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QUANTITATIVE ANALYSIS OF LARVAL BODY GROWTH IN THE SILKWORM, *BOMBYX MORI* (LEPIDOPTERA: BOMBYCIDAE)

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ABSTRACT Quantity-wise and rate-wise growth changes in the larval body mass vis-à-vis log-based growth curves were analyzed in *Bombyx mori*. The body mass accumulation followed a progressively increasing trend from third to fifth instar, registering higher values in growth index (GI) and mean growth rate (MGR). But, the corresponding compound periodical growth rates and mean specific growth rates declined remarkably during the same period. The growth increments in body mass are in consonance with Hutchinson's investment policy and accordingly the silkworm accumulates energy reserves and structures that are necessary for future use during metamorphosis. Higher growth ratios recorded in the present study indicate that the silkworm systematically violates the Dyar's constant rule throughout the growth regime. The log-based growth curves were further analyzed to determine the critical and threshold body sizes that trigger moulting and metamorphosis in the silkworm.

KEYWORDS : *Bombyx mori*, critical size, growth curves, larval growth, threshold size.

INTRODUCTION

Insect growth is a complex process involving in its life cycle, morphologically distinct larval, pupal and adult stages with intervening molting processes (Esperk & Tammaru, 2004). The acquisition of appropriate body size at maturity is an important life history trait of insects (Grunert *et al*., 2015). The larva undergoes multi-dimensional body growth through active mobilization of energy and resources from surroundings and it is primarily determined by food intake and the rate of metabolism (Maino and Kearney, 2015). It is imperative to have at reliable estimates of the cost of large body size and trace the existing interrelationships between growth and maturity in insects (Gotthard, 2004). Further, majority of insects show inconsistencies in body mass accumulation that posed major challenges for the correct analysis of their growth patterns during different stages of its life cycle. Therefore, the quantitative analysis of exponential growth curves of insects remained unresolved. Similarly, a detailed account on silkworm growth and quantitative profiles of its growth curves is limited and the available data is inconclusive. Hence, the objective of research in insect growth should encompass the systematic analysis of growth curves, besides understanding the anatomical and physiological bases of insect body size (Llandres *et al*., 2015). In his pioneering report, Woodruff (1939), suggested a practical and realistic model for the analysis of growth curves in insects that remained a valid model for growth analysis in insects till to date. Against this backdrop, it was considered necessary to have an in-depth knowledge of the quantitative profiles of silkworm growth, as it could have positive implications for evolving appropriate strategies of rearing silkworms and other economic insects. Obviously, the present study aims at analyzing systematically the growth in the larval body mass of silkworm and the resultant growth curves with a view to arrive at a broad understanding of growth intricacies underlying its metamorphosis.

MATERIAL AND METHODS

The growth studies were carried out on the PM x CSR_2 hybrid silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). The silkworms were reared on the M_5 variety of mulberry leaves giving 5 feeds per day at 6AM, 10AM, 2PM, 6PM and 10PM, under standard environmental conditions of 28°C, 85% RH, 12 h light and 12 h dark conditions as per Krishnaswami (1986). Day-to-day quantitative changes in the larval body weight (LBW) during third, fourth and fifth instars were measured by weighing 25 randomly selected worms in an electric balance (ELICO; Model LBL-22OH) and the same was expressed in grams.

Statistical Analysis

The experimental data were statistically analyzed by mean, standard deviation (SD), percent change (PC) and test of significance using M.S. Excel platform and relevant online software packages (www, Graph pad. com / quick calcs / index cfm / and www.percent change com / index php). In order to draw meaningful conclusions, the larval growth was statistically analyzed in terms of following parameters.

1) Growth Index (GI): It refers to the actual growth capacity of insect larva during metamorphosis. It was computed by the formula $W_f - W_i / W_i$ where, W_i and W_f represent the initial and final body weights of silkworm larva in each instar.

2) Mean growth rate (MGR): It refers to the average growth of larval body within an instar. It was obtained by dividing the growth increase in LBW in an instar by number of days between moults and expressed in percentage.

3) Mass-specific growth rate (MSGR): It refers to the rate of increase in biomass of a larval form per unit of biomass concentration. It was obtained by dividing the mean growth rate of an instar by mid-instar mass and expressed in percentage.

4) Compound Periodical Growth Rate (CPGR): It explains day-to-day growth trends during the life cycle of an insect. Its value was computed using relevant online packages as given by Sivaprasad (2012) and expressed in percentage.

5) Critical size: It represents the optimal larval body size within an instar that must be passed in order to trigger its commitment to moulting. It was measured in terms of critical weight (CW) and expressed in grams. In each instar, the corresponding CW value was identified at the inflection point of the log curve on the exponential phases of the growth trajectory as suggested by Sharma (2005).

6) Threshold size; It represents the optimal larval body size of the final instar that must be passed in order to trigger its commitment to pupation. It was measured in terms of threshold weight (TW) and expressed in grams. Its value was identified at the inflection point of the log curve of the overall growth trajectory (Fig.2) as given by Sharma (2005).

7) Dyar's coefficient: The Dyar's coefficient (also called Growth ratio) is a calculated value arrived at by dividing the LBW of one instar by the LBW of the preceding instar as given by Thakur (2016).

RESULTS AND DISCUSSION

The larval body mass could be used as a realistic indicator of growth and metamorphosis in *Bombyx mori*. The silkworm passes through a 17-day vegetative growth phase with five characteristic instars and four molts before the onset of pupation. After each molt, the larval body weight (LBW) recorded significant elevations from one instar to the other. From a low of 0.035g at the beginning of third instar, the LBW increased to 0.093 (~166%) at the end of third instar, 0.68g (~1843%) at the end of fourth instar and 2.98 g (~8414%) at the end of fifth instar, gaining a mass of about 2.95g (2.98 – 0.035) during the entire period of larval development (Table 1). The growth trends reflect several interesting features of silkworm metamorphosis as illustrated in the following heads.

The Larvae Accumulate Body Mass In Day-specific And Instar-specific Manners

The present study amply demonstrates that the body mass accumulation in silkworm follows a definitive time sequence with day-specific and instar-specific variations. Predominantly, the day-specific variations are quantity-based and the instar-specific variations are rate-based (Table 1). Evidently, the silkworm accumulated a small quantity of body mass (~3%) in 3-day long third instar, a moderate quantity (~23%) in 4-day long fourth instar and a bulk of body mass (~74%) in 7-day long fifth instar. The growth index (GI), which also indicates the overall growth rate, recorded a higher value of 1.66 in third instar, a moderate value of 1.52 in fourth instar and a maximal value of (2.87) in fifth instar. The day-wise accumulation trends showed that the LBW grew maximally on 3rd day in third instar (~98%), 2nd day in fourth instar (~67%) and again 3rd day in fifth instar (~58%). The compound periodical growth rate (CPGR), which represents the average daily growth trends, showed a declining trend from third to fourth and from fourth to fifth instar. Obviously, the LBW recorded the highest CPGR of 63% in third instar, a moderate CPGR of 36% in fourth instar and a very low CPGR of 25% in the fifth instar (Table 1). The mean growth rate (MGR) and the mean specific growth rate (MSGR), which act as the indicators of digestive efficiency and physiological function of larval growth showed inverse relationship with instar growth. While the former showed an elevator trend, the latter projected a declining trend during larval growth. Significantly the MGRs increased from about 2% in third instar to about 10% in fourth instar and to about 32% in fifth instar. Conversely, the MSGR declined significantly from a original value of about 42% in third instar to 20% in fourth instar and about 15% in fifth instar. The present study demonstrates that the silkworm grows more vigorously during third instar, compared to fourth and fifth instars. Nonetheless, the larva accumulates most of the body mass during the final fifth instar, probably in tune with its voracious feeding habit coupled with high power of digestibility [Hou *et al.,* 2010].

Table 1: Growth In The Larval Body Mass Of Silkworm, *Bombyx Mori* During Metamorphosis, Together With Computed / Observed Statistical Parameters.

(A) EXPERIMENTAL DATA				
Day	Statistical tool	Third Instar	Fourth Instar	Fifth Instar
1	Mean SD (±)	0.035 ±0.001	0.27 ±0.005	0.77 ±0.005
2	Mean PC (%) SD (±)	0.047 34.3 ±0.005*	0.45 66.7 ±0.005*	0.91 18.2 ±0.005*
3	Mean PC (%) SD (±)	0.093 97.9 ±0.005*	0.59 31.1 ±0.005*	1.44 58.2 ±0.005*
4	Mean PC (%) SD (±)	-	0.68 15.2 ±0.005*	2.05 42.4 ±0.006*
5	Mean PC (%) SD (±)	-	-	2.60 24.0 ±0.005*
6	Mean PC (%) SD (±)	-	-	2.92 12.3 ±0.005*
7	Mean PC (%) SD (±)	-	-	2.98 1.21 ±0.009*

(B) COMPUTED/OBSERVED STATISTICAL PARAMETERS			
Mean LBW (g)	0.058	0.497	1.95
Growth index	1.66	1.52	2.87
CPGR (%)	63.01	36.1	25.3
MGR (%)	1.93	10.25	31.57
MSGR (%)	42.06	19.71	15.40
Dyar's coefficient	-	8.568	3.924
CW (g)	0.076	0.64	2.30
CT (days)	2.54	3.50	4.50
TW (g)	-	-	1.75
TT (days)	-	-	3.50

*Statistically significant (P value <0.001); ** statistically not significant. *Each value of LBW (larval body weight) is a mean, ± standard deviation of four individual observations on a larval group comprising 25 worms. Growth trends in LBW were analyzed in terms of percent change (PC), growth index (GI), mean growth rate (MGR), mass-specific growth rate (MSGR), compound periodical growth rate (CPGR), critical weight (CW), critical time (CT), threshold weight (TW) and threshold time (TT). The critical values (CW & CT) were calculated for the third (3 days), fourth (4 days) and fifth (7 days) instar larvae, while the threshold values (TW & TT) were computed for overall growth of larvae in all the three instars.*

Silkworm Growth Curves Are Truncated Trajectories

A previous report projected a logarithmic increase in the body mass of silkworm during larval growth (Venugopal Reddy *et al.*, 2015). The present study shows that the silkworm growth curves are truncated Gompertz trajectories (Figs.1 and 2). In each instar the growth followed an exponential path to reach its peak but declines at the end as the larva prepares for the next molt. It implies that the larval growth within an instar is initially exponential but the exponent declines gradually making the overall growth trajectory sigmoidal.

Woodruff (1939) suggested a log-based ratio analysis technique for fitting insect growth curves in terms of relative magnitudes (proportionate increases) rather than in their absolute magnitudes (actual increases) in body mass. Accordingly, dual purpose charts with exponential and logarithmic functions were drawn using iterative software algorithms available on MS Excel platform in order to evaluate the hypothesis of exponential growth in body mass (Figs. 1A, B, C). In such curves, the slope or curvature of log-based trend line was used as an indicator of growth vis-à-vis the acquisition of instar specific body mass. Thus, the curve helps in deciphering the silkworm growth at a glance for descriptive and predictive purposes.

Further, the Woodruff's log curve gives an idea of how many data points fall within the regression equation and thereby facilitates the identification of coefficient of determination (R^2) that could be used as an indicator of better goodness of fit. If the curve bends upwards (towards left) it was assumed that the growth rate is increasing and if it bends downwards (towards right) the growth rate is decreasing.

A close look at the log curve of the body mass of silkworm indicates that the slope of the curve predominantly shifted left (upwards) and hence the third instar recorded higher values of CPGR (63%) and MSGR (42%), compared to those in fourth (CPGR;36%; MGR:20%) and fifth instars (CPGR;25%; MSGR:15%).

The movement of log curve initially to the left and latter to the right indicates that the growth increases exponentially during the first half of instar but declines latter during the second half of the instar at the inflection point prior to entering molting phase (Fig. 2). This anomaly is explained in terms of Hutchinson's investment policy.

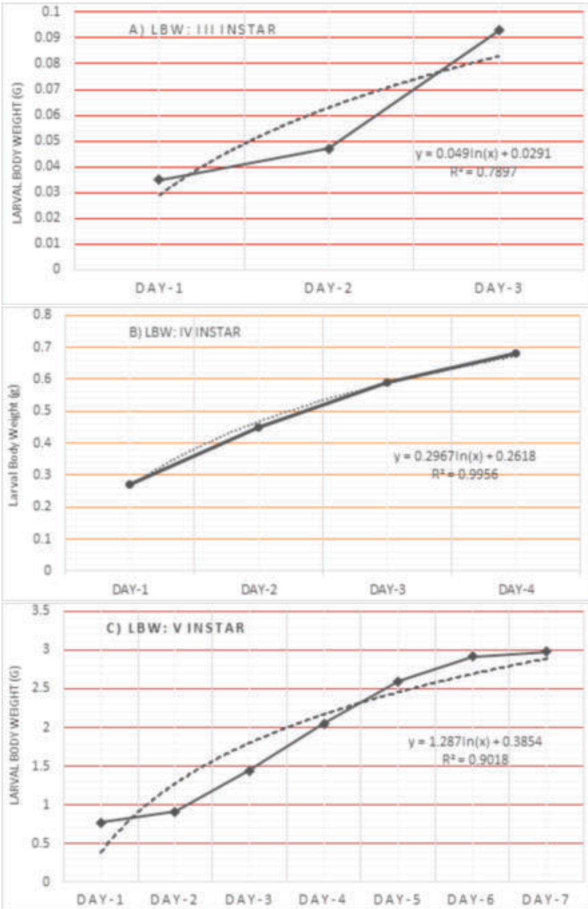

*Fig. 1: Growth curves of larval body weight (LBW) of **Bombyx mori** during third (A), fourth (B) and fifth (C) instar development (Source: Table 1).*

Silkworm Growth Follows Hutchinson Investment Principle

The Hutchinson's investment principle (HIP), as illustrated by Hutchinson *et al*., in 1997, provides an adaptive explanation for the body mass accumulation in insects. It assumes that growth in molt-dependent larval soft bodies represents investment of energy resources for future use. Supported by Maino and Kearney (2015), the principle delineates insect biomass into components of structure (size) and energy reserve (nutritional condition) and that the growth in the former is always accompanied by growth in the latter leading to higher production efficiency in later stages of development. It further assumes that insects, whose feeding rate depends on the size of structures that grow by molt, monotonously display declining trends in size increments and increasing trends in instar durations.

The growth trends observed in the larval body mass of *Bombyx mori* are in compliance with the energy reserve and structure concept of HIP. Accordingly, this insect accumulates energy reserves in a phased manner during larval stage to be used in the ensuing non-feeding pupal and adult stages. As predicted by the HIP, the larval growth vis-a-vis body mass accumulation in silkworm depends on three factors; larval duration, diet volume and feeding time. The greater the larval duration, the greater the feeding time and greater would be the feeding volume. Since, the silkworm has longer duration of 7 days in fifth instar with longer feeding time, it accumulated more mass during this stage, compared to that of 3-day period in third instar and 4-day period in fourth instar. Despite progressive increase in body mass from the third through fifth instar, the overall larval growth trajectory of silkworm (Fig. 2), deviates from the exponential growth path and its growth exponent declines at the end of each instar, much like that in ***Manduca sexta*** (Sears *et al.,* 2012). Though, the anatomical and physiological bases of body mass growth in silkworm are not examined in the present investigation, the principle is attributable to the selection pressure caused by emerging energy demands, tracheal expansions, fat body depositions and other structural components required for post-embryonic development, silk production and metabolic acceleration (Greenlee *et al*., 2013; Helm and Davidowitz, 2013; Nijhout and Callier, 2015). Obviously, the larval silkworm augments its respiratory structures during growth by continuously increasing the volume and delivery capacity of the tracheae during larval growth as it is crucial for meeting the energy needs of aerobic metabolism during larval growth and attainment of optimal body size. Further the falling trends in CPGR and MGR, observed in the silkworm are probably caused by pre-molt growth interruptions that are initiated at the inflection point of respective Gompertz trajectories. Apparently, such growth interruptions are attributable to three independent limitations on the functional roles of exoskeleton, tracheal system and the gut that would impose additional restrictions on larval growth (Grunert *et al.,* 2015).

Silkworm Growth Violates Dyar's Rule

It is customary to analyze insect growth rates in terms of an innovative concept called Dyar's constancy rule proposed by Dyar and Rhinebeck, in 1890. The DCR, asserts that the pre-molt/ post-molt growth ratios remain constant from instar to instar during ontogeny in insects and other arthropods. The Growth ratio (also called Dyar's coefficient) is defined as the size of one instar divided by the size of the preceding instar. The silkworm recorded relatively higher growth ratios of 9.0 between the third and fourth instar and 3.98 between fourth and fifth instar (Table 1B), while the insects that follow DCR, showed growth ratios in the range of 1.199 and 1.222 or 1.255 and 1.277 in between two successive instars (Thakur, 2016). Thus, the log-transformed exponential growth trajectories (exponential regressions) of *Bombyx mori* are violative of the DCR, a fact that indicates that the larval growth increment is not constant, but increases from instar to instar, a feature common to all lepidopteran insects (Grunert *et al.,* 2015).

The concepts of DCR and HIP represent two contradictory facets of silkworm growth. While the former assumes regular geometrical progression in the growth of head capsule and other sclerotized parts that optimize the rate of feeding, the HIP emphasizes the molt-dependent growth of soft bodies that is governed by investment principle that lead to the accumulation of body mass in the form of structures and energy reserves. Thus, while the DCR expects constancy of growth ratios, HIP predicts that larger growth ratios can occur under conditions of the risk of molt-related mortality. Obviously, there is a progressive increase in size increment in silkworm, from instar to instar during the period of study. In addition, the CPGR, the instar-specific growth rate declines throughout the growth phase in a nutrition-dependent manner. The constancy principle of DCR holds good for the linear measure of exoskeleton of silkworm (not examined in the present study), but not for the other growth dimensions of soft tissues of the larval body. The higher R^2 values (ranging from 0.7897 to 0.996) for regression, observed in the present investigation would be generally taken as an evidence for the goodness of the fit (Fig.1). Thus, our study demonstrates that the size increment of silkworm actually increases from third instar to fifth instar reflecting systematic deviations from the apparent constancy of size increment proposed in DCR, but meticulously obeys the energy investment principle envisaged by Hutchinson *et al.,* (1997).

Critical And Threshold Sizes Control Moulting And Metamorphosis

The moulting and metamorphosis in insects are logically controlled by two important body size parameters called critical and threshold sizes (D'Amico *et al*., 2001). In this article, the silkworm growth rates have been analyzed with particular reference to the attainment of these two size parameters.

Critical Size:

The critical size is defined as the minimal larval body size limit that must be passed in order to trigger commitment to moulting and further metamorphosis, which is equivalent to 55% of the larval body mass (Davidowitz *et al.*, 2003). The attainment of CS is a time sensitive-process that sets-in a time interval for the cessation of larval growth leading to molting. The critical time (the time of acquisition of critical size), occurs on a specific day in every instar. After a molt, this size becomes initial size for the next instar and growth resumes from that point. Aptly, the CS could be used as an indicator of silkworm growth that helps predict its molting and metamorphosis. Realistic methods for the determination of critical size are not available. Nonetheless, it could be equated with critical weight (CW) and its corresponding values were located at the inflection points of log curves on the Gompertz trajectories of all the three larval instars at their peak level (Fig. 1A, B, C). The CW value and its time of acquisition differ from instar to instar. The current study demonstrates that the silkworm attains a CW of 0.076g, (R^2=0.7897) on the 2.5th day in third instar, a CW of 0.64g (R^2=0.9956) on 3.5th day in fourth instar and a CW of 2.3g (R^2=0.9018) on 4.5th day in fifth instar (Table 1B). Obviously, the acquisition of critical size is time-dependent and once it is achieved the growth in silkworm is not affected by further nutritional supplementations. It is likely that the silkworm acquires the critical size by appropriately modulating higher day-wise growth rates within an instar, as for instance the day 3 in third instar, day 4 in fourth instar and day 5 in fifth instar. Once, the larva reaches this optimal size, the physiological mechanism gears-up for the next molt (Browder *et al.*, 2001). The knowledge of CS and CT is indispensable for all those involved in silkworm rearing as any discrepancies in feeding conditions would drastically affect the larval growth and sericulture productivity.

Threshold Size:

Also called minimal variable size (MVS), the threshold size (TS) is defined as the minimum body mass required for larval-pupal metamorphosis (De Moed *et al.*, 1999). In sericulture, it represents the minimal body size of silkworm in the final fifth instar, at which further feeding and growth are not required in its journey towards pupation and metamorphosis. In the present study, the TS was considered equivalent to threshold weight (TW) and the same has been spotted at the inflection point of log curve on the compound Gompertz trajectory at its peak level (Fig. 2). Evidently, the silkworm attained a TW of 1.75 g, (R^2=0.6556) on the 3.5th day of fifth instar (Fig. 2 &Table 1B). This, it does so substantially by modulating instar-specific growth rates during larval development. Clearly, significant trends in GI (1.66), CPGR (63%), MGR (1.93%) and MSGR (42%), observed in third instar could have laid strong foundation for the acquisition of threshold size dimensions in the silkworm and it was further reinforced by modulation of these growth rates in subsequent instars. The TS is substantially smaller than the CS and the silkworm attains it on the 3.5th day, one day earlier than that of CS, which is acquired on the 4.5th day during fifth instar development. These observations lend support to the similar methods of acquisition of threshold growth dimensions by the larval of *Manduca sexta* (Kingsolver, 2007).

A word of caution: Quite often, the threshold size (TS) is misconceived as the critical size (CS). It should be kept in mind that that the CS represents an '**immediate cause**' that triggers a metamorphic change (moulting or pupation) in insects, while the threshold size represents a genetically determined '**long-term cause**' that initiates a cascade of developmental events that determine the timing of larval-pupal metamorphosis and acquisition of adult body size.

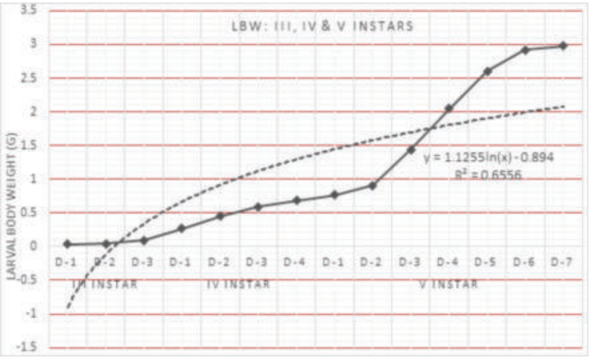

Fig. 4. Compound growth curve of *Bombyx mori*, showing growth in the larval body weight (LBW) during the developmental period from third to fifth instar (Source: Tables 1).

CONCLUSION

The silkworm growth kinetics reveal interesting features of larval body size regulation. The silkworm larvae grow in size and accumulate body mass in day-specific and instar-specific manners. The larval growth in *Bombyx mori* systematically follows Hutchinson's investment principle (HIP), but deviates from Dyar's constancy rule (DCR). Firstly, as enunciated in HIP, the molt-dependent growth in silkworm is associated with the investment of energy reserves and development of structures that are necessary for metamorphosis, post-embryonic development, silk production, metabolic acceleration and reproduction. Secondly, the larval growth systematically violates the DCR in that it recorded higher growth ratios (9.0 between third and fourth instar and 3.98 between fourth and fifth instar) in the larval body mass. More importantly, the body mass accumulation in silkworm is directed to ensure the acquisition of requisite body size (critical or threshold size) on a specific day of instar at specific time that triggers moulting and metamorphosis in silkworm.

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