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Correlation between yield and biochemical parameters in the mulberry silkworm, *Bombyx mori* L

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Abstract. A detailed study was carried out on six biochemical parameters and four yield attributes using multiple regression analysis to investigate their relationship in the mulberry silkworm, *Bombyx mori*. The study generated new information on the importance of digestive amylase activity for the survival of the silkworm and revealed the inability of other enzymes to affect this relationship. Data also substantiate the observations made earlier on the genetic variability of amylase in the mulberry silkworm. Analyses extend the positive role of alkaline phosphatase and invertase in the expression of the other yield traits studied and indicate the definite possibility of using biochemical markers for silkworm breeding.

Key words: Silkworm – Biochemical genetics

Introduction

Intensive studies on the correlation between yield and biochemical parameters, as well as isozyme markers, have been carried out for both crop plants (Tanksley and Rick 1980; Tanksley et al. 1982; Van Geyt et al. 1990) and domestic animals (Bulfield et al. 1988; Jung et al. 1989). These studies have opened new areas of research in animal and plant breeding (Tanksley et al. 1982).

Previous work (Hirata 1974; Kuroda 1979) has examined the correlation between specific biochemical parameters (e.g., amylase in digestive juice or α-ketoglutaric acid in blood) and yield parameters in the silkworm. Analyses have also been carried out to ascertain the correlation between different individual yield components (Gammo et al. 1985; Nino et al. 1990; Shibukawa, personal communication). But no attempt has been made to analyse correlations between multiple biochemical parameters and yield in the silkworm. Moreover, the earlier studies were primarily based on high-yielding bivoltine breeds of Japanese or Chinese origin and their hybrids. Thus, a detailed investigation was planned to generate information on the relationship between multiple biochemical parameter(s) and yield in silkworm races of both tropical and temperate regions, and also to identify biochemical parameters having a definite correlation with yield components.

This report presents an analysis of the correlation between six biochemical parameters among four yield traits and the correlation amongst all ten variables in a selected group of mulberry silkworms. The biochemical parameters are amylase, invertase, alkaline phosphatase, and protease in the digestive fluid and the trehalose content of the blood. These traits were chosen on the basis of earlier findings of their involvement in digestion and also for their suggestive role in insect resistance. For example, the findings of Aratake and Ueno (1973), Funakoshi and Aizawa (1989), Yungen (1989) and Zhange and Eguchi (1989) demonstrated the antiviral role of alkaline protease and alkaline phosphatase in the digestive juice of the silkworm. Similarly, the importance of carbohydrate metabolism in the growth and differentiation of the silkworm has been highlighted by various studies (Horie 1961; Sakator 1970; Chapman 1982).

Our ultimate objectives were to identify the specific biochemical marker(s) useful for improving survival

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and/or the specific yield component(s) and so pave the way for establishing linkage between specific biochemical markers and the yield components of *Bombyx mori* L.

Materials and methods

The fifty-four choosen races and breeds of the mulberry silkworm, selected to represent collections from different countries, can be broadly classified into four groups on the basis of origin, yield and diapause (Table 1). In selecting the breeds or races, attention was given to include the whole range of yield potential of the species (Table 1) under tropical conditions.

Following standard practice (Krishnaswami 1978), the rearing of early age and late age larvae was done using mulberry leaves from two seasons: January–February (Season I) and April–May 1990 (Season II). Fifteen layings were brushed *en masse* for each stock on chopped (approximately 1 cm^2) mulberry leaf. Feeding was three times a day. Subsequent to the third moult, larvae were divided into three replications of 450 each. Late age rearing was done in 2′ × 3′ trays for each replication.

Yield parameters

The four yield parameters considered are: (1) weight (g) of ten mature larvae prior to spinning (LWT); (2) weight (mg) of a single cocoon (CWT); (3) weight (mg) of a single cocoon shell (SWT), and (4) the effective rate of rearing (ERR), calculated as follows:

$$\frac{\text{Total no. of cocoons harvested}}{\text{Total no. of larvae kept after moult III}} \times 100$$

The assessment of cocoons was done individually for a minimum of 30, and the mean was computed as the estimate for that particular replication.

Biochemical parameters

Subsequent to the 15th feeding after moult IV, haemolymph and digestive juice were collected in chilled Eppendorf tubes (Chatterjee et al. 1988, 1992) from five larvae and, for each replication, three such collections were made. All the samples were collected in the presence of thiourea and were kept at −20 °C untill the time of analysis.

Amylase activity was estimated after Bernfeld (1955) using 0.2% starch in phosphate buffer as substrate. The 3–5-dinitrosalicylic acid used was from E. Merck, Germany. The amylase in silkworm has been characterized as an α-amylase (Kanekatsu 1978) and, for the present study, amylase activity was expressed as μg of maltodextrin released per 30 min per 20 μl of sample used. Invertase was assayed following the method of Ishaaya and Swirski (1970) with sucrose as a substrate, and the enzyme activity was expressed as μg of glucose released per 20 μl of digestive juice per 30 min. Protease activity was measured after Eguchi et al. (1972) using casein as a substrate both at neutral (7.0) and alkaline (10.0) pH after testing the activity at several pH increments between 6.8 and 11.0. Incubation was at 37 °C for 30 min, and the OD was measured at 660 nm by using a Philips UV/VIS spectrophotometer. Alkaline phosphatase activity was determined by the method of Mihara et al. (1988) using p-nitrophenyl phosphate as a substrate at pH 10.8. The reaction mixture was incubated at 37 °C for 30 min, and the OD was recorded at 400 nm. Trehalose was estimated after the method of Roe (1955) using anthrone reagent and the colour was read at 620 nm.

Statistical analysis

A correlation matrix was obtained from the average estimates of ten variables in two seasons separately, whereas combined estimates of two seasons were used for the "stepwise regression". For all statistical analyses, the computer software SPSS/PC+ (M. J. Norusis, SPSS Inc., Chicago, Ill.) was utilised. Based on the correlation matrix, the relative importance of various independent variables (biochemical parameters in this analysis) was ascertained, and the significance of the stepwise addition of variables in the forward selection programme was calculated. As suggested by Afifi and Clark (1984), the maximum value of 0.990 for PIN was used to examine the full sequence of stepwise regression analysis.

The various statistics used in the text are defined as follows:

Beta = BSx/Sy, where B is the regression coefficient (also denoted as slope), Sx and Sy are the standard deviations of independent (x) and dependent (y) variables.

$$\text{Adjusted } R^2 = R^2 - \frac{p(1 - R^2)}{(N - p - 1)}$$

Table 1. Brief description of the four groups of silkworm stocks (A–D) as detailed in Materials and methods

Group: title	Cocoon shape	Cocoon colour	Cocoon wt (cg)	Shell wt (mg)	SR%	Country of origin
I. Non-Hibernating						
A. Low yielding	Oval, spindle, dumbell	White, cream yellow (1, 2)	80–120	90–160	9–14	India
B. Medium yielding	Spindle and oval	Whilte, cream, yellow	110–145	120–220	11–15	India
II. Hibernating						
C. Low and medium yielding	Spindle, dumbell, oval	White and yellow	98–140	120–230	10–16	India, China, France, South East Asia
D. High yielding	Oval and dumbell	White	140–230	250–450	18–22	India, China, Japan, France

where "p" is the number of independent variables. The adjusted R^2 attempts to correct R^2 to closely reflect the goodness of fit of the model to the population.

The partial correlation coefficient $= R^2 - R_i^2/1 - R_i^2$ and can be interpreted as the correlation between ith independent variable and the dependent variable when the linear effect of other independent variables has been removed from both X_i and Y.

F change = R^2 change $(N - p - 1)/q(1 - R^2)$ where "p" is as defined as above and "q" is the number of independent variables entered at that particular step.

Results

Mean estimates of six independent and four dependent variables along with standard deviations and ranges for the two seasons are presented in Table 2. The estimates for most of the characters in both seasons are very close to each other except for trehalose. Trehalose content in season II (April–May) was found to be significantly ($t = 4.17$; $P < 0.001$) lower than that realised in season I (January–February 1990), and the difference in the trehalose content was obvious for most of the races belonging to the four groups detailed earlier.

Table 3 presents the correlation matrix of six independent variables and four dependent yield attributes. It was evident from the matrix that correlations among three yield parameters (weight of mature larva, cocoon and cocoon shell) were highly significant and positive ($r > 0.8$) for both seasons. However, the correlations between biochemical parameters reflect a seasonal influence as r values show a significant difference between seasons. The correlation between invertase and amylase (−0.332 and −0.133), alkaline phosphatase and invertase (−0.399 and +0.088), and that between protease at pH 10.0 and protease at pH 7.0 (+0.173 and +0.776), may be cited as examples. However, correlations between certain parameters were similar in both seasons, e.g., the correlation between amylase and protease at pH 7.0 (+0.281 and +0.317).

Data on correlations between biochemical and yield parameters reveal a number of interesting features. In season I, amylase showed highly-significant ($P < 0.001$) correlations with all four yield parameters, whereas trehalose gave a negative correlation ($P < 0.05$) with the effective rate of rearing (ERR). However, other independent (biochemical) variables in season I did not reveal a significant correlation with any of the yield parameters. In season II, the ERR was positively correlated with both digestive amylase and digestive alkaline protease. Larval weight (LWT), on the other hand, showed negative correlations with protease at pH 7.0 (−0.378) and 10.0 (−0.314), but had a strong positive correlation (+0.49) with alkaline phosphatase. Both single 'cocoon weight' (CWT) and 'shell weight' (SWT) were negatively correlated with protease activity at pH 7.0 and 10.0. However, positive correlations were observed with alkaline phosphatase for the same two characters (CWT and SWT). Digestive amylase showed strong correlations with all the attributes in season I, but in season II was correlated only with the effective rate of rearing (+0.58) and shell wt. (−0.298).

Table 2. Mean estimates of six biochemical parameters and four yield components of 54 races and breeds in two seasons

Variables	Season	Mean	SD	Minimum	Maximum
Amylase	I	502.4	282.6	29.6	1049.6
	II	442.5	275.2	61.4	939.7
Invertase	I	553.2	70.8	177.1	1022.5
	II	548.8	158.3	161.4	968.7
Trehalose	I	132.1	41.4	26.2	223.4
	II	91.8	29.2	45.3	210.5
Protease (pH7)	I	8.85	1.33	5.57	12.35
	II	9.03	1.59	5.86	12.06
Protease (pH10)	I	10.58	5.05	7.01	33.53
	II	9.82	1.51	5.61	12.86
Alkaline	I	10.58	5.05	7.01	33.53
phosphatase	II	9.82	1.51	5.61	12.86
Wt. of 10 larvae (gm)	I	23.73	6.78	12.79	37.70
	II	25.78	5.59	15.22	41.31
Single	I	1068.9	260.6	592.0	1585.0
cocoon wt. (mg)	II	1109.3	203.2	666.0	1518.0
Single	I	177.2	64.5	79.0	311.0
shell wt. (mg)	II	187.7	54.5	87.0	312.0
Effective	I	84.2	11.6	37.2	97.5
rate of rearing	II	77.3	11.8	37.2	93.5

Table 3. Correlation matrix of six independent and four dependent variables estimated for 53 and 54 genetic stocks of mulberry silkworm raised in RBD during January–February (Season I) and April–May 1990 (Season II) at CSRTI, Mysore

Season	ERR	LWT	CWT	SWT	AML	INV	TRE	PRO7	PRO10
I									
LWT	−0.089								
CWT	−0.042	0.939****							
SWT	−0.138	0.930***	0.941***						
AML	0.364**	−0.500***	−0.449***	−0.463***					
INV	−0.234	0.087	0.075	0.186	−0.332*				
TRE	−0.288*	−0.069	−0.059	−0.057	−0.028	0.100			
PRO7	0.053	−0.174	−0.205	−0.175	0.281*	−0.216	−0.096		
PRO10	−0.067	0.187	0.165	0.233	−0.106	0.071	0.002	0.173	
ALK	−0.216	0.165	0.182	0.157	0.139	−0.399**	0.081	0.292*	0.183
II									
	ERR	LWT	CWT	SWT	AML	INV	TRE	PRO7	PRO10
LWT	−0.082								
CWT	−0.034	0.855***							
SWT	−0.201	0.874***	0.922***						
AML	0.581***	−0.242	−0.243	−0.298*					
INV	−0.245	0.103	0.177	0.358**	−0.133				
TRE	−0.161	−0.055	0.002	0.144	0.079	0.172			
PRO7	0.265	−0.373**	−0.386**	−0.402**	−0.317*	−0.091	−0.073		
PRO10	0.313*	−0.314*	−0.302*	−0.325*	0.252	0.029	−0.180	0.776***	
ALK	0.010	0.490***	0.427**	0.521***	−0.064	0.088	0.099	−0.310*	−0.254

LWT, CWT and SWT denote the weight of mature larvae, single cocoon, and single cocoon shell respectively, while AML, INV, PRO7, PRO10, ALK denote estimates of amylase, invertase, protease at pH 7 and 10, and alkaline phosphatase activity in digestive juice, respectively. TRE denotes trehalose content in blood and ERR indicates effective rate of rearing as explained in the text. ** and *** denote r value significant at > 5% (r > 0.267), 1% (r > 0.346) and 0.1% (r > 0.435), respectively

Stepwise multiple regression analysis

Multiple stepwise regression analysis was adopted with 107 entries to estimate the relative significance of correlated independent (biochemical) variables in determining the regression line for separate dependent variables.

All four dependent variables had highly significant adjusted R^2 values ($P < 0.005$) at all stages of stepwise selection and the final R^2 values (after including all variables in the equation) was highest (0.316) for shell-weight.

With regard to the stepwise selection of independent variables, the results presented in Table 4 indicate the significant contribution of digestive amylase. As for all four yield variables, it was selected in the first step. The adjusted R^2 at the first step was highest for the effective rate of rearing (ERR). In the second step, INV (invertase) was selected for ERR while ALK (alkaline phosphatase) was selected for the other three independent variables.

The addition of a second variable to explain the ERR, however, resulted in insignificant change (F change) in R^2 (0.0165). The addition of other variables at subsequent steps also failed to produce any significant increase in the adjusted R^2 value. The results presented in Table 5 further showed that the slope (B) of amylase over ERR was significantly linear and positive, which is also supported by a high value of 'Beta'. The small difference between the correlation (0.484) and the partial correlation (0.467) index indicated little contribution of other independent variables to both ERR and amylase (AML). For ERR, the slopes of other independent variables were negative but not significant. However, the correlation for INV was significant at $P < 0.05$, although no other correlation or partial correlation was found to be significant.

For larval weight, the addition of ALK at step 2 alone significantly changed the adjusted R^2 (from 0.146 to 0.247). But, an effect of TRE (trehalose content of blood) on larval weight cannot be ignored as the partial correlation was significant (Table 5) and the slope was almost significant ($P = 0.053$).

With regard to CWT and SWT, the addition of ALK also caused a significant change of multiple R (adjusted R^2). The similarity of correlation and partial correlation values suggested the absence of any large effect of other independent variables on both X (CWT) or SWT) and Y (ALK). At step 3, PRO7 was selected for CWT, but it failed to change the adjusted R^2 significantly and the same happened with the addition of other variables at subsequent stages. For SWT, INV was selected at step 3 with a significant change in the multiple regression coefficient. The contribution of PRO7 to the expression cannot be ignored as is evident

Table 4. Result of multivariate stepwise regression analysis. The analysis is based on 107 entries for two seasons

Step[a]	Adj Rsq	F(Eqn)	FCh	Sig Ch
Effective rate of rearing				
AML	0.227	32.143	–	***
+INV	0.236	17.417	2.294	NS
+ALK	0.239	12.096	1.340	NS
+TRE	0.237	9.255	0.801	NS
+PRO10	0.234	7.488	0.575	NS
+PRO7	0.228	6.227	0.213	NS
Weight of mature larva				
AML	0.146	19.154	–	***
+ALK	0.247	18.388	15.057	***
+TRE	0.259	13.383	2.753	NS
+PRO7	0.272	10.882	2.711	NS
+PRO10	0.272	8.939	1.120	NS
+INV	0.267	7.422	0.195	NS
Weight of single cocoon				
AML	0.124	15.955	–	***
+ALK	0.209	14.993	12.312	***
+PRO7	0.226	11.335	3.343	NS
+TRE	0.229	8.851	1.301	NS
+PRO10	0.231	7.357	1.281	NS
+INV	0.227	6.197	0.557	NS
Weight of single cocoon shell				
AML	0.142	18.607	–	***
+ALK	0.259	19.532	17.529	***
+INV	0.302	16.315	7.456	**
+PRO7	0.312	13.043	2.509	NS
+PRO10	0.319	10.953	2.054	NS
+TRE	0.316	9.177	0.544	NS

*** and ** denote significance with $P = 0.001$ and 0.01, respectively. The step indicates the selection of a biochemical (independent) variable in the respective stages of stepwise regression analysis. '+' symbol indicates the addition of the next variable in the equation. Abbreviation of variables used are the same as indicated in Table 3. Adj Rsq = adjusted R^2; F(Eqn) = F statistic of adjusted R^2; FCh = measure of change of R^2; Sig Ch = significance of FCh; NS = not significant

from the significance of the slope and the correlation and partial correlation coefficients (Table 5).

Discussion

Based on preliminary observations of the correlations between digestive amylase and yield attributes, Chatterjee and Datta (1989) suggested the importance of carbohydrate metabolism towards yield realisation in silkworm, and this contention is substantiated by the results of the present study. Of the six biochemical traits considered here, the enzymes which were correlated with yield parameters (amylase, invertase = sucrase, and alkaline phosphatase) are all primarily related with carbohydrate metabolism in general and sugar metabolism in particular (Horie and Tanaka 1957; Horie 1961; Wyatt 1961; Applebaum 1985).

The selection of "amylase" at the first stage for all four yield parameters highlighted its importance in the silkworm and confirmed the earlier observations of Chatterjee et al. (1988) and others (Hirata 1971, 1974; Moon and Seol 1983). Chatterjee et al. (1992) presented data to show a positive correlation between digestive amylase and survival on one hand and a negative correlation with larval span, weight of mature larva, cocoon and cocoon shell on the other. The present report further elaborates this and shows that five other biochemical parameters were not able to significantly alter the linear relationship between amylase activity and the effective rate of rearing (ERR). The application of discriminant analysis (Chatterjee, unpublished) also led to identification of the same independent variables (AML, INV, ALK) having a significant contribution. This result suggests the need for further study on the genetical and physiological aspects of amylase.

The enzyme invertase catalyses the breakdown of sucrose into glucose and fructose (Morrison and Boyd 1983); how this action negatively influences survival and positively influences the expression of "shell weight" in the silkworm is not understood. However, the important relationship between "sugar metabolism" and high energy requirement (e.g., during flight) in insects is well established (Steele 1981). The positive influence of invertase on shell weight is of particular importance because an improvement of this yield component is a priority area.

The results presented in this report clearly show the importance of alkaline phosphatase (ALP) in the expression of larval weight, cocoon weight, and shell weight. Yungen (1989) also presented data indicating a positive correlation between the activity of ALP and cocoon characters. But nothing specific is known about the actual pathways of alkaline phosphatase in the digestive fluid with regard to its influence on the above mentioned characters. In this context, the suggested role of ALP as an ATPase (Azuma et al. 1991) implies a positive effect on the growth and activity of the silk gland.

Earlier work of Funakoshi and Aizawa (1989) indicated the importance of alkaline protease in antiviral activity. Based on those observations, it was presumed that this enzyme may have a significant correlation with the survival index. But the present result does not reveal any effect significant enough to modify the relationship with digestive amylase. It is possible that only under the condition of viral infection will the significance of the enzyme be realised, as reflected by the recent observations on the differential response of protease activity in the larval digestive juice of susceptible and highly-tolerant silkworm stock subsequent to NPV inoculation at instar V (Sen et al. 1992).

Table 5. Estimates from stepwise regression analysis of B (slope), standardized regression coefficient (Beta), correlation and partial correlation indices for the different independent variables

Variable	B	Beta	Correl	Partial correl
Effective rate of rearing				
AML	1.315E-04***	0.477	0.484***	0.462**
INV	−5.577E-05	−0.135	−0.228*	−0.150
ALK	−9.259E-04	−0.098	−0.074	−0.113
TRF	−1.561E-04	−0.083	−0.069	−0.095
PRO10	1.598E-03	0.077	0.047	0.086
PRO7	−2.305E-03	−0.044	0.150	−0.046
Weight of mature larva				
AML	−7.454E-03***	−0.333	−0.393***	−0.348***
ALK	0.249***	0.326	0.316***	0.360***
TRE	−0.025	−0.167	−0.136	−0.192*
PRO7	−0.726	−0.169	−0.255**	−0.180
PRO10	0.150	0.089	0.061	0.101
INV	1.284E-03	0.038	0.089	0.044
Weight of single cocoon				
AML	−0.242***	−0.290	−0.363***	−0.302**
ALK	8.428***	0.296	0.293**	0.323***
PRO7	−32.155***	−0.201	−0.280**	−0.208*
TRE	−0.644	−0.113	−0.075	−0.128
PRO10	6.044	0.096	0.060	0.106
INV	0.083	0.066	0.121	0.074
Weight of single cocoon shell				
AML	−0.063***	−0.295	−0.388***	−0.323***
ALK	2.542***	0.350	0.339***	0.395***
INV	0.068*	0.214	0.270**	0.248**
PRO7	−7.318*	−0.180	−0.282**	−0.198*
PRO10	1.981	0.124	0.105	0.145
TRE	−0.088	−0.061	−0.002	−0.073

Abbreviation of variables explained in Table 3
*, ** and *** denote significance at $P < 0.05$, 0.01 and 0.001, respectively

Regarding the trehalose content of the haemolymph, this study did not reveal any significant role for it in the expression of the four yield attributes. Nonetheless, its correlation with larval weight cannot be ignored. Further, the significantly lower content of trehalose during the warmer season indicates its importance in thermotolerance. In this connection, the finding of Pelham (1986) on the similarity of response of biological material towards temperature shock and glucose metabolism may be relevant.

While it is true that information on the relationships between yield and biochemical parameters within races may throw light on genetical aspects of yield, the material used in the present study has some limitations. As the collection of digestive juice or blood strongly affects the expression of yield parameters, it is not possible to correlate biochemical parameters and yield attributes in the same individual. Consequently, emphasis was directed to understanding the genetic relationship across races. Moreover, the study also aimed at identifying surrogate biochemical parameters which might be utilised in a specific breeding programme, an expectation justified by the present results. The identification of invertase and alkaline phosphatase as surrogate markers for the expression of shell weight is very pertinent, especially with reference to their relationship with amylase, the most important biochemical marker for survival. These aspects should be of value in adopting a more directional approach to silkworm breeding.

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