Influence of host plant (*Terminalia arjuna*) defences on the evolution of feeding behaviour in the tasar silkworm

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Both under indoor and outdoor rearing conditions, early instars of *Antheraea mylitta* showed differential preference for eating towards developmentally different leaves of host plant, *Terminalia arjuna*. Semi-mature leaves were preferred by first, second and third instar of *A. mylitta*. Nutritional-value study of leaves of different age groups with respect to soluble protein and electrophoretic profile showed that young leaves are nutritionally rich compared to semi-mature and mature leaves. However, growth response and survival of larvae were better on semi-mature leaves compared to young and mature leaves. When analysed, semi-mature leaves showed protease inhibitor activity intermediate between young and mature leaves. This observation suggests optimal defence theory, where young and semi-mature leaves having high fitness and high probability of attack tend to have higher concentration of defence metabolites. Differential inhibition of midgut and bovine proteases by host plant protease inhibitor indicates that the tasar silkworm might have detoxified or evolved proteases that are insensitive to the leaf protease inhibitor of the host plant. Thus the differential feeding behaviour of larvae of tasar silkworm is an adaptation for coexistence of the insect and its host plant.

Keywords: *Antheraea mylitta*, larval growth, midgut proteases, tasar silkworm, *Terminalia arjuna*.

THE plant phenological age or development age hypothesis[1] predicts that herbivores prefer and perform better on developmentally young plants than on old plants, because nutritional quality of plant material decreases with age. As a general rule, the nutrient level, i.e. nitrogen and water decreases, while the non-nutrient chemicals and leaf toughness increase with plant age[2].

Similarly, age-dependent variation in the distribution of chemical defences within and among plants also has bearing on herbivore fitness and behaviour[3]. Protease inhibitors are a class of compounds found in a wide range of plant families and are studied for their activity as anti-herbivore compounds[4]. In fact, protease inhibitors constitute a significant part of the chemical changes that occur during development of plants leading to increased resistance against herbivores[5].

Several studies have documented the variability in insect performance and host plant selection based on plant genotype[6], plant age[1], leaf age[7] and various aspects of plant–insect interaction[8,9].

Antheraea mylitta (Lepidoptera: Saturniidae) is a polyphagous species that feeds on foliage of *Terminalia arjuna*, *T. tomentosa* and *Shorea robusta*, which are its primary food plant species. But different ecoraces inhabiting diverse eco-climatic zones and feeding on different host plants have become host-specific and thus monophagous in behaviour.

Little is known about the chemical basis of *A. mylitta*–*T. arjuna* host plant interaction (Figure 1 *a* and *b*), although *A. mylitta* has been extensively reared on *T. arjuna* for its tasar silk. For many insect taxa, this type of research is the cornerstone of chemical ecology, as it has direct bearing on insect fitness and host resistance. Many of the central nutritional questions are unanswered for *A. mylitta*. For example, what is the comparative role of primary nutrient verses anti-herbivore compounds in determining the feeding behaviour of *A. mylitta*? Does *A. mylitta* follow 'co-evolutionary theory' in that species with limited host range and are they more influenced by anti-herbivore compounds produced by hosts than are generalist species[10]? In this article we have attempted to answer these questions.

Materials and methods

Bovine trypsin, bovine chymotrypsin, TAME (*p*-toluenesulfonyl-L-arginine methyl ester) and BTEE (benzoyl-1-tyrosin ethyl ester), were procured from Sigma Chemical Co, USA.

Insect behaviour in response to developmentally different leaves

Leaves were delimited as young, semi-mature and mature based upon morphological and physiological characteristics. The age of the leaves was evaluated by measuring the leaf

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area along with fresh and dry weight. Leaf area was calculated using graph paper. To determine the dry weight, leaves were oven-dried at 70°C for more than 24 h till constant weight was obtained.

An experiment of indoor rearing was conducted to determine the growth performance of tasar silkworm larvae with developmentally different leaves. Larvae were reared under room condition (27–32°C temperature and 65–80% relative humidity). The per cent mortality and growth of larvae (measured as mass gain) were taken as the index of rearing performance. Three replicates of each treatment, i.e. young, semi-mature and mature were maintained. Ten newly hatched larvae in each replicate were provided with developmentally different leaf diets, reared up to the fifth instar and weighed every morning before giving fresh leaves from the field.

Study of protein variability in developmentally different leaves

Fifty grams of each leaf sample was homogenized in 250 ml ice-cold extraction buffer (0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM $Na_2S_2O_3$ and 20 mM *b*-mercaptoethanol). Soluble protein concentration was determined in the supernatant after centrifuging the homogenate at 10,000 rpm for 10 min at 4°C using the dye-binding assay[11]. Bovine Serum Albumin (BSA) was used as standard. Electrophoretic variation in soluble proteins among young, semi-mature and mature leaves of host plant was also assessed. About 20 µg of ammonium sulphate-precipitated protein from young, semi-mature and mature leaves was electrophoresed on SDS–PAGE (12%)[12] and the gels were silver-stained[13].

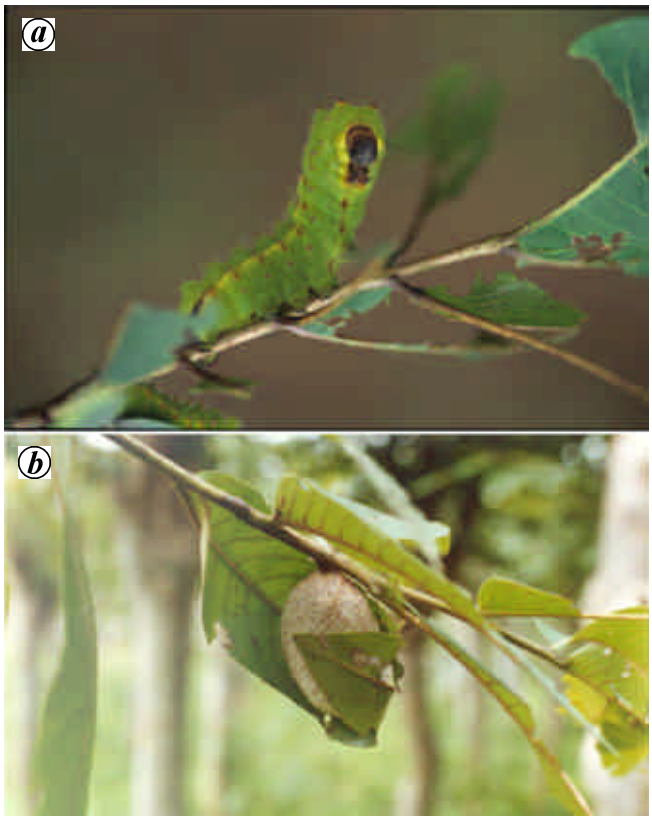


Figure 1. Twigs of *Terminalia arjuna* showing 4th instar of tasar silkworm (***a***) and cocoon formed on the lower surface of mature leaves (***b***).

Extraction and partial purification of protease inhibitor

Crude extracts of different leaf samples as prepared above were centrifuged and the protein from supernatant was precipitated using ammonium sulphate (0–60%). The precipitated protein was used as a semi-purified inhibitor, after dialysis.

Larval midgut protease extraction

The midgut protease of fifth instar larvae reared under outdoor conditions on *T. arjuna* plants maintained in the experimental plots of Central Tasar Research and Training Institute (CTRTI), Ranchi, India, was extracted following the method of Lee and Anstee[14]. Ammonium sulphate (0–80%) was added to this aqueous fraction at 4°C to precipitate the proteins. Trypsin and chymotrypsin activities were checked after dialysis in the fraction following Birk[15].

Assay of plant protease inhibitor activity

Host plant protease inhibitor activity was determined by pre-incubating 50 µg of partially purified protease inhibitor obtained from each of the three developmently different leaves, with bovine trypsin (25 µl) and chymotrypsin (30 µl) from a stock of 1 mg/ml each in 1 mM HCl. After incubation, 100 µl of this mixture was taken and assayed for trypsin and chymotrypsin activity following Birk[15]. The ability of host plant protease inhibitors to inhibit the larval mid-gut trypsin and chymotrypsin activity was determined by incubating midgut protease (activity equivalent to 25 µg bovine trypsin) with 50 µg of partially purified host protease inhibitor sample for 5 min and then testing for trypsin and chymotrypsin activity as mentioned above. All these assays were carried out at 25–30°C using pre-incubation mixture with pH 9.0.

Statistical analysis

Indoor rearing was done in triplicate and the difference in growth and mortality of different larval instars between leaves of different developmental stages was analysed using Two Way Analysis of Variance (ANOVA). Protein quantification and protease inhibitor analysis was done in young,

semi-mature and mature leaves of ten trees. Single factor ANOVA was used to evaluate the significance of the results at different probability levels.

Results

Delimitation of leaves as young, semi-mature and mature

Young leaves were morphologically small, varying in size from 21 to 32 cm^2, mostly folded and pale green, thin and fresh ones weighed about 1.54 g. Semi-mature leaves were unfolded, expanding, thin leaves of about 104.4 ± 6.7 cm^2 size with fresh weight of 4.2 g. Mature leaves were tough, fully expanded about 137.3 ± 7.4 cm^2 in size having fresh weight of 5.5 g and were not senescent. Surface area, fresh weight and dry weight of leaves increased with increase in age (Figure 2). The leaf area showed statistically significant correlation (at $P < 0.05$) with both fresh and dry weight of leaves (Figure 2).

Effect of leaf diet on larval growth and development

Significant variation was observed in amount of young, semi-mature and mature leaves consumed by different larval instars (Figure 3). Larvae also showed significant variation in growth (mass gain) and per cent mortality when fed on the leaves of three age groups (Figure 3). Young and mature leaves had a significant retarding effect on growth and survival of larvae. For mass gain and per cent mortality, the differences in means between leaf samples, between instars and interaction between leaf samples and instars were statistically significant at $P < 0.001$ (Table 1).

Soluble protein in young, semi-mature and mature leaves

Quantity of soluble protein was found to decrease from young to mature leaves. Maximum protein concentration (mg/g fresh wt of leaves) was obtained in young leaves followed by semi-mature leaves and lowest in mature leaves (Figure 4 *a*). Differences among leaf samples with respect to soluble protein were statistically significant at $P < 0.001$ (Table 2). Protein bands having molecular weight ranging from 14.4 to 94.0 kDa were detected on SDS–PAGE across leaves of different age groups (Figure 4 *b*). Mature leaves showed more high molecular weight protein bands compared to young and semi-mature leaves. The number of protein bands between 20.1 and 43 kDa decreased with age of leaves. Young leaves contained more low molecular weight protein bands compared to semi-mature and mature leaves (Figure 4 *b*).

Effect of protease inhibitor on midgut protease

Midgut trypsin and chymotrypsin showed diverse level of susceptibility towards partially purified protease inhibitor from leaves of different age groups. The midgut trypsin was inhibited maximum (~ 65% inhibition) by leaf protease inhibitors derived from young leaves in contrast to 39% inhibition by the inhibitor derived from mature leaves. About 57% inhibition was observed with inhibitors

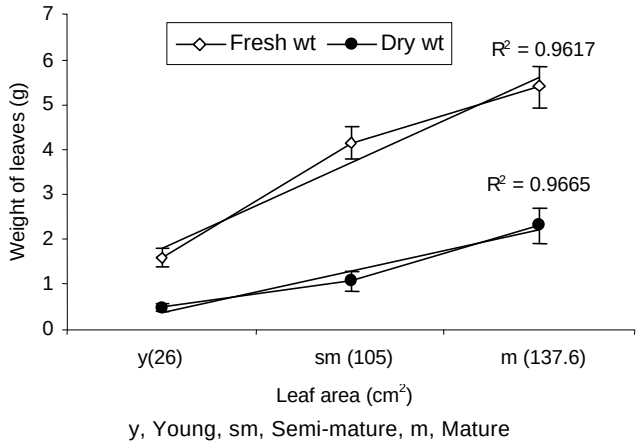


Figure 2. Graph showing relationship between leaf area of young, semi-mature and mature leaves, and their fresh and dry weight.

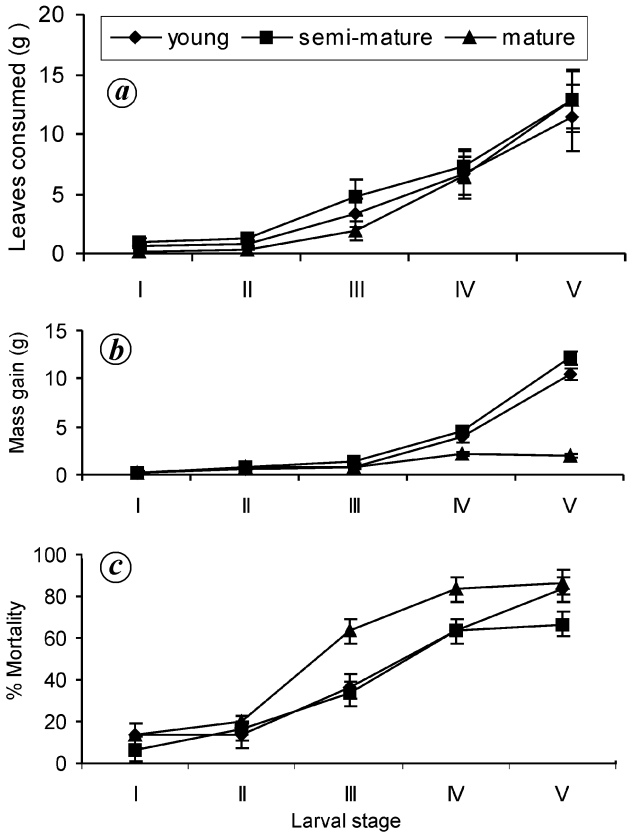


Figure 3. Diagram showing amount of leaves consumed (***a***), mass gained (***b***) and per cent mortality (***c***) of different instars of *A. mylitta* fed on young, semi-mature and mature leaves of *T. arjuna* under indoor conditions.

Table 1. Two-way analysis of variance of per cent mortality and mass gain of different larval stages with respect to young, semi-mature and mature leaves

Food utilization index	Source of variation	*F*	*P*	F_{crit}
Mass gain	Between leaf samples	146.59	1.16E-10	4.35
	Between instars	115.09	1.66E-13	2.86
	Interaction	71.72	1.43E-11	2.86
Per cent mortality	Between leaf samples	64	1.17E-07	4.35
	Between instars	187.61	1.49E-15	2.86
	Interaction	5.94	0.0025	2.86

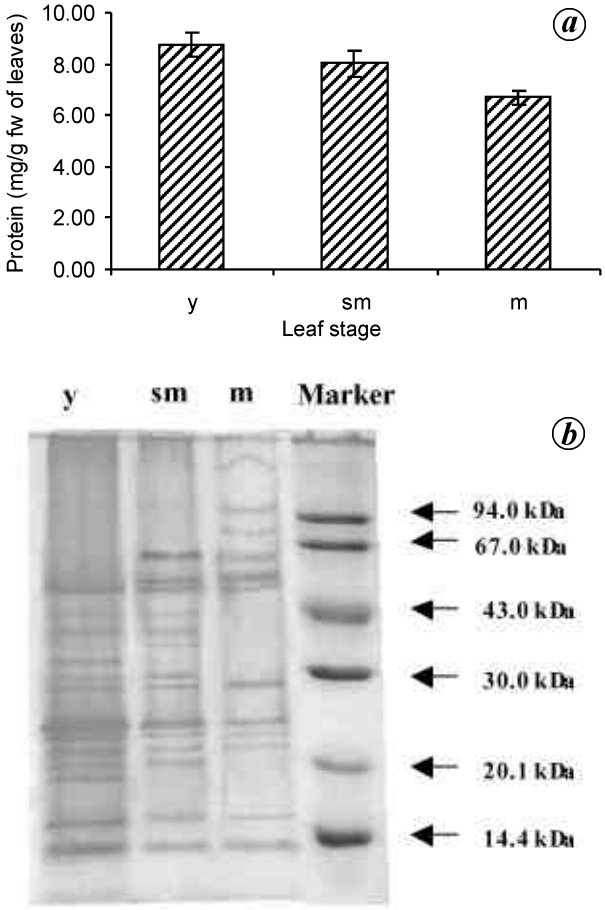
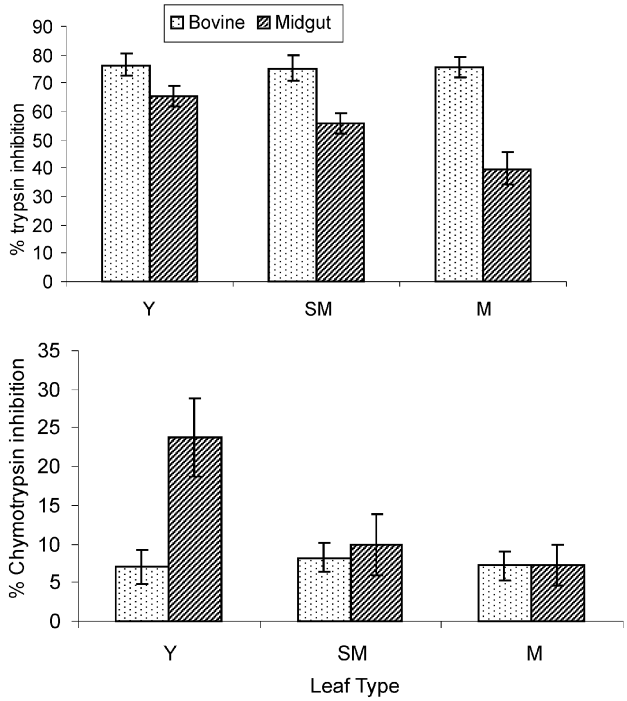


Figure 4. ***a***, Histogram showing variation in soluble proteins of leaves of different age groups of *T. arjuna*. ***b***, 12% SDS–PAGE gel showing electrophoretic variation in soluble proteins of young, semi-mature and mature leaves of *T. arjuna* and marker proteins.

Table 2. One-way analysis of variance of different biochemical traits with respect to young, semi-mature and mature leaves

Trait	*F*	*P*	F_{crit}
Protein quantity	63.6	6×10^{-11}	3.35
Per cent midgut trypsin inhibition	45.3	$2.5 \times 10^{-0.6}$	3.8
Per cent midgut chymotrypsin inhibition	24.4	$5.9 \times 10^{-0.5}$	3.8



Y, Young, SM, Semi-mature, M, Mature

Figure 5. Histogram showing inhibitory activity of protease inhibitor derived from young, semi-mature and mature leaves of *T. arjuna* on midgut and bovine proteases.

derived from semi-mature leaves (Figure 5). Differences in the inhibition profiles of inhibitor derived from leaves of different age groups were statistically significant at $P < 0.001$ (Table 2). The pattern observed for inhibition of chymotrypsin activity by the protease inhibitor derived from leaves of different age groups was same, except that the inhibition of chymotrypsin was low (7.5–25%; Table 2). No differences were observed in the inhibition profiles of bovine trypsin and chymotrypsin by the protease inhibitor derived from leaves of different age groups, although the leaf protease inhibitor was more active on bovine proteases compared to midgut proteases of tasar silkworm.

Discussion

Semi-mature leaves were preferred by first, second and third instar of *A. mylitta* for feeding compared to young and mature leaves. Fourth and fifth instar larvae without any reservation fed on all the leaves irrespective of the stage of leaf development. Visual field observations on the feeding behaviour of different instars also supported the observa-

tions made during indoor rearing of different instars on leaves of different age groups.

The nutritional value[2] study with respect to soluble proteins and their electrophoretic profiles showed substantial variation among leaves of different age groups. Young leaves were nutritionally rich compared to mature leaves. The semi-mature leaves were intermediate between young and mature leaves. This is evident from the fact that young and semi-mature leaves showed high amount of soluble proteins compared to mature leaves. They also showed high amount of electrophoretic variation compared to mature leaves in terms of band polymorphism. These observations indicate substantial diversity in amino acid composition of soluble proteins among leaves of different age groups and band polymorphism can be used as a marker for identification of age of leaves.

To explain the selective feeding behaviour of early instars on semi-mature leaves in spite of high nutritive value of young leaves, we examined the distribution of protease inhibitor as anti-herbivore compound among leaves of different age groups. Protease inhibitors have been known as anti-nutritional factors that negatively affect the growth of animals[16]. In the present study, the presence of protease inhibitor was observed in the leaves of *T. arjuna*, but its distribution varied in different developmental stages. Maximum protease inhibitor activity was observed in young leaves and lowest in mature leaves; semi-mature leaves showed intermediate protease inhibitor activity between young and mature leaves. Optimal defence theory argues that defence metabolites are allocated preferentially to tissues with high fitness value and a high probability of attack. Young leaves, stem and reproductive parts tend to have the highest concentration of defense metabolites, whereas roots and old leaves the lowest[9]. Our observations also support optimal defense theory. Young and semi-mature leaves have high fitness and high probability of attack because of high nutritive value. Thus the selective feeding of all the instars ranging from 1st to 5th instars on semi-mature leaves is a kind of strategy adopted by tasar silkworm for its optimum survival and coexistence with the host plant.

The partially purified leaf protease inhibitor from *T. arjuna* showed differential activity on midgut trypsin and chymotrypsin of *A. mylitta* compared to that on bovine trypsin and chymotrypsin, suggesting that it acts as an anti-herbivore compound but not specific to tasar silkworm. These observations also indicate that the tasar silkworm might have detoxified it and/or evolved proteases that are insensitive to the leaf protease inhibitor of the host plant. Pests such as *Helicoverpa* spp. frequently feed on plants expressing protease inhibitors because their digestive system is resistant to the presence of protease inhibitors[17]. The selection pressures on insects to evolve proteases that are largely insensitive to host plant protease inhibitors have been documented[18].

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