

Aflatoxin B₁ Contamination of Shrimp Feeds and its Effect on Growth and Hepatopancreas of Pre-adult *Penaeus monodon*

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Abstract: A survey of aflatoxin B₁ (AFLB₁) levels in commonly used commercial shrimp finisher feeds in the Philippines showed a various range of values from not detected to 120 µg kg⁻¹ using high-performance thin-layer chromatography. Six experimental diets were prepared to contain various levels of AFLB₁ based on survey results to determine the effects of such contamination in pre-adult shrimp *Penaeus monodon* (17.5 ± 0.6 g). Results showed that shrimps fed diets containing AFLB₁ greater than or equal to 73.8 µg kg⁻¹ gave comparatively poor growth rate and higher susceptibility to shell diseases. No AFLB₁ residues were detected in sampled whole shrimp tissues after 62 days of exposure to AFLB₁ containing diets indicating a low potential for transmission of the toxin from edible shrimp tissues to consumers. Histopathological alterations in the hepatopancreas of shrimp chronically exposed to AFLB₁ were observed in all samples. The degree of alterations correlated with the level of AFLB₁. Based on growth performance, pre-adult shrimps can tolerate AFLB₁ levels of up to 52.3 µg kg⁻¹ in the feeds although histopathological changes were already evident in the tissues of shrimps given diets with 26.5 µg kg⁻¹ AFLB₁.

Key words: aflatoxin B₁, hepatopancreas, *Penaeus monodon*.

INTRODUCTION

The increased demand for shrimp in the world market has led to intensification of the shrimp industry wherein the animals depend exclusively on formulated feeds. This development led to the proliferation of commercial diets in the market. Shrimp farmers, being more concerned with seasonal availability of commercial feeds and fluctuations in prices rather than quality, buy the feeds in bulk without realizing the harmful consequences of improper storage and handling.

Storing feeds at high relative humidity (above 65%) and under unhygienic conditions (presence of insects) may result in the growth of aflatoxin-producing fungi, *Aspergillus flavus* and *Aspergillus parasiticus* (Smith and

Moss 1985). Wiseman *et al* (1982) reported that aflatoxin (AFL) could be produced *in situ* in penaeid feeds which are not properly stored. AFL contamination in animal feed is already a global problem which is more seriously affecting the warm areas in the tropics.

Developed countries have already set allowable levels of aflatoxin B₁ (AFLB₁) contents in animal feeds and feedstuffs. The limits imposed vary depending on the country, feedstuff and whether or not the commodity is destined for human or animal use. In the European Community (EC), the maximum limit of AFLB₁ content varies from 0 to 60 µg kg⁻¹ and 10-50 µg kg⁻¹ for human use and animal feeds, respectively (Jewers *et al* 1989). However, the value depends on both the age and kind of animal being fed as well as the type of feed (Jewers 1982). The US Food and Drug Administration action level for both human foods and animal feeds is

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20 $\mu\text{g kg}^{-1}$. In feeds that are used for breeding cattle, swine and mature poultry, a level of 100 $\mu\text{g kg}^{-1}$ AFLB₁ content is allowable (Task Force Report 1989).

AFLB₁, produced by *Aspergillus flavus* is of greatest concern in the Philippines because of its toxic and carcinogenic properties as well its frequent occurrence. Feed-stuffs which are commonly used as shrimp feed ingredients such as groundnut, cottonseed, coconut, Soya bean meal, corn, and cereals are highly susceptible to AFLB₁ contamination (Widiastuti *et al* 1988; Sutikno 1990). The allowable level of AFLB₁ in shrimp feeds has not yet been established. This study was therefore focused on determining the extent of AFLB₁ contamination in commercial shrimp feeds. The effects of AFLB₁ were monitored based on growth and histological structure of the hepatopancreas of shrimp. The functions of the crustacean hepatopancreas have been extensively studied and, because of its importance in the general body economy, physiological abnormalities caused by various disease agents are often visualised in this organ and its interstitial tissues (Johnson 1980; Lightner *et al* 1982; Vogt *et al* 1985; Baticados *et al* 1987). Present findings are aimed at establishing the tolerable limit of AFLB₁ in *Penaeus monodon* diets for pre-adult shrimps.

MATERIALS AND METHODS

Survey of AFLB₁ levels of commercial shrimp feeds in the Philippine market

Samples of commercial finisher shrimp feed were taken from feed mills and shrimp farms in different areas in Luzon (northern Philippines: Batangas, Quezon, Bulacan, Pampanga); Visayas (central Philippines: Cebu, Iloilo, Negros Occidental, Capiz); and Mindanao, (southern Philippines: Davao, South Cotabato, Agusan del Norte) for AFLB₁ analysis. Finisher pellets are given to shrimps with average weights of more than 15 g. Sampling was done during the months of August 1990 to February 1991 considered as rainy and cold months in the country.

Feed manufacture and preparation of AFLB₁ stock and standard working solution

Six batches of practical diets (1 kg of each) using the SEAFDEC formulation (Bautista *et al* 1992) were prepared. All dry ingredients were analysed prior to use and were found to be free from AFLB₁. The dry feed-stuffs, except bread flour, were mixed thoroughly in a Hobart mixer (Hobart Corporation, Troy, Ohio, USA) for 10 min. Preparation of AFLB₁ stock and standard working solutions was done following the methods described in AOAC (1984). Analytical reagent grade AFLB₁, purchased from Sigma Chemical Co (St Louis,

MO, USA) was prepared accordingly, mixed with the liquid ingredients, and then added to the dry ingredients to come up with final concentrations of 25, 50, 75, 100 or 200 $\mu\text{g kg}^{-1}$ AFLB₁ in the feeds. The sixth batch of feed was not contaminated with AFLB₁ solution and served as the control. The ingredients were mixed for another 5 min. Warm water (25 ml) was added to give a smooth consistency to the feed and mixing was continued for another 15 min. The bread flour was gelatinised at 100°C, allowed to cool, and added to the mixture to get an even consistency. The diet was extruded through a 3 mm pellet die in a Hobart meat extruder. The extruded moist pellets were dried in an oven at 40°C, for 5–6 h, cut into 3 cm lengths, and stored at 10–20°C in a well-ventilated hygienic room. All prepared feeds were analysed for proximate composition and AFLB₁ content.

AFLB₁ and proximate composition analysis

Proximate composition of the test feeds was performed using the methods described in AOAC (1984). AFLB₁ levels in commercial feed samples obtained during the survey and in those formulated for the feeding experiments were determined using the modified Natural Resources Institute phenyl bond-elution bi-directional high-performance thin-layer chromatography (HPTLC), a modified method of Coker *et al* (1984). At least 50 incremental samples per 5 kg feed or equivalent were obtained and ground finely with a mill grinder, then mixed in a rotary cascade divider, and extracted in 70% acetone in a shaker for 40 min. A 5 ml portion of the extract was applied to a phenyl substituted silica column in methanol/acetic buffer using lead acetate to clean up the column. The AFLB₁ absorbed in the column was eluted with 7.0 ml of chloroform and the eluate was dried at 45°C under nitrogen in a sample concentrator. The dry AFLB₁ film was dissolved in benzene/acetonitrile (98 : 2, v/v) and then readied for spotting. AFLB₁ standards and sample duplicates were precision spotted on HPTLC silica-coated plates with a minimum sensitivity level of 5 $\mu\text{g kg}^{-1}$. Spots were developed in the normal direction with chloroform/xylene/acetone (6 : 3 : 1, v/v). The area and height data were integrated by scanning of peaks to determine the fluorescent intensity of the standards and the sample spots. Calculation of AFLB₁ levels of the samples was made with reference to the standards. The same method was used to determine AFLB₁ in whole shrimp tissue that gave 98% recovery with 99% repeatability.

Biological assessment

Penaeus monodon (17.5 ± 0.6 g) were obtained from brackish water ponds in Leganes (Iloilo, Philippines). These animals were acclimatised in the laboratory for 1 week and stocked at 7 individuals/tank in 48 60-litre

rectangular tanks, provided with flowing sea water at a flow rate of 12 litres h⁻¹. Each treatment had eight replicates. Shrimps were fed twice daily (half of the ration at 09:00 to 10:00 h and other half at 16:00 to 17:00 h) at 5–6% of the total biomass for 62 days. Faeces and excess feeds were removed daily. Animals were weighed every 15 days. The exoskeletons removed during moulting were also weighed. The amount of ration was adjusted accordingly. Changes in physical appearance and behaviour of the shrimp were monitored weekly. Water salinity and temperature were maintained at 31–32 ppt and 28–32°C, respectively. Water pH, nitrite-nitrogen, and ammonia nitrogen were monitored weekly (Strickland and Parsons 1972).

Histological analysis

Five shrimps from each treatment were taken every 15 days for histological analysis. Samples were injected with Davidson's fixative. The cephalothorax region was longitudinally bisected, dehydrated in alcohol series, and embedded in paraffin. These were then cut into 3–4 µm sections. Tissues were stained with haematoxylin and eosin (Humason 1972) and examined by light microscopy following structural illustrations and terminologies of Johnson (1980), Vogt *et al* (1985), and Bell and Lightner (1988).

Statistical analysis

Treatment means for growth were tested for significance using analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) (Gomez and Gomez 1984) with SPSS software. Differences were deemed significant at $P < 0.05$.

RESULTS AND DISCUSSION

AFLB₁ levels of commercial and experimental shrimp feeds

A total of 62 shrimp samples taken randomly from different feed mills and shrimp farms in Luzon, Visayas, and Mindanao had AFLB₁ levels of not detected (ND) to 120 µg kg⁻¹ (Table 1). Higher levels of AFLB₁ (60–120 µg kg⁻¹) were found in samples taken from shrimp farms in Quezon and Batangas, both from the Luzon area, due to improper handling and storage of the feed. The feeds came from shrimp farms far from the main road so that feeds had to be transported using motorised boats. This means of transportation increases the chances of accidentally spilling water into the boat and wetting the feeds. This factor could have contributed to the development of AFLB₁-producing fungi in the feed. Storing feeds with high moisture content (>10%) at high relative humidity (>65%) may result in the growth

TABLE 1
Survey results of aflatoxin levels of commercial shrimp fisher feeds in the Philippines (values are number of feed samples)

Area	AFL levels (µg kg ⁻¹) ^a			
	ND ^b	10–20	30–40	60–120
Luzon				
Batangas		2	2	
Quezon		2	2	
Bulacan		2	3	
Pampanga		3	2	
Visayas				
Cebu		4	2	
Iloilo		3		
Negros		5		
Occidental				
Capiz		4	2	
Mindanao				
Davao		4	2	
South		4	2	
Cotabato				
Agusan del Norte	—	3	2	—
Total	3	36	21	2

^a Average of two replications/treatment.

^b ND, not detected.

of AFLB₁-producing fungi (Smith and Moss 1985). Furthermore, proper storage procedures were not practised in those farms. Poor ventilation resulted in an increase in room temperature causing rapid condensation of water in the feed bags. (Wiseman *et al* 1982) reported that AFB could be produced *in situ* in penaeid feeds which are not properly stored especially under warm and humid conditions. The risk potential for AFLB₁ formation in a tropical country such as the Philippines is considered doubly alarming because of the prevailing high temperature and relative humidity which favour fungal growth (Garcia 1987).

AFLB₁ contents of the experimental diets are shown in Table 2. The analysed values of AFLB₁ were very close to the intended amount that was incorporated during diet preparation. This shows the precision of mixing AFL and feed that was employed during feed preparation.

Biological assessment

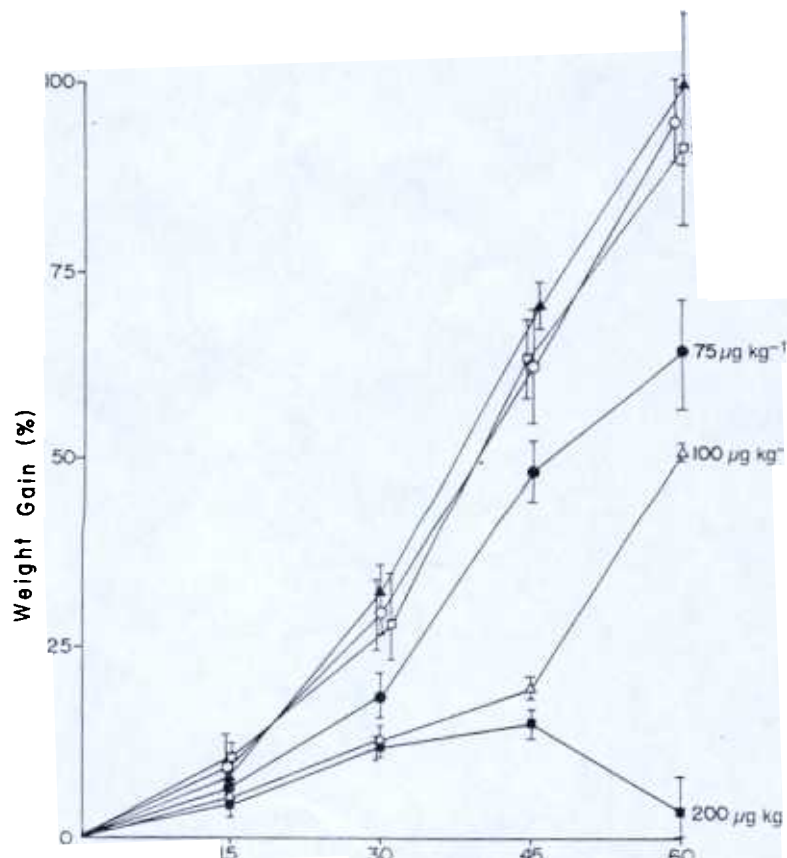
Shrimps fed diets containing 0, 26.5 or 52.3 µg kg⁻¹ levels of AFLB₁ gave significantly higher growth compared with those given the 73.8, 100.8 or 202.8 µg kg⁻¹ AFLB₁ (Fig 1). Weight gain started to slow down at day 30 for shrimps fed diets with 73.8, 100.8 or 202.8 µg kg⁻¹ AFLB₁. Shrimp fed a diet with 202.8 µg kg⁻¹ AFLB₁ started to lose weight after 45 days. However, the final average weight gain was still

TABLE 2
Proximate composition and AFLB₁ content of experimental diets

AFLB ₁ content	Diet					
	1	2	3	4	5	6
AFLB ₁ , $\mu\text{g kg}^{-1}$ (amount incorporated in the diet)	0	25	50	75	100	200
AFLB ₁ , $\mu\text{g kg}^{-1}$ (amount after analysis)	0	26.5	52.3	73.8	100.8	202.8
Nutrient	Proximate composition (%)					
Moisture	4-5					
Crude protein	40-41					
Crude fat	12-13					
Crude fibre	3.0					
Nitrogen-free extract (NFE)	31-33					
Ash	11.0					

positive, although it was significantly lower than the other treatments. No AFLB₁ residues were detected in whole shrimp tissues after the feeding trial. The non-detection of AFLB₁ residues after the feeding experiments was attributed to either low dosage of

incorporation of AFLB₁ to the feed samples to cause retention in the body or this AFLB₁ could have been converted to other metabolites or by-products which are less toxic and could have been excreted by the shrimp. Divankaran *et al* (unpubl) observed histopatho-



logical changes in the hepatopancreas and antennal gland of *Penaeus vannamei* fed diets with 25–2500 $\mu\text{g kg}^{-1}$ AFLB₁ contamination but they did not detect the same toxin in shrimp tissues at the end of the experiment. This result indicates a very low potential for AFLB₁ transmission from edible shrimp tissues to humans.

Animals that were exposed to a higher AFLB₁ diet showed lower resistance and higher susceptibility to shell disease, as shown by the many lesions on the exoskeleton. Discolorations in the ventral part of the exoskeleton were already evident during the third week of rearing in shrimps fed diets containing 73.8, 100.8, or 202.80 $\mu\text{g kg}^{-1}$ AFLB₁. The body and appendages changed from whitish to yellowish and eventually had a reddish discoloration. Faecal matter turned from brownish to brownish red. These changes were not seen in shrimps fed diets with 0, 26.5, or 52.3 $\mu\text{g kg}^{-1}$ AFLB₁ even during the last weeks of rearing. The slow growth of shrimp fed diets containing 73.8, 100.8, or 202.8 $\mu\text{g kg}^{-1}$ AFLB₁ might be due to alteration of some nutrient metabolism and might have imposed nutritional stress on the animal that resulted in reduced feed intake. Based on growth performance, pre-adult shrimps were able to tolerate AFLB₁ levels of up to 52.3 $\mu\text{g kg}^{-1}$ in the feeds although histopathological changes were already evident in the shrimp tissues given diets with 26.5 $\mu\text{g kg}^{-1}$ AFLB₁.

Histological observations

Significant histological alterations in the hepatopancreas of shrimps chronically exposed to AFLB₁ through the experimental diets were observed after 62

days of feeding, especially in shrimps fed 73.8, 100.8, or 202.8 $\mu\text{g kg}^{-1}$ AFLB₁, where various degrees of inflammation were observed. Mild inflammatory response was also observed in shrimps given 52.30 $\mu\text{g kg}^{-1}$ AFLB₁. With 26.5 $\mu\text{g kg}^{-1}$ AFLB₁ in the diet, the only observed hepatopancreatic structure alteration was in the form of reduced storage R-cell vacuoles.

The normal shrimp hepatopancreas is organised in a tubular pattern (Bell and Lightner 1988). The differences in the height and structure of the epithelial cells lining the tubules account for the four- to five-pointed star appearance of the tubule lumen (Fig 2). This organ has been observed to be very sensitive to different diets (Vogt *et al* 1985; Bautista *et al* 1992) and water-borne pollutants (Baticados *et al* 1987; Vogt 1987; Baticados and Tendencia 1991), thus it is used as a monitor organ to determine the effects of various toxicants. The histopathology of acute and subacute aflatoxicosis in *Penaeus stylirostris* and *Penaeus vannamei* was investigated by Lightner *et al* (1982) who found that the principal lesions occur in the hepatopancreas and the mandibular organs of these shrimps.

At 26.5 $\mu\text{g kg}^{-1}$, a reduction in the lipid reserves in the hepatopancreas as a result of R-cell atrophy was observed. R-cells have been found to be the most readily and severely affected cell type in the tubular epithelia of the hepatopancreas (Vogt 1990). However, no inflammatory responses were observed in the hepatopancreas of shrimps in this treatment. At 52.3, 73.8, 100.8, or 202.8 $\mu\text{g kg}^{-1}$ AFLB₁ level of incorporation in the diet however, dose and time-related inflammatory responses were observed in addition to epithelial cell atrophy. The earliest sign of inflammation was haemocytic infiltration in the interstitial spaces in between the

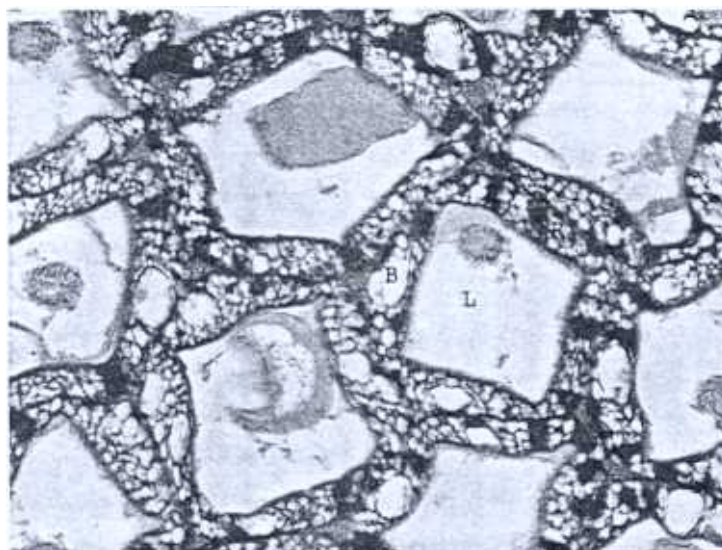


Fig 2. Transverse section through the medial region of the hepatopancreas of a shrimp given a diet without aflatoxin. The tubules are lined by well-vacuolated secretory (B), and absorptive and storage (R) epithelial cells. B, Blasenzellen or B-cells; R, Restzellen or R-cells; L, lumen. The heavily stained non-vacuolated cells are Fibrillenzellen or F-cells (H & E stain, original magnification $\times 200$).

tubules (Fig 3). More advanced lesions were in the form of fibrosis and melanized encapsulation of necrotic tissues, either in the tubule itself or the sinuses around it (Figs 3 and 4). These lesions were most common in the proximal region of the hepatopancreas conforming to the observations of Lightner *et al* (1982) that subacute and acute aflatoxicosis in *P. stylirostris* and *P. vannamei* were expressed as necrosis in the hepatopancreatic tubule epithelium that proceeded from the proximal portion to the peripheral tubule tips. This study however, used significantly lower levels of AFLB₁ which was introduced with the daily feed ration. Expectedly, the hepatopancreatic lesions observed were not severe.

The alterations in the tubular structure of the hepatopancreas will directly affect its absorptive, storage, and secretory functions such that severely atrophied and inflamed organs may not be expected to perform normally. The growth of shrimps fed diets with 73.8, 100.8, 202.8 $\mu\text{g kg}^{-1}$ AFLB₁ was significantly lower because of the prominent lesions that have caused a dysfunction.

These results demonstrate in part the effects of chronic exposure of *P. monodon* to AFLB₁. Growth of shrimps exposed to 0, 26.5, or 52.3 $\mu\text{g kg}^{-1}$ AFLB₁ were not significantly different (Fig 1). However, the presence of structural alterations in the R-cell at 26.5

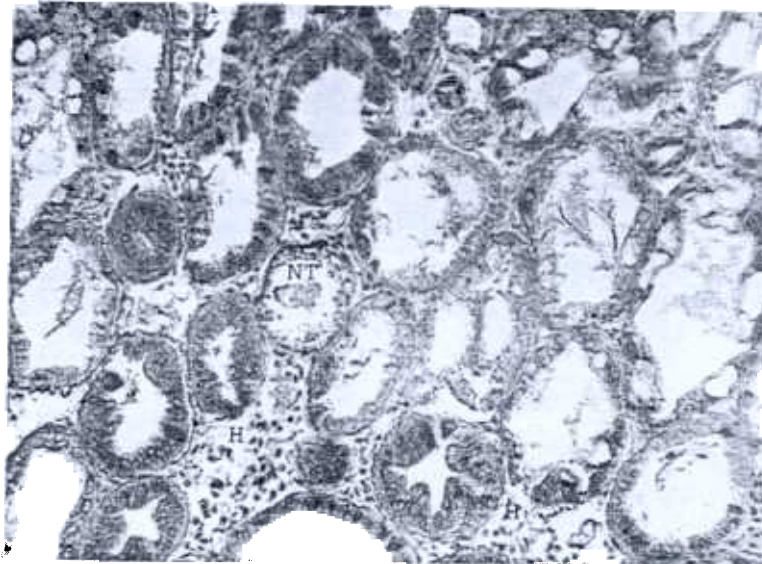


Fig 3. Section of the hepatopancreas of shrimp fed a diet with 200 $\mu\text{g kg}^{-1}$ AFLB₁. Some tubules are surrounded by aggregating haemocytes (H) in the interstitial sinuses. A necrotic tubule (NT) loses its epithelial cells and contain tissue debris in the lumen (H & E stain, original magnification $\times 100$).

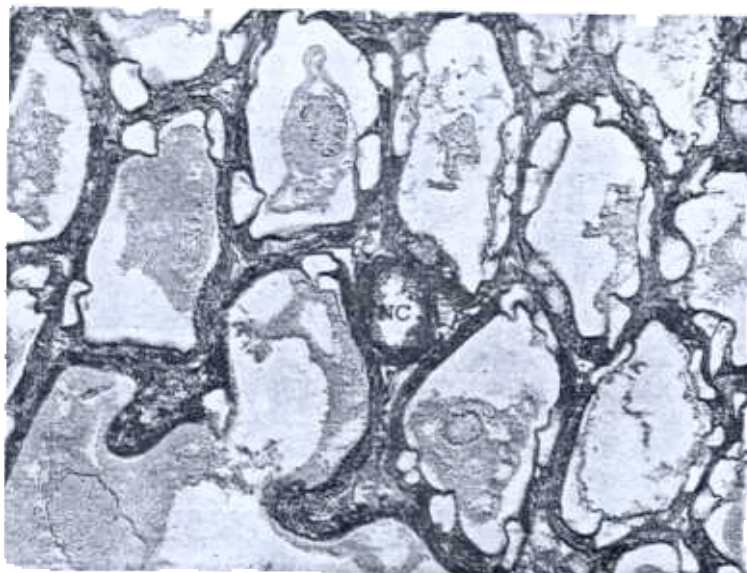


Fig 4. Section of the hepatopancreas of shrimp fed a diet with 100 $\mu\text{g kg}^{-1}$ AFLB₁ showing atrophied R-cells containing no storage vacuoles. The necrotic centre (NC) in the interstitial sinus is gradually being encapsulated by fibre cells and melanin (H & E stain, original magnification $\times 200$).

and 52.3 $\mu\text{g kg}^{-1}$ AFLB₁ levels after 62 days of feedings, warrants further study of the long-term effects of chronic exposure to low levels of AFLB₁. The findings stress further the importance of histology as a tool in determining the nutritional quality of a diet. From the practical point of view, these indicate that feeding of shrimps with diets containing up to 52.3 $\mu\text{g kg}^{-1}$ AFLB₁ will not significantly affect the growth of culture shrimps after 62 days. Survey results showed that 92% of commercial shrimp finisher feed samples had AFLB₁ levels of 40 $\mu\text{g kg}^{-1}$ or below indicating an acceptable margin of safety for the users. This level of contamination, however, should warn the farmers about proper handling and storage so that no further increases in AFLB₁ levels will occur in their feeds.

CONCLUSION

Based on growth performance, pre-adult shrimps (average weight = 17.5 \pm 0.6 g) are able to tolerate AFLB₁ levels of up to 52.3 $\mu\text{g kg}^{-1}$ in the feeds although histopathological changes are already evident in the tissues of shrimps given diets with 26.5 $\mu\text{g kg}^{-1}$ AFLB₁. This shows that histology is an important component in assessing the value of feeds because the effects that are not expressed at the organism levels can already be detected in the tissues. Survey results of commercial shrimp feeds showed that 92% contained 40 $\mu\text{g kg}^{-1}$ and below indicating an acceptable though narrow margin of safety for the users. Shrimp farmers should be careful about proper storage and handling of the feed in order not to have AFLB₁ contamination related problems.

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