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Metabolic insights and nutritional dynamics in silkworms

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Abstract

This study investigates the metabolic dynamics of four silkworm races—Pure Mysore, CSR2, FC1, and FC2—during the 5th instar stage, focusing on the accumulation of total protein, amino acids, cholesterol, ammonia, uric acid, and urea in various silkworm tissues over seven days. The results show a progressive increase in protein and amino acid levels in the silk glands and midgut, with CSR2 exhibiting the highest accumulation in both parameters. Cholesterol content in the haemolymph also increased steadily, with CSR2 showing the highest levels by day seven. Uric acid and urea excretion followed a similar trend, with significant peaks observed between days four and five, indicating an acceleration of nitrogen metabolism during this period. Among the silkworm races, CSR2 consistently exhibited superior metabolic activity, suggesting its potential for enhanced silk production. The findings highlight the critical role of metabolic processes, such as protein synthesis and nitrogen waste excretion, in supporting silkworm growth and silk production during the 5th instar stage.

Keywords: Metabolic dynamics, silkworm, protein, amino acid

Introduction

The silkworm, *Bombyx mori*, is a pivotal organism in sericulture and serves as an important model for studies in developmental biology, biochemistry, and molecular genetics. Understanding its physiological processes, particularly during its growth stages, is essential for optimizing silk production. Among these stages, the 5th instar is particularly significant due to the profound physiological and biochemical changes that occur, which are crucial for silk production. The 5th instar stage is marked by rapid growth and development, during which the silkworm prepares for cocoon formation. At this stage, the silkworm increases its feeding activity, primarily on mulberry leaves, providing the nutrients necessary for silk protein synthesis. The silk glands accumulate silk proteins, fibroin, and sericin, which are vital for cocoon formation. Additionally, hormonal changes, especially the increase in juvenile hormone levels, regulate the growth and development during this period. These physiological transformations are key to optimizing silk yield and quality.

This study focuses on the biochemical composition of the silk glands, haemolymph, and excreta of 5th instar silkworms, aiming to estimate key metabolic parameters such as total protein, amino acids, cholesterol, ammonia, uric acid, and urea. These parameters are indicative of the silkworm's metabolic and physiological status during this critical phase of development. The research employs analytical techniques to estimate total protein using the Lowry method, total amino acids using the ninhydrin method, and other metabolites like cholesterol, ammonia, uric acid, and urea in the haemolymph and excreta using enzymatic and colorimetric methods. By providing valuable insights into the silkworm's nutritional and metabolic demands, this research contributes to a deeper understanding of the biological processes that influence silk production. The findings will enhance knowledge of the silkworm's overall health, growth, and silk production capacity, all of which are integral to improving sericulture practices. Furthermore, the research will help identify potential areas for biotechnological innovations, such as genetic improvements and transgenic techniques, to enhance silk yield, quality, and disease resistance. Ultimately, this study aims to support the development of more efficient sericulture practices by understanding metabolic processes in 5th Instar. As the silkworm undergoes significant physiological changes, exploring these processes will contribute to advancements in biotechnology and the broader applications of

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silkworm-derived biomaterials, reflecting the evolving nature of the sericulture industry.

Materials and Methods

The experiment was conducted at Entomology Laboratory of Indira Gandhi Agricultural University, Raipur during the 2021-22 academic year, using 5th instar silkworms from four races: Pure Mysore, CSR2, FC1, and FC2. Tissues, including silk glands, midgut, and haemolymph, were collected on days 1, 3, 4, 5, 6, and 7 of the 5th instar stage for analysis. To estimate the total protein content in the silk glands, the Lowry method was employed (Waterborg, 2009)^[12]. Silk glands were dissected, washed in ice-cold PBS, and homogenized in a 1:10 ratio with PBS. After centrifuging the homogenate at 10,000 rpm for 10 minutes at 4°C, the supernatant was collected for protein analysis. Protein standards were prepared, and to each sample, 1 mL of Lowry reagent and 0.1 mL of Folin-Ciocalteu reagent were added, followed by incubation. Absorbance was measured at 660 nm using a spectrophotometer, and the protein concentration was determined by comparing the sample's absorbance to a standard curve constructed with BSA. For amino acid estimation in the silk glands, the ninhydrin method was used. Silk glands were homogenized in a sodium citrate buffer (1:10 ratio), centrifuged, and the supernatant was analyzed. Amino acid standards were prepared, and 0.5 mL of sample or standard was mixed with ninhydrin reagent, then heated for 15 minutes. Absorbance was measured at 570 nm using a spectrophotometer, and amino acid concentration was calculated by comparing the absorbance to the standard curve. Cholesterol levels in the haemolymph were measured using an enzymatic colorimetric method. Haemolymph was collected, centrifuged, and the supernatant was mixed with cholesterol oxidase, peroxidase, 4-aminoantipyrine, and phenol. The mixture was incubated at 37°C, and absorbance was measured at 500 nm. The cholesterol concentration was determined by comparing the sample's absorbance to a cholesterol standard curve. Ammonia content in the excreta was quantified using the phenol-hypochlorite method. The excreta was homogenized in distilled water and centrifuged. The sample was mixed with phenol reagent, sodium nitroprusside, and sodium hydroxide, and the mixture was incubated. Absorbance was measured at 640 nm, and ammonia concentration was determined from the standard curve. For uric acid measurement, an enzymatic method using uricase was applied. The excreta was homogenized and centrifuged, and the sample was mixed with uricase enzyme, peroxidase, 4-aminoantipyrine, and phenol. After incubation, absorbance was measured at 520 nm. Uric acid concentration was determined by comparing the sample's absorbance to a standard curve. Urea content in the excreta was measured using a urease-based colorimetric method. Excreta was homogenized, and after centrifugation, the sample was mixed with urease enzyme. Following incubation, phenol reagent and sodium hypochlorite were added. Absorbance was measured at 520 nm, and urea concentration was determined by comparing the absorbance to the standard curve.

Results and Discussion

The study analysed the total protein content in the silk glands of four silkworm races—Pure Mysore, CSR2, FC1,

and FC2—over a seven-day period during the 5th instar stage. The findings indicate a progressive increase in protein levels across all races, with variations in accumulation rates. Among the tested races, CSR2 exhibited the highest protein content on each day, reaching 0.260 mg by the seventh day. This suggests a superior protein synthesis capacity, which is likely attributed to its genetic makeup and metabolic efficiency. Conversely, FC1 displayed the lowest protein content throughout the period, ending at 0.240 mg, implying relatively slower protein accumulation. The protein content in Pure Mysore and FC2 followed a similar trend, with values closely aligned. However, FC2 showed slightly higher levels than Pure Mysore on most days, indicating better silk gland productivity (Table 1). On the first day, protein concentrations were comparable across races, but from the third day onward, the variations became more pronounced. The sharpest increase was observed between the fourth and fifth days, suggesting a critical phase in silk protein synthesis.

The study examined the total protein content in the midgut of four silkworm races—Pure Mysore, CSR2, FC1, and FC2—over seven days during the 5th instar stage. The results indicate a consistent increase in protein levels across all races, suggesting active metabolic processes and protein assimilation during this period. Among the studied races, CSR2 exhibited the highest protein content, reaching 0.230 mg by the seventh day. This trend suggests that CSR2 has a more efficient digestion and absorption mechanism, leading to enhanced protein availability for silk production. Pure Mysore closely followed CSR2, with a final protein concentration of 0.220 mg, indicating a comparable but slightly lower efficiency in protein metabolism (Table 2). FC1 showed the lowest protein accumulation, ending at 0.210 mg, while FC2 exhibited intermediate levels, reaching 0.215 mg by the final day. The relatively lower protein content in FC1 may be due to genetic factors influencing nutrient absorption and metabolic activity in the midgut.

The variations in protein accumulation across silkworm races reflect genetic and physiological differences in silk gland metabolism. CSR2 exhibited the highest protein content, indicating its potential for superior silk yield and quality, making it a preferred choice in commercial sericulture. In contrast, FC1 showed lower protein levels, which may suggest slower growth or less efficient metabolism, potentially affecting silk quality. These results align with studies showing that hybrid and genetically improved races typically accumulate more protein due to enhanced metabolic efficiency. The peak in protein accumulation around days five and six highlights the importance of optimising nutritional and environmental conditions during this period to maximise silk production. Genetic factors, including specific quantitative trait loci (QTL) and genes involved in epigenetic modifications, influence silk gland metabolism and protein synthesis. The quality of mulberry leaves also plays a key role, as nutrient-rich hybrids have been shown to enhance protein synthesis. Environmental stressors, such as viral infections, can alter protein synthesis pathways, further influencing protein accumulation. Therefore, managing genetic, nutritional, and environmental factors, particularly during peak protein accumulation, can optimise silk yield and quality.

Table 1: Quantity of total proteins in 5th instar of silkworm tissue (Silk gland)

S. No	Silkworm Race	1 st Day	2 nd Day	3 rd Day	4 th Day	5 th Day	6 th Day	7 th Day
1.	Pure Mysore	0.110	0.130	0.160	0.180	0.220	0.230	0.250
2.	CSR2	0.120	0.140	0.170	0.190	0.230	0.240	0.260
3.	FC1	0.100	0.120	0.150	0.170	0.210	0.220	0.240
4.	FC2	0.115	0.135	0.165	0.185	0.225	0.235	0.255

Table 2: Quantity of total proteins in 5th instar of silkworm tissue (Midgut)

S. No	Silkworm Race	1 st Day	2 nd Day	3 rd Day	4 th Day	5 th Day	6 th Day	7 th Day
1.	Pure Mysore	0.12	0.135	0.15	0.165	0.19	0.205	0.22
2.	CSR2	0.125	0.14	0.16	0.175	0.2	0.215	0.23
3.	FC1	0.11	0.125	0.14	0.155	0.18	0.195	0.21
4.	FC2	0.115	0.13	0.145	0.16	0.185	0.2	0.215

The analysis of total amino acid content in the silk gland and midgut tissues of four silkworm races—Pure Mysore, CSR2, FC1, and FC2—revealed a consistent increase in amino acid levels over the seven-day period. CSR2 demonstrated the highest amino acid accumulation, reaching 0.300 mg by the seventh day, followed closely by Pure Mysore, which peaked at 0.290 mg. These two races showed similar trends, with a steady rise in amino acids throughout the 5th instar stage. The highest increase was observed between the fourth and fifth days, where a noticeable jump in amino acid levels occurred in all races. FC1 exhibited the lowest amino acid levels, with a final concentration of 0.280 mg on day seven, showing a more gradual increase compared to the other races. FC2 showed moderate amino acid accumulation, with a final value of 0.285 mg, slightly higher than FC1 but lower than CSR2 and Pure Mysore (Table 3). Across all silkworm races, amino acid levels showed a consistent increase from the first to the seventh day, reflecting the ongoing metabolic activity and protein synthesis during the 5th instar stage. This rise in amino acids is crucial for the growth and development of the silk gland and midgut, both essential for silk production. Proteomic analyses of the silk glands during this stage have shown dynamic changes in protein expression, with 60% of identified proteins varying across different days (Bovilla *et al.*, 2016) [1]. Proteins involved in metabolism and protein homeostasis were particularly prevalent, emphasising the active metabolic processes during this period. The increase in key amino acids, such as glycine, serine, and alanine, is vital for silk protein synthesis, as these amino acids are integral to fibroin, the primary component of silk (Bovilla *et al.*, 2016; Majima *et al.*, 1975) [1, 5]. The elevated tRNA acceptor activities for these amino acids during the 5th instar stage facilitate efficient protein synthesis in the silk glands (Majima *et al.*, 1975) [5]. Additionally, high rates of protein synthesis in the fat body tissue of larvae further highlight the continuous incorporation of amino acids into proteins. Amino acids also play a broader role in silkworm physiology, as their presence in the hemolymph contributes to the expression of vitellogenin, a key protein in reproductive processes (Liu *et al.*, 2019) [4]. This underscores the importance of amino acids not only in silk production but also in other metabolic functions crucial for silkworm development.

Table 3: Quantity of total amino acids in 5th instar of silkworm tissue (silk gland and midgut)

S. No	Silkworm Race	1 st Day	2 nd Day	3 rd Day	4 th Day	5 th Day	6 th Day	7 th Day
1.	Pure Mysore	0.22	0.23	0.24	0.25	0.27	0.28	0.29
2.	CSR2	0.21	0.22	0.24	0.26	0.27	0.29	0.3
3.	FC1	0.2	0.21	0.22	0.24	0.26	0.27	0.28
4.	FC2	0.215	0.225	0.235	0.245	0.265	0.275	0.285

The total cholesterol content in the haemolymph of four silkworm races—Pure Mysore, CSR2, FC1, and FC2—was monitored over a seven-day period during the 5th instar stage. The data revealed a consistent increase in cholesterol levels across all races, with the highest increase observed between the fourth and fifth days (Table 4). CSR2 exhibited the highest cholesterol content, reaching 55.2 mg by the seventh day. This indicates a faster accumulation of cholesterol compared to the other races, with a steady increase from the first to the seventh day. The Pure Mysore race had a final cholesterol concentration of 49.7 mg, which, while lower than CSR2, still showed a consistent rise over the seven days. FC1 and FC2 demonstrated similar trends, with FC1 ending at 52.8 mg and FC2 at 53.6 mg by the seventh day. Both races displayed a gradual increase in cholesterol content, with FC2 showing slightly higher levels than FC1. The difference between these two races was minor, indicating similar metabolic processes regarding cholesterol synthesis and accumulation.

Table 4: Quantity of total cholesterol in 5th instar of silkworm tissue (Haemolymph)

S. No	Silkworm Race	1 st Day	2 nd Day	3 rd Day	4 th Day	5 th Day	6 th Day	7 th Day
1.	Pure mysore	28.4	32.7	37.1	41.5	45.9	48.3	49.7
2.	CSR2	31.2	36.8	41.9	46.3	50.7	53.8	55.2
3.	FC1	29.8	34.6	39.4	44.1	48.5	51.4	52.8
4.	FC2	30.1	35.2	40.1	44.7	49.2	52.1	53.6

The study measured the total ammonia content in the tissue excreta of four silkworm races—Pure Mysore, CSR2, FC1, and FC2—over seven days during the 5th instar stage. The results show a progressive increase in ammonia excretion across all races, with significant increments observed as the silkworms continued their development (Table 5). Among the races, CSR2 exhibited the highest ammonia content, reaching 4.5 mg by the seventh day. This race showed a steady increase in ammonia excretion, with the most pronounced rise occurring between the fourth and fifth days. FC2 demonstrated the second highest ammonia levels, peaking at 4.6 mg on day seven. This race showed a similar pattern of increase, with ammonia levels rising progressively over the week. Pure Mysore showed a relatively slower increase in ammonia excretion, with a final concentration of 4.0 mg on day seven. This was the lowest among the tested races, suggesting slightly lower metabolic activity or nitrogen waste production during the developmental phase. FC1 followed closely behind, ending at 4.3 mg by day seven, demonstrating a similar trend to FC2 but with marginally lower ammonia content. The observed increase in cholesterol content from the fourth to the fifth day in silkworms indicates a significant shift in lipid metabolism, associated with developmental changes. This period marks an acceleration in lipid synthesis and

mobilization, essential for supporting growth and preparing the silkworm for the energy-intensive processes of silk production. The sustained rise in cholesterol levels across all races reflects continuous metabolic activity, vital for these processes. Lipid metabolism plays a crucial role in silkworm development, with dynamic changes in lipid profiles across different growth stages. Various lipid molecular species, such as triacylglycerol (TG), phosphoethanolamine (PE), and phosphocholine (PC), highlight the complexity of lipid metabolism in silkworms. The regulation of lipid homeostasis through processes like lipolysis and lipid synthesis is essential for silkworm growth and reproductive success. Diet significantly impacts lipid metabolism, with different feeding regimens influencing gene expression related to lipid synthesis and metabolism. For example, silkworms fed on artificial diets show alterations in lipid-related gene expression, potentially affecting their metabolic efficiency and growth (Wang *et al.*, 2023) ^[11]. The conversion of dietary α -linolenic acid to eicosapentaenoic acid (EPA) also suggests a sophisticated lipid biosynthetic pathway that supports neuronal functions, extending the role of lipid metabolism beyond energy storage. Furthermore, changes in antioxidant capacity and protein profiles during silkworm pupal development are linked to lipid metabolism. The increase in fatty acids and the regulation of proteins involved in mTOR signaling indicate a coordinated metabolic response to support the silkworm's growth and development (Liu, 2023) ^[13].

Table 5: Quantity of total Ammonia in 5th instar of silkworm tissue excreta

S. No	Silkworm Race	1 st Day	2 nd Day	3 rd Day	4 th Day	5 th Day	6 th Day	7 th Day
1.	Pure mysore	1.2	1.5	2.1	2.8	3.3	3.8	4.0
2.	CSR2	1.4	1.8	2.4	3.1	3.7	4.2	4.5
3.	FC1	1.3	1.7	2.3	3.0	3.5	4.0	4.3
4.	FC2	1.3	1.8	2.5	3.2	3.8	4.3	4.6

The total uric acid content in the tissue excreta of four silkworm races—Pure Mysore, CSR2, FC1, and FC2—was measured over seven days during the 5th instar stage. The results revealed a steady increase in uric acid excretion across all races, with the most substantial rise observed between the fourth and fifth days. CSR2 showed the highest uric acid content, reaching 6.6 mg by the seventh day. This race demonstrated a consistent increase in uric acid levels, with the sharpest rise occurring between the fifth and sixth days. FC2 followed closely, reaching 6.4 mg on day seven, showing a similar trend in uric acid excretion but slightly lower than CSR2. Pure Mysore exhibited the lowest uric acid levels, with a final concentration of 5.8 mg on the seventh day. However, the increase in uric acid content over the week was still considerable, indicating ongoing metabolic activity. FC1 had levels slightly higher than Pure Mysore, peaking at 6.1 mg by day seven, indicating a similar metabolic pattern to FC2 but with marginally lower excretion than CSR2. The observed increase in uric acid levels in silkworms during the 5th instar stage is closely associated with their developmental phase, especially as they prepare for pupation. This rise indicates heightened nitrogen metabolism, essential for processing waste products from protein and amino acid breakdown. The consistent increase in uric acid across all races reflects a typical physiological response during periods of intensive

growth, facilitating the excretion of nitrogenous waste and supporting the silkworm's survival and development. Uric acid, the final product of nitrogen metabolism in silkworms, accumulates in the epidermis, contributing to the characteristic white appearance of larvae. Key genes, such as Bmpali, Bmb1, and Bmcap, are involved in uric acid granule formation, and mutations in these genes can result in a transparent phenotype due to reduced uric acid production. The uric acid pathway also plays a significant role in silkworms' resistance to Bombyx mori nucleopolyhedrovirus (BmNPV). Supplementing silkworms with inosine, a precursor in the uric acid pathway, has been shown to enhance their viral resistance by modulating apoptosis and other metabolic pathways (Su *et al.*, 2023; Li *et al.*, 2023) ^[10, 2]. This increase in uric acid aligns with the peak of metabolic activity during the 5th instar, supporting silkworm growth and development. Genetic factors, such as mutations affecting uric acid metabolism, can influence silkworm susceptibility to environmental stressors and pathogens, as observed in certain mutant strains (Su *et al.*, 2023) ^[10]. Furthermore, research in Drosophila suggests that uric acid metabolism is influenced by environmental factors like diet and crowding, which may also apply to silkworms (Morimoto, 2022) ^[6]. The increase in uric acid levels during this critical developmental phase highlights the complex relationship between genetic factors and environmental conditions.

Table 6: Quantity of total Uric acids in 5th instar of silkworm tissue excreta

S. No	Silkworm Race	1 st Day	2 nd Day	3 rd Day	4 th Day	5 th Day	6 th Day	7 th Day
1.	Pure mysore	2.3	2.8	3.5	4.2	5.0	5.4	5.8
2.	CSR2	2.7	3.3	4.0	4.8	5.6	6.2	6.6
3.	FC1	2.5	3.0	3.7	4.5	5.3	5.7	6.1
4.	FC2	2.6	3.2	3.9	4.7	5.5	6.0	6.4

The total urea content in the tissue excreta of four silkworm races—Pure Mysore, CSR2, FC1, and FC2—was assessed over seven days during the 5th instar stage. The results indicate a steady increase in urea excretion across all races, with the most substantial rise occurring between the fourth and fifth days (Table 7). CSR2 exhibited the highest urea concentration, reaching 4.0 mg by the seventh day. This race showed a continuous increase in urea excretion, with notable growth observed between the fifth and sixth days, suggesting a peak in nitrogen metabolism during this phase. FC2 followed closely, with a final urea concentration of 4.1 mg, showing a similar trend but slightly higher levels than CSR2 by the end of the observation period. FC1 exhibited a lower urea content compared to CSR2 and FC2, reaching 3.9 mg on day seven. Despite this, FC1 demonstrated a steady increase in urea excretion, with the most significant rise occurring between days four and five. Pure Mysore had the lowest urea excretion, with a final concentration of 3.5 mg, indicating a slower but consistent accumulation over the study period. Overall, the most significant increase in urea excretion was observed between days four and five, consistent with the period of heightened metabolic activity associated with the 5th instar stage. The progressive accumulation of urea across all races suggests that the silkworms are effectively processing and excreting nitrogenous waste products, which are essential for their metabolic functions during development. The significant

increase in urea excretion between days four and five of the 5th instar stage in silkworms corresponds with heightened metabolic activity. This period is critical for silkworm development, as it involves intense protein metabolism and the processing of nitrogenous waste. The accumulation of urea across all races reflects efficient nitrogen metabolism, crucial for supporting both growth and silk production. This shift in nitrogen excretion demonstrates the silkworm's adaptation to the demands of its developmental stage, as supported by studies on other insect species. During the 5th instar, silkworms increase excretion of nitrogenous waste, including urea, which is indicative of active metabolic and protein synthesis (Shamitha & Rao, 2008) ^[9]. The decrease in uric acid levels during this stage suggests a metabolic shift towards silk synthesis, necessitating more efficient nitrogen processing (Shamitha & Rao, 2008) ^[9]. This change supports the silkworm's energy-intensive production of silk, underscoring the importance of nitrogen metabolism in developmental processes. Insects, including silkworms, typically convert ammonia, a toxic byproduct of protein catabolism, into less toxic compounds like uric acid or urea for excretion (Weihrauch & O'Donnell, 2021) ^[13]. This conversion process is supported by various transporters and enzymes that regulate nitrogen waste excretion, ensuring homeostasis and supporting growth (Weihrauch & O'Donnell, 2021) ^[13]. Developmentally, nitrogen excretion mechanisms evolve across different life stages. The ontogeny of nitrogen metabolism in silkworms is marked by peak metabolic activity during the 5th instar, requiring efficient nitrogen excretion to facilitate rapid growth and tissue development (Nagata & Yoshitake, 1989) ^[8]. Similar patterns are seen in other insect species, such as fish, where nitrogen excretion also adapts throughout ontogeny (Wright & Fyhn, 2001) ^[14]. While urea excretion plays a crucial role in the 5th instar stage, the roles of other nitrogenous waste products, such as uric acid and its metabolites, should not be overlooked. These compounds can function as nitrogen stores, pigments, or antioxidants, illustrating the complexity of nitrogen metabolism in insects (Mauchamp & Lafont, 1975; Weihrauch & O'Donnell, 2021) ^[6, 13].

Table 7: Quantity of total Urea in 5th instar of silkworm tissue excreta

S. No	Silkworm Race	1 st Day	2 nd Day	3 rd Day	4 th Day	5 th Day	6 th Day	7 th Day
1.	Pure mysore	1.1	1.5	2.0	2.5	3.0	3.3	3.5
2.	CSR2	1.3	1.8	2.3	2.8	3.4	3.8	4.0
3.	FC1	1.2	1.7	2.2	2.7	3.2	3.6	3.9
4.	FC2	1.3	1.8	2.3	2.9	3.5	3.9	4.1

Conclusion

The study reveals that metabolic processes, including protein synthesis, amino acid accumulation, and nitrogen metabolism, are pivotal during the 5th instar stage of silkworm development. CSR2 emerged as the most metabolically efficient race, with higher protein, amino acid, cholesterol, and nitrogenous waste levels, indicating superior growth and silk production potential. The sharp increase in nitrogenous waste excretion, particularly urea and uric acid, between days four and five underscores the critical metabolic shifts that occur in preparation for pupation. While FC1 showed relatively lower metabolic efficiency, the overall trend across all races highlights the importance of genetic and metabolic factors in determining

silkworm growth and productivity. These findings provide valuable insights into the metabolic mechanisms driving silkworm development and offer potential strategies for optimizing sericulture practices.

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