EFFECT OF PHOTOPERIODS ON ELECTOPHORETIC STUDIES ON *BOMBYX MORI* L.

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Abstract

Insects' heads play a vital role in receiving environmental stimuli and processing information in the brain and other nervous organs. In this study, we aimed to investigate the effect of photoperiods on the hemolymph and brain proteins of the silkworm, *Bombyx mori* L, using SDS-PAGE electrophoresis. We found that photoperiod influenced the number, mobility, and intensity of protein bands in the hemolymph of treated larval instars. Interestingly, the electrophoretogram of hemolymph and brain proteins showed that protein bands observed in larvae disappeared at the pupal stage. These findings suggest that key components of the silkworm's circadian system reside in the neurons of the larval head and that additional hierarchically arranged oscillators contribute to overt pace-making. The results of this study provide valuable insights into the role of photoperiod in regulating the silkworm's protein expression, which could contribute to the development of new strategies for improving silkworm rearing practices. This study's significance lies in its potential to shed light on the mechanisms underlying the circadian rhythms of silkworms and their response to environmental cues, which could have practical applications in sericulture.

Keywords: LxCSR2, Hemolymph**,** SDS-PAGE, environmental stimuli

INTRODUCTION

A variety of processes in living organisms are regulated by endogenous clocks. Circadian clocks serve as internal timing mechanisms that generate daily rhythms in a wide range of organisms, from cyanobacteria to humans. These circadian clocks synchronize with environmental time cues, such as light and temperature, for their entrainment (Rusak *et al*., 1993). Insects' heads play a pivotal role in receiving environmental stimuli and processing information in the brain and other nervous organs. Additionally, they comprise essential endocrine organs that are crucial for various physiological processes in insects, including growth, reproduction, diapause, and metamorphosis (Li *et al*., 2010). Moreover, environmental conditions, primarily photoperiod and temperature, profoundly impact the endocrine system (Itoh *et al*., 1995). Through gel electrophoresis and mass spectrometry, numerous proteins, including heat shock proteins, have been identified in the heads of *B. mori* (Li *et al*., 2009a, 2009b; Manjunatha *et al*., 2010).

It is a well-known fact that storage proteins belong to the most prominent family of insect hemolymph proteins (Slovak and Repker, 1993). Their native molecular weight is approximately 500,000 (Wyatt and Pan, 1978). The hemolymph of insect larvae contains several high molecular weight proteins, such as lipophorins (Shapiro *et al*., 1988) and arlyphorin (Telfer *et al*., 1983), in addition to the storage proteins. The hemolymph of *B. mori* comprises multiple proteins with the highest activities and has been found to contain the 30k proteins, which belong to a specific type of plasma protein called storage proteins (Kim *et al*., 2001). *B. mori* has the largest number of these proteins in comparison to other insects. Insects possess small heat-shock protein genes (Li *et al*., 2009b). A tissue-specific protein, lysozyme, has been demonstrated to be released into the hemolymph but remains undetectable in other tissues (Tan *et al*., 2003). Ultrasound has been employed as an exogenous modulator for manipulating protein metabolism, specifically targeting the content of structural proteins within specific tissues (Jyothi *et al*., 2010).

MATERIALS AND METHODS

Poly acrylamide gel electrophoresis (PAGE)

The hemolymph and brain proteins of *B. mori*, exposed to varying photoperiods, were analyzed utilizing SDS-PAGE electrophoresis.

Sample preparation

Haemolymph was collected from both the treated and control caterpillars (LXCSR2). Brain samples from treated and control pupae were then washed with double-distilled water and homogenized using insect Ringer solution. The tissues were homogenized in a glass homogenizer with a Teflon pestle in 150 µl of homogenizing buffer (Tris-EDTA, pH 6.8). The homogenization process was conducted in an ice bath under freezing conditions. Each sample was homogenized separately. After homogenization, the samples were centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatants were collected and further centrifuged at 5000 rpm for 20 minutes in a cooling centrifuge. Ten µl of the resulting supernatant were diluted with 90 µl of ion-free double-distilled water. From this, 20 µl of the sample was mixed with 20 µl of sample buffer and loaded into each well. The remaining supernatant was mixed with an equal volume of sample buffer and stored at -4°C.

Final protein sample

The supernatant samples were mixed with equal volumes of sample buffer, which contained 0.15 M Tris HCl (pH 6.8), 10% SDS, glycerol, β-mercaptoethanol, and traces of bromophenol blue. The mixture was then boiled for 2 minutes and immediately placed on ice to prevent denaturation from overheating. Subsequently, the samples were centrifuged again at 8000 rpm for 5 minutes at 4°C prior to loading into the precasted gel.

SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis) was conducted following the method established by Laemmli (1970), utilizing 10.5% separating gels and 6.5% stacking gels. Each well was loaded with 50 µl of the sample. A constant current of 60 volts was applied for stacking, while 120 volts were used for the running gel, both maintained for a duration of 3 hours. Following the electrophoresis, the gels were stained overnight with

Coomassie Brilliant Blue R-250. After the staining process, the gels were destained, stored in 7% acetic acid, photographed, and subsequently documented using a computerized gel documentation unit to identify and locate the different densitometric peaks.

RESULTS

SDS-PAGE was conducted on the haemolymph of V instar larvae and the brain of pharate *B. mori* in order to compare their protein patterns. The protein pattern of the haemolymph from V instar larvae treated with different photoperiods is depicted in Plate 11. The protein extracted from the haemolymph exhibited multiple bands with molecular weights ranging from 201 to 3 kDa in SDS-PAGE. The staining intensities were notably high in the range of 201-128 kDa, followed by 14-10 kDa.

The PAGE analysis of haemolymph proteins from the treated larval instars demonstrated that photoperiod had an impact on the number of bands, band mobility, and band intensity. During normal development, the total number of bands ranged from 3 to 4. However, after treatment, the number of bands varied from 5 to 7, with the highest number observed at LD 8:16 h (seven bands) of photoperiod.

In the control electropherogram of haemolymph from V instar larvae, four bands of varying intensities were observed. Among these bands, two (with Rf values of 0.098 and 0.178) appeared to be the major haemolymph proteins, characterized by their thickness. The remaining protein bands were minor bands with varying intensities (Plate 11 and Lane-2). Under LD 24:0h. photoperiod conditions, the haemolymph exhibited five distinct bands with varying intensities (Plate 11 and Lane-3). Among these bands, one band with a high molecular weight of 12 kDa (Rf value 0.663) stood out with higher intensity compared to the other bands. *B. mori* larvae exposed to LD 16:8h. photoperiod conditions displayed a total of six bands, showing some differences compared to the other haemolymph patterns (Plate 11 and Lane-4). The first band, prominent in this pattern, had a molecular weight of 13 kDa and an Rf value of 0.644. Additionally, new bands emerged. Under LD 12:12h. photoperiod conditions, larval haemolymph exhibited a distinct protein pattern, although further details were not provided. Under LD 8:16h. photoperiod conditions, the larval haemolymph displayed the maximum number of bands, reaching a total of seven bands, each with varying intensities (Plate 11 and Lane-6). Among these bands, band 1 and band 2 exhibited the highest intensities, with molecular weights of 14 kDa and 10 kDa, respectively. The Rf values ranged from 0.627 to 0.873. On the other hand, when exposed to LD 0:24h. photoperiod conditions, the haemolymph protein profile of *B. mori* revealed that two bands showcased maximum molecular weights of 148 kDa and 128 kDa, displaying better band width intensity compared to the other four bands (Plate 11 and Lane-7).

The protein profile of the brain in both the control and photoperiod-treated larvae is presented in Plate 12. The brain haemolymph exhibited a few bands, which were notably weaker compared to the haemolymph proteins and did not show significant variation among themselves. However, all these brain proteins appeared as thin bands. The brain of the pharate larvae (Plate 12 and Lane-2 - control) displayed a total of 5 bands, with molecular weights ranging from 89 to 4 kDa (with respective Rf values of 0.156, 0.311, 0.458, 0.816, and 0.868). Larvae exposed to different photoperiods showed a decrease in the number of bands, resulting in only 3 bands being observed. These bands were prominent and exhibited nearly equal staining intensity. Specifically, larvae exposed to LD 12:12h. photoperiod conditions showed only 2 bands, with molecular weights of 81 kDa and 8 kDa, respectively. The

electropherogram of the haemolymph and brain revealed that the protein bands observed in the larvae started to disappear at the pupal stage. From the 5th instar to the pharate adult stage, there was a marked reduction in the number of proteins.

Plate - 11. Effect of different photoperiods on protein profile of haemolymph in *B.mori.* SDS-PAGE

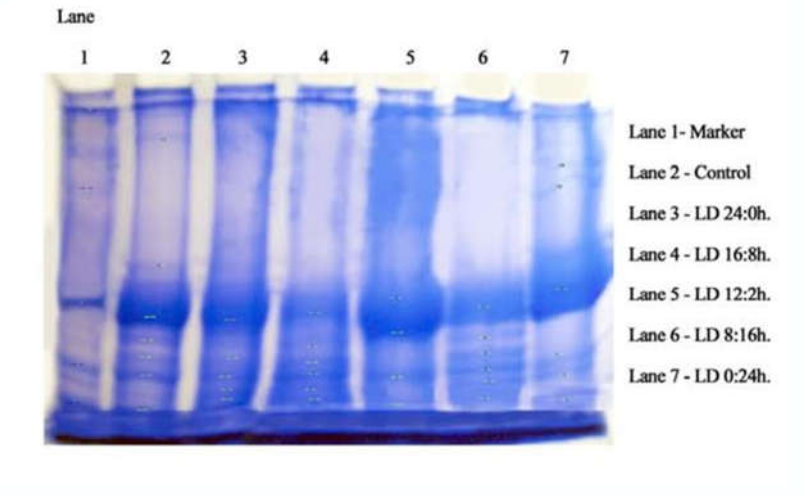

Plate - 12. Effect of different photoperiods on protein profile of brain in *B.mori.* SDS-PAGE

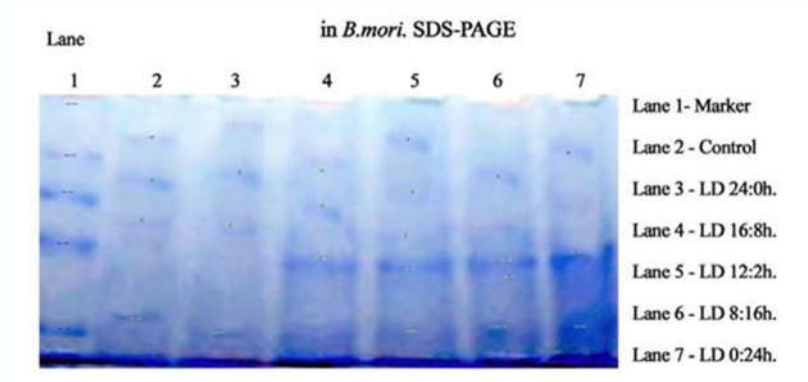

Table 2.10

Effect of photoperiods on protein profile of haemolymph in *B. mori* (SDS-PAGE)

Lane 1 (Marker)

Band	Position	Mol.wt. kDa	Rf
1	112	201	0.098
2	349	29	0.307
3	704	14	0.619
4	890	6	0.782
5	1008	3	0.886

Lane 2 (Control)

Band	Position	Mol.wt. kDa	Rf
1	202	173	0.178
2	586	51	0.515
3	746	12	0.656
4	816	9	0.717
5	869	7	0.764
6	925	5	0.813

Lane 3 (LD 24:0h)

Band	Position	Mol.wt. kDa	Rf
1	754	12	0.663
2	874	7	0.768
3	928	5	0.815
4	968	4	0.851
5	997	3	0.876

Lane 4 (LD 16:8h)

Band	Position	Mol.wt. kDa	Rf
1	733	13	0.644
2	837	9	0.736
3	885	7	0.778
4	920	6	0.808
5	968	4	0.851
6	977	3	0.876

Lane 5 (LD 12:12)

Band	Position	Mol.wt. kDa	Rf
1	688	19	0.605
2	794	10	0.698
3	930	5	0.817
4	1013	3	0.890

Lane 6 (LD 8:16)

Band	Position	Mol.wt. kDa	Rf
1	714	14	0.627
2	810	10	0.712
3	810	10	0.712
4	856	8	0.752
5	906	6	0.796
6	941	5	0.827
7	994	3	0.873

Lane 7 (LD 0:24h.)

Band	Position	Mol.wt. kDa	Rf
1	280	148	0.246
2	344	128	0.302
3	661	28	0.581
4	861	8	0.757
5	925	5	0.813
6	1000	3	0.879

Effect of photoperiods on protein profile of brain in *B. mori* (SDS-PAGE)

Lane 1 (Marker)

Band	Position	Mol.wt. kDa	Rf
1	28	201	0.033
2	188	29	0.222
3	300	14	0.354
4	460	6	0.542
5	724	3	0.854

Lane 2 (control)

Band	Position	Mol.wt. kDa	Rf
1	132	89	0.156
2	264	19	0.311
3	388	10	0.458
4	692	8	0.816
5	736	4	0.868

Lane 3 (LD 24:0)

Band	Position	Mol.wt. kDa	Rf
1	236	23	0.278
2	344	12	0.406
3	508	6	0.599

Lane 4 (LD 16:8)

Band	Position	Mol.wt. kDa	Rf
1	140	81	0.165
2	436	8	0.514

Lane 5 (LD 12 : 12h.)

Band	Position	Mol.wt. kDa	Rf
1	300	14	0.354
2	540	5	0.637
3	732	4	0.863

Lane 6 (LD 8:16)

Band	Position	Mol.wt. kDa	Rf
1	252	20	0.297
2	512	6	0.604
3	716	3	0.844

DISCUSSION

The haemolymph protein pattern exhibited multiple protein bands when larvae were exposed to different photoperiods, indicating qualitative and quantitative changes in protein profiles. In the control group, four bands were observed, while the treatment groups revealed the presence of a new band. LD 8:16h. of photoperiod exhibited seven bands, whereas LD 12:12h. showed only four bands. The electropherogram clearly indicates that exposure to different photoperiods leads to the disappearance of certain proteins and the emergence of new ones. Mohan and Muraleedharan (2001) conducted a comparative analysis of the haemolymph electropherograms of *D. cingulatus*, revealing an increase in the number of protein bands in the haemolymph, fat body, and ovary from the 0th day of the 5th instar to the 6th day, followed by a subsequent decrease. Sohal and Rup (1997) reported similar findings in *Lipaphis erysimi* (Kalt), where an increase in the number of protein bands and changes in their mobility and intensity were observed following methoprene treatment, indicating both protein synthesis stimulation and the initiation of new protein synthesis. In the current study, changes in the number of protein bands were also observed, with a subsequent decrease until the final day. After eclosion, the number of protein bands in the haemolymph decreased, suggesting their involvement in cuticle formation. Previous studies have identified various molecular forms of juvenile hormone binding proteins in *B. mori* , including 33 kDa and 35 kDa forms, as well as a homogenous haemolymph protein with a molecular weight of 19 kDa in adult *B. mori* . In the present study, a 19 kDa band was identified in *B. mori* larvae exposed to a 12:12h photoperiod, with an Rf value of 0.605.

Sohal and Rup (1997) reported that in *Lipaphis erysimi* (Kalt), the appearance of an increased number of protein bands and changes in their mobility, intensity, and size after methoprene treatment indicated not only the stimulation of protein synthesis but also the initiation of synthesis for new proteins. Similarly, in the present study, notable changes were observed in the protein count throughout the days, with a gradual decrease in the number of protein bands observed until the final day. Furthermore, immediately after eclosion, a decline in the number of protein bands in the haemolymph indicated their involvement in cuticle formation. Chulho *et al*. (1996) identified two molecular forms of juvenile hormone binding proteins in the haemolymph of *B. mori ,* with molecular weights of 33 and 35 kDa. Yoshida *et al*. (1996) also obtained a homogeneous haemolymph protein of 19 kDa from adult *B. mori*. In the present study, a band of 19 kDa was identified in *B. mori* larvae exposed to a 12:12h photoperiod, with an Rf value of 0.605.

When *B. mori* larvae were exposed to different photoperiods, the haemolymph exhibited distinct protein bands with varying molecular weights and Rf values. The highest molecular weight protein band (173 kDa with an Rf value of 0.178) was obtained under LD 24:0 (continuous light) photoperiod, while the lowest molecular weight protein band (3 kDa) appeared at five positions with an Rf value of approximately 0.876. A total of forty proteins displaying seventeen banding patterns were identified. In a separate study on *B. mori* haemolymph proteins, Kawaguchi *et al*. (1993) also isolated three types of protein bands. Rajathi *et al*. (2010) investigated the impact of RH. 2485 on the protein profile of haemolymph, noting significant changes in storage proteins (80 kDa) and 30 kDa proteins in the haemolymph. The concentration of storage proteins increased during the active feeding period, reaching a peak on day 0 of spinning, followed by a sharp decline during the larval-pupal transition. Furthermore, Rhee *et al*. (2009) discovered that the silkworm haemolymph protein with the highest apoptotic inhibiting activity was a 3 kDa protein, which was one of the 30 kDa proteins.

Heat shock proteins are produced as a result of the heat shock response and other stress-related mechanisms. Their primary role is to act as molecular chaperones, safeguarding proteins from denaturation under extreme conditions. The heat shock response exhibits considerable variation among different silkworm races, as documented by Manjunatha *et al*. (2010). Additionally, in many species, specific heat shock proteins have evolved distinct functions adapted to various environmental conditions (Li *et al*., 2009b).

The PERIOD proteins in *B. mori* were analyzed by conducting a comprehensive comparison of their entire structures and aligning the sequences with other previously reported PERIOD proteins. When examining the full length sequences, it was observed that *B. mori* PERIOD exhibited significant similarities with other insect PERIOD proteins, as indicated by Levine *et al*. (1995) and Takeda *et al*. (2004).

The insect head comprises vital sensory systems that facilitate communication with both the internal and external environment, as well as endocrine organs like the brain and corpus allatum, which play a crucial role in regulating insect growth and development. To gain a comprehensive understanding of how these components function and interact within the head, it is essential to investigate their molecular basis at the protein level. In the present study, proteins extracted from silkworm larval heads were analyzed using SDS-PAGE. A total of 22 proteins, ranging in molecular weight from 201 kDa to 3 kDa, were identified. These findings align with the research conducted by Li *et al*. (2010), who employed SEQUEST and X Tandem algorithms, along with Tran's proteomic pipeline validation, to identify a total of 539 proteins with a low false discovery rate from an in-house database. Moreover, 43 proteins were found to have a theoretical isoelectric point greater than 10, making their separation challenging through two-dimensional gel electrophoresis. Among the identified proteins were four chemosensory proteins, one odorant binding protein, two diapause-related proteins, and a substantial number of cuticle proteins. These proteins are involved in various processes, including nervous system development, stress response, apoptosis, and more, and are closely linked to the physiological status of the head.

In the current investigation, the protein profile of the brain was examined in *B. mori* larvae exposed to different photoperiods. In the control group, five bands were observed, with the first band having a molecular weight of 89 kDa and an Rf value of 0.156. In the treated groups, the protein bands exhibited lower molecular weights ranging from 81 kDa to 3 kDa. Tan *et al*. (2003) identified a tissue-specific protein, a lysozyme, with a molecular weight of 27K in the suboesophageal body (SB) of the silkworm *B. mori*. This P27K protein shares 56.5% identity with a hemolymph glycoprotein of *M. sexta*.

Furthermore, Li *et al*. (2009b) investigated three organs of the silkworm larval endocrine system, including the brain, salivary glands (SG), and prothoracic gland (PG). A total of 3430, 2683, and 3395 proteins were identified in these organs, respectively. These findings reveal a collaborative mechanism among the three endocrine organs in regulating various physiological and developmental processes. Additionally, the study suggests that organ-specific proteins may play a fundamental role in the functional differentiation of these organs.

In another study by Schadova *et al*. (2004), the neuronal architecture of the circadian system in the cephalic ganglia of adult silkworms was examined. The results indicate that essential components of the silkworm circadian system are located in the Ia neurons, and additional hierarchically arranged oscillators contribute to the overall pace-making process.

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