of moths b-Moths emerged from cacoons c-Male and Female moths

d-Healthy moths e-Moths at under development with westigial wings

Figure-3. Emergence of moths and morphology of *A. mylitta* daba BV

Figure-4. Different combinations of moths in coupling

Mating behavior and coupling duration:

Moths emerged of *spd 100 µM, spm 100 µM, spm 50 µM, spd 50 µM spd 150 µM, spd 150 µM, put 50 µM, put 100 µM and put 150 µM* treated larvae of V instar observed the actively flying, attracting towards opposite-sex partner, coupling behavior and time, periodically, noted.

Mating Combinations (Cross-fertilization)

Moths emerged from various groups were allowed for cross-fertilization (selective breeding) in different 12 combinations (fig. v). *spm100 µM ♂ X spd 100 µM ♀, spd 100 µM ♂ X spd 100 µM ♀, Spd 100 µM ♂ X spm 100 µM ♀, spm 100 µM ♂ X spm 100 µM ♀, Spd 100 µM ♂X control ♀, Spm 100 µM ♂ X control ♀, Control ♂ X spd 100 µM ♀, Control ♂ X spm 100 µM ♀, Control ♂X control♀, Put 50 µM ♂X control ♀, Control ♂ X put 50 µM ♀, Put 50 µM ♂ X put 50 µM ♀.* Moths were separated after 24 hours of copulation and the females were placed in discrete mud cups for laying eggs. Eggs laid by various moths were counted and the outcomes were analyzed statistically.

Fecundity - Egg-laying capacity:

After copulation, the male and female partners separated and kept in small plastic trays separately at RT in the laboratory till laying eggs. Females laid eggs for 3- 4 intervals and the eggs were attached with gum-like material, the muconium (Figure-5). Eggs laid by each female were counted and the mean and STDEV calculated at the end of the activity.

Disinfecting the eggs:

The eggs were wet and would be exposed to pathogenic contamination. To avoid this, the eggs were washed in 5% formalin for 3-4 minutes followed by further washing in normal running tap water two times. (Figure-5).

Figure-5. Eggs produced in Ovary and Eggs after cleaning with 0.5% NaCl

Egg weight:

To estimate the average weight of egg, ten of each experimental group was randomly chosen and the weight of each egg was measured accordingly. The values were used to calculate the mean along with the standard deviation.

Incubation of eggs:

Disinfected eggs placed in different earthen bowls and small cotton cloth bags (good temperature regulators) and preserved in the laboratory at RT and relative humidity was optimum at 75-85 % (Figure-5)

Hatching eggs:

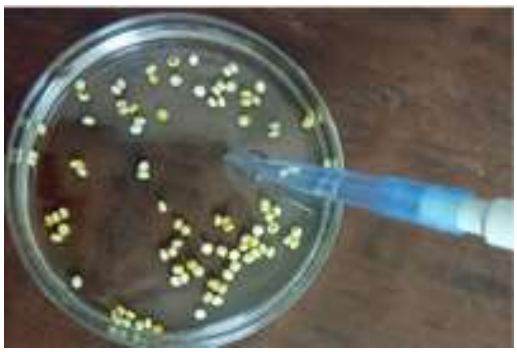
The young worms comes out from the eggs by breaking the shell, especially during the morning hours, and firmly holds to the *Terminalia arjuna* leaves and twigs already kept in the earthen bowls and cotton cloth bags (Figure-6b).

Brushing the Larvae:

Young worms which were at i- instar (fig. ib) were brought to the culturing field and were mounted onto the chawki (young plant with tendered leaves) *Terminalia arjuna* with a soft brush (Figure-6d). The silkworms were at instar- I and crept along the tree and occupied the whole plant and started feeding leaves.

Statistical Analysis-

Significance, Regression and Correlation in between Pupal weight and Fecundity



a-Disinfecting eggs with NaCl

b-Eggs at incubation

c-Hatched larvae from eggs

d-Brushing larvae to chawki rearing

Figure-6. Eggs, Hatching and Brushing of *A. mylitta*, Daba BV

ANOVA single factor analysis of excel determined the *p-value* and *f-distribution* in between two variables, the silkworm pupal growth and development (independent variable) and fecundity (dependent variable), the significant relationship. Rejection of *Null Hypothesis* (H_0) denoted the significance in all the experimental combinations. The regression and co-relation (at y=mx+c) studies explored the proximity and strength of relative interdependence in between the pupal development and egg laying capability.

RESULTS

Fecundity of selective breeding combinations

Among the Selective breeding combinations of the experimental groups result shown the egg laying capacity (fecundity) as, *spm100 µM ♂ X spd 100 µM ♀(193.8)> spd 100 µM ♂ X spd 100 µM ♀(157.3) > Spd 100 µM ♂ X spm 100 µM ♀(147.2) > Spm 100 µM ♂ X Spm 100 µM ♀(145.3) > Spd 100 µM ♂X control ♀(129.7) > Spm 100 µM ♂ X control ♀(121.2) > Control ♂ X spd 100 µM ♀(119) > Control ♂ X spm 100 µM ♀(110.3) > Control ♂ X Control♀ (99.3) > Put 50 µM ♂X control ♀(77.2) > Control ♂ X put 50 µM ♀(75.5) > Put 50 µM ♂ X put 50 µM ♀(64.02)* (Figure-7)

Pupal Weight and Fecundity

Pupal weight of the experimental groups disclosed that, *spd with 50 µM* and *100 µM* and spm with *100 µM* concentration shown elevated results, whereas *spm* by *50 µM* concentrations slightly reduced the outcomes. The rest of the PAs at any concentration remain declined results, yet. (Figure-8).

Morphological Observations of Emerged Moths:

The moths emerging from the cocoons (Figure-4) of V instar larvae of silkworm nourished by the supplements of *spd 100 µM, Spm 100 µM, spm 50 µM, spd 50 µM* treated larvae exhibited normal development in wings structure (fig-ive). Contrastingly, moths emerged from the cocoons of *spm 150 µM, spd 150 µM, put 50 µM, put 100 µM* and put *150 µM* treated were with vestigial wing development, sluggish movement (fig.ive) and most of them died without reaching their mating stage.

Mating Behavior and Coupling Duration:

Moths emerged from the cocoons of silkworm larvae of V-instar by feeding treatment of *spm100 µM ♂ X spd 100 µM ♀, spd 100 µM ♂ X spd 100 µM ♀, Spd 100 µM ♂ X spm 100 µM ♀, spm 100 µM ♂ X spm 100 µM ♀* (Figure-5) participated in copulation actively by at least for 12 to 24 hours, Whereas moths of control along with the other *PAs* combinations were hesitated to reach their opposite sex partner and few of others spent for very few hours in copulation and left over dwindled consequences.

Hatching Percentage of Eggs

The experimental groups (*PAs* treated and *control*) exhibited hatching percentage as, *spm100 µM ♂ X spd 100 µM ♀ > spd 100 µM ♂ X spd 100 µM ♀ > spm 100 µM ♂ X spm 100 µM ♀ > spd 100 µM ♂X control ♀ > spm 100 µM ♂ X control ♀ (121.2) > Control ♂ X spd 100 ♀ µM > Control ♂ X spm 100 µM ♀ > Control ♂ X Control♀*. Contrastingly, lowest hatching percentage left over by *put 50 µM ♂ X control ♀ > control ♂ X put 50 µM ♀ > put 50 µM ♂ X put 50 µM ♀* (graph. iv).

Significance, Regression and Correlation Statistics of Pupal Weight to Fecundity

Figure-7. Percentage of Fecundity (Selective mating), treated by Pas & control, A. mylitta, Daba BV

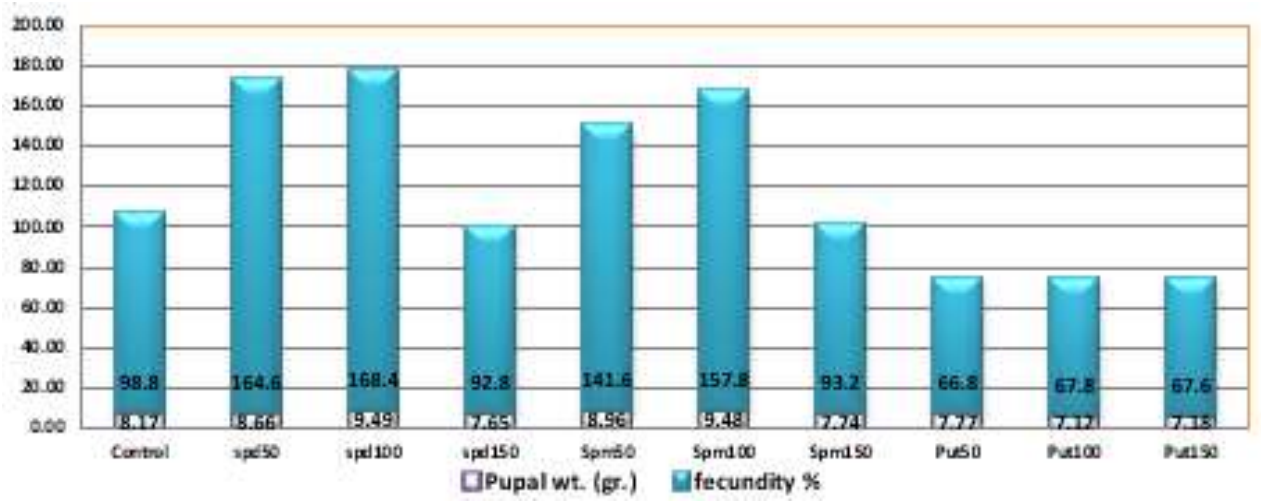


Figure-8. Pupal weight vs fecundity

In all the experimental groups (9 *PA*s treated and one *control*), pupal weight to the fecundity was significant ($a \leq 0.05$) and *f> f- Crit* Value in all cases. Regression analysis (graph. iii), exploring that a relationship between the two variables with Perfect +Ve shown by the *spd 50 µM, spd 100 µM, spm 50 µM, spm 100 µM*, whilst a moderate relationship established by control and *spd 150 µM* (graph 3.2). Yet, the rest of the batches had no relationship. (Table. 1).

DISCUSSION

Effect of Polyamine on Fecundity

Polyamines play a vital role in the cell metabolism including effects on the structure of cellular macromolecules DNA, RNA and gene expression, protein metabolism, protein synthesis, and protection from oxidative damage (Pegg, 2014). *Sprmidine and spermine* enhance the testicular development in *B. mori* (Mysarla *et al*., 2016), spermine in the semen of sea lampreys act as sex pheromone (Scott *et al*., 2019). Spermine enhanced the embryonic fibroblast development in mice (Mackintosh & Pegg, 2000). *Spd* protects the gonads from oxidative damage (Shahin *et al*., 2019), Considering that,in this present study, the *spm* male and *spd* female combinations produced more fecundity than all others. Highest rate of fecundity noticed by *spd 100 µM* treated female (♀) mate with *spm 100 µM* treated males even than their counter combination *spd* male with100 and *spm* female with 100 (graph. i). Moreover, all females with *spd* and all males with *spm* produced more fecundity than all males with *spd* and females with *spm*. The elevated egg production by the *spd* female silkworms mating combinations disclosed that the polyamine spd involve in ova improvement in ovaries and oogenesis. *Control* moths in any mating combinations with *spd 100 µM* and *spm 100 µM* produced less fecundity than , *spm 100with spd 100 combinations, yet,* better results than any *put 50 µM* treated combinations. Contrastingly, mating in between the *put 50 µM* combinations, the results were more detrimental (graph. i). The reason might be the antagonistic interference of *put at 50 µM, 100 µM* and *150 µM* inside the cells.

Consequently, *spm 100 µM* male with *spd 100 µM* female produced the highest level of fecundity than all, even *spd 100 µM* male with *spm100 µM* female that is indicating that *spm 100 µM* shown considerable development on testicular development and spermatogenesis. The same phenomenon also observed in the percentage of hatching. The hatching percentage of *spm µM 100 ♂ X spd 100 µM ♀ (96.47%), spm 100 µM ♂ X spm 100 µM ♀ (94.95%)* and *spm 100 µM ♂X control (94.91%). Spm* treated male combination with any other combination increased hatching percentage. *Spd* female in all combinations showed increased percentage in egg laying, contrastingly, less hatching percentage than *spm* male combination (Figure-9).

Whereas any another male (♂) combinations except *spm 100 µM ♂* lower levels of hatching percentage although the elevated egg production by *spd* ♀ amalgamates with any partner. These results revealed the male-specific gametogenic enhancement promoted by *spm 100 µM* and female-specific gametogenesis enhanced by *spd 100 µM*.

Spd and *Spm* inoculation into chick embryos during the 10 days development increased the DNA and RNA levels (Caldarera *et al*., 1965). Inhibition of *spm* and *spd* experiments in rats arrest the fibroblast development (Rupniak & Paul, 1978). Inhibition of *Spermine* and *Spermidine* during mouse embryonic development causes cavity formation in tissues of uterine walls (Alexandre, 1979).considering that, in the present study, highest the egg weight was observed in the test organisms treated with *spd50 µM, 100 µM* and *spm 50 µM and 100 µM* (graph. v) which is indicating the concern Polyamines enriched the biochemical entity.

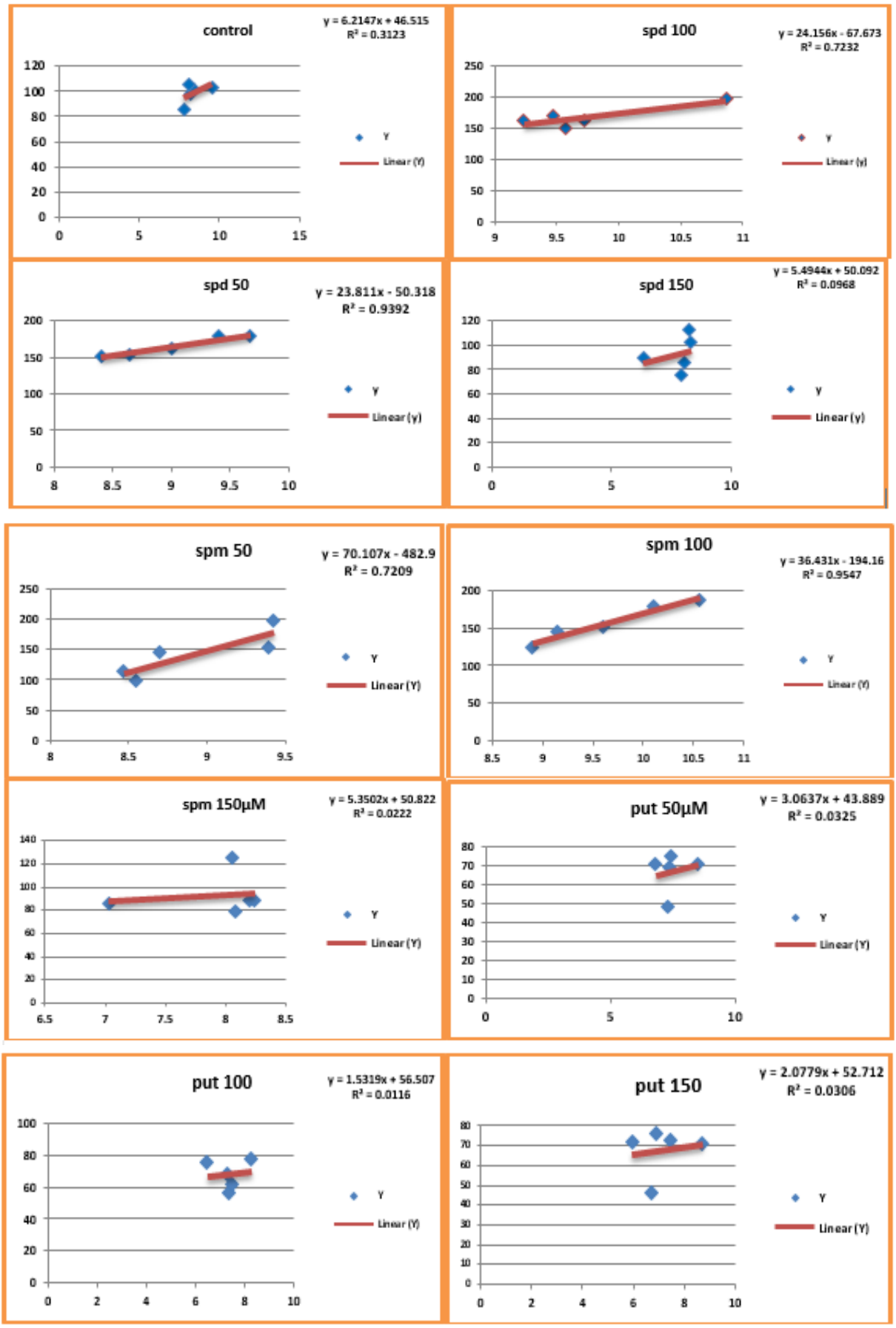


Figure-9. Showing the regression curves of PAs treated (9) and control (1) groups.

PAs	correlation (ρ)	coefficient determination (r^2) %	Pearson correlation coefficient * %	p-value (α=0.05)	F ratio to F crit.	result of p & F values	Regression curve
control	less +Ve	0.31	0.56	< 0.00001	702.28 > 5.32	significant	Y=6.2147X+46.515
spd 50	perfect +Ve	0.93	0.96	< 0.00001	733.11 > 5.32	significant	Y=23.811X-503.18
spd 100	perfect +Ve	0.72	0.85	< 0.00001	383.35 > 5.32	significant	Y=24.156X-67.673
spd 150	less +Ve	0.96	0.31	< 0.00001	173.48 > 5.32	significant	Y= 5.4944X+50.092
spm 50	perfect +Ve	0.72	0.85	< 0.00001	5.59 > 5.32	significant	Y=70.107X-482.9
spm 100	perfect +Ve	0.95	0.97	< 0.00001	170.74 > 5.32	significant	Y=36.431X-194.16
spm 150	No Relation	0.02	0.14	< 0.00001	110.20 > 5.32	significant	Y=5.3502X+50.822
put 50	No Relation	0.03	0.17	< 0.00001	152.21 > 5.32	significant	Y= 3.0637X+43.889
put 100	No Relation	0.01	0.1	< 0.00001	221.02 > 5.32	significant	Y= 1.5319X+56.507
put 150	No Relation	0.03	0.17	< 0.00001	121.46 > 5.32	significant	Y= 2.0779X+52.712

Table-1. Correlation statistics between pupal weight and fecundity of PA's treated (Selective mating). A. mylitta, Daba BV.

Significance, Regression, and Correlation of pupae to fecundity

Larval development contributes effective metamorphosis of pupae, enhanced levels of fecundity. Quality nutrients in the larval stage determines the fate of the total life cycle of Tasar silkworm *Antheraea mylitta* (Rath *et al*., 2009).In our previous study, significant relationship and positive correlation exhibited in between larval and silk gland weight as well as cocoon and shell weight U.Swamy & G.Shamitha 2021a and b).The fecundity correlated with the significant pupal weight *(a ≤ 0.05)* and the correlation of co-efficient exhibited the highest positivity (r = 0.97 in *spm 100 µM*) and (r= 0.96 in Spd 50) along with both the *spd 100 µM* and *spm 50 µM* (r=0.85) (table. i). The pupal weight determines the genuine estimator of fecundity (Barah & Sengupta, 1991) . In this present study, a strong parallel Positive (+ve) developmental relationship established again by *PAs*, viz., *spd 50 µM, 100 µM*, and spd *50 µM, 100 µM* treated larval lines. Yet, Putrescine and *spm at 150 µM* concentrations were left over the declined relationship. . *spd 150 µM, spm 150 µM*, and all put fed larvae were recording low-level results from larval, cocoon, and silk yield characteristics which were continued in the fecundity also.

CONCLUSION

The rate of fecundity explored the superiority of *spd 100 µM* on female reproductive organs (ovaries) and oogenesis, while the rate of hatching declared that the *spm 100 µM* indulge in development of male reproductive organs (testes) and promote spermatogenesis and contribute to produce eggs with fertility.

Conflicts of Interest

Authors declare that there is no conflict of interests regarding the publication of this paper.

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