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Haemolymph composition of *Ancylostomia stercorea* Zeller (Lepidoptera:Pyralidae) larvae with particular reference to proteins and amino acids

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ABSTRACT:

Haemolymph of 3rd, 4th and 5th larval instars of *A. stercorea* was extracted and used in the identification of amino acids by electrophoresis, total protein content by Lowry's method and proteins by SDS-PAGE. Potassium, sodium and glucose levels were also determined. Seventeen amino acids were identified in all three larval instars. Thirteen protein bands were present in the haemolymph of 4th and 5th larval instars, five of which had molecular weights of >45.4 kDa while ten protein bands were present in the haemolymph of 3rd larval instar. Sodium:Potassium ratios in 3rd, 4th and 5th larval instars were 0.05, 0.14 and 0.13 respectively. Glucose concentrations in 3rd, 4th and 5th larval instars were 0.16, 0.33 and 0.57 mg/ml respectively. The results provided critical information that can be used to create artificial diets for *in-vitro* rearing of *A. stercorea* parasitoids.

Keywords:
Ancylostomia stercorea, haemolymph, larvae, *Cajanus cajan.*

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INTRODUCTION

Pigeonpea (C*ajanus cajan* L. Millsp.) is a leguminous shrub that originated in India (van der Maesen, 2006). It was found in West Africa about 2000BC and the slave trade redistributed it to the West Indies, where it's use as a bird feed led to the name "pigeonpea" in 1692. Pigeonpea forms an essential part of the protein diet found within the Caribbean and can also be used for a number of purposes including animal feed and firewood (Shanower *et al.,* 1999). Furthermore, pigeonpea improves the soil through its extensive root system, nitrogen fixation and mulch provided by the fallen leaves (Van der Maesen, 2006).

The plant is plagued by numerous insect pests throughout the Caribbean and other areas where it is grown (Khan *et al*, 2003). They attack all stages and parts of the plant from seedling to harvest and beyond. However, damage caused by lepidopterous pod borers is very significant since they attack the edible seeds rendering them unfit for consumption. The most important pod borer pest of pigeonpea in the Caribbean and particularly Trinidad and Tobago is *Ancylostomia stercorea* Zeller (Lepidoptera:Pyralidae) which causes significant damage to green seeds. Buckmire (1979) reported that in Trinidad, over 90% of the green pods could be infested by *A. stercorea*. Bennett (1960) described the pest as a multivoltine species, depositing its eggs on the young pods of pigeonpea; the larvae then feed on the developing seeds and later pupate in soil. The life cycle is completed in 26-32 days.

Major concerns regarding pesticide overuse and its impact on human health and the environment have arisen. A great deal of emphasis has therefore been placed on finding alternative solutions (e.g. biological control) to large scale use of synthetic insecticides. Guerra *et al* (1993) notes that mass production of parasitoids for use in biological programmes requires very costly *in vivo* techniques which involve mass production of host insects. Researchers have attempted to develop artificial diets containing insect haemolymph on which to rear parasitoids, and so should be able to cut production costs dramatically. Matthews (1974) concurred that more attention should be paid to the mass rearing efficiency of parasitoids by inducing species to oviposit directly and to successfully develop upon artificial nutrient media. However, there are several limiting factors hindering the development of these artificial diets, including the biochemical interactions between the insect host and parasitoid and the lack of understanding of entomophage physiology and metabolism. The objective of this study was to determine the haemolymph composition of *A. stercorea* with particular reference to protein, amino acid and ion composition as a pre-requisite step in artificial rearing of its major parasitoid, *Bracon thurberiphagae* Muesbeck (Hymenoptera:Braconidae).

MATERIALS AND METHODS

The experiment was carried out during the months of March – May 2010. Pigeonpea pods from fields were collected with 3rd, 4th and 5th larval stages of *A. stercorea* and kept in cages. Haemolymph from 3rd, 4th and 5th instar larvae was extracted by piercing the cuticle with a thin, sharp insect mounting pin (No.3) and separately collected in Corning Pyrex® 5µl disposable micropipettes (Corning Science Products, USA).

Zone electrophoresis of amino acids

Electrophoresis of amino acids in the haemolymph of *A. stercorea* larvae was done using a Gelman® Semi-micro Electrophoresis chamber with a 180V DC supply. Phosphate buffer (150ml of 0.1M KH_2PO_4 was added to 90ml of 0.1M NaOH and diluted to 600ml giving a pH of 7.02) was poured into the chamber tray until almost half filled. The tray was gently tilted to ensure that the phosphate buffer was equalized in all compartments. Strips (6 cm x 14 cm) of Whatman® No.1 filter paper were placed on clean paper and a light pencil line was drawn across the centre of

each strip. One end was marked (+) to be immersed in the anode and the other (-) to be immersed in the cathode. Three equally spaced small dots were marked on the centre line for application of two standard amino acid samples (0.2% (w/v) amino acid solutions preserved with a drop of toluene) and the other for the haemolymph sample. 5 µl of each sample was transferred to its appropriate spot and the strip placed in the chamber and ran for 75 minutes at the end of which a glass rod was used to remove the paper. The paper was then dried with a hair dryer for about 3-4 minutes and dipped in 0.3% Ninhydrin solution (0.3% (w/v) solution in acetone + one drop of pyridine added per 50mL of solution) and dried for a further 4-5 minutes. The appearance of coloured spots on the paper indicated the position of each amino acid. Retention factor (R_f) values were then calculated from the results collected, using the following formula: R_f = Distance moved by spot / Distance moved by solvent front. These values were compared to standard values of the amino acids used and the amino acids present in the haemolymph samples were identified.

Protein content and separation

Total protein concentration in the haemolymph was determined using the Lowry *et al.* (1951) method. Bovine Serum Albumin (200 µg/mL) was used as the standard protein. Proteins were separated using PAGE with a running buffer of pH 8.3 (0.6g Tris, 2.88g glycine in 1000ml of de-ionized water) and a protein stain of 0.2% Coomassie Blue R-250 in 50% methanol : 10% acetic acid (0.2g Coomassie Blue R-250, 50ml of methanol and 10ml of acetic acid in 100ml of water). Gels were de-stained using a solution of 30% methanol: 10% acetic acid. A 7.5% separating gel (4.95ml of de-ionized water, 2.50ml of 1.5M Tris-HCl (pH 8.8), 2.50ml of Acrylamide – BIS (30% stock), 50µl freshly prepared 10% Ammonium Persulphate and 5µl TEMED and a 4% stacking gel (6.2ml de-ionized water, 2.5ml 0.5M Tris-HCl (pH 6.8), 1.33ml Acrylamide – BIS (30% stock), 50µl freshly prepared 10% Ammonium Persulphate and 10µl of TEMED). Ten wells each with 10µL /well was used for the determination of proteins present in the haemolymph of 3rd, 4th and 5th larval instars with each sample comprising: 3µL Sample + 3µL Sucrose + 3µL Bromophenol Blue + 1µL Running Buffer. α-Lactalbumin from Bovine milk (14.2kDa), Carbonic Anhydrase from Bovine Erythrocytes (29kDa), Albumin from Chicken egg white (45kDa), and Urease from Jack Bean (545kDa) were used as markers.

Sodium, Potassium and Glucose determination

Sodium and potassium haemolymph concentrations were determined using a Sherwood Flame Photometer Model 410 (Sherwood Scientific Ltd, UK). The values presented represent the means of five replicates per instar. Glucose concentrations for each instar were determined using the method of Folin-Wu (1919).

RESULTS AND DISCUSSION

Amino acids

Free amino acids, proteins and other organic ions are very important components of parasitoid diets (Baker and Fabrick, 2000). Research on the nutritional components of host larvae which can be utilized by ectoparasitoids to support their larval growth and development is useful for the development of optimal artificial diets for the *in vitro* rearing of these ectoparasitoids. There were seventeen amino acids identified in all three larval instars of *A. stercorea* (Table 1). Leucine was present in 4th and 5th larval instars but absent in 3rd larval instar. Isoleucine was present in 3rd and 5th larval instars and absent in 4th larval instar while tryptophan was present in 4th and 5th larval instars but absent in 3rd larval instar **(Table 1).** Chen (1962) noted that ten amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) have been proven to be essential for insect growth and development, all of which were present in the insect larvae investigated. Duke (1983)

Table 1 Amino acids identified by paper chromatography in haemolymph of *Ancylostomia stercorea* Zeller (Lepidoptera:Pyralidae) 3rd, 4th and 5th instar larval stages.

Amino acid	3rd Instar	4th Instar	5th Instar
Alanine	√	√	√
Arginine	√	√	√
Asparagine	√	√	√
Aspartic acid	√	√	√
Cysteine	√	√	√
Glutamine	√	√	√
Glutamic acid	√	√	√
Glycine	√	√	√
Histidine	√	√	√
Isoleucine	√	X	√
Leucine	X	√	√
Lysine	√	√	√
Methonine	√	√	√
Phenylalanine	√	√	√
Proline	√	√	√
Serine	√	√	√
Threonine	√	√	√
Tryptophan	X	√	√
Tyrosine	√	√	√
Valine	√	√	√
√ – Presence X - Absence			

revealed the presence of 20 amino acids in *Cajanus cajan* (pigeonpea) namely alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. Most of these were found in the larval haemolymph of *A. stercorea* investigated indicating that the larvae of *A. stercorea* may have derived most of its amino acid content from *C.cajan* seeds.

Protein content and separation

Haemolymph protein levels were not significantly different ($p<0.05$, Tukey-Kramer Multiple Comparisons Test) between 3rd, 4th and 5th instars of *A. stercorea* (Table 2) since the same proteins may be needed for growth and development in each larval stage however, they may be present in different concentrations. Indeed changes in the haemolymph biochemistry of *Plodia interpunctella* after treatment with *Bacillus thuringiensis* revealed that there was insignificant variation in the total proteins in normal untreated larvae (Aboul-Ela *et al*, 1991). Thirteen protein bands were present in the haemolymph of 4th and 5th larval instars, five of which had molecular weights >45.4 kDa. Ten protein bands were present in 3rd instar larvae, however most of the bands were found in very low concentrations except for a prolific band with a molecular weight of 18.7 kDa that was also present in the other larval instars. Generally high molecular weight proteins were present in 4th and 5th instars while proteins of low molecular weights were found mainly in 3rd larval instar although they were also present in the other two larval instars but in very low concentrations **(Plate 1).** One probable explanation for the numerous protein bands in 4th and 5th instar haemolymph may be that most insects require larger amounts of protein for further growth and development. Nation (2008) and Kanagalakshmi (2011) confirm that some insects (larvae of Diptera and Lepidoptera) synthesize large quantities of one or more storage proteins during late larval life and this was particularly evident in the haemolymph of 4th and 5th instar *A. stercorea* larvae **(Table 2).** Chen (1962) also suggested that the total haemolymph protein content increased during larval development and was most rapid during the period approaching pupation. The large number of protein bands present in 4th and 5th instars may be as a result of protein accumulation to sustain growth during the pupal stage. Interestingly though, a distinct protein band of a molecular weight of 18.7 kDa was very prolific in all three larval stages and could possibly be a major protein needed for growth and development in all

Table 2 Potassium (K^+), sodium (Na^+), glucose and total protein content (± SE) in haemolymph of 3rd, 4th and 5th larval instars of *Ancylostomia stercorea* (Zeller)

Instar	Na^+ (mg/ml)*	K^+ (mg/ml)*	$Na^+ : K^+$ Ratio	Glucose (mg/ml)*	Total protein (mg/ml)*
3rd	0.092 ± 0.003[a]	1.692 ± 0.002[a]	0.05	0.16 ± 0.06[a]	66.0 ± 27.0[a]
4th	0.153 ± 0.006[b]	1.128 ± 0.005[b]	0.14	0.33 ± 0.11[ab]	85.5 ± 22.5[a]
5th	0.146 ± 0.002[b]	1.128 ± 0.003[b]	0.13	0.57 ± 0.09[b]	73.5 ± 20.5[a]

***Values followed by the same letters in a column are not significantly different from each other based on Tukey- Kramer multiple comparisons test (p< 0.001).**

life stages of the insect.

Potassium, Sodium and Glucose

Potassium concentrations were not significantly different (p> 0.001) between 4th and 5th instars; however 3rd instar haemolymph had significantly higher (p< 0.001) potassium levels than the other two instars. A similar trend was observed for sodium haemolymph concentrations (Table 2). Ratios of Na^+: K^+ are generally very low (≤ 0.3 – 0.1) in Lepidoptera, phytophagous Coleoptera, Hemiptera, Homoptera and Hymenoptera and may be correlated with the food habits of these groups (Nation 2008). Glucose haemolymph concentrations were significantly different (p<0.05) between 3rd and 5th instars (Table 2) and may be interpreted as the later larval instars requiring larger quantities of glucose for increased metabolism as the insect approaches pupation (Aboul-Ela *et al*, 1991; Nation 2008; Malik and Malik 2009). The results from the experiment provided critical information that can be used to strengthen the gaps that exist in creating artificial diets for *in-vitro* rearing of parasitoids of *A. stercorea*.

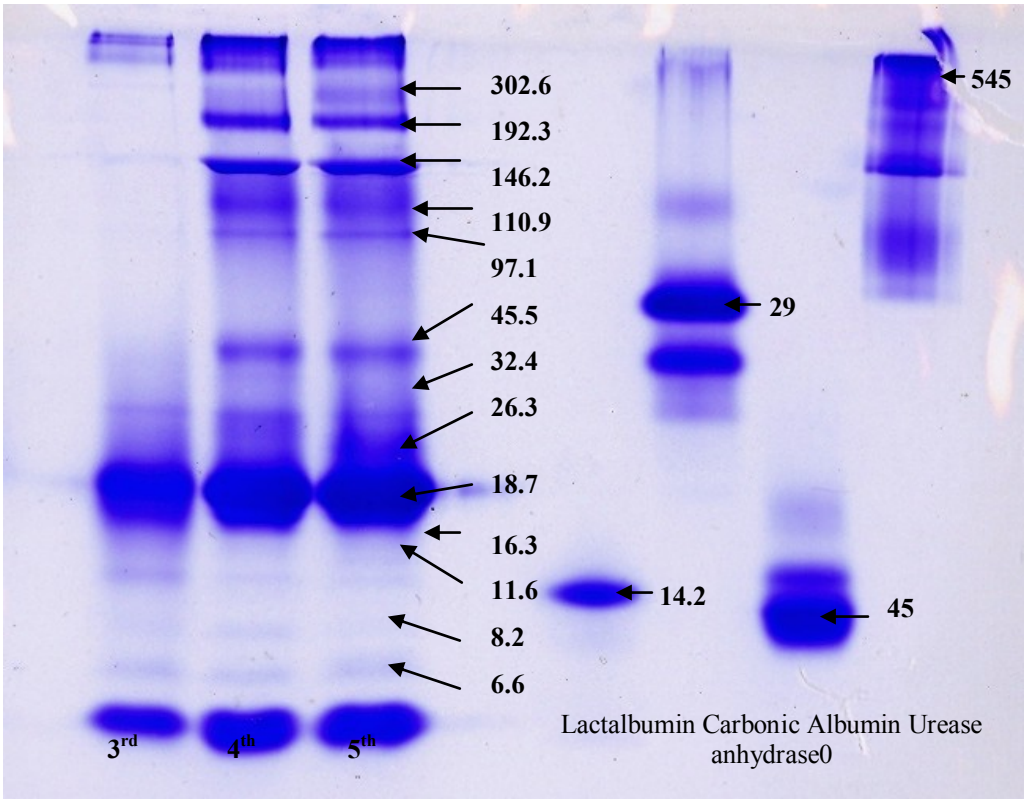

Plate 1 Electrophoresis gel of proteins present in haemolymph of 3rd, 4th and 5th larval instars of *A. stercorea* (values are given in kDa, markers used were Lactalbumin, Carbonic anhydrase, Albumin and Urease)

REFERENCES

Aboul-Ela R, Kamel MY, Salama HS, El-Moursy A, Abdel-Razek A. 1991. Changes in the biochemistry of the haemolymph of *Plodia interpunctella* after treatment with *Bacillus thuringiensis*. *Journal of Islamic Academy of Science* 4(1):29-35.

Baker JE, Fabrick JA. 2000. Host haemolymph proteins and protein digestion in larval *Habrobracon hebetor* (Hymenoptera:Braconidae). *Insect Biochemistry and Molecular Biology* 30 (10):937-946.

Bennett FD. 1960. Parasites of *Ancylostomia stercorea* (Zell.) (Pyralidae:Lepidoptera), a pod borer attacking pigeon pea in Trinidad. *Bulletin of Entomological Research* 50:737-757.

Buckmire KU. 1979. Pests of Grain Legumes and their control in the Commonwealth Caribbean. In Singh SR, van Emden HF, Taylor TA (Eds) *Insect Pests of Grain Legumes: Ecology and Control*. Academic Press, London UK. 454.

Chen PS. 1962. Amino acid and protein metabolism in insect development. *Advances in Insect Physiology* 3:53-132.

Duke JA. 1983. *Handbook of energy crops*. http://www.hort.purdue.edu/newcrop/duke_energy/Cajanus_cajun.html.

Folin O, Wu H. 1919. A system of blood analysis: Supplement I - A simplified and improved method for determination of sugar. *Journal of Biological Chemistry* 41:367-374.

Guerra AA, Robacker M, Martinez S. 1993. Free amino acid and protein titers in *Anthonomus grandis* larvae venomized by *Bracon mellitor*. *Entomophaga* 38 (4):519-525.

Kanagalakshmi K. 2011. Fluctuation of protein level in haemolymph, ovary and hepatopancreas during non-reproductive and reproductive molt cycle of *Albunea symmista*. *Journal of Research in Biology* 1(2): 68-72

Khan A, Baker PS, Pollard GV. 2003. Semi-field tests of the effects of three insecticides on *Ancylostomia stercorea*. *Tests of Agrochemicals and Cultivars (Annals of Applied Biology, 141, Supplement):*23:4-5.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin-Phenol reagent. *Journal of Biological Chemistry* 193:265-275.

Malik MA, Malik AF. 2009. Ontogenic changes in haemolymph biochemical composition in the silkworm, *Bombyx mori* L under thermal stress. *Academic Journal of Entomology* 2(1):16-21.

Matthews RW. 1974. Biology of Braconidae. *Annual Review of Entomology* 19:15-32.

Nation JL. 2008. *Insect Physiology and Biochemistry*. 3rd Edition CRC Press, USA. 496.

Shanower GT, Romus J, Minja ME. 1999. Insect pests of pigeonpea and their management. *Annual Review of Entomology* 44:77-96.

Van der Maesen LJG. 2006. *Cajanus cajan* (L.) Millsp. http://database.prota.org/search.htm.