



Research Article

## DIFFERENTIAL ADULT LIFESPAN AND STARVATION STRESS RESPONSES IN LABORATORY EVOLVED BIVOLTINE SILKWORM BREEDS OF *BOMBYX MORI* L.

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### ABSTRACT

Silkworm breeding is majorly focused on balancing the progress of productive trait to generate profitability. However, with recurrent commercialization and utilization of such productive trait through heterosis may decline with drastic climatic variations. In order to achieve and generate profitability in sericulture, paradigm shift is essential through transition from balanced breeding to sustainable breeding via selection of breeding lines with better adult moth longevity which confers the benefits by coping with stressful environment. In the present investigation, CSR bivoltine silkworm breeds were screened to understand the adult moth longevity among them which have attained two decades of evolution. Differential longevity was recorded among the selected breeds. The maximum and minimum longevity was observed in CSR 51 and CSR17 respectively than other breeds. The short-listed breeds were exposed to starvation stress, wherein, interestingly, the CSR51 was highly tolerant compared to other breeds and CSR17 being short-lived was susceptible to starvation stress. The data obtained suggest that the relatively longer lifespan may be rendering more tolerance towards the starvation stress mediated lethality, as evident by the induced starvation stress assay. This would help us to focus on the generation of long-lived breeding lines with better longevity which could intrinsically determine for better abiotic and biotic stress tolerance

**Keywords:** Bivoltine, Breeding, Longevity, Starvation, Survivorship.

### INTRODUCTION

The silkworm is endowed with a potential ability providing a preponderant foundation for research with many avenues, one among that is breeding and genetics. Silkworm breeding has been the main focus since decades in the virtue of improving crop production and productivity in sericulture sector. Breeding experiments were initiated at Central Sericultural Research and Training Institute, Mysore to evolve bivoltine silkworm races suitable to tropical conditions for achieving the promotion and adoption of bivoltine sericulture to reach high productivity. With this aim, hybridization programme was initiated. Hybridization is considered as one of the important evolutionary force, and through which many productive and qualitatively superior bivoltine hybrids were developed by utilizing Japanese commercial breeds as breeding resource material at CSRTI Mysuru (Basavaraja *et al.*,

1995). The CSR breeds of bivoltine silkworm evolved during that period serve as an interesting system to understand extended longevity. Longevity (life expectancy) is one of the components of fitness which is extensively being used to assess the genetic characteristic of this trait in different organisms (Durbin and Yoon, 1986). The evolution of longevity says that, improvement in life span is dependent on an increased ability to resist environmental stresses. Selective breeding of *Drosophila melanogaster* populations for longevity had demonstrably increased life span and also altered a number of other traits, such as resistance to starvation, desiccation, and ethanol fumes, and the ability to sustain longer flight (Luckinbill, 1998).

A detailed investigation utilizing several temperate silkworm races in order to understand the relevance of lifespan with commercial characters had been attempted by Kang *et al.* (1999). However, in tropical silkworm races

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detailed investigation on utilising the breeding lines for the trait longevity for the betterment of breeding programme is sparse. Despite major progress in our understanding of the genetic basis of aging and life history, especially in model organisms such as yeast, *C.elegans*, *Drosophila*, and Mice (Guarente and Kenyon, 2000; Tatar et al., 2003; Partridge and Gems, 2006; Kenyon 2010; Flat and Heyland, 2011) ample research has been undertaken in silkworm. Silkworm experiences both abiotic and biotic stresses which deteriorates the survival and lifespan by ultimately affecting the crop production. As *Bombyx mori* silkworm is domesticated, inevitably, Mulberry leaves are fed by the silkworm rearing people. Sometimes, there may be variation in the time schedule for feeding the silkworms by different farmers, though the time and schedule is known in the managerial practices (Krishnaswami, 1979), depending upon their convenience. Hence, there are many instances, where silkworm faces starvation stress when they are not properly given food in the stipulated time before exhaustion of the provided food and silkworms also experience the starvation load when the quality of mulberry leaves is poor with manifestation of different mulberry pests and varying climatic conditions. One of the most causes of stress, may be at least for silkworms like other animals, is shortage or suboptimal quality of mulberry leaves. Silkworms must cope with periodical malnutrition or starvation, and though the mulberry leaves may seem abundant it may be limited by availability of specific nutrients. Likewise, though the breeds with special qualities exists, many a times in farmers field there could be combination of all the biotic and abiotic stresses which could drastically accounts for crop loss due to accumulation of endogenous stress levels.

The organism with determined healthy and better lifespan is accelerated with strong immune system and the selection of such lines in breeding has greater impact. However, the CSR breeds had been evolved through hybridization since two decades and it becomes important to study the lifespan, as it will have the constraints in breeding if ignored. Hence, in this study, adult lifespan of CSR breeds have been assessed to answer the few questions; whether there is variation in the adult lifespan of bivoltine breeds? Is there any sexual difference in the longevity? How do the breeds respond to starvation stress? The answers to these questions will provide insights and enrich the breeding programmes to develop long-lived breeding lines to strengthen the silkworm breeding programme as the long-lived strains are proven in *Drosophila* to cope up with different biotic and abiotic stress conditions.

## MATERIALS AND METHODS

### Study Material

Twelve Bivoltine silkworm breeds viz., CSR2, CSR4, CSR6, CSR16, CSR17, CSR26, CSR27, CSR50, CSR51, CSR52, CSR53 and S8 (Table 1) maintained at Silkworm Bivoltine Breeding Laboratory, CSRTI, Mysuru were utilized for the assessment of adult lifespan.

### Assessment of Longevity

Rearing was conducted following the standard method (Krishnaswami, 1979) for three consecutive generations. Assessment was carried out with twenty unmated male and female moths which were placed on the Bivoltine egg sheets in assigned numbers and each moth was covered with cellulose. Likewise, 20 unmated male and female moths (in three replicates of each breed) were collected separately and pair mating was done. Once the moths were decoupled the mated male moths were transferred to an egg sheet with assigned number and the mated female moths were collected and transferred to a separate egg sheet with assigned number after 24hrs of oviposition. Observations were carried out thrice daily at 9:30 Am., 12:30 Am., and 4:30pm, and the mortality was recorded every 24hrs by considering no movement of moth when poked by chop stick.

### Starvation Stress Assay

The short-listed breeds for maximum longevity and minimum longevity were exposed to starvation stress. After fourth moult, the first day of fifth instar larvae (20 larvae of each breed in three replicates) of the short-listed breeds was subjected to starvation stress by not providing mulberry leaves, whereas, control was maintained by ad-libitum feeding of Mulberry leaves. Mortality was recorded every 24hrs until larvae were fully dead.  $LT_{50}$  was calculated through probit analysis.

### Statistical analysis

One-Way ANOVA was carried out using SPSS version 20.0. Log-rank test was conducted wherein, Survivorship ( $l_x$ ) was measured, which is a measure of the proportion of individuals which survive to the beginning of age category  $x$ , and it was estimated as  $l_x = n_x / n_0$ , where  $n_x$  is the number of individuals in the study population which survive to the beginning of age category  $x$ , and  $n_0 = N$  (the total population size). (<http://mathworld.wolfram.com/LifeExpectancy.html>). Survivorship was calculated for each larva of all the selected breeds.

## RESULTS AND DISCUSSION

Lifespan assessment in twelve bivoltine silkworm breeds revealed differences in the average lifespan ranging from  $204 \pm 5.64$  to  $318 \pm 18.74$ ,  $177.6 \pm 8.77$  to  $260.4 \pm 11.18$ ,  $193.2 \pm 8.95$  to  $270 \pm 14.34$  and  $162 \pm 11.13$  to  $234 \pm 9.51$  hours in virgin females, unmated males, mated females and mated males respectively (Table 2). The maximum and minimum longevity was observed in CSR51 and CSR17 in both unmated, mated females and males respectively when compared to all the other breeds under study. One-Way ANOVA of lifespan between virgin females and unmated males as well as, between mated females and mated males of each race showed significant differences with  $P$ -value  $< 0.001$ . One-Way ANOVA of lifespan among virgin

females, unmated males, mated females and mated males indicated significant differences with  $P$ -value < 0.001 (Table 2). The survivorship depicted that, among all the breeds under study, the survivorship of unmated females/virgin females of CSR2, CSR53, S8 and CSR51 ended at 384hr, 408hr, 432hr and 480hr respectively, whereas, in CSR17, it ended at 264hr (Figure.1a). In unmated males, the survivorship of CSR2, CSR51 and CSR53 ended at 360hr and S8 at 312hr respectively, whereas in CSR17 it ended at 240hr (Figure.1b). In mated females, the survivorship of CSR2, S8 ended at 336hr and 312hr respectively and survivorship of CSR51 and CSR53 ended at 360hr the highest survivorship was noticed in CSR6 with 384hr (Figure 2a). In mated males the survivorship of CSR2 and S8 ended at 288hr and CSR51, CSR53 ended at 312 hr respectively, whereas in CSR17 it ended at 216hr (Figure 2b). The average lifespan of both unmated and mated males and females of CSR17 was observed with lowest survivorship compared to all other breeds and CSR51 (among Dumbbells), S8 (among Ovals) was observed with highest survivorship compared to all other breeds under study. The time course mortality response obtained among the breeds CSR2, CSR17, S8 and CSR51 exposed to starvation stress is presented in Fig. 3. Among the breeds

exposed to starvation, mortality initiated in CSR17 at 72hr, and it was progressive (100% at 144hr). On the other hand, the check breed CSR2 and S8 showed the maximum mortality at 168hr (100%). Interestingly, no mortality occurred upto 72hr in the breed CSR 51 and ensued the maximum mortality at 192hr (100%). With extended survival CSR 51 appeared to be relatively less susceptible to starvation stress compared to other breeds. For this study, we selected CSR2 (as a check breed), Short-lived breed (CSR 17), S8 (Long-lived oval breed) and CSR 51 (Long-lived Dumbbell breed) exposing these to starvation stress for different time intervals since initiation of starvation from 24hr is presented in Fig. 3. The check breed CSR 2 showed no mortality up to 48hr, 70% mortality at 120hr and progressed to 100 % at 168hr. The statistically computed  $LT_{50}$  was ~110.3. Interestingly, starvation exposure even at the lowest time interval induced significant (90%) mortality and (100%) mortality at 144hr, clearly suggesting the higher vulnerability of the short-lived breed CSR 17. The statistically computed  $LT_{50}$  was ~107.64. In contrast, long-lived breed CSR 51 showed no mortality up to 72hr. However, only a mortality of 55%-70% ensued with higher concentrations. The statistically computed  $LT_{50}$  value was ~137.47.

**Table 1.** Details of Breeds utilized for the study.

Breed	Parentage	Larval marking	Cocoon shape
CSR 2	Shunrei x Shogetsu	Plain	Oval
CSR4	(BN18 x BCS25) x NB4D2	Plain	Dumbbell
CSR6	Shunrei x Shogetsu	Marked	Dumbbell
CSR16	(C135 x N134) x J14	Marked	Dumbbell
CSR17	(N137 x C146) x A24	Plain	Oval
CSR26	C135 x N134	Marked	Dumbbell
CSR27	Thaihei x Choan	Plain	Oval
CSR50	CSR202 x CSR46	Plain	Oval
CSR51	9HT x CSR16	Marked	Dumbbell
CSR52	Diazo x 10HT	Plain	Oval
CSR53	CSR203 x CSR47	Marked	Dumbbell
S8	CSR204xCSR27	Plain	Oval

In silkworm, though research has been focused on screening breeds and hybrids for different quantitative and qualitative traits, study on longevity trait in line of generating breeding lines is sparse. The adult lifespan in silkworm *Bombyx mori* is one of the important biological characters in the life history of silkworm, and "longevity of moths" can be used as an important "breeding index" in the evolution of races (Murakami 1989a, 1989b). The species and strain difference in longevity have been reported in a few silkworm races (Umashankara and Subramanya, 1999; Rohith and Subramanya, 2010). Sexual differences in moth longevity in few multivoltine breeds have been studied (Veeranna *et al.*, 2016; Ramya and Jagdeesh Kumar, 2020). To meet the demand for quality silk in the country attempts

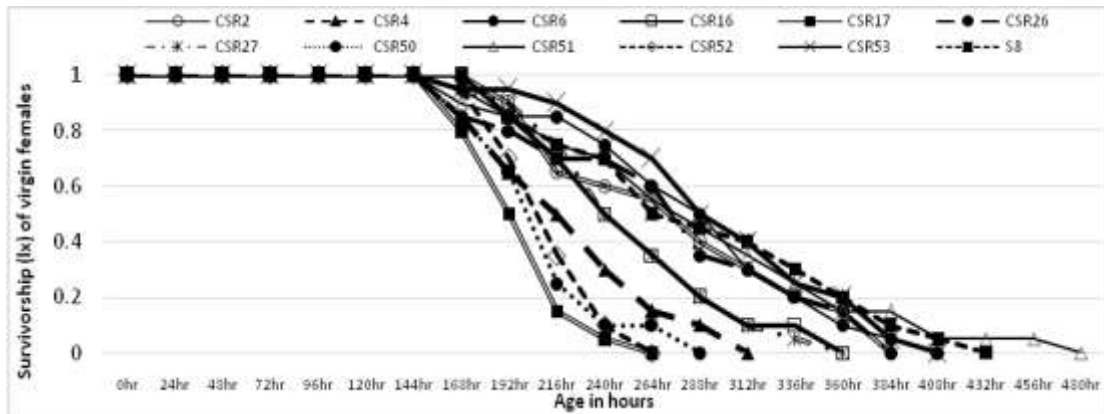
have been made to evolve highly productive CSR bivoltine breeds which have potential to produce international grade silk (Basavaraja *et al.*, 1995; Datta *et al.*, 2001; Suresh Kumar *et al.*, 2002, 2006; Dandin *et al.*, 2006). The CSR breeds utilized in the present investigation have evolved in laboratory attaining two decades since hybridization and in concurrence with the studies reported on silkworm longevity, in the present study it has been recorded that both unmated and mated females lived longer than the unmated and mated males in all the breeds under study and the unmated moths longevity is higher compared to the mated moths. Better longevity determines the efficiency of an organism to cope with the endogenous and exogenous stresses, which is evident in the present study wherein, the

genetic plasticity have been observed among the CSR breeds. CSR 51 attained maximum adult moth longevity (Long-lived) with higher survivorship and CSR 17 attained minimum longevity (Short-lived) with lesser survivorship

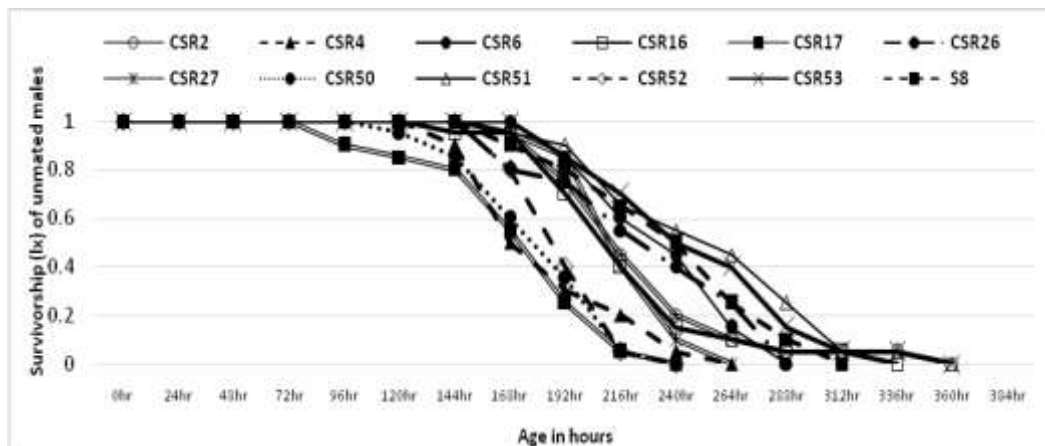
when compared to all other breeds. CSR 51 exhibited elevated tolerance to starvation stress when compared to other breeds.

**Table 2.** Mean lifespan  $\pm$  SE of selected Bivoltine silkworm breeds. (\*) denotes  $P$ -value $<0.05$  and (\*\*) denotes  $P$ -value $<0.001$  statistically by One-Way ANOVA of lifespan between both unmated (female & male) and mated (female & male) moths and among virgin females, unmated males, mated females, mated males of all breeds respectively.

Races	Adult Moth Lifespan			
	Virgin Female	Unmated Male	Mated Female	Mated Male
CSR2	282 $\pm$ 14.96	231.6 $\pm$ 9.42*	247.2 $\pm$ 10.01	216 $\pm$ 8.70*
CSR4	229.2 $\pm$ 10.07	190.8 $\pm$ 7.68*	210 $\pm$ 7.36	177.6 $\pm$ 8.77*
CSR6	291.6 $\pm$ 14.49	241.2 $\pm$ 7.27*	262.8 $\pm$ 13.65	224.4 $\pm$ 8.4*
CSR16	266.4 $\pm$ 13.47	224.4 $\pm$ 9.09*	238.8 $\pm$ 14.61	204 $\pm$ 7.88*
CSR17	204 $\pm$ 5.90	177.6 $\pm$ 8.77*	193.2 $\pm$ 8.95	162 $\pm$ 10.42*
CSR26	279.6 $\pm$ 16.26	234 $\pm$ 9.98*	256.8 $\pm$ 13.49	217.2 $\pm$ 10.22*
CSR27	259.2 $\pm$ 10.81	223.2 $\pm$ 5.25*	238.8 $\pm$ 7.27	213.6 $\pm$ 7.15*
CSR50	214.8 $\pm$ 7.483	187.2 $\pm$ 6.87*	199.2 $\pm$ 10.01	169.2 $\pm$ 10.07*
CSR51	318 $\pm$ 18.74	260.4 $\pm$ 11.18	270 $\pm$ 14.34	234 $\pm$ 9.51*
CSR52	218.4 $\pm$ 5.74	198 $\pm$ 4.56*	210 $\pm$ 8.32	180 $\pm$ 8.44*
CSR53	304.8 $\pm$ 14.04	256.8 $\pm$ 10.31*	259.2 $\pm$ 13.44	224.4 $\pm$ 9.42*
S8	295.2 $\pm$ 17.06	244.8 $\pm$ 9.78*	253.2 $\pm$ 10.07	222 $\pm$ 9.51*
	df=11 F-value=8.52**	df=11 F-value=10.34**	df=11 F-value=5.58**	df=11 F-value=7.26**



**Figure1a.** Survivorship (lx) of virgin female moths of all the bivoltine mulberry silkworm breeds.



**Figure1b.** Survivorship (lx) of unmated male moths of all the bivoltine mulberry silkworm breeds.

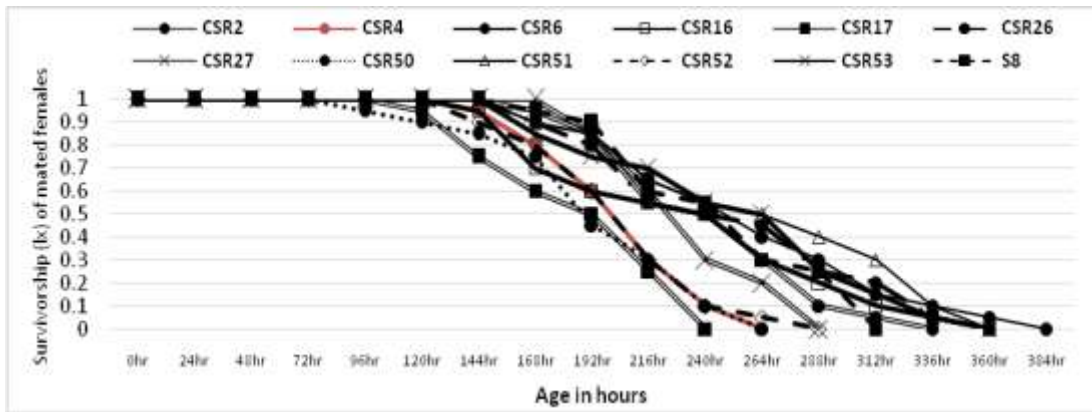


Figure 2a. Survivorship (lx) of mated female moths of all the bivoltine mulberry silkworm breeds.

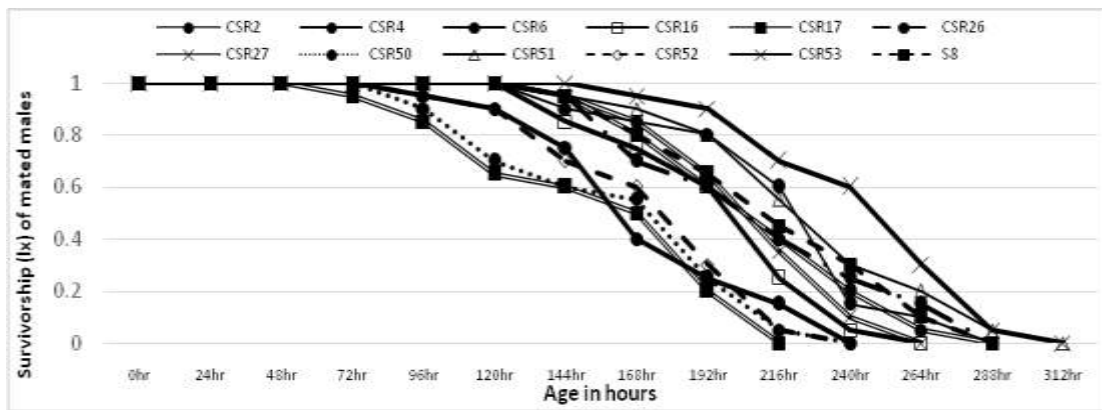


Figure 2b. Survivorship (lx) of mated male moths of all the bivoltine mulberry silkworm breeds.

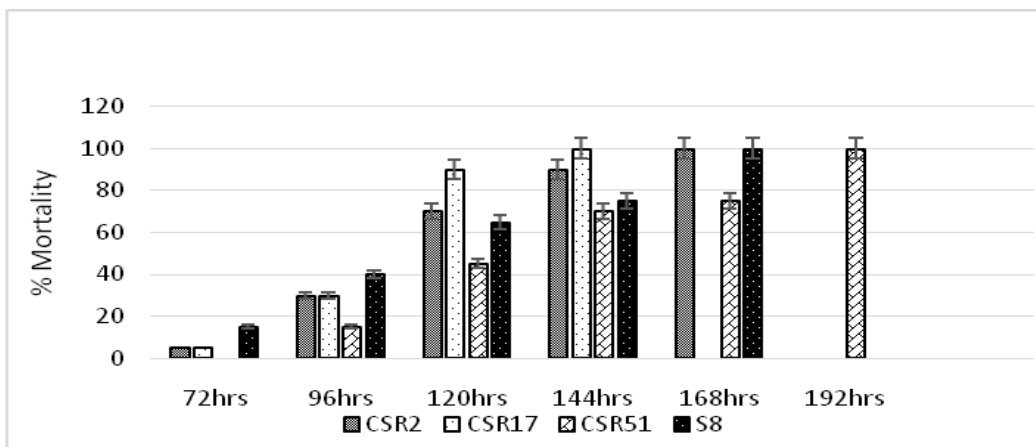


Figure 3. Percent mortality in breeds exposed to induced starvation stress.

Interestingly, we also obtained a similar trend of results in our  $LT_{50}$  determination experiment among selected breeds. There was a 30-fold higher susceptibility of short-lived breed CSR 17 to starvation stress as compared to CSR 51, long-lived breed. The results obtained are evident with

Service *et al.*, (1985) studies, which revealed that the genetically increased life span in *D.melanogster* populations appears to result proximally from greater resistance to mortality arising from environmental stress. Populations which displayed postponed senescence/

longevity were significantly more resistant than control flies to desiccation, starvation, and the vapor of a 15% ethanol solution (Rose *et al.*, 1992). According to the study conducted by Luckinbill (1998) the longer-lived individuals are more robust with respect to environmental stress from low temperature resistance that appeared to be specific to particular stage in the life history of the animal. Recent findings of Fabian *et al.*, (2018) conducted in *Drosophila melanogaster* with long-lived flies experienced the down regulation of expression of antimicrobial peptides upon infection with age yet survived fungal, bacterial, and viral infections significantly better, consistent with alleviated immunosenescence when compared to control.

Increased stress resistance has been often observed in *Drosophila melanogaster* lines selected for increased longevity (Service *et al.*, 1985, 1987; Leroi *et al.*, 1994; Force *et al.*, 1995; Harshman *et al.*, 1999), in mutants (Lin *et al.*, 1998), and in phenotypic manipulation experiments (Zwaan *et al.*, 1991). This association holds in other species such as mice and nematodes (Longo and Fabrizio 2002). Similarly, selection on shorter longevity also results in decreased starvation resistance (Zwaan *et al.*, 1995). These results strongly suggest that starvation resistance shares, at least in part, genetic regulatory mechanisms with longevity (Baldal *et al.*, 2006). It is evident in the present study also wherein, the breed CSR17 which attained minimum longevity is susceptible to the starvation stress and the breed CSR51 with maximum longevity is tolerant to starvation stress. Natural selection favor genotypes capable of shifting their physiology towards greater starvation resistance in response to cues heralding a period of starvation, such as crowding or declining food quality or quantity (Rion and Kawecki, 2007). Silkworm being a holometabolous insect undergoes complete metamorphosis, the larval period is a determined metamorphic event and hence, for longevity study among insects the adult longevity is intrinsically considered for generating the breeding lines. With the overwhelming need for expanding new model organisms in the whole biological and medical research, the silkworm has been emerging as an organism of choice for research into the molecular regulation of aging and longevity (Song *et al.*, 2016; 2018).

## CONCLUSION

The short-lived and long-lived breeds evaluated with an evident susceptibility and tolerance to starvation stress respectively, would serve as an important resource material, which could be utilized for enriching the silkworm breeding lines with molecular mediated approach for better silk production and crop sustainability, as well as, a choice of model system for aging studies.

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