Advances in Bioresearch
Adv. Biores., Vol 15 (1) January 2024: 133-140

Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.15.1.133140

Advances in Bioresearch

ORIGINAL ARTICLE

Estimation of Haemolymph Protein Concentration of Different Larval Stages of Eri Silkworm, *Samia Ricini* (Donovan)

Anjela Ahmed*, Brishnita Borah, Shatabdi Biswas, Abhijit Chandra Roy, Bhuban Chandra Chutia
[1]Department of Zoology, Nowgong College (Autonomous), Nagaon- 782001, Assam, India
***Corresponding author**: Anjela Ahmed
Email address- aanjela747@gmail.com

ABSTRACT

The present investigation was conducted to study the concentration of haemolymph protein in different developmental stages of the Eri silkworm, Samia ricini Donovan. It deals with the study of the concentration of haemolymph protein in different developmental stages during moulting stage until the exit of the moult-out stage. Study reveals that protein concentrations varied significantly in the developmental stages. The lowest protein concentration was found to be in the initial stage of 3rd instar recorded as 0.2 mg/ml. The highest protein concentration was found in the pre-pupal stage recorded as 6.4 mg/ml. The present study also reveals that there was a decrease in protein concentration from the pre-pupal to the pupal stage.

Keywords*: Feeding, sericogenous, morphology, hemocyanin, silkworm rearing.*

Received 24.09.2023 Revised 01.10.2023 Accepted 11.12.2023

How to cite this article:
Anjela Ahmed, Brishnita Borah, Shatabdi Biswas, Abhijit Chandra Roy, Bhuban Chandra Chutia. Estimation of Haemolymph Protein Concentration of Different Larval Stages of Eri Silkworm, *Samia Ricini* (Donovan). Adv. Biores., Vol 15 (1) January 2024: 133-140

INTRODUCTION

The northeastern region has several sericogenous insects, of which a total of four different silk varieties are in focus for production. The state of Assam traditionally produces Muga, Eri, and Mulberry silks. Out of all the different varieties major emphasis is given to the production of Muga and Eri Silk which are popularly known as Vanya silk. Eri silk is a product of *Samia ricini,* which is domesticated and found majorly in North East India and different parts of China and Japan. It mainly feeds on castor leaves. Two varieties of castor plants red and green are found abundantly in the hills and plains of Assam including the other northeastern states of the country. The concentration of protein in the haemolymph of larvae being fed green castor leaves was found to be significantly higher than that of red –leaf fed larvae [19] Other host plants include Kesseru (*Heteropanas fragrans* Roxb), Ailanthus, Ligustram, Prunus, etc. Castor and Kesseru are used extensively for feeding during the rearing of these insects. It is a known fact that nearly 70% of proteins are directly derived from the mulberry leaf which is the source of silk protein production by silkworms [1-4]. It is observed that the supplementation of proteins through mulberry leaves improves the cocoon quality and reduces larval mortality [5]. The Eri silkworm *Samia ricini* is both a sericogenous insect as well as one of the economic insects like *Bombyx mori* and *Antheria assama* and it belongs to the family Saturniidae and order Lepidoptera. The domesticated Eri is very different from its wild type and consists of 6 different strains. They are- Greenish blue plain (GBP), Greenish blue-spotted (GBS), Greenish blue zebra (GBZ), Yellow plain (YP), Yellow-spotted (YS), and Yellow zebra (YZ). The development of holometabolous insects, including the silkworm, is under hormonal control and includes major morphological changes in the conversion of larvae to adults. The process involves the degradation of most larval tissues and organs and the formation of adult tissues and organs. The hemolymph serves as the basic environment of cells, tissues, and organs [38, 39]. Hemolymph is a circulating fluid that functions as blood in various arthropods. It circulates freely around the different cells of haemocoel and is

considered a dynamic tissue that is never static [6]. This is an indication of its usefulness as a barometer in determining the biological status of a developing insect. Hemolymph consists of a high concentration of free amino acids and water. It consists of haemocyanin that turns blue when oxygenated instead of the iron-based haemoglobin in red blood cells found in vertebrate, when not oxygenated haemolymph quickly loses its colour and appear grey. The haemolymph of lower invertebrates including most insects and arthropods is not used for oxygen transport because these animals respirate directly from their body surface to the air. Haemolymph proteins have always been an interesting tool for insect biochemists because of their pertinent role in development, morphogenesis, and almost all intermediary metabolic pathways in insects [7-12]. Proteins in insects especially the ones circulating in the blood are seen to be the major contributors to the insects' successful survival. Consequently, measuring the protein concentration of a crustacean's blood can provide valuable information to identify its condition [34]. Hemolymph is a critical site in the host immune response and is considered "sterile" without proliferating microorganisms in healthy animals [37]. Owing to their open-type circulatory system the haemolymph in insects soaks all the major organ systems in the body and thus making it an important body fluid for the regulation of homeostasis [18]. Haemolymph is composed of water, inorganic salts (Mostly Na+, Cl-, K+, and Ca2+), and organic compounds (Mostly carbohydrates, proteins, and lipids). The primary oxygen transportation molecule is hemocyanin, and one of the important parts of hemolymph is the haemolymph protein. Dynamic metabonomic changes in silkworm hemolymph were closely associated with the silkworm larva development involving multiple metabolic pathways [49]. Such changes are showcased by the degradation of the old skins and the formation of new skins during moulting processes and metamorphosis. The mean pH of larval hemolymph after the collection was found to be 6.45, that of pupal hemolymph, 6.57; *in vivo* values may be slightly lower in the silkworm, *Bombyx mori* [44]. Haemolymph can contain nucleating agents that confer extracellular freezing protection, such nucleating agents have been found in the haemolymph of insects of several orders like Coleoptera (Beetle), Diptera (Files), and Hymenoptera. There is a fairly good number of records regarding haemolymph , but only a few definitive studies have been conducted on haemolymph protein concentration in silkworms, hence the current systemic study has been carried out for proper investigation.

MATERIAL AND METHODS

The experiment was carried out in the Department of Zoology, Nowgong College (Autonomous). Disease-free eggs of *Samia ricini* were collected from Sericulture Farm Senchowa, Nagaon, Assam on 24th March 2022. These eggs hatched after 3 days in the rearing room and are fed on a diet of castor leaves during all the developmental stages including the 3rd,4th, and 5th instar larvae of the insect. Every possible condition was taken care of and kept under strict hygienic conditions and made sure none of the hatched larvae were sick and showing signs of lethargy. Castor leaves collected were from the Nowgong college campus itself and the nearby area. The larva up to the 3rd instar were fed tender leaves, and aged leaves were fed from the 4th instar.

The following materials are required for hemolymph protein estimation-

1. Bradford reagent.
2. 10% TCA.
3. Distilled w.ater
4. Ethanol.
5. Ether.
6. 0.1N NAOH.
7. Standard BSA 0.1%.
8. Equipment- test tube, beaker, micropipette, hot water bath, Eppendorf tube, measuring cylinder, vernier caliper, etc.

Fig 1: Pictures of developmental stages of Eri silkworm *Samia ricini* Donovan during rearing.

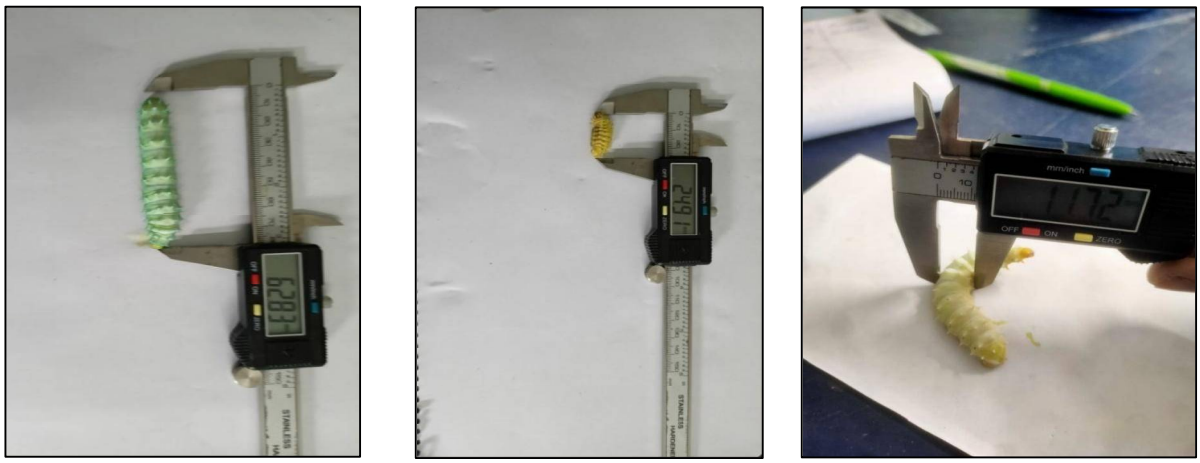

Fig 2: Pictures of measuring Eri silkworm larvae and pre-pupa using a vernier caliper.

ESTIMATION OF HAEMOLYMPH PROTEIN

The protein concentration of early larval stages (I and II) is not determined because of the extremely inadequate volume of hemolymph protein present during these periods. Haemolymph protein was obtained from 3rd,4th, and 5th instar larvae including pre-pupae and pupal stages. Hemolymph was extracted from all the above stages by piercing one of the prolegs of larvae from each developmental stage with a pin and gently squeezing the body. 50 micro litre hemolymph is collected in chilled Eppendorf tubes. The tubes contain 1 ml of 10% trichloroacetic acid and 0.95 ml of distilled water to make a volume of 2 ml. The solution is then centrifuged for 10 minutes at 4000-5000 rpm. The precipitate was then treated with ethanol: ether at a 3:1 ratio. The precipitate formed is dissolved with 0.1N NaOH

which is then kept in a hot water bath overnight at 45 ºC. The protein estimation is then carried out using Bradford Method and crystalline Bovine Serum Albumin (BSA) as standard.

RESULTS

Table 1: Haemolymph protein concentration in Eri silkworm entire body from 3rd to 5th instar larva.

Sl no	Developmental stage	Heamolymph protein concentration (mg/ml)	
		Moult in	Moult out
1	3rd instar	0.2	0.2
2	4th instar	1.0	1.6
3	5th instar	3.0	4.6

Table 2: Haemolymph protein concentration in Eri silkworm entire body from pre-pupal stage to pupa

Sl no	Developmental stage	Initial stage protein concentration (mg/ml)	Final stage protein concentration (mg/ml)
1	Pre pupa	6.4	5.9
2	Pupa	5.4	5.2

Length, breadth, and weight of the silkworms in the different developmental stage was also taken using a vernier caliper and readings were observed as follows-

Table 3: Morphometric characteristics of Eri silkworm *Samia ricini* Donovan from 3rd instar to 5th instar when they enter the moulting phase.

Sl no	Developmental stage	Moult in		
		Length	Breadth	Weight
1	3rd instar	1.29cm	3.5mm	0.21gm
2	4th instar	3.52cm	6.8mm	1.27gm
3	5th instar	5.89cm	1.02cm	3.70gm

Table 4: Morphometric characteristics of Eri silkworm *Samia ricini* Donovan from 3rd instar to 5th instar when they exit the moulting phase.

Sl no	Developmental stage	Moult out		
		Length	Breadth	Weight
1	3rd instar	2.77cm	5.1mm	0.56gm
2	4thinstar	4.42cm	9.35mm	2.42gm
3	5th instar	6.02cm	1.01cm	4.52gm

Table 5: Morphometric characteristics of Eri silkworm *Samia ricini* Donovan in pre-pupa and pupa stages.

Sl no	Developmental stage	Length	Breadth	Weight
1	Pre pupa	2.49cm	1.05cm	4.89gm
2	Pupa	2cm	1.02cm	3.38gm

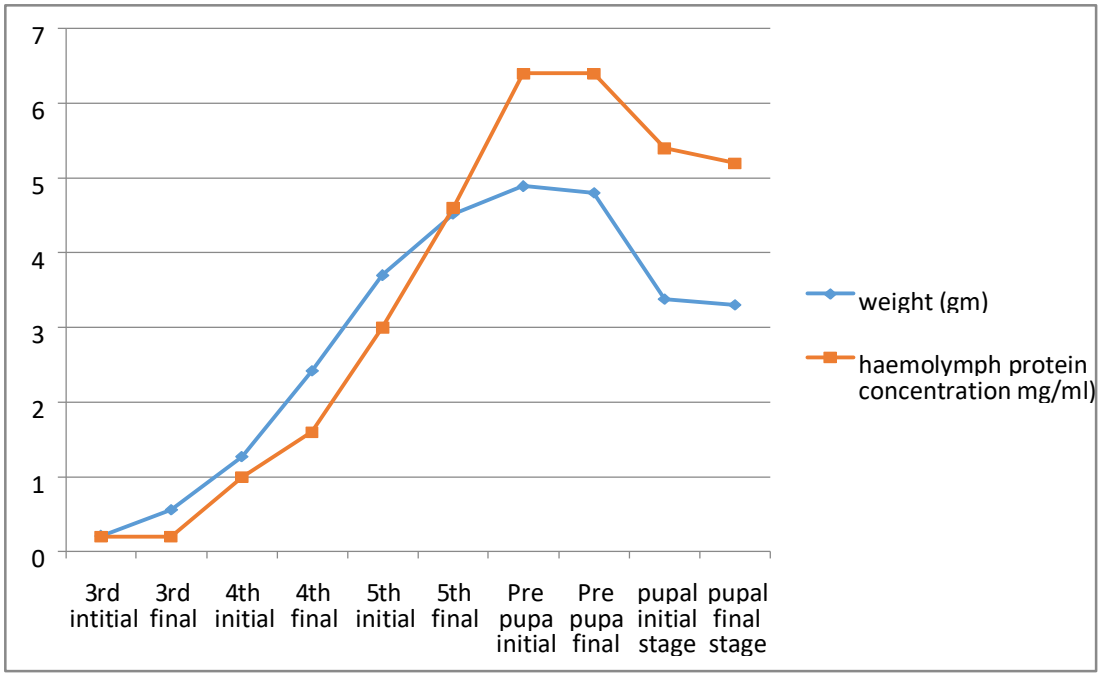

Fig 3: Line graph showing the relationship between body weight and haemolymph protein concentration in the different developmental stages of Eri silkworm.

DISCUSSION

In the present study, the results of haemolymph protein concentration in the Eri silkworm indicate that the protein concentration in the Eri silkworm increases gradually and reaches the maximum level at the ripening period during which the larva prepares itself for spinning. The increase in the concentration of protein is considered to be related to the growth of larvae because during growth and particularly at metamorphosis and extensive synthesis of protein is known to take place. Higher body protein concentration in Eri silkworm larvae is perhaps due to the increased consumption of castor leaves. It results in a high rate of conversion and accumulation of proteins in the silkworm larval body. The works of *B.mori* show that the concentration of blood protein rises gradually from 1.2% in the early 3rd instar to 5.3% [22-33]. Protein concentration increases rapidly from the 1st instar and reaches the maximum level at the end of the 4th instar. Similar findings were also observed by [4]. This was also seen by [34] that in all the strains the concentration of protein was found highest at ripening when the worm is fully matured, which is the 5th instar. In the present study it was observed that although the hemolymph protein concentration increased linearly from the 3rd instar up to the 5th instar, the was a drop in the hemolymph protein concentration after the end of larval stages. Similar results were observed by [5] in which it was also found that during the development of the 5th instar larva of *Samia ricini*, there is a rapid increase in the hemolymph protein concentration which attained its peak at the end of larval life irrespective of the season. This may be due to reduced feeding by infected larva as well as due to the metabolism of protein and amino acid of hemolymph by developing pathogen. However, hemolymph carbohydrates decreased significantly at the end of larval life compared to the control. Nutrition plays an important role in the development and metamorphosis, particularly in lepidopteran insects where the adult is in a nonfeeding stage [35]. High concentrations of hemolymph proteins have been correlated with high consumption of mulberry leaves and subsequently the high rate of conversion and their accumulation in the hemolymph of Bombyx mori [4, 5, 1]. The importance of haemolymph protein as a reserve is also illustrated by changes during starvation. Thus when *Celerio euphorbiae*, *Bombyx mori*or, *Simex lutaria* are subjected to enforced starvation hemolymph protein falls markedly while non-proteins remain unchanged. This presumably reflects the hydrolysis of protein to maintain amino acids and thus osmotic pressure [36, 37]. Larval growth is largely dependent on dietary proteins. When the larvae were reared on a diet containing weakly nutritive proteins such as gluten and zein, haemolymph protein was decreased and uric acid excretion was markedly accelerated. The free amino acid composition of the haemolymph manifested characteristic patterns according to the kinds of dietary protein. The supplementation of gluten and zein with their limiting amino acids resulted in a rise in haemolymph protein and a drop in uric acid excretion. [21]. A high dose of vitamin B_3 in the silkworm diet interrupts larval feeding and normal growth. High

mortality of larvae occurs during molting and they cannot complete this process normally. Also, the larvae exhibit nicotinamide hypervitaminosis symptoms such as immobility, dyspepsia, darkening of the skin, inability to excrete normally, exerting brownish fluid from the anus, and swelling of rectal muscles. [12]. Changes in the composition of haemolymph reflect the physiological and biochemical transformations taking place in the insect tissues [38]. Moulting, reproduction, nutritional state, infection, stress response, hypoxia, and salinity variations are some of the factors affecting the relative proportions and total quantities of the hemolymph proteins [19-25]. When the larvae and pupae were exposed to selected higher temperatures, a significant decrease in the protein levels of haemolymph was noticed and the order of decrease was found to be more at 36°C than at 31°C Relatively higher increase in the free amino acid levels in the haemolymph presumably provides protective cover to tissues against high temperature by an increase in osmolarity and reduction in evaporative water loss [31]. The exoskeleton of crustaceans and insects is formed by cells of the hypodermis, but several hemolymph proteins contribute to the synthesis of the new exoskeleton. These hemolymph proteins share a surprising degree of sequence similarity and are members of the hemocyanin gene family. The members of the hemocyanin gene family play vital roles during molting, including the transport of oxygen to the tissues to support enhanced protein synthesis, transport of proteins for inclusion in the new exoskeleton, and incorporation of oxygen into substrates for hardening of the new covering [44]. When hemolymph, HemaP levels exceed a yet undefined threshold level, *B. mori* larvae initiate locomotor activation and exhibit the characteristic behaviors of motivated feeding. The idea of a threshold HemaP level for triggering locomotor activation corresponds well with the simulation model in locust [35, 40, 45]. In the silkworm, *Bombyx mori*, two storage proteins named SP-1 and SP-2 were shown to decline in concentration in the haemolymph and increase in the fat body during the larval-pupal transformation, when protein granules are formed in the fat body at the same time as the degeneration of mitochondria and endoplasmic reticulum. The concentration of protein granules in the fat body cytoplasm is much greater in females than in males, and the granules in females have partially crystalline inner zones. This is different from males where granules with the non-crystalline structure are most numerous [12, 13, 45-49].

CONCLUSION

The present study revealed the variation in haemolymph protein concentration in different larval stages of Eri silkworm (*Samia ricini*). Besides these, the morphometric variation in all the developmental stages was revealed. The study also revealed that the haemolymph protein concentration reached its peak in the 5th instar, pre-pupal and pupal stages, and then in the later stage of pre-pupa and pupa it again decreases. All these haemolymph protein concentrations in the developmental stages reflect the biochemical and physiological transformations taking place in the tissues of the silkworm.

COMPETING INTERESTS

All the authors agree to the publication of this paper and they don't have any conflict of interest with any party. They have no involvement that might raise questions of bias in this reported work or its conclusions.

ACKNOWLEDGEMENT

The authors are grateful to the Head of the Department of Zoology, Nowgong College (Autonomous), for providing laboratory facilities, support, and ambiance to work on this paper. We are also thankful to Dr. Sarat Borkataki, Principal of Nowgong College (Autonomous), Nagaon, Assam, India for his constant encouragement.

AUTHOR'S CONTRIBUTION

This work was carried out in collaboration among all the authors. All the authors contributed to the design of the study. Authors Anjela Ahmed and Brishnita Borah have done the laboratory work. Author Anjela Ahmed wrote the first draft of the manuscript. Author Shatabdi Biswas helped in the Methodology part of the study. Author Bhuban Chandra Chutia contributed to the management and execution of the study and supervised the whole work. Author Abhijit Chandra Roy analyzed the data and revised the whole manuscript. All the authors read and approved the final manuscript.

REFERENCES:

1. Amin, A. R., Haque, A., Rahman, M. A., Chen, Z. G., Khuri, F. R., & Shin, D. M. (2015). Curcumin induces apoptosis of upper aerodigestive tract cancer cells by targeting multiple pathways. *PloS one*, *10*(4), e0124218.

2. Bevivino, G., Arcà, B., & Lombardo, F. (2021). Effects of local and systemic immune challenges on the expression of selected salivary genes in the malaria mosquito Anopheles coluzzii. *Pathogens*, *10*(10), 1300.
3. Bimonte, S., Barbieri, A., Palma, G., Rea, D., Luciano, A., D'Aiuto, M., ... & Izzo, F. (2015). Dissecting the role of curcumin in tumour growth and angiogenesis in mouse model of human breast cancer. *BioMed research international*, *2015*.
4. Borphukan, A., & Bhola, R. K. Hemolymph Protein and Carbohydrate Alteration during Larval Development in Samia ricini Infected with Nosema.
5. Chu, F. J., Jin, X. B., & Zhu, J. Y. (2011). Housefly maggots (Musca domestica) protein-enriched fraction/extracts (PE) inhibit lipopolysaccharide-induced atherosclerosis pro-inflammatory responses. *Journal of atherosclerosis and thrombosis*, *18*(4), 282-290.
6. Cremonez, P. S., Marco, H. G., Andrello, A. C., Neves, P. M., & Pinheiro, D. O. (2021). The effect of pyriproxyfen on the concentration of circulating metabolic fuel molecules and chemical elements in the hemolymph of Acraea horta L.(Lepidoptera: Nymphalidae): A quantitative analysis. *Pesticide Biochemistry and Physiology*, *177*, 104907.
7. Cremonez, P. S., Marco, H. G., Andrello, A. C., Neves, P. M., & Pinheiro, D. O. (2021). The effect of pyriproxyfen on the concentration of circulating metabolic fuel molecules and chemical elements in the hemolymph of Acraea horta L.(Lepidoptera: Nymphalidae): A quantitative analysis. *Pesticide Biochemistry and Physiology*, *177*, 104907.
8. Cao, X., Zhou, M., Wang, C., Hou, L., Li, Y., & Chen, L. (2012). Musca domestica pupae lectin improves the immunomodulatory activity of macrophages by activating nuclear factor-κB. *Journal of Medicinal Food*, *15*(2), 145-151.
9. DIMARCQ, J. L., Hoffmann, D., Meister, M., Bulet, P., Lanot, R., REICHHART, J. M., & Hoffmann, J. A. (1994). Characterization and transcriptional profiles of a Drosophila gene encoding an insect defensin: a study in insect immunity. *European Journal of Biochemistry*, *221*(1), 201-209.
10. El-Garawani, I., El-Seedi, H., Khalifa, S., El Azab, I. H., Abouhendia, M., & Mahmoud, S. (2020). Enhanced antioxidant and cytotoxic potentials of lipopolysaccharides-injected musca domestica larvae. *Pharmaceutics*, *12*(11), 1111.
11. El-Garawani, I. M., El-Sabbagh, S. M., Abbas, N. H., Ahmed, H. S., Eissa, O. A., Abo-Atya, D. M., ... & El-Seedi, H. R. (2020). A newly isolated strain of Halomonas sp.(HA1) exerts anticancer potential via induction of apoptosis and G2/M arrest in hepatocellular carcinoma (HepG2) cell line. *Scientific reports*, *10*(1), 14076.
12. Etebari, K., Bizhannia, A. R., Sorati, R., & Matindoost, L. (2007). Biochemical changes in haemolymph of silkworm larvae due to pyriproxyfen residue. *Pesticide Biochemistry and Physiology*, *88*(1), 14-19.
13. Etebari, K., & Matindoost, L. (2004). Effects of hypervitaminosis of vitamin B 3 on silkworm biology. *Journal of Biosciences*, *29*, 417-422.
14. Frederich, M., DeWachter, B., Sartoris, F. J., & Pörtner, H. O. (2000). Cold tolerance and the regulation of cardiac performance and hemolymph distribution in Maja squinado (Crustacea: Decapoda). *Physiological and biochemical zoology*, *73*(4), 406-415.
15. Fu, P., Wu, J., & Guo, G. (2009). Purification and molecular identification of an antifungal peptide from the hemolymph of Musca domestica (housefly). *Cellular & molecular immunology*, *6*(4), 245-251.
16. Arcos, G. F., Ibarra, A. M., Vazquez-Boucard, C., Palacios, E., & Racotta, I. S. (2003). Haemolymph metabolic variables in relation to eyestalk ablation and gonad development of Pacific white shrimp Litopenaeus vannamei Boone. *Aquaculture Research*, *34*(9), 749-755.
17. Ozbay, G., & Riley, J. G. (2002). An analysis of refractometry as a method of determining blood total protein concentration in the American lobster Homarus americanus (Milne Edwards). *Aquaculture Research*, *33*(8), 557-562.
18. Greenwell, M., & Rahman, P. K. S. M. (2015). Medicinal plants: their use in anticancer treatment. *International journal of pharmaceutical sciences and research*, *6*(10), 4103.
19. Hwangbo, J., Hong, E. C., Jang, A., Kang, H. K., Oh, J. S., Kim, B. W., & Park, B. S. (2009). Utilization of house fly-maggots, a feed supplement in the production of broiler chickens. *Journal of Environmental Biology*, *30*(4).
20. Hou, L., Shi, Y., Zhai, P., & Le, G. (2007). Antibacterial activity and in vitro anti-tumor activity of the extract of the larvae of the housefly (Musca domestica). *Journal of Ethnopharmacology*, *111*(2), 227-231.
21. Horie, Y., & Watanabe, K. (1983). Effect of various kinds of dietary protein and supplementation with limiting amino acids on growth, haemolymph components and uric acid excretion in the silkworm, Bombyx mori. *Journal of Insect Physiology*, *29*(2), 187-199.
22. Kumar, D., Pandey, J. P., Jain, J., Mishra, P. K., & Prasad, B. C. (2011). Qualitative and quantitative changes in protein profile of various tissue of tropical tasar silkworm, Antheraea mylitta Drury. *International journal of zoological research*, *7*(2), 147.
23. Łoś, A., & Strachecka, A. (2018). Fast and cost-effective biochemical spectrophotometric analysis of solution of insect "blood" and body surface elution. *Sensors*, *18*(5), 1494.
24. Liu, B., Ezeogu, L., Zellmer, L., Yu, B., Xu, N., & Joshua Liao, D. (2015). Protecting the normal in order to better kill the cancer. *Cancer medicine*, *4*(9), 1394-1403.
25. Liu, B., Qi, X., Han, Q., Jia, L., Xiang, Z., & He, N. (2012). A silkworm hemolymph protein is a prophenoloxidase activation blocker.
26. Ocampo, L., Patiño, D., & Ramírez, C. (2003). Effect of temperature on hemolymph lactate and glucose concentrations in spiny lobster Panulirus interruptus during progressive hypoxia. *Journal of Experimental Marine Biology and Ecology*, *296*(1), 71-77.

27. Lorenzon, S., Martinis, M., & Ferrero, E. A. (2011). Ecological relevance of hemolymph total protein concentration in seven unrelated crustacean species from different habitats measured predictively by a density-salinity refractometer. *Journal of Marine Biology*, *2011*.
28. Lu, X., Jin, X., Wang, J., Chu, F., & Zhu, J. (2014). Antihepatitis B virus activity of a protein-enriched fraction from housefly (Musca domestica) in a stable HBV-producing cell line. *The Scientific World Journal*, *2014*.
29. Murthy, V. Y. (2015). Estimation of protein concentration in different tissues of popular silkworm (Bombyx mori L.) races. *International Journal*, *3*(1), 254-261.
30. Morishima, I., Horiba, T., Iketani, M., Nishioka, E., & Yamano, Y. (1995). Parallel induction of cecropin and lysozyme in larvae of the silkworm, Bombyx mori. *Developmental & Comparative Immunology*, *19*(5), 357-363.
31. Malik, M. A., & Malik, F. A. (2009). Ontogenic changes in haemolymph biochemical composition in the Silkworm, Bombyx mori L under thermal stress. *Academic journal of Entomology*, *2*(1), 16-21.
32. Mahmoud, S., Hassab El-Nabi, S., Hawash, A., El-Seedi, H. R., Khalifa, S. A., Ullah, S., ... & El-Garawani, I. M. (2022). curcumin-injected musca domestica larval hemolymph: Cecropin upregulation and potential anticancer effect. *Molecules*, *27*(5), 1570.
33. Tabunoki, H., Dittmer, N. T., Gorman, M. J., & Kanost, M. R. (2019). Development of a new method for collecting hemolymph and measuring phenoloxidase activity in Tribolium castaneum. *BMC research notes*, *12*, 1-7.
34. Nakayama, S. (1990). Osmotic pressure of hemolymph in the silkworm, Bombyx mori: its changes during development. *Agricultural and biological chemistry*, *54*(3), 797-798.
35. Nagata, S., Morooka, N., Asaoka, K., & Nagasawa, H. (2011). Identification of a novel hemolymph peptide that modulates silkworm feeding motivation. *Journal of Biological Chemistry*, *286*(9), 7161-7170.
36. Pietrzyk, A. J., Bujacz, A., Mueller-Dieckmann, J., Łochynska, M., Jaskolski, M., & Bujacz, G. (2013). Crystallographic identification of an unexpected protein complex in silkworm haemolymph. *Acta Crystallographica Section D: Biological Crystallography*, *69*(12), 2353-2364.
37. Potgieter, M., Bester, J., Kell, D. B., & Pretorius, E. (2015). The dormant blood microbiome in chronic, inflammatory diseases. *FEMS microbiology reviews*, *39*(4), 567-591.
38. Pemberton, R. W. (1999). Insects and other arthropods used as drugs in Korean traditional medicine. *Journal of ethnopharmacology*, *65*(3), 207-216.
39. Scherfer, C., Karlsson, C., Loseva, O., Bidla, G., Goto, A., Havemann, J., ... & Theopold, U. (2004). Isolation and characterization of hemolymph clotting factors in Drosophila melanogaster by a pullout method. *Current biology*, *14*(7), 625-629.
40. Sharma, P., & Kalita, J. C. (2017). Haemolymph protein estimation in six different strains of Eri silk worm, Samia ricini (Donovan).
41. Saravanan, M., Selvi, S., Veeranarayanan, M., & Nadanam, S. (2011). Modulations in the haemolymph of silkworm [Bombyx mori (l).(Lepidoptera: Bombycidae)] fed with mulberry leaves augmented with cowpeas (Vigna unguiculata). *International Journal of Nutrition, Pharmacology, Neurological Diseases*, *1*(1), 64.
42. Simpson, S. J., & Abisgold, J. D. (1985). Compensation by locusts for changes in dietary nutrients: behavioural mechanisms. *Physiological Entomology*, *10*(4), 443-452.
43. Theopold, U., Li, D., Fabbri, M., Scherfer, C., & Schmidt, O. (2002). The coagulation of insect hemolymph. *Cellular and Molecular Life Sciences CMLS*, *59*, 363-372.
44. Terwilliger, N. B. (1999). Hemolymph proteins and molting in crustaceans and insects. *American Zoologist*, *39*(3), 589-599.
45. Tojo, S., Nagata, M., & Kobayashi, M. (1980). Storage proteins in the silkworm, Bombyx mori. *Insect Biochemistry*, *10*(3), 289-303.
46. Ueno, Y., Takao, M., He, N., Yamamoto, K., Banno, Y., & Fujii, H. (2006). Genetic analysis of basic chymotrypsin inhibitors in the hemolymph of the silkworm, Bombyx mori. *Journal of insect biotechnology and sericology*, *75*(2), 65-69.
47. Zhang, X., Sun, Z., Zhang, X., Zhang, M., & Li, S. (2018). Hemolymph microbiomes of three aquatic invertebrates as revealed by a new cell extraction method. *Applied and environmental microbiology*, *84*(8), e02824-17.
48. Zhang, J. Y., Tao, L. Y., Liang, Y. J., Chen, L. M., Mi, Y. J., Zheng, L. S., ... & Fu, L. W. (2010). Anthracenedione derivatives as anticancer agents isolated from secondary metabolites of the mangrove endophytic fungi. *Marine drugs*, *8*(4), 1469-1481.
49. Zhou, J., Kong, L., Fang, N., Mao, B., & Ai, H. (2016). Synthesis and functional characterization of MAF-1A peptide derived from the larvae of housefly, Musca domestica (Diptera: Muscidae). *Journal of medical entomology*, *53*(6), 1467-1472.