International Journal of
Research in Chemistry and Environment
Available online at: www.ijrce.org
ISSN 2248-9649

Research Paper

Acid Phosphatase Activity in Relation to Reproductive Behaviour in Males of *Antheraea mylitta* Drury During Different Development Stages and Impact of use of Male Moth for Repeated Mating

***Lokesh G., Srivastava A.K., Srivastava P.P., Kar P.K., Sinha A.K. and Sahay Alok**
Silkworm Breeding & Genetics, Central Tasar Research & Training Institute,
Central Silk Board, Ranchi, Jharkhand- 835303, INDIA

(Received 05th January 2016, Accepted 12th February 2016)

Abstract: *Comparative Acid phosphatase (ACP) activity was studied in two ecoraces of Antheraea mylitta viz., Daba and Laria during different developmental stages of lifecycle to analyze the reproductive behavior of the male tasar silkworm and also the effect of repeated use of male moths for mating. It was found that higher protein concentration was recorded in the pupal hemolymph of both the tasar silkworm races i.e., Daba (135.33± 3.21mg/ml) and Laria (131± 3.60 mg/ml) compared to testis. Also an increased protein concentration was recorded in the testis tissue from the larval stage to moth before coupling. Higher ACP activity was found in the testis tissue of the moth (55.2 ± 1.92 µg p-nitrophenol/mg protein/hr) followed by pupal hemolymph with similar trend in both the ecoraces studied but comparatively low activity in the Laria. Fecundity and hatching was affected when the male moths were used for second mating. About 6-8% reduction in the fecundity and 10-16% in hatching was observed. Also difficulty of mating and needs hand coupling method to establish the mating. Hence, second time using of males during scarcity and in exigent cases is suggested to improve total layings in the grainages to meet the demand.*

Keywords: Acid phosphatase, *Antheraea mylitta*, Daba, Laria, Repeated mating, Testis.

Introduction

Tropical tasar silkworm *Antheraea mylitta* represent variation for a number of qualitative and quantitative traits of basic biological and economic interests, such as silk quality, fecundity, disease resistance and tolerance to various environmental entities in its populations [1]. It is polyphagus in nature, though primarily adapted to three important food plants, but feeds on number of other host plants in different geographical regions of India. The biochemical parameters have been proved to be valuable tools for studying genetic variation in natural populations [2] and have been used as useful indicators in plant and animal breeding programmes[3]. Variation in some vital biochemicals in different species form the basis to understand the phylogeny and to establish the genetic basis of taxonomic relationships between species and enzyme polymorphism are known to be extremely common and easy to find in most of insect species. The different races of silkworm of Indian origin, exotic, multivoltine, bivoltine and their hybrids have been tested for a few enzyme systems which bring about certain groups of chemical reactions among silkworm races, in which amylases, proteases, esterases and others have been detected by a technique specially developed for the purpose[4]. The present study was aimed at finding the level of protein biosynthesis and activity of Acid phosphatases in the hemolymph and testis during the different developmental stages of *A.mylitta.* Acid phosphatase (ACP) is a lysosomal enzyme that catalyzes the hydrolysis of variety of phosphate monoesters and phosphoprotein in an acidic medium. Phosphatases are capable of transphosphorylation in addition to hydrolysis. Phosphatases thus play an important role in the metabolism of carbohydrates, phospholipids and nucleotides[5]. Spermatogenesis is an extremely active replicative process capable of generating high number of gametes in seconds which takes place in the testis. This high profile physiological activity requires large amount of energy and protein biomolecules for an efficient

synthesis of active sperms. The protein metabolism is regulated in the cells of different tissues by the appropriate mechanism of phospharylation and dephospharylation. Hence, in the present study, acid phosphatase activity was recorded in the different development stages of the tasar silkworm.

Material and Methods

In the present work two ecoraces of tropical tasar silkworm *Antheraea mylitta* D viz., semi-domesticated Daba and wild ecorace Laria were used for the analysis of Protein and Acid phosphatase (ACP) activity during different developmental stages viz., 5th instar Larva, Pupa and Moths (before coupling & after coupling). Also, the consequence of repeated use of male moths on the coupling behaviour, fecundity and larval hatching performance was analyzed.

I. Biochemical analysis

Preparation of Samples for biochemical studies:

The larvae of semi-domesticated Daba and wild ecorace Laria during 5th instar were obtained from field laboratory of Silkworm Breeding and Genetics, CTR&TI, Ranchi, hemolymph and testis from male larvae was collected. Subsequently, Male pupae were obtained after harvesting of cocoons for the collection of hemolymph and testis. Further, testis was also collected from some male moths after emergence and some moths after coupling for six hours.

Hemolymph was collected by cutting the pro leg of silkworm larvae and anterior part of the pupa in a pre-cooled micro-centrifuge tube containing a pinch of phenylthiourea as an anticoagulant and centrifuged at 5,000 rpm for 5 min at 4°C. The supernatant collected and stored at -20^{o}C until further use.

Testis was collected in the male silkworm Larvae, pupae and moth. Dissection was carried out in ice cold phosphate buffer. Homogenization of testis was done in a homogenizer using phosphate buffer pH 7. The homogenate was transferred to a clean centrifuge tube and processed the sample as above.

Estimation of total protein: The total protein was estimated using the method of [6] with Bovine Serum Albumin (BSA) as standard in the hemolymph of Larva & pupa and testis tissue of larva, pupa and in moth.

Acid Phosphatase activity: The testis tissue was homogenised in 0.25 M sucrose containing 1mM EDTA. The homogenate was centrifuged at 10,000 rpm the pellets were discarded and the supernatant was diluted appropriately with 150mm Sodium acetate buffer and immediately used for the assay following the method of [7].

II. Study of Grainage behaviour

About 30 male moths were collected separately, 10 male moths were used for testis collection and 20 male moths each were used for coupling with females of both the ecoraces. After 6-7 hours of mating, 10 coupled male moths were collected for second time mating and remaining 10 moths were used for the collection of testis. Female moths were allowed for egg laying according to the standard practices. Collected 10 male moths were mated repeatedly second time with fresh female moths. Subsequently, allowed female moths for egg laying for three days and decoupled male moths were discarded. On 4th day of laying, eggs were analyzed for the fecundity [8]. Further, eggs were washed and maintained as per recommended procedures up to hatching and assessed the hatching performance[9] in both Daba and Laria.

Results

In the present study, the comparative analysis of biochemicals in the hemolymph and testis of Daba and Laria ecorace of *A. Mylitta* and the impact analysis of males used for second time mating on fecundity and hatching was observed and recorded as below.

a. Protein concentration in different developmental stages: Protein concentration in the hemolymph and testis of different stages in the Daba and Laria ecoraces of *A. mylitta* were observed, which indicated that, protein content increased with the development in the hemolymph of the silkworm from larva to pupae. Similar increase was also recorded in the protein concentration of testis up to early stage of moth but lowered significantly in the testis of moth after coupling (Table 1). This trend was noticed in both Daba and Laria ecoraces despite significant variations ($p<0.05$) in the protein concentration between the races. Higher values were observed in the Daba pupal hemolymph (135.33± 3.215mg/ml) followed by uncoupled moth testis (99.33±3.512), larval hemolymph (97.33±5.033), pupal testis (94.33± 3.512), larval testis and testis of coupled moth (84.67±2.082 & 62.67± 2.082). Comparatively similar trend in the protein concentration was recorded with significant variability in the Laria ecorace Viz., pupal hemolymph (131± 3.6065mg/ml) followed by uncoupled moth testis (94.33±1.15), larval hemolymph (86.67±5.5), pupal testis (90.67± 2.517), larval testis and testis of coupled moth (74.67±3.512 & 58.00± 2.64).

Acid phosphatase activity: Acid phosphate activity showed a positive correlation with the protein concentration in the testis during different developmental stages of tasar silkworm. Higher activity was recorded in the testis of moth (55.2 ± 1.92 µg p-nitrophenol/mg protein/hr) followed by pupal hemolymph, pupal testis, larval testis and larval hemolymph. Lowest was observed in the testis of coupled moth (32.0 µg p-nitrophenol/mg protein/hr) in the Daba. Difference in the activity between semi-domesticated Daba and Wild Laria ecoraces was

well pronounced (Table 2). Where, higher acid phosphatase activity was recorded in Daba ecorace than Laria 50.2 ± 2.16 µg p-nitrophenol/mg protein/hr in the testis of uncoupled moth and 30.60 ± 2.07 in the testis of coupled moth (Table 2).

Table 1: Total protein content in hemolymph and testis during different stages in Daba and Laria tasar silkworm ecoraces

Tissues studied	Daba				Laria			
	Mean	SEM	t value	P value	Mean	SEM	t value	P value
Larval hemolymph	97.33*	5.033	4.028	0.01	86.67*	5.508	3.18	0.05
Larval testis	84.67	2.082			74.67	3.512		
Pupal hemolymph	135.33**	3.215	14.915	0.001	131**	3.606	15.89	0.001
Pupal testis	94.33	3.512			90.67	2.517		
Moth testis before coupling	99.33**	3.512	15.556	0.001	94.33**	1.155	21.8	0.001
Moth testis after coupling	62.67	2.082			58.00	2.646		

**Highly significant, *Significant, NS Non-significant

Table 2: Acid Phosphatase activity (µg p-nitrophenol/mg protein/hr) in hemolymph and testis during different stages in Daba and Laria tasar silkworm ecoraces

Tissues studied	Daba				Laria			
	Mean	SEM	t value	P value	Mean	SEM	t value	P value
Larval hemolymph	43.6	3.647	1.832	NS	40.4	2.074	0.703	NS
Larval testis	39.8	2.864			39.4	2.408		
Pupal hemolymph	51.2*	2.864	4.148	0.010	46.0	4.472	1.177	NS
Pupal testis	44.4	2.302			43.0	3.536		
Moth testis before coupling	55.2**	1.924	19.269	0.001	50.2**	2.168	14.609	0.001
Moth testis after coupling	32.0	1.949			30.60	2.074		

**Highly significant, *Significant, NS Non-significant

Table 3: Fecundity and Hatching performance of Daba and Laria (mean ± SD)

		Daba	Laria
Fecundity (Nos.)	1st mating Males	235 ± 12	211 ± 8
	2nd time mating Males	222 ± 10	190 ±11
Hatching (%)	1st mating males	85 ± 2	73 ± 3
	2nd time mating Males	68 ± 4	61 ± 4.5

II. Comparative assessment of fecundity and hatching in the males used for first and second time mating:

In the present study, the coupling behaviour was analyzed in relation to the number of time male moths were utilized for mating. It was observed that, 90% self coupling was achieved in the first mating in the Daba, but self coupling was reduced to 50% when the males used for second time mating. About 30% was mated with hand couple technique. 20% did not couple even tried with induced methods of coupling. In contrast, Laria ecorace did not recorded self coupling, in both first and second time use of males for mating. But, it completely relied up on induced coupling. Achieved 60% hand coupling in case of males used for second time.

Fecundity and Hatching

Higher fecundity was recorded with Daba ecorace on the first mating (235± 12) followed by Daba ecorace on second time used male for coupling (222 ± 10), Laria (211± 8) and Laria second time used male (190±11). The low percentage of hatching was observed in the batches used the males second time for mating in both Daba and Laria ecorace. 85% of hatching was recorded in the Daba ecoraces, Laria recorded with 73% and lower hatching in the batches where males used for second time mating in both Daba (68%) and Laria (61%) was observed.

Discussion

Proteins have been well studied in insects by many workers that, proteins affect various life stages of insects [10-13]. Stage / age specific changes in protein concentration of different tissue during post-embryonic development of *A. mylitta* might be for different performances for various biological activities in the developmental stages and for the maintenance of various physiological functions in different tissues[14,15]. Higher proteins in the mature larval hemolymph is due to the accumulation of proteins and higher rates of metabolism during the development of vital organs in the body viz., silk glands, reproductive organs and etc., during final larval stages [16,17]. Accumulation of protein in the non-feeding pupal hemolymph acts as the reserve for the development of various organs in the adult and also to prepare for the subsequent reproductive activity in the moth stage. Increased concentration of proteins in the testis during development and metamorphic stages larval, pupal and moth showing development based concentration of proteins and also reflects the level of biosynthesis of male gametes through the process of spermatogenesis in the gonads (testis) for subsequent process of reproduction [18]. Similar reports were available with [13]. In contrast, the level of proteins recorded less in the gonads of moth after decoupling this may be due to the discharge of gametes during mating and very low level of protein concentration and slow synthesis rate of gametes.

In the present study, higher concentration of the protein recorded in the hemolymph and testis of Daba ecorace of *A mylitta* compared to Sal based Laria is attributed to the fact that this ecorace is being well adapted to the present rearing environment and amenable for human handling thus it has been recognized as one of the semi-domesticated ecoraces of *A mylitta.* Also higher observed higher food consumption in turn efficiently converted the dietary food during its feeding stage the corresponding level of proteins were maintained in the hemolymph and testis. Many authors have been recorded similarly the higher proteins in the hemolymph earlier [19,13,20,21]. The differences in Daba and Laria depict the differential feeding rate and physiological activity among different ecoraces and adaptively for the geographical region and food plant [22]. It was reported that, the rearing performance, and expression of phenotypic characters of tasar silkworm largely depends on type of the food plant, geographical attribute such as altitude, latitude and other environmental factors [23, 24].

The level of phosphatase activity in the hemolymph is correlated with that of silk protein synthesis level and absorption capacity of digested food in the silkworm larvae [25]. The higher ACP activity in the hemolymph was attributed that, mid gut is the chief site for activity of Phosphatases and some amount of the enzymes percolated in to the circulatory system, thus hydrolyses and transphosphorylase activity of ACP is takes place at significant level in the hemolymph [26]. The differential level of activity in different tissues ie., hemolymph and testis attributed that, the level of metabolic process and need for the tissue development. Since, hemolymph is the principal medium of circulatory system in the insect larvae and it consists of complex mixture of biomolecules leads higher level of phosphatase activity.

Fecundity is considered as one of the most desired quantitative traits of commercial importance in silkworms [27]. The genotype-environment interaction has highly significant influence on the fecundity of the silkworms. The healthy and robustness of the mating male-female moths are very important for the subsequent quality and quantity of egg laying. Also the male of different breeds can influence the egg laying performance in silkworms [28,29]. In the present study, the fecundity and the hatching performance was recorded lower in case of male used for second time in the mating. This could be due to the higher discharge of healthy and viable sperms during first mating compared to second mating. This has consequence in the fecundity of the mother moth and also in the hatching percentage.

The differential performance of the Daba and Laria with reference to fecundity attributed to the genetic endowment of the two ecoraces. Similar results were reported earlier in domesticated silkworm [30]. A synonymous effect was also observed in case of fertility rate of eggs and hatching performance with differential impact in Laria and Daba ecoraces. From the present study, it is understood that, transphosphorylation is an important metabolic activity that is driven by the level of synthesis of acid phosphatases in different tissues of the silkworm. The increased level of activity during different developmental stages enumerates the preparation of the silkworm for subsequent reproduction in the adult stage. The variation in the protein concentration and ACP activity found in Daba and Laria can be used as markers to analyze the genetic variability in the tasar silkworms. Second time using of male moth for mating/coupling has direct implications on the fecundity and hatching performance of the silkworm. Thus, this could be applied in exigent cases during male moth scarcity.

References

1. Suryanarayana N. , and Srivastava A.K., Monograph on Tropical Tasar Silkworm, Central Tasar Research and Training Institute, CSB, India, **(2005)**

2. Adams W.J., In:Isozymes in plant genetics and breeding, *Elsevier Science*, 381-400, **(1983)**

3.Tanksley K., Maruyama K. and Rick C.M., Plant genetic and breeding ; *Heredity*, **49**, 11-25, **(1982)**

4. Hirobe T., Evolution, differentiation and breeding of the silkworm- the silkroad, past and present –Genetics in Asian countries, *XII Int. Cong. Genet*., 25-36, **(1968)**

5. Bhawane G.P., Bhanot R.K., Alkaline and acid phosphatases of cockchafers *Holotrichia fissa* Brinske, *Indian Journal of Entomology*, **56 (4)**, 342-346, **(1994)**

6. Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J., Protein measurement with Folin phenol reagent, *J. Biol. Chem*., **193**: 267-275, **(1951)**

7. Wilson K. and Walker J., Kinetics of Alkaline Phosphatase In, Practical Biochemistry, Principles and Techniques, BL-304, 1-3, **(2000)**

8. Sinha B.R.R.P., Survey of genetic resources and study of the genetic divergence in *Antheraea mylitta* Drury. Ph.D. Thesis, Ranchi University, Ranchi, India **(1998)**

9. Saheb Biram N.M, Tribhuwan Singh and Beera Saratchandra, Occurrence of Unfertilized eggs in the mulberry silkworm *Bombyx mori* L (Lepidoptera: Bombycidae), *Int. J. Indst. Entomol*, **18(1)**, 1-7, **(2009)**

10. Brown J. J., Hemolymph protein reserves of diapausing and non-diapausing Codling moth larvae, Cydia pomonella L (Lepidoptera: Tortricidae), *J. Insect. Physiol.*, **26**, 487-491, **(1980)**

11. Koopmanschap B., Lammers H. and de Kort S., Storage proteins are present in the hemolymph from larvae and adults of the Colorado potato beetle, *Aech. Insect. Biochem. Phsiol.*, **20**, 119-133, **(1992)**

12. Kumar N.S., Basavaraja H.K., Joge P.G., Kalpana G.V. and Malreddy N., Heterosis studies on hybrids of cocoon color sex-limited breed of the silkworm, *Bombyx mori* L. under different environments of temperature, *J. Entomol. Res. Soc*., **10**, 1-12, **(2008)**

13. Kumar D., Pandey J.P., Jain J., Mishra P.K., and Prasad B.C., Qualitative and Quantitative changes in protein profile of various tissue of tropical tasar silkworm, *Antheraea mylitta* Drury, *Int. J. Zool. Res.*, **7(2)**, 147 -155, **(2011)**

14. Damara, M.D. and Gupta A.D., Identification of 86 kDa protein as methionine rich hexamerin in rice moth, Coreyra cephalonica, Comparative biochem. Physiol. Part B; *Biochem. Mol. Biol.*, **15**7, 1071-1077, **(2010)**

15. Ann C. and Kanost M.R., Manduca Sexta serpin-5 regulates prophenoloxidase activation and the Toll signaling pathway by inhibiting hemolymph proteinase HP6, *Insect. Biochem. Mol. Biol.*, **(2010)**

16. Lokesh G., Putkho Paul Pao, Madhusudhan K.N., Kar P.K., Srivastava A.K., Sinha M.K., Manohar Reddy R., Muniswamy Reddy P.M. and Prasad B.C., Study of Phenotypic Variability in Silk Gland Characters in Three Ecoraces of Tropical Silkworm *Antheraea mylitta* Drury, *Asian Journal of Animal and Veterinary Advances*, **7(1)**, 80-84, **(2012)**

17. Lokesh G. and AnanthaNarayana S.R., Changes in the Protein profile of silkworm *Bombyx mori*. L (Lepidoptera: Bombycidae) in response to the chemical mutagen, *Int. J.Sci. Nat.*, **2(3)**, 559 -563, **(2011)**

18. Scaraffia P.Y., Zhang Q., Thorson K., Wysocki V.H. and Miesfeld R.L., Differential ammonia metabolism in Aedes aegypti fat body and midgut tissues, *J. Insect. Physiol.*, **56**, 1040-1049, **(2010)**

19. Babu K.R., Ramakrishna S., Reddy Y.H.K., Lakshmi G., Naidu N. V., Basha S.S. and Bhaskar M., Metabolic alterations and molecular mechanism in silkworm larvae during viral infection: A review, *Afr. J. Biotechnol*., **8**, 899-907, **(2009)**

20. Lokesh G. and Anantha Narayana S.R., Changes in the activity of digestive enzymes in response to the chemical mutagen Diethyl Sulfate in the silkworm *Bombyx mori*. L (Lepidoptera: Bombycidae), *Asian Journal of Applied Sciences*, **5 (6)**, 431 -437, **(2012)**

21. Srivastava, A. K., Kar P.K., Naqvi A.H., Sinha A.K., Singh B.M.K., Sinha B.R.R.P. and Thangavelu K. Biochemical variation in ecoraces of *Antheraea mylitta* : A review, *Perspectives in Cytology & Genetics*, **10**, 359-346, **(2001)**

22. Venugopal Pillai S., Krishnaswami S. and Kashivishwanathan K., Growth studies in silkworm, *Bombyx mori*. L. under tropical conditions. II. Influence of agronomical methods of mulberry on growth, cocoon crop and fecundity of silkworm, *Ind. J. Seric*. **26(1)**, 38-45, **(1987)**

23. Srivastava A.K., Sinha A.K., Sinha B.R.R.P., Descriptor of tropical tasar silkworm, *Antheraea mylitta* Drury (Lepidoptera Saturniidae), CTR&TI, Ranchi, 1-14, **(2003)**

24. Srivastava A.K., Naqvi A.H., Sinha A.K., Vishwakarma S.R. and Roy G.C., Genotype and environment interaction in *Antheraea mylitta* Drury and its implications. *Perspectives in Cytology and Genetics*, **11**, 219- 224, **(2004)**

25. Gaikwad Y.B., Gaikwad S.M., Bhawane G.P., Effect of induced oxidative stress and herbal extracts on acid phosphatase activity in lysosomal and microsomal fractions of midgut tissue of the silkworm, *Bombyx mori. Journal of Insect Science*, **10**, 113, **(2010)**

26. Singh Anita, Ratnesh Kr Sharma and Bechan Sharma, Low temperature induced alterations in certain biochemical constituents of 5th instar larvae of *Philosamia ricini* (Lepidoptera: Satunidae) Open Access Insect Physiology, **2** 11–16, **(2010)**

27. Reddy R.M., Sinha M.K. and Prasad B.C., Application of Parental Selection for Productivity Improvement in Tropical Tasar Silkworm *Antheraea mylitta* Drury-A Review. *Journal of Entomology*, 7, 129-140, **(2010)**

28. Kumar Kishore C M. and Paul D.C., Effect of age of males on ejaculation and behaviour of spermatozoa in the internal reproductive organs of the female moth, *Bombyx mori* L. (Lepidoptera: Bombycidae), *Sericologia*, **33(2)**, 219 - 222, **(1993)**

29. Krishna Prasad N K., Sannappa B. and Varalakshmi R., Effect of multiple mating on grainage performance of newly evolved bivoltine breeds of *Bombyx mori* L., *Bulletin of Indian Academy of Sericulture*, **6(2),** 50-55, **(2002)**

30. Omana J. and Gopinathan K.P., Heat shock response in mulberry silkworm races with different thermo tolerances, *J. Biosci*., **20**, 499-513, **(1995)**.