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Quantitative Variation of Lipids in Various Organs during Larval Development of Normal and Aposymbiotic *Chironomus barbatitarsis* (Diptera: Chironomidae)

A ISLAM and S ROY

Entomology Laboratory, Zoology Department, University of Burdwan, Burdwan 713104

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Ontogenetic variation of total lipids, phospholipids, fatty acids and cholesterol were analysed in the integument, gut, fat body and haemolymph during larval development of *Chironomus barbatitarsis* in normal and aposymbiotic conditions. The pattern of fluctuation of lipids appeared to be organ-specific and instar-specific. Total lipids level in gut and haemolymph showed an increasing trend from early to late periods; lipids level in the integument maintained more or less uniform levels throughout the moulting cycle and fat body lipid level depicted a positive fluctuations in relation to different physiological states of the moulting cycles. Considerable depletion of lipids was noted accompanying each moult. Aposymbiotic insects depicted declining levels of almost all lipids except increasing contents of fatty acids.

Key Words: Lipids, Larva, Moulting cycles, Aposymbiosis, *Chironomus* sp.

Introduction

Patterns of variation of different nutrients during post-embryonic development in insects have generated much interest among the workers in the past few decades (Gilbert & Schneiderman 1961, Bade & Wyatt 1962, Agrell 1964 and Islam & Roy 1981). This type of investigation facilitates interpetations of the balance of synthesis, storage and degradation in response to developmental needs. The importance of lipids in the metabolic processes and their role to provide energy for almost all endergonic processes along with their participation in the maintenance of structural and physiological integrity of cellular and subcellular organelles have prompted workers to investigate quantitative and qualitative variation of lipid contents during post embryonic developments (D'e Costa & Birt 1966, Gilbert 1967, Kim & Kyung 1975, Dunphy et al. 1977 and Pant et al. 1978). However, most of the investigators have considered the developmental profiles of lipids either in the whole insects or only in the fat body and haemolymph. The present work, reports the fluctuation of total lipids, phospholipid, fatty acid and cholesterol in the integument, the gut, the fat body and the haemolymph of *Chironomus barbatitarsis* during it's larval development.

As the intracellular bacteria in insects provide essential nutrients to the host, the present paper also elucidates the possible role of

symbiotic bacteria in the synthesis of lipids during the larval developmental periods.

Materials and Methods

All experiments were performed with an inbred stock of the fly, reared in the laboratory. The adults (females) were collected in the night and each kept separately in a vial containing 3ml presterilized water and a paper strip to serve as a substratum for oviposition After oviposition, egg masses were transferred to a petri dish. The larvae, hatched after an incubation period of 48 hr were reared in polypropylene tanks in a synthetic culture medium containing a thin film of mud (autoclaved), sterilized water, a pinch of yeast hydrolysate and fed with some algae. The larvae were classified into four instars on the basis of the length of head capsule (ventral) and basal segment of the mandible (Nandi 1981). Intermoult stages were classified into early, mid and late periods on the bases of chronological age and external characters. Durations of four larval instars were 4, 4, 5 and 12 days. In each case, the first day after the preceeding moult was considered as early, the last day as late and the intermediate days as mid instar periods. Regarding fourth instar larvae, 11th day was considered as late and 12th day as pharate pupa stage.

The larvae were rendered aposymbiotic by the incorporation of aqueous extracts of garlic (*Allium sativum*) and terramycin (Dey's Med. stores) in the culture medium. Garlic extract was prepared following the technique of Augusti and Mathew (1973). Thus three sets of culture media were maintained, viz. one without and additive one with 5%, 10% and 20% aqueous extract (v/v) of garlic and another with the same concentrations of terramycin. The treated larvae were allowed to complete one generation and a fresh generation was started from the eggs laid by the aposymbiotic adults. Experiments with the aposymbiotic larvae were carried out with the larvae of the second generation. For the detection of bacteria, larvae were rinsed in 0.1% $HgCl_2$ solution for 15 min and then homogenized in sterilized ringer solution. Occurrence of intracellular bacteria was confirmed by staining the total body homogenate smears in gram stain. Fluctuations in the number of bacteria harboured, in normal and aposymbiotic larvae, were determined by counting the number through a haemocytometer using a phase contrast microscope following the formula of Noda (1974).

Biochemical estimation of total larvae were done from second instar onwards and organ-specific estimations were carried out from third instar larvae. Larvae were dissected out under ringer solution. After dissection, the integument, the gut and the fat body from 5–8 larvae (depending upon developmental stages) were collectively considered for a single estimation. Haemolymph was collected in a graduated capillary tube from the excised prolegs and the pooled haemolymph from 9–14 larvae was used for each estimation. Dry-weights of the tissues were determined following standard method.

Total lipid was estimated by the sulphophosphovanilin method (Barnes & Blackstock 1973). For the estimation of phospholipids, ether:ethanol (1:3) was used for extraction and the method of Connetry et al. (1961) was followed. Total fatty acid was estimated following the photometric method of Chakrabarty et al. (1969). Total cholesterol was estimated by the method of Zak (1957). All experiments were replicated 7 times to minimize errors. Results are expressed as mg/100mg dry tissue weight and in case of haemolymph as mg/100 µl.

Results

Total lipid and other lipoid fractionse xhibited a gradual increase corresponding with the age

Table 1 *Relation of total lipids, phospholipids, fatty acids and cholesterol contents (mg/100mg dry weight) to age during larval periods in* C. barbatitarsis. *Data are mean±SE of 7 replications*

	Age in days	Total lipids	Phospholipids	Fatty acids	Cholesterol
2nd	1	9.21±0.4	1.91±0.1	0.19±0.04	0.29±0.06
Instar	2	10.19±0.6	2.32±0.2	0.25±0.07	0.38±0.03
	3	13.89±0.9	3.88±0.4	0.47±0.09	0.55±0.05
	4	15.21±0.4	4.12±0.8	0.59±0.08	0.48±0.02
3rd	1	13.45±0.9	3.92±0.3	0.47±0.03	0.34±0.08
Instar	3	17.56±0.9	6.68±0.5	0.72±0.07	0.48±0.04
	5	19.22±0.5	6.87±0.8	0.75±0.03	0.42±0.02
4th	1	15.91±0.3	5.36±0.1	0.54±0.06	0.35±0.05
Instar	2	18.59±0.6	6.14±0.5	0.83±0.07	0.48±0.03
	4	21.56±0.7	9.64±0.2	1.08±0 09	0 67±0.06
	6	25.29±0.7	11.81±0.8	1.33±0.07	0.88±0.07
	8	24.72±0.8	11.98±0.4	1.33±0.06	0.88±0·04
	10	23.96±0.9	11.11±0.9	1.29±0.08	0.79±0.03
	12	24.61±0.4	12.63±0.3	2.61±0.09	0.95±0.05

of the larvae considering the contents during the mid instar periods (table 1). Organ-specific variation showed highest lipid content in the fat body (32–43%) followed by the haemolymph (24–32%), the gut (14–29%) and the integument (8–12%) in that order. The highest phospholipid content (as % of total lipid of the organs concerned) was noted in the gut (42–85%) followed by the integument (40–70%), the fat body (42–62%) and the haemolymph (4–4.9%). The highest fatty acid contents was noted in the integument (8.8–11.9%) followed by the haemolymph (3.2–8.9%), the gut (3.3–4.6%) and the fat body (1.8–3.6%). Similarly cholesterol content was (1.8–4.8%) in the integument, 2.8–4.6% in the gut (2.2–3.6%) in the fat body and (1.8–2.7%) in the haemolymph.

Fluctuation of lipids within the moulting cycles also presented organ-specific variations. In the integument, total lipid content maintained at more or less uniform level throughout the development. Gut tissues depicted a steady lipid accumulation up to late developmental period in the third instar larvae, while in the fourth instar after attaining maximum concentration in the mid periods it maintained uniformity in rest of the periods. In the third instar lipid content in the fat body showed maximum concentration in the mid stadium, while in the fourth instar an increasing trend up to 8th day followed by a steady decline in the late periods was observed. In pharate pupa stage the lipid level in the fat body showed an increase while the haemolymph lipid level depicted declining level (figure 1 A).

A gradual increase in the level of phospholipid was observed up to late periods in the fourth instar larvae notwithstanding the maximum concentrations of phospholipids in the mid periods. The gut tissues showed a gradual increasing trend. In the fat body a considerable higher level was noted in fed mid instar

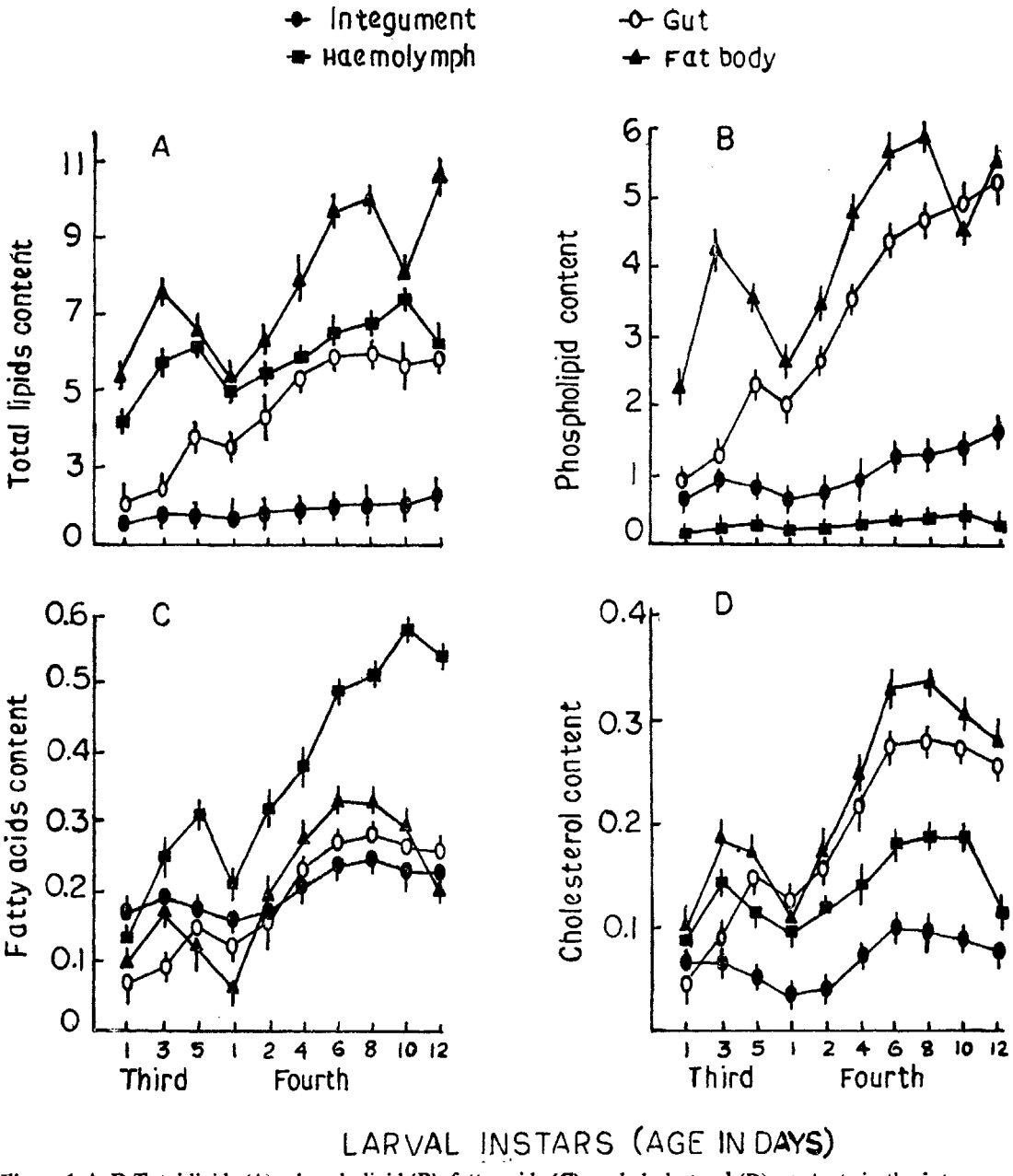

Figure 1 A–D Total lipids (A), phospholipid (B), fatty acids (C), and cholesterol (D) contents in the integument, the gut, the fat body and the haemolymph during third and fourth larval instars of *C. barbatitarsis*

periods followed by a sharp decline. In the pharate pupa, however it showed a sharp peak. The phospholipid content of the haemolymph remained more or less uniform throughout the periods (figure 1 B).

Fatty acid levels in the fat body and the integument were maximum during mid instars, while in the gut-tissues a gradual increase was observed in the third instar. In the fourth instar, after attaining highest level in mid instar periods, it showed a plateau up to pharate pupa stage. Fatty acid level in the haemolymph presented a gradual increase up to late fourth instar periods followed by a moderate decline in pharate pupa (figure 1C). Fatty acid contents in the haemolymph and the fat body showed significant decline during moulting.

Cholesterol in almost all organs was the highest during the mid instar period. In the fourth instar after attaining maximum level, the cholesterol contents remained uniform for sometimes and then declined in pharate pupa (figure 1 D).

Both 3rd and 4th instars showed rapid growth up to mid instars followed by a plateau and a slight decrease on the last day (table 2)

In aposymbiotic larvae, the number of bacteria/larva was notably lower; among the three doses 20% garlic extract and terramycin were found to be the most effective in reducing the number of bacteria/larva (table 3). The bacteria appeared to be gram-positive. In aposymbiotic larvae there was a marked decline of total lipids, phospholipid and cholesterol coupled with a slight increase of fatty acids. The aposymbiotic larvae failed to pupate.

Discussion

A gradual increase of total body lipid content and other lipoid constituents (as % of dry weight) in relation to larval age confirmed the reports of Perincott (1960), D'eCosta and Birt (1966), Mukherjee and Guppy (1973) and Islam and Roy (1981). Fluctuation of different lipids in various organs clearly shows that the synthesis, storage and degradation of lipids correspond with the specific dynamics of the developmental patterns of the concerned system. Rate of food consumption also affects lipid fluctuation during ontogenetic periods as has also been reported by Woodring et al. (1977).

Further, except for th e fat body, the lipid level in almost all organs progressively increased up to late periods due to constant influx from the food which resulted in a high rate of lipid accumulation. The moderate increase in the lipid level during post mid instars is suggestive of transformation of other food stuffs, particularly carbohydrates to lipids (Gilbert 1967). Marked depletion of lipids in the fat body and the haemolymph during moulting indicates that some of the stored lipids are utilized during apolysis for energy. The pharate pupa was characterized by high rate of lipid synthesis—lipid remaining stored mainly in the fat body and almost all other organs. Such a high level of lipids might be owing to rapid transformation of carbohydrates into lipids (Mukherjee & Guppy 1973) or release of all protein bound lipids at the onset of pupation (Gilbert 1967). Increased accumulation of lipids in the fat body and other organs in pharate pupa and also high rate of lipid utilization in this stage for various synthetic purposes collectively lowered the lipid levels in the haemolymph.

Declining levels of phospholipids in the fat body and the integument during late larval periods indicated phospholipid mobilization from these organs (Fast 1966). Gradual increase in the phospholipid content in the gut can be explained as a storage site of phospholipid. Increased phospholipid accumulation in almost all organs in pharate pupa stage indicated conservation of phospholipid prior to larval-pupal moult to be used up for various

Table 2 *Relation of total body wet weight and dry weight in third and fourth larval instars of* C. barbatitarsis. *Weights are expressed as mg±SE of 7 replications. Data within the parentheses represent the number of insects used in each replication**

Age in days	Third instar		Fourth instar	
	Wet wt.	Dry wt.	Wet wt.	Dry wt.
1	5.1±0.1 (25)	1.1±0.02 (25)	6.5±0.2 (25)	1.26±0.03 (25)
3	6.8±0.2 (26)	1.8±0.03 (22)	6.8±0.6 (22)	1.79±0.04 (22)
5	6.9±0.3 (25)	1.5±0.04 (25)	9.7±0.7 (25)	2.91±0.03 (25)
7	—	—	10.3±0.3 (29)	3.4±0.02 (29)
9	—	—	10.7±0.7 (26)	3.7±0.05 (26)
11	—	—	10.9±0.4 (22)	3.6±0.06 (22)
12	—	—	10.8±0.2 (21)	3.5±0.08 (21)

**Larval duration in case of third larval instar is 5 days and in case of fourth instar is 12 days*

Table 3 *Total lipids (LIP), phospholipid (PL), fatty acids (FA) and cholesterol (CHOL) contents (mg/100mg dry wt) in relation to the number of bacteria (BN, data × 10^7) in normal, garlic and terramycin treated* C. barbatitarsis *during third and fourth larval (mid) instars. Data are mean±SE of 7 replications*

		Third instar					Fourth instar				
		BN	LIP	PL	CHOL	FA	BN	LIP	PL	CHOL	FA
Control		65 ±1.8	17.56 ±0.6	3.92 ±0.2	0.47 ±0.02	0.34 ±0.05	71 ±1.6	25.29 ±0.7	11.81 ±0.4	1.33 ±0.9	0.88 ±0.06
	5%	7.5 ±0.1	12.86 ±0.7	1.82 ±0.04	0.27 ±0.01	0.89 ±0.06	8.3 ±0.08	15.89 ±0.5	8.99 ±0.6	0.46 ±0.03	1.22 ±0.07
Garlic treated	10%	6.1 ±0.5	12.09 ±0.3	1.82 ±0.07	0 21 ±0.01	0.93 ±0.09	7.4 ±0.04	15.28 ±0.4	8.62 ±0.2	0.41 ±0.02	0.29 ±0.05
	20%	4.1** ±0.06	10.79** ±0.2	1.39* ±0.03	0.19* ±0.01	0.95* ±0.05	5.4** ±0.06	14 07** ±0.8	8.31* ±0.9	0.33* ±0.02	1.34* ±0.03
	5%	7.2 ±0.06	12.79 ±0.8	1.84 ±0.03	0.26 ±0.01	0.89 ±0.08	8.1 ±0.06	15.88 ±0.9	8.97 ±0.5	0.45 ±0.04	1.21 ±0.06
Terra-mycin treated	10%	6.8 ±0.02	12.11 ±0.9	1.75 ±0.05	0.24 ±0.09	0.92 ±0.02	7.6 ±0.03	15.35 ±0.4	8.71 ±0.1	0.42 ±0.01	1.31 ±0.03
	20%	4.3** ±0.03	10.81** ±0.3	1.61* ±0.04	0.16* ±0.08	0.96* ±0.06	5·5** ±0.08	14.12** ±0.3	8.39* ±0.5	0.32* ±0.07	0 38* ±0.06

**P*<0.1; ***P*<0.05 *in comparison to control*

structural reorganisations at the early pupal life (Niemierko 1956).

Fatty acid contents showed a direct relationship to energy requirement of the larvae. Up to mid instar periods in both the fat body and the haemolymph the level increased initially after which an inverse relationship in the levels in the fat body and haemolymph was observed. In fact, in pre-mid instar periods, rapid influx from the food raised the level but in post mid instars, high rate of phospholipid mobilization from the fat body raised the haemolymph phospholipid level to meet the energy requirement (Turunen 1973).

Cholesterol levels in almost all organs appeared high in mid-instar periods followed by a plateau and decline in late periods. Increased accumulation of cholesterol in the first of respective moulting cycles corresponds with the feeding periods, because of it's dietary source. Relatively lower contents in the late moulting periods indicated mobilization of cholesterol for various structural reorganisation and synthetic activities.

Garlic extract is known as a potent antimicrobial agent (Augusti & Mathew 1973). The reduction in the number of bacteria/larva in garlic and terramycin-treated larvae and quantitative variation of lipids in these larvae suggest that intracellular bacteria play an important role in the metabolism of lipids (Brooks & Kringen 1972 and Brown & Chippendale 1975). Aposymbiotic larvae showed significant decrease in the phospholipid and the cholesterol content and simultaneous increase of fatty acids (table 3). Reduced level of phospholipid and increased fatty acid contents might indirectly be attributed to the lack of glucose utilization (Brooks & Kringen 1972 and Nogge 1975), since a blockage in transfer of glycolytic end products in the TCA cycle may result in the premature utilization of lipid reserves (Brown & Chippendale 1975). Any specific role of symbiotic bacteria in the metabolism of cholesterol still appears puzzling, however level of cholesterol in aposymbiotic larvae apparently indicates that symbionts are cholesterogenic in nature (Clayton 1970, Augusti & Mathew 1973 and Noda et al. 1979). It may be also possible that they help in the conversion of dietary sterol into cholesterol by supplementing some enzymes and this process might have been impaired in aposymbiotic larvae resulting in it's decreased level (Islam & Roy 1982*b*). Lack of pupation in aposymbiotic larva indicates the possible role of intracellular bacteria in the synthesis of specific protein (Brooks & Kringen 1972) or amino acids essential for normal growth and pupation (Islam & Roy 1982*a*).

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