Nutrition, Haemolymph Changes and Ovarian Maturation of Rat fleas *Xenopsylla cheopis* (Rothschild) and *X. astia* (Rothschild) (Insecta: Siphonaptera)

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Female fleas, *Xenopsylla cheopis* (Rothschild) and *X. astia* (Rothschild) fed directly on a host (*in vivo*) started laying eggs by the 2nd day, while those fed *in vitro* (membrane fed) on whole blood seldom laid eggs even after 5 days, eventhough they had yolk-laden oocytes. Neither plasma nor sucrose diet induced yolk deposition. A comparison of unfed and *in vivo* fed fleas showed that concentrations of haemolymph protein and sugars increased while that of triglyceride decreased after feeding. Triglyceride is suggested to be the major source of energy reserve in unfed fleas. PAGE of haemolymph of *in vivo* fed fleas showed 11 coomassie positive fractions. All these were conjugated proteins. Immunological studies showed selective sequestration of two of these fractions into the developing oocytes. This sequestration and their absence in the male suggest them to be vitellogenins. These two fractions are present in the haemolymph albeit in low concentrations even before the female fleas take blood meal. This suggests that initiation of vitellogenin synthesis is not under the control of adult feeding. Though the haemolymph of whole-blood fed (*in vitro*) female fleas showed same number of bands as *in vivo* fed fleas, plasma or sucrose fed fleas showed only 9 fractions. Haemolymph protein concentration is positively linked with vitellogenesis in these fleas.

**Key Words:** Rat fleas, *Xenopsylla cheopis*, *X. astia*, Nutrition, Haemolymph changes, Ovarian maturation, Vitellogenesis

**Introduction**

Earlier studies on rat fleas *Xenopsylla cheopis* and *X. astia* have shown that blood meal is essential for ovarian maturation and that the protein content in diet influences the oocyte development and egg laying (Kamala Bai & Prasad 1976, 1981). Further, while the whole blood or blood cells resuspended in isotonic saline would trigger yolk deposition, rat blood plasma did not do so except when it was fortified with the rabbit albumin.

Studies on several other insects have helped to establish the sequence of events following feeding and haemolymph changes leading to oocyte maturation, e.g. the synthesis of vitellogenin in the fat body, its subsequent appearance in the haemolymph and its sequestration into the developing oocyte (Telfer 1954, 1960, Brookes 1969, Engelmann 1969, Pan et al. 1969, Hagedorn & Judson 1972, Hagedorn & Fallon 1973, Hagedorn et al. 1973).

In the present study colorimetric, electrophoretic and immuno-electrophoretic analyses of the haemolymph of *X. cheopis* and *X. astia* were carried out to ascertain the effect of nutritionally-induced haemolymph changes on ovarian maturation.

**Materials and Methods**

**Fleas used for Experiments**

Subcultures of *X. cheopis* and *X. astia* were prepared

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from the parent laboratory colonies. Dried and powdered beef blood and yeast constituted the larval diet. Pupae as and when appeared were removed and adults emerged were used for experiments approximately 24 hr after emergence.

In vivo Feeding
Fleas collected from the subcultures were released on a one-month-old white rat (Rattus norvegicus albino). After 24 hr they were collected and starved for another day. They were then fed on a rat of the same age and species and haemolymph was collected after 18–20 hr. Thus, in vivo fed fleas from which haemolymph was drawn were approx. 4-day-old.

In vitro Feeding
The technique used for artificial feeding (membrane feeding) of whole blood and blood plasma of white rat and sucrose was the one described by Kamala Bai and Prasad (1976). Fleas were fed once daily for four consecutive days and haemolymph was collected 18–20 hr after the last feed. Only well-fed fleas were used for haemolymph collection.

Withdrawal of Haemolymph and Collection of Egg Yolk
Fleas were lightly etherized, sexed, washed in distilled water and wiped on filter paper. Withdrawal of haemolymph was carried out under a binocular microscope (× 20). A puncture was made on the thorax with a sharp needle and the flea was gently pressed with a blunt forceps. The expressed haemolymph was drawn into a microcapillary tube graduated to 1 μl. Nearly 35–40 females and 80–100 male fleas were required to collect 1 μl of haemolymph sample. Phenylthiourea was added to prevent darkening of the haemolymph as a result of tyrosinase activity. Chorionated eggs were dissected out from fleas and washed in isotonic saline. The yolk was extracted in 10% saline by puncturing and pressing the eggs with forceps.

Estimations of Protein, triglyceride and Sugars
Protein from haemolymph (1 μl) was precipitated in 0.5 ml of 0.3M trichloroacetic acid and quantitatively estimated using the technique of Lowry et al. (1951). Bovine serum albumin was used as the standard. Van Handel’s (1965) microseparation and estimation techniques were used for estimation of triglyceride and sugars.

Polyacrylamide Gel Electrophoresis
Flea haemolymph and egg yolk were fractionated in 7.5% polyacrylamide gel at 3.5 mA/tube and 100–150 volts. One microliter sample was fractionated in all cases. Proteins were stained with coomassie blue, lipid with sudan black and polysaccharides with PAS (Clarke 1964).

Antibody Preparation
Yolk from about 150 eggs was centrifuged at 5000 RPM for 15 min, and the supernatant was used for immunizing the rabbit. For the first injection 0.5 ml of the supernatant solution was thoroughly mixed with 0.5 ml Freund’s complete adjuvant and injected into the thigh muscles of a one-year-old male rabbit. Injections were repeated twice at an interval of one week. Antigen for the 2nd and 3rd injections were mixed with 0.5 ml Freund’s incomplete adjuvant. Anti-egg serum was collected from the immunized rabbit one week after the last injection. This was kept in the refrigerator for further use, after adding 1% sodium azide to prevent bacterial growth (Telfer 1954, Weir 1978).

Immunodiffusion
Agarose plate (1% in veronal buffer pH 8.3) on microscope was used for immunodiffusion. After 48 hr, development the plates were washed for 72 hr in an antiseptic buffer (sodium borate 2g; NaCl 8.8g; Distilled water 1000 ml) to remove the unprecipitated proteins. The slides were then stained for 2 hr in 0.5% Coomassie blue and dried under Whatman No. 1 filter paper at room temperature (Crowle 1973).

Disc-immunoelectrophoresis
Yolk or haemolymph, as the case may be, was subjected to polyacrylamide gel electrophoresis. Immediately after the run the gels were removed and each was slit longitudinally into two halves. One-half was stained with coomassie blue. The other half was implanted in a 1% agarose plate prepared as for immunodiffusion. The anti-egg serum then placed in a trench cut parallel to the implanted gel. Forty-eight hours after, the positions of the stained bands and the precipitin bands of the respective halves were compared. In another test, individual discs from the polyacrylamide electrophorograms were transversely cut and inserted into wells punched in 1% agarose plate. Positions of the discs in unfixed and unstained gels were located by comparing with fixed and stained gels with the same material run simultaneously. Anti-egg serum was kept in wells cut near the implanted disc. These slides were kept in a refrigerator and observed for precipitin bands.

Statistical Analyses
Analysis of Variance was done to find statistical significance of data relating to in vivo and in vitro fed fleas. Student’s t was used for paired comparisons of artificial diets.
Results
Female fleas left on a live host (white rat) laid eggs by the 2nd day at the rate of 8–10 eggs/female, whereas only few of those fed in vitro once daily on whole blood of white rat laid eggs by the 5th day @1–2 eggs/female, even though all females sacrificed had yolk-laden oocytes. With rat blood plasma diet, though there was slight growth in the oocytes, no yolk granules were visible. Sucrose diet did not initiate any growth in the oocytes.

Haemolymph proteins, triglyceride and sugars
Colorimetric analyses of haemolymph protein concentrations of female X. cheopis and X. astia fed in vitro on different diets or in vivo showed that: There was almost a two-fold increase in in vivo fed fleas of both species compared to newly emerged unfed fleas, the results being statistically significant (table 1). Female fleas fed in vitro on whole blood of white rat had significantly higher haemolymph protein concentration than rat blood plasma or sucrose-fed fleas, but comparison of plasma and sucrose-fed fleas showed no statistical significance (table 2). No statistically significant difference was detected in haemolymph protein concentration of in vivo fed fleas compared to in vitro whole-blood fed fleas. Haemolymph protein concentration of fresh unfed female fleas of both species was not statistically different from either plasma or sucrose-fed fleas. An interspecific comparison of haemolymph concentration showed no significant difference.

Haemolymph triglyceride concentration presented a different picture. Newly emerged unfed female fleas of both species showed a significantly higher concentration compared to fed ones. In general, in vitro fed fleas has a higher haemolymph triglyceride concentration compared to in vivo fed ones. Analysis of variance showed no statistical significance between different diets and hence paired comparisons were not done (table 2).

In in vivo fed female fleas, concentration of haemolymph sugars showed a significant increase compared to unfed female fleas of both species (table 1). Curiously in vitro fed fleas compared among themselves showed statistically significant least value in sucrose fed fleas. Whole-blood fed fleas had a significantly higher haemolymph sugar concentration compared to the other two (table 2).

Electrophoretic Analyses
The haemolymph of unfed and fed male fleas of both species resolved into 10 coomassie blue positive fractions whereas that of unfed and fed females of both species showed 12 fractions (figure 1) though there was no numerical variation in the fractions, yet there was a considerable difference in the thickness of the discs and staining intensity. All the fractions, except fractions 1 and 8, were more intensely stained in fed fleas indicating an increase in the concentration as a result of feeding. Fraction 10 seen in female was not represented in male and there was only a very faint suggestion of fraction 11 in male and this fraction disappeared after feeding (figure 1 d,e). All the fractions were more intensely stained in females (both unfed and fed) compared to males indicating higher concentrations. Fractions 1, 2, 6, 10, 11 and 12 of the in vivo fed female fleas were lipoglycoproteins and fraction 8 was glycoprotein. In the case of newly emerged females, fractions 2, 6, 11 and 12 were lipoglycoproteins and 1 was lipoprotein. Fractions 8 and 10 were negative for

<table>
<thead>
<tr>
<th>Table 1 Haemolymph analysis of in vivo fed fleas</th>
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<tr>
<td>Species and Nutritional Status</td>
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<td>-----------------------------------------------</td>
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<tr>
<td><strong>Xenopsylla cheopis</strong></td>
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<tr>
<td>Newly emerged unfed females</td>
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<td><em>In vivo fed</em></td>
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<td><strong>Xenopsylla astia</strong></td>
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<tr>
<td>Newly emerged unfed females</td>
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<td><em>In vivo fed</em></td>
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*Values given are averages of 5 replicates
* *Between treatment significant at 5% level, * *Between treatment significant at 1% level. *1* Between replicate not significant, *2* Between replicate significant.
Table 2  Haemolymph analyses of in vitro fed fleas

<table>
<thead>
<tr>
<th>Species &amp; Diet</th>
<th>Analysis of variance</th>
<th>t-test</th>
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<tr>
<td></td>
<td>F(1,4) ratio</td>
<td>P</td>
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<table>
<thead>
<tr>
<th>Protein</th>
<th>mg/100 ml</th>
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<tr>
<td>Whole blood</td>
<td></td>
<td></td>
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<tr>
<td>X. astia</td>
<td></td>
<td></td>
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<tr>
<td>Plasma (Pl)</td>
<td>8.92 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>Sucrose (Su)</td>
<td>10.57 ± 0.45</td>
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<tr>
<td>Triglyceride</td>
<td>μg/100 ml</td>
<td></td>
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<tr>
<td>Whole blood</td>
<td>14.15 ± 0.63</td>
<td></td>
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<tr>
<td>Sugars</td>
<td></td>
<td></td>
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<tr>
<td>Whole blood</td>
<td>19.89 ± 1.23</td>
<td></td>
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<tr>
<td>Plasma</td>
<td>14.07 ± 0.41</td>
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<tr>
<td>Sucrose</td>
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<thead>
<tr>
<th>Protein</th>
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<tr>
<td>Whole blood</td>
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<tr>
<td>X. cheopis</td>
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</tr>
<tr>
<td>Plasma</td>
<td>8.10 ± 0.23</td>
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<tr>
<td>Sucrose</td>
<td>9.74 ± 0.26</td>
<td></td>
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<tr>
<td>Triglyceride</td>
<td>μg/100 ml</td>
<td></td>
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<tr>
<td>Whole blood</td>
<td>11.29 ± 0.35</td>
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</tr>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
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<tr>
<td>Whole blood</td>
<td>16.16 ± 0.50</td>
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</tbody>
</table>

P, Probability; ++, Between treatment significant at 5% level; +, Between treatment significant at 1% level; @, Between treatment not significant; 1, Between replicate not significant

Sudan black, while fraction 11 gave a mildly positive reaction (figure 2). No fractions of the haemolymph of both species were comparable in electrophoretic mobility to those of blood plasma of white rat, which is used as the host for laboratory rearing of these fleas. Yolk from both species of fleas resolved into 9 fractions of which some were very faintly stained. Two prominent fractions in the yolk had electrophoretic mobility and concentration (based on visual comparison of colour intensity) similar to fractions 10 and 11 of fed females. Fractions 7, 8 and 9 seen in fed females were absent in yolk. All other fractions of the haemolymph of fed females were represented in yolk, though only very faintly stained (figure 10). Fractions 2, 3, 6, 10, 11 and 12 were lipoglycoproteins. The following details were noticed when the haemolymph of artificially fed fleas were electrophoretically analysed. All the 12 fractions seen in the in vivo fed females were discernible in the in vitro whole-blood fed female X. cheopis. However, haemolymph of in vitro whole-blood fed female X. astia resolved into 15 fractions. The additional bands were towards the cathodic end and the profile of the rest of the bands were the same as described above. On visual
Figure 1 Electrophorogram of haemolymph protein fractions of *Xenopsylla cheopis*

- a, Newly emerged unfed female
- b, Fed female
- c, Yolk
- d, Newly emerged unfed male
- e, Fed male

A—E Diagrammatic representations of a—e

Figure 2 Schematic representations of the haemolymph and egg fractions of *Xenopsylla cheopis* female

- A, Coomassie blue positive fractions of fed female
- B, C & D, Sudan black positive fractions of newly emerged unfed and fed females and yolk respectively
- E, F & G, PAS positive fractions of newly emerged unfed and fed females and yolk respectively
comparison all the fractions of the in vitro whole-blood fed ones appeared to be less dense compared to those of the in vivo fed ones. In particular fractions 4, 7 and 9 were diffused and faintly stained. Only 9 fractions were noticed in plasma-fed ones. Fractions 4, 7 and 9 of whole-blood fed fleas were not detected. In sucrose-fed fleas the pattern was the same as the plasma-fed ones, but the bands were more diffused and faintly stained. Striking differences were noticed in the composition of haemolymph fractions 1, 3, 6, 8, 10 and 11 of in vitro fed groups. Fraction 1 was a lipoprotein in in vitro fed fleas, whereas it was a lipoglycoprotein in in vivo fed ones. Fraction 3 was an unconjugated protein except in whole-blood fed fleas, where it was a glycoprotein. Fraction 6 was an unconjugated protein in plasma and sucrose-fed fleas, whereas it was a glycoprotein in whole-blood fed fleas and lipoglycoprotein in in vivo fed fleas. Fraction 8 was an unconjugated protein in all the in vitro fed fleas whereas it was a glycoprotein in in vivo fed fleas. Fraction 10 was a lipoprotein in all the in vitro fed groups while it was lipoglycoprotein in in vivo-fed ones. Fraction 11, a lipoglycoprotein in in vivo and in vitro plasma and sucrose fed fleas, appeared to be a glycoprotein in in vitro whole-blood fed fleas.

Immunodiffusion of total haemolymph against anti-egg serum showed two precipitin bands, one of which was prominent while the other was faint (figure 3). In disc-immunodiffusion only fractions 10 and 11 of the haemolymph of fed female showed positive reaction against anti-egg serum indicating that these fractions were common in egg and haemolymph. Fraction 10 gave a strong precipitin reaction whereas fraction 11 was comparatively faint (figure 4). Fractions similar to 10 and 11 in electrophoretic mobility were also present in pupal haemolymph.

**Discussion**

The results show a clear correlation between haemolymph protein concentration and vitellogenesis. Fleas fed on diets nutritionally inadequate (rat blood plasma and sucrose) to trigger vitellogenesis had low haemolymph protein concentrations. Hill (1962) reported that yolk deposition in locust begins only when the haemolymph protein concentration reaches about 4g/100 ml. Failure of yolk deposition when fleas were fed on rat blood-plasma or sucrose and oocyte resorption when ‘laying females’ were put on a rat blood-plasma diet (Kamala Bai & Prasad 1976) are suggestive of such a link between dietary protein concentration and vitellogenesis.

One of the striking features of the present study is the comparatively high levels of triglyceride in newly emerged unfed female fleas (probably linked up with its sustenance until it meets a host and has an opportunity to feed) and its subsequent reduction after feeding. The low and high haemolymph concentrations of sugars and triglyceride respectively in unfed fleas suggest that the newly emerged unfed fleas use triglyceride as the major source of energy reserve. Candy and Kilby (1975) have stated that although the lipid content of the haemolymph is usually small compared to that of fat body, nevertheless it constitutes an important energy reserve. Another striking point is the low haemolymph protein concentration of these fleas compared to other insects like hymenopterans (5g/100 ml) coleopterans (3-4g/100 ml), lepidopterans (2g/100 ml) and orthopterans (1g/100 ml) (Florkin 1931; cited by
Florkin & Jeuniaux 1974). In Triatoma infestans, the haemolymph protein level rose from 1.5±0.8g% immediately after feeding to 4.8±0.8g% (after 14 day).

As in Hylophora cecropia (Telfer 1965) and Leptinotarsa decemlineata (DeLoof & DeWilde 1970 a,b), the majority of haemolymph proteins of these fleas have the same electrophoretic mobility as those in the yolk, yet the only fractions that gave precipitin reaction in immunoelectrophoresis were 10 and 11, suggesting their selective sequestration into the developing oocytes as has been shown in other insects (Bell 1970, Telfer 1954, 1960, 1965). Absence of these fractions in males and their precipitin reaction in female indicate them to be vitellogenins. The low concentrations of fractions 10 and 11 in rat blood-plasma or sucrose-fed fleas, where no yolk deposition was detected, indicate the pivotal role played by these fractions in vitellogenesis. It is also clear that initiation of vitellogenin synthesis is not under the control of feeding, because of the presence of fractions 10 and 11 in the haemolymph of pupa and unfed female fleas. As in the case of other haemolymph fractions, the concentrations of these two also increase after feeding. This is unlike that of anaautogenous mosquitoes where vitellogenin synthesis is initiated only after feeding (Hagedorn & Fallon 1973, Hagedorn 1985) and probably similar to the condition described in Musca (Kelly et al. 1986). Lipoglycoprotein nature of these fractions indicates their similarity to those of other insects (Wyatt & Pan 1978). However, histochemical studies have shown that the major yolk granules seen in the oocytes (vitellin) of fleas are a protein-polsaccharide complex (Prasad 1969). Techniques to expose lipid remaining in masked state in the major yolk granules (if any) gave negative results indicating that these granules are devoid of lipid moiety. Further, the triglyceride globules present in mature oocytes are not conjugated with any other biomolecule (Kamala Bai 1972). However, the fractions in the yolk corresponding to fractions 10 and 11 of the haemolymph were lipoglycoproteins. It appears that within the maturing oocytes, the sequestered vitellogenin undergoes modifications in its chemical composition. Reports show that in Philosamia cynthia, vitellogenin is richer in lipid than vitellin, particularly with reference to diacylglycerol and sphingomyelin (Chino et al. 1976). The vitellogenin lipid may be used to construct oocyte membranes or as energy source during oocyte development (Hagedorn & Kunkel 1979). The significance in ovarian development of the striking differences noticed in the biochemical profiles of the haemolymph fractions between and among in vivo and in vitro fed fleas is not clear and needs further study.

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