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Impact of bacterial immune response on growth indices of mulberry silkworm, *Bombyx mori.* L

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Abstract
Silkworm is one of the best commercial and beneficial insect in the invertebrates which produce cocoons that act as a source for textile industry in silk and fabric production. In this studies, silkworm nutritional growth indices for two bivoltine pure silkworm races (CSR2, CSR4), bivoltine hybrid (CSR2 X CSR4) and cross hybrid (PM X CSR2) with respect to immunity response against Kanamycin resistant *Escherichia coli* were studied in third, fourth and fifth instar. The immunity response measured in terms of growth indices *viz.*, food consumption index, digestibility, conversion of ingested food, efficiency of conversion of digested food, silkworm growth rate were analyzed. The results revealed that growth rate of the larvae was affected significantly in CSR2 x CSR4 and PM x CSR2 (0.289 and 0.325) but not in pure races CSR2 and CSR4 (0.300 and 0.303). Approximate digestibility values showed significant difference between control and immunized larvae in the crossbreeds / hybrids, PM x CSR2 and CSR2 x CSR4 (82.857 and 84.064) but not in the pure races CSR2 and CSR4 (84.235 and 83.854). ECI was significantly influenced by immunization, as there was distinct difference between control and immunized set in all the breeds evaluated. ECD values also reflected similar trend depicting the strong negative influence of immunization on this nutritional index. Hence, immunity varies among the different age group of the insect and also the different race.

Keywords: silk worm race, *Bombyx mori*. L., growth indices and *Escherichia coli*

Introduction
Insect immune response is an evolutionary trait. Evolutionary ecology seeks to explain the design feature of immune system by analyzing their effects in individual fitness. There are two types of costs involved in immune response *viz.*, absolute cost and opportunity cost. Absolute cost of immune response is the quantity of nutrients required. Opportunity costs related to the fitness loss if another task cannot be met. Therefore measurement of energy demands of an immune response unfolds interesting facts on the influence of immune response on biological parameters of insects (Schmid-Hempel, 2005) [23]. There are few studies on the relationship between insect diet and immune function. Larvae of *Rhodinus prolixus* kept on plasma instead of whole blood had a lower production of (cecropin-like) antimicrobial peptides, reduced lysozyme activity and nodule formation, fewer haemocytes and were less resistant when experimentally infected with bacteria (Feder *et al*., 1997) [10].

The physiology of the organism has evolved to maintain an efficient immune response at the expense of some other traits (Hazel, 2002) [12]. Induction of immune responses has resulted in negative influence on several biological traits in different species of insects. Examples are slower larval development and reduced egg viability in Indian meal moth resistant to granulosis virus (Boots and Begon, 1993) [6], loss of competitiveness over food in *Drosophila* larvae resistant to common parasitoids (Kraaijevel and Godfray, 1997) [15], reduced fecundity in *D. melanogaster* (Fellowes *et al*., 1999), reduced survival rate in bumble bees (Moret and Schmid- Hempel, 2001) [20], reduced fecundity in mosquitoes (Barnes and Siva-Jothy, 2000) [5] and shorter life span of *Tenebrio molitor* (Armitage *et al.*, 2003) [4].

In silkworm, innate immune system activates the cells against microbial pathogens during their life periods. Innate immune system comprising AMPs, lysozymes, melanization and phagocytosis plays a major role in prevention of microbial infections (Zhang *et al.*, 2017; Kausar *et al*., 2018) [28, 14]. In silkworm both positive and negative bacteria strongly stimulate immune responses (Lemaitre and Hoff Mann, 2007; Kausar *et al.*, 2018) [17, 14]. Specific bacterial strains strongly stimulates a particular antimicrobial peptides AMPs in silkworm body against them that is *Streptococcus aureus* expressed Cecropin XJ (Xia *et al.*, 2013) [26];

Escherichia coli and *Bacillus subtilis* activated Defensin B in fat body (Kaneko *et al.*, 2008) [13]; *Pseudomonas aeruginosa* stimulates defensin, attacin, cecropin, lebocin, gloverin and moricin in fat body, *Bacillus bombysepticus* (Gram positive bacteria) induce lebocin, attacin, enbocin, moricin and gloverin in the gut of *B. mori*. In the present study, the different races of mulberry silkworm were immunized with gram negative bacteria *Escherichia coli*, kanamycin resistant and their growth indices were noticed in silkworm pure races, bivoltine and multivoltine hybrids such as CSR2, CSR4, PMxCSR2, CSR2xCSR4.

2. Materials and methods

2.1Rearing of silkworm races

Two bivoltine pure races (CSR2, CSR4),bivoltine hybrid (CSR2 x CSR4) and cross d(PM x CSR2)mulberry silkworm races were procured from CSGRC, Hosur for carry out the experiment. The silkworm races were reared on fresh V1 mulberry leaves, maintained at 25-28 °C with relative humidity of 60-80% and photoperiod of 12 hours light and 12 hours darkness. Growth indices were measured during III, IV and V instar of silkworm larvae.

2.2 Bacterial broth media and immunisation

Kanamycin resistant *Escherichea coli* strain was used in the studies. Nutrient agar medium and nutrient broth were prepared for culturing of *E.coli* K^R. Freshly collected mulberry leaves were dipped in 50 ppm *E.coli* K^R broth culture filtrate (10^6cfu / ml), shade dried and fed to III, VI and V instar silkworm immediately after moult.

2.3 Maintenance of larvae

Two sets of larvae (100 Numbers/ Set) were maintained in separate trays with one set being control and others being immunized set. Immunization was done with *E. coli* K^R as described earlier. Growth and development of larvae were monitored in both the sets to study the growth indices of silkworm.

2.4 Measurement of growth indices

The fresh weight of silkworm larvae from each replication of every treatment were weighed and recorded at third, fourth and fifth instar of each races. The randomly selected silkworm larval weight was recorded as batch wise before giving the first feed after moulting as initial larval weight and just before moult for III and IV instar, on seventh day before spinning for V instar as final weight.

Other silkworm growth indices of consumption and digestion indices such as ingested food (I.F), digested food (D.F), consumption index (C.I), growth rate (G.R), approximate digestibility (A.D), efficiency of conversion of ingested food (E.C.I), efficiency of conversion of digested food (ECD) were computed by gravimetric method (Waldbauer, 1968) [25] as follows.

i. Ingested food = Weight of fresh leaves offered to larvae - Weight of fresh remnants
ii. Digested food =Weight of fresh food ingested – Weight of fresh excreta produced
iii. Consumption index = Fresh weight of food eaten / Duration of feeding period (days) x Mean fresh weight of larvae during feeding period Mean weight of larvae = (Initial weight of larva + final weight of larva in each instar)/2
iv. Growth rate = Fresh weight of larvae during feeding period/ (Duration of feeding period (days) x Mean fresh weight of larvae during feeding period) Weight gain of larvae = (Weight of larvae before going to moult- weight of larvae after month)/ Number of larvae taken per sample
v. Approximate digestibility = (Weight of food ingested+ weight of faecal pellets/ Weight of food ingested) x 100
vi. Efficiency of conversion of ingested food = (Growth rate / Consumption Index) x 100
vii. Efficiency of conversion of digested food = (Weight gained/ Weight of food ingested- weight of faecal pellets) x 100

Table 1: Immune response of silkworm races against *E. coli* ^{R} in third instar

S. No.	Growth indices *	Races	CSR2	CSR4	CSR2 x CSR4	PM x CSR2
1.	CI	Control	1.290^a	1.267^a	1.410^a	1.474^a
		Immunized	1.274^b	1.266^{ab}	1.240^b	1.295^b
2.	GR	Control	0.313^a	0.311^a	0.34^a	0.374^a
		Immunized	0.300^{ab}	0.303^{ab}	0.289^b	0.325^b
3.	AD	Control	84.271^a	84.287^a	84.119^a	84.175^a
		Immunized	84.235^{ab}	83.854^b	84.064^{ab}	82.857^b
4.	ECI	Control	24.273^a	24.545^a	23.353^a	25.363^a
		Immunized	23.596^b	23.960 ^{b}	20.807^b	24.579 ^{b}
5.	ECD	Control	28.803d^a	29.121c^a	28.698^a	30.130^a
		Immunized	28.012^b	28.573^b	27.780^b	29.664^b

* Each value is the mean of three replications with 100 silkworms in each replication.
Mean values followed by a common (small) letter are not significantly different at 5% level by DMRT.

Table 2: Immune response of silkworm races against *E.coli*^{R} in fourth instar

S. No.	Growth indices *	Races	CSR2	CSR4	CSR2 x CSR4	PM x CSR2
1.	CI	Control	0.43^a	0.378^a	0.388^a	0.404^a
		Immunized	0.409^b	0.359^b	0.363^b	0.382^b
2.	GR	Control	0.213^a	0.242^a	0.231^a	0.262^a
		Immunized	0.203^{ab}	0.22^{ab}	0.213^b	0.245^b
3.	AD	Control	87.308^a	84.389^a	83.954^a	86.495^a
		Immunized	86.773^b	82.132^b	82.655^b	84.876^b
4.	ECI	Control	49.270^a	63.795^a	59.634^a	64.916^b
		Immunized	49.017^{ab}	61.075^b	58.635^b	64.259^a

5.	ECD	Control	56.712[a]	71.031[a]	75.586[a]	76.483[a]
		Immunized	56.143[ab]	70.939[ab]	74.360[b]	74.294[a]

* Each value is the mean of three replications with 100 silkworms in each replication.
Mean values followed by a common (small) letter are not significantly different at 5% level by DMRT.

Table 3: Immune response of silkworm races against *E. coli*[R] in fifth instar

S. No.	Growth indices *	Races	CSR2	CSR4	CSR2 x CSR4	PM x CSR2
1.	CI	Control	0.76[a]	0.64[a]	0.60[a]	0.60[a]
		Immunized	0.73[b]	0.60[b]	0.558[b]	0.57[b]
2.	GR	Control	0.06[a]	0.074[a]	0.099[a]	0.13[a]
		Immunized	0.05[ab]	0.067[ab]	0.090[ab]	0.12[ab]
3.	AD	Control	65.79[a]	66.40[a]	66.38[a]	66.09[a]
		Immunized	65.37[ab]	66.10[ab]	66.43[b]	65.52[ab]
4.	ECI	Control	7.30[a]	11.38[a]	16.35[a]	21.71[a]
		Immunized	6.55[b]	11.17[b]	16.11[ab]	21.51[ab]
5.	ECD	Control	11.10[a]	17.18[a]	24.62[a]	32.85[a]
		Immunized	10.022[ab]	16.907[b]	24.253[b]	32.824[ab]

* Each value is the mean of three replications with 100 silkworms in each replication.
Mean values followed by a common (small) letter are not significantly different at 5% level by DMRT.

3. Results and Discussion

Nutritional indices of silkworm breeds were worked out at third, fourth and fifth instars in control as well as immunized larvae and the results are furnished in Table 1, 2 and 3. Four breeds *viz.,* CSR2, CSR4, CSR2 x CSR4 and PM x CSR2 were chosen for the study. Nutritional indices *viz.,* consumption index, growth rate, approximate digestibility, efficiency of conversion of ingestion and efficiency of conversion of digestion were estimated.

Several authors have earlier recorded that induction of immune response actually resulted in negative biological traits. Boots and Bragon (1993) [6] recorded slower larval development and reduced egg viability in Indian meal moths to granulosis virus. Injection of bacterial cell wall component reduced fecundity in mosquitoes (Ahmed *et al.*, 2002) [2]. As every physiological function or biochemical pathway needs energy, the absolute cost of immune response interms of nutrient requirement, energy demand actually hampered the growth and development (Schmid-Hempel, 2003) [22]. Ardia *et al.* (2012) [3, 8] also reported strong direct evidence that an immune response entails energetic and physiological costs in range of insect speciesand a corresponding decrease in antimicrobial activity linked with increased levels of PO. In invertebrates increased handling with the resultant stress can lead to increased metabolic rates and neuroendocrine interactions that modify immunity (Demas *et al*., 2011) [9].

During the third instar, immunized larvae recorded significantly lower consumption indices in CSR2(1.274), CSR2 x CSR4 (1.240) and PM x CSR2 (1.295) but CSR4 (1.266) was on par with control (1.267). Growth rate of the larvae was affected significantly in CSR2 x CSR4 and PM x CSR2 (0.289 and 0.325).In pure races, immunized CSR2 and CSR4 (0.300 and 0.303) were not affected when compared to control. Approximate digestibility values showed significant difference between control and immunized larvae in the crossbreeds / hybrids, PM x CSR2 and CSR2 x CSR4 (82.857 and 84.064) but not in the pure races CSR2 and CSR4 (84.235 and 83.854). ECI was significantly influenced by immunization, as there was distinct difference between control and immunized set in all the breeds evaluated. ECD values are reflected as similar trend depicting the strong negative influence of immunization on this nutritional index.

Estimations have been done on comparative nutritional indices of control and immunized larvae during fourth and fifth instars. In general, perusal of the data showed that during fourth instar for all the five nutritional indices were strongly influenced by immunization in CSR2 x CSR4 and PM x CSR2 barring ECD (70.939 and 74.294) in the former. With regard to pure races, CSR 2 and CSR4 it is seen that growth rate (0.203 and 0.220) and ECD (56.143 and 70.939) were unaffected by immunization. Strong negative influence of immunization on CI (0.499 and 0.359), AD (86.773 and 82.132) and ECI (49.017 and 61.075) were explicit.

During fifth instar also it was observed that growth rate was affected to a lesser extent by immunization when compared to other nutritional indices. ECD values were on par in control and immunized sets in PM x CSR2 (32.85 and 32.824) and CSR2 (11.10 and 10.022) and AD values were also on par in control and immunized sets in PM x CSR2 (66.09 and 65.52), CSR2 (65.79 and 65.37) and CSR4 (66.40 and 66.16)

Waldbauer nutritional indices were estimated to measure the growth and development of larvae in control as well immunized larvae. Perusal of the results showed that during third instar, significantly lower Consumption index (1.240 and 1.295), Growth rate (0.289 and 0.325), Approximate digestibility (84.064 and 82.857), Efficiency of conversion of ingested food **(**20.807 and 24.579) were recorded in immunized larvae of CSR2 x CSR4, PM x CSR2. During fourth and fifth instars also immunization resulted in significantly lower values of nutritional indices.

Many of the biochemical pathways are shared between immune response and other physiological functions. PPO cascade was more intensely related with melanin transport to cuticle (Levin and Strand, 2002) [16]. Excess triggering of PPO cascade led to internal melanization of tissues (Gregorio *et al.,* 2002) [11]. The immune response of insects begins with the fat body and haemocytes, which are the main tissues responsible for insect innate immunity (Lü, 2008) [18]. The fat body of insects, the homologue of the mammalian liver, is a major organ involved in innate immunity and can produce antimicrobial peptides (AMPs), reactive oxygen species (ROS) and other humoural response factors (Aggrawal and Silverman, 2007) [1]. The present study proved that immunized silkworm showed significant reduction in ingestion, consumption and digestion.

Based on the results, it could be inferred that immunization resulted in slower growth and development of larvae during third and fourth instars than in fifth instar. Moreover, immunized larvae of PM x CSR2 and CSR2 x CSR4 registered significantly lower values of nutritional indices especially during third and fourth instar. However, growth rate of the larvae was not affected due to immunization while consumption, digestion and ingestion were greatly reduced in immunized larvae. Negative influence of immune response on nutritional indices of silkworm was explicit.

The present results supported the growing evidence that immune responses entail specific energetic and corresponding physiological costs. Thus the reported reduction in nutritional indices in silkworm breeds could be attributed as the biological cost of immune response. A detailed study on the influence of immune responses on several biological parameters like larval duration, fecundity, egg hatchability and cocoon economic parameters would throw light on the absolute cost of immune response in silkworm breeds.

Ravinder *et al.* (2015) [21] showed that the proteins and amino acids levels were significantly elevated and 18 individual free amino acids were found in the haemolymph after challenge with gram –ve and gram +ve bacteria when compared to control and sterile haemolymph. It might be due to direct involvement in the antimicrobial immune response of Eri silkworm innate immunity. Yang *et al.* (2018) [27] reviewed that the latest research progress on silkworm immune mechanisms, including phenoloxidase-dependent melanization and apoptosis, which is conducive to improve understanding of the silkworm immune mechanism.

4. Conclusion

Immunization in silkworm resulted in slower growth and development of larvae during third and fourth instar than in fifth instar. Also, immunization effects were more in cross breeds such as PMxCSR2 and CSR2X CSR4 than pure breeds of CSR2 and CSR4. However, growth rate of the larvae was not significantly affected due to immunization while consumption, digestion and ingestion were greatly reduced in immunized larvae. This immune development study could be useful for identifying disease resistant silkworm races based on immune response among the different races and age.

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