Histological alterations in the hepatopancreas of *Penaeus monodon* Fabricius (1798) given aflatoxin B₁-incorporated diets

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**Abstract**

Aflatoxin is a toxic contaminant produced by toxigenic fungi of the genus *Aspergillus* during the processing and storage of feeds and feed ingredients. Aflatoxins can cause abnormalities such as poor growth, physiological imbalances and histological changes that result in a reduction in the yield and profitability of shrimp culture. Histological changes in *Penaeus monodon* sub-adults fed different doses of aflatoxin B₁ were studied. The doses of aflatoxin B₁ administered in the diets were 50, 100, 150, 500, 1000 and 2000 ppb. At the end of the fourth and the eighth weeks of the experiment, the shrimps were sampled and the cephalothorax was observed for histological changes. Significant changes were observed in the different treatment groups at the fourth and eighth weeks. The severity of pathological changes was proportional to the increase in the concentration of aflatoxin fed to the shrimps. Histological changes in the hepatopancreas were loss of structure of the cells and tubules, nodule formation, cell elongation, desquamation, rounding of cells, fibrosis, necrosis, haemocytic infiltration and cellular inflammation.

**Keywords:** aflatoxin B₁, AFB₁ incorporated feed, *Penaeus monodon*, hepatopancreas, histological changes

**Introduction**

Aflatoxins are particularly important in aquaculture because their presence exerts a negative economic impact on relevant commerce as well as severe health problems after exposure to contaminated food and feed. Among the mycotoxins, aflatoxins are the most toxic and are of considerable interest in the fields of agriculture, livestock and aquaculture. Aflatoxins are extremely biologically active secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus*. Aflatoxins are receiving increasing attention from researchers, the food industry and the general public — firstly because they reduce production and secondly they remain as residues in animal tissues, which in turn affects the human metabolic system on being consumed.

To date, only three species of the fungi have been reported to produce aflatoxins. These are *A. flavus*, *A. parasiticus* and *Pencillium tuberculum*. The toxins produced by moulds are broadly classified as nephrotoxins, hepatotoxins and neurotoxins depending on the haematological effects and general digestive disorders they cause. Aflatoxin comes under the category of hepatotoxins and targets its activities mainly on the liver (Spensley 1963). Aflatoxins are polycyclic unsaturated compounds with a coumarin molecule flanked on one side by a bisfuran moiety and on the other by either a pentanone for B series or a six-membered lactone for G series (Coulombe 1991).

The culture of *Penaeus monodon* (Fabricius 1798) is constantly hampered by outbreaks of bacterial, viral and/or parasitic diseases and also by environmental and nutrition-related diseases. One such constraint is the disease caused by fungal contamination of feed that often causes secondary infections. Experimental studies of aflatoxicosis in shrimps were restricted to *Penaeus vannamei* (Lightner, Redman, Price & Wiseman 1982), *P. stylirostris* (Wiseman, Price, Lightner & Williams 1982; Ostrowski-Meissner, LeaMaster, Duerr & Walsh 1995) and *P. monodon*, the most cultivated penaeid shrimp in India and...
elsewhere (Boonyaratpalin, Supamattaya, Verakun-piriya & Suprasert 2001).

Only a few species of crustaceans like Brine shrimp, *Artemia salina* (Harwig & Scott 1971), copepod, *Cyclops fuscus* (Reiss 1972), and water flea, *Daphnia pulex* (Sinnhuber & Wales 1978), have been tested for aflatoxin sensitivity. Red disease or discoloration, the prominent diagnosis of aflatoxicosis, was first reported in *P. monodon* by Liao (1977) in Taiwan; the principal lesion types observed were marked atrophy and necrosis of the hepatopancreas, accompanied by an intense cellular inflammatory response. Histological observations in shrimps fed with a diet containing aflatoxin (150 and 200 g·g⁻¹ of feed) indicated severe damage to the hepatopancreas (Cruz & Tendencia 1989). The histopathological alterations in *P. stylirostris* and *P. vannamei* were time and dose dependent in the hepatopancreas, mandibular organ and in the haematopoietic organs (Lightner et al. 1982). Juvenile *P. vannamei* fed 50–300 ppm aflatoxin B₁ showed lesions in the hepatopancreas, mandibular organ and haematopoietic organ. Lavilla-Pitogo, Bautista and Subosa (1994) observed histopathological changes in *P. monodon* juvenile fed AFB₁ (26.5–202.8 ppb for 60 days).

Ostrowski-Meissner et al. (1995) observed growth rate and sub-lethal effects in *P. vannamei* juveniles fed AFB (3–15 ppm). Boonyaratpalin et al. (2001) observed atrophic changes and necrosis in hepatopancreas tubules in *P. monodon* fed 50–2500 ppb AFB₁ for 60 days. Kalaimani, Ali, Shanmugasundaram and Sarathchandra (1998) have reported the presence of aflatoxin in imported and indigenous shrimp feeds in the range 10–130 ppb collected from shrimp farms in Andhra Pradesh in India. The USFDA has regulated the levels of AFB₁ in food commodities to be processed into foods and has established an action guideline of 20 ppb for total aflatoxin (FDA 1989). The objective of the present study was to determine the histological changes caused by feeding different doses of AFB₁-incorporated diets in *P. monodon*.

### Table 1 Dosage of AFB₁ in shrimp feeds for study on histological changes in *Penaeus monodon*

<table>
<thead>
<tr>
<th>Doses of AFB₁ (ppb)</th>
<th>Working solution of AFB₁ (mL)</th>
<th>AFB₁ in feed per 500 g (μg)</th>
</tr>
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<tbody>
<tr>
<td>50</td>
<td>0.125</td>
<td>25</td>
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<tr>
<td>100</td>
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<td>1000</td>
<td>2.5</td>
<td>500</td>
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<td>2000</td>
<td>5</td>
<td>1000</td>
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### Materials and methods

#### Experiment protocol

Two hundred sub-adult *P. monodon* of size 7.5 ± 0.72 g brought from a farm at Narakkal, Ernakulam district, Kerala, were acclimatized to 20 ± 0.5 g·L⁻¹ salinity for 1 week in holding tanks of 2-tonne capacity. One control and six treatment groups were selected for the experiment of 60 days duration. The doses of aflatoxin selected were 0, 50, 100 ppb, 150, 500, 1000 and 2000 ppb (Table 1). Shrimps were weighed and about 26 were segregated into separate 1-tonne fibreglass-reinforced plastic tanks of 2 m length, 1 m width and 0.5 m depth. The shrimps stocked in one tank were taken as one treatment, and after 4 weeks of feeding AFB₁, 13 shrimps were sacrificed for analysis and the rest of the 13 were sacrificed at the end of the experiment. Three shrimps from each group were taken for normal histological analysis at the start of the experiment, and samples were made in triplicate at the fourth and the eighth weeks for individuals exposed to each dose.

#### Experimental diets

**Preparation of a stock solution and a working solution of aflatoxin B₁**

A pure crystalline powder of aflatoxin B₁ was obtained from Sigma chemical company, St Louis, MO, USA (Product Name A6636). Aflatoxin B₁ (50 mg) was dissolved in 5 mL of chloroform to form a stock solution containing 10 mg AFB₁/mL of chloroform. From this, a working solution was prepared by adding 1 mL of the stock solution to 49 mL of chloroform (10 mg aflatoxin in 50 mL of chloroform). The working solution was stored in amber-coloured bottles sealed tightly with teflon and cello tape and stored under refrigeration. Before addition of the toxin to experimental feeds, the required amount of toxin dissolved in chloroform from the working solution was taken in a glass beaker, evaporated in a water bath and replaced with equal volumes of ethanol.

**Feed formulation and preparation**

The feed ingredients chosen for preparing the experimental diets were fish meal, shrimp meal, clam meal, soya bean meal as protein sources, wheat flour as a
carbohydrate source and cod liver oil as a lipid source. Moisture, crude protein and crude fibre levels in the feed and feed ingredients were determined as per AOAC (1990). Feed formulation was carried out using the standard protocol of AOAC (1990). Shrimp feed formulation containing 38% crude protein was used to prepare experimental diets (Table 2). All the feed ingredients were taken to prepare 500 g each of seven types of feed viz, control diet, and six test diets: 50, 100, 150, 500, 1000 and 2000 ppb.

### Data collection

The tanks were labelled with a clipboard indicating details of the experiment such as dose of aflatoxin and day of experiment for record maintenance. The tanks were monitored daily for any unusual behaviour and feeding activity. The feeding rate was 4.2% for the first 30 days and 3.5% for next 30 days. Small amounts of feed were added at a time to ensure complete feeding to avoid feed wastage and contamination. The data collected from each group and replicate tanks included feed given and consumption, mortality and salinity, temperature and dissolved oxygen in the water. Water exchange was carried out daily at a 50% level. Water quality, temperature, dissolved oxygen and salinity were monitored daily. The salinity was maintained at 20 ± 0.5 g L⁻¹, the temperature at 26 ± 2 °C and the dissolved oxygen at 5 ± 2 mL L⁻¹ for the entire experimental duration. The shrimps were observed for any unusual behaviour and morphological changes. The cephalothorax region was used for histological study.

### Histology

Three cephalothoracic regions from each treatment were fixed in Davidson’s fixative overnight at the fourth and the eighth weeks of the treatment. Tissues were processed for paraffin embedding in a Leica TP 1020 automatic tissue processor (Leica Microsystems, Nussloch, Germany) and sections of 5–6 μm thickness were cut in a Leica RM 2145 semi-automatic rotary microtome. Longitudinal and transverse sections of the tissues were taken. The paraffin sections taken on glass slides were cleared in xylene, hydrated with descending grades of alcohol, stained in haematoxylin, passed through acid alcohol, Scott’s top water and then stained by eosin. The stained sections were dehydrated in ascending grades of alcohol, cleared in xylene before mounting with DPX and observed under a light microscope (Leica DMLS).

### Results

The cephalothorax of the control shrimp revealed normal architecture. Hepatopancreas sections alone showed drastic changes in the treatment groups. The lymphoid organ, mandibular organ and antennal gland revealed only mild lysis. The hepatopancreas was selected for detailed examination because it was the most affected target organ.

In the control group, the hepatopancreas was normal (Fig. 1) along with the proximal and the apical regions, specific components of the stomach and midgut namely, the gastric sieve and the lappets. The lumen contained a granular material and the lumen-facing surface of the tubule was covered with a microvillus border. The tubular apex contained undifferentiated embryonic cells (E cells). Proceeding away from the apex, the cells began to differentiate into storage cells (R cells/Restzellen). In the median region, R cells and F cells (Fibrillazellen) were observed. Those F cells farthest from the tubular apex were more basophilic and larger than those nearest the apex. F cell nuclei were larger than those of R cells and each typically contained one prominent nucleus. The cytoplasm of R cells characteristically contained numerous nuclei. The proximal region of the tubule...
contained large distinctive secretory cells (B cells/Blasenzellen), each of which contained one larger vacuole and a convex luminal surface. The lymphoid organ and the antennal gland in the control group revealed normal structures.

In the 50-ppb treatment group, at 4 weeks, there was lysis of tubules and loss of architecture in a few areas, a change in the structure of tubules, loss of brush-border appearance (arrow head), H&E × 200. TS, tranverse section; H&E, haemotoxylin and eosin.

Figure 1 Tranverse section (TS) of the hepatopancreas of the shrimp in the control group. Note the normal structure of tubules, lumen and cells. H&E × 100. H&E, haemotoxylin and eosin.

Figure 3 TS of hepatopancreas of 50 ppb aflatoxin B₁-fed shrimp at 4 weeks. Note the haemocytic nodule formation in the tubules (arrow), H&E × 200. TS, tranverse section.

Figure 2 TS of hepatopancreas of the shrimp at 4 weeks given 50 ppb aflatoxin B₁, revealing a change in structure of tubules (arrow) and loss of brush-border appearance (arrow head), H&E × 200. TS, tranverse section; H&E, haemotoxylin and eosin.

Figure 4 TS of hepatopancreas of shrimp given 50 ppb aflatoxin B₁ at 8 weeks, showing desquamation of tubules (arrow) and loss of cells (arrow head). H&E × 200. TS, tranverse section; H&E, haemotoxylin and eosin.

Figure 5 TS of hepatopancreas of 100 ppb aflatoxin B₁-treated shrimp at 4 weeks revealing cellular inflammatory response (arrow head) and loss of cells (arrow). H&E × 200. TS, tranverse section; H&E, haemotoxylin and eosin.

and necrotic cells in the lumen. There was desquamation, thickening of intertubular tissue and haemocytic infiltration in the hepatopancreas after 4 weeks. Formation of peculiar elongated cells and destruction of E cells were observed. R cells had almost disappeared in some areas. After 8 weeks, severe necrosis,
loss of tubules and fibrosis were found (Fig. 6). There was reduction in the number of R cells and B cells and rounding of cells in the hepatopancreas.

In the shrimps treated with 150 ppb AFB1, after 4 weeks, there was complete loss of the structure of cells and tubules of the hepatopancreas. Necrotic cells were seen in the lumen and loss of brush-bordered appearance. Complete detachment of cells was observed. There was degeneration of focal areas and beginning of fibrous tissue growth (Fig. 7). After 8 weeks, necrosis and lysis became extensive; more fibrous growth (Fig. 8) and desquamated cells were noticed in the lumen; and an inflammatory reaction was observed in between the lobules.

Shrimps fed with 500 ppb AFB1 after 4 weeks showed extensive fibrosis, degeneration, cell elongation and loss of cells in the distal end of the hepatopancreas (Fig. 9). After 8 weeks, the hepatopancreas showed destruction of the tubular structure in the distal region and necrosis (Fig. 10). In the shrimps dosed with 1000 ppb AFB1, fibrosis, necrosis and degeneration were intense (Fig. 11). Inflammatory reaction and cell elongation were the peculiar features observed in the hepatopancreas. After 8 weeks, many lumen interconnections, cystic hyperplasia and dilation were noticed. There was necrosis of the antennal gland.
and an inflammatory reaction in the lymphoid organ.
Other changes observed in the hepatopancreas were fibrous tissue growth around the tubules, haemocytic infiltration and cell detachment (Fig. 12).
Shrimps fed with 2000 ppb AFB1 histologically showed severe necrosis, extensive fibrosis, fibrous tissue growth, haemocytic infiltration (Fig. 13) and intense papillomatous growth in the hepatopancreas after 4 weeks. After 8 weeks, a cellular inflammatory response was observed. There was severe necrosis and complete loss of architecture of the entire focal area (Fig. 14). Fibrous tissue growth had replaced the tubules and cells. Apoptosis or rounding of cells was also observed in a few areas.

**Discussion**
The histological analysis of the cephalothoracic region of the control shrimps conformed to the structure described by Bell and Lightner (1988). Histological study of the cephalothoracic region of the AFB1 treatment groups revealed progressive damage to the hepatopancreas with increasing concentration of aflatoxin B1, with only mild changes in the lymphoid organ and the antennal gland. The midgut gland or the hepatopancreas is considered to be the central organ of digestion in crustaceans. It is a system of blind-ending tubules consisting of four cell types (Loizzi 1971). The E cells at the summit of the tubules develop into R cells (absorption and storage of nutrients), F cells (production of digestive enzymes) and B cells (presumed to be secretory in function).

Besides, the effect of aflatoxins on the hepatopancreas appears to be directly correlated with the concentration of aflatoxins and the duration of feeding. The experimental study clearly shows that high doses of AFB1 are detrimental to the shrimps as the changes in the hepatopancreas were severe and intense like complete fragmentation, apoptosis,
inflammation and desquamation. Smaller doses of the toxin (50 and 100 ppb) induced the onset of necrosis and fibrosis around the tubules of the hepatopancreas, and slight necrosis in the antennal gland. It is evident that even with mild doses of AFB1, hepatopancreas becomes damaged.

The first sign of toxicity observed in the present investigation was the atrophy of hepatopancreatic tubules, followed by the destruction of E, R and B cells, desquamation, cellular inflammation, papillomatous growth, apoptosis in a few areas, necrosis and infiltration of fibroblastic tissue between the tubules of the hepatopancreas. Only mild necrosis was observed in the antennal gland, the gills and the lymphoid organ. Similar changes were reported in penaeid shrimps fed AFB1 (Lavilla-Pitogo et al. 1994; Boonyaratpalin et al. 2001). As observed by Boonyaratpalin et al. (2001), AFB1 levels above 100 ppb caused inflammation, necrosis, severe degeneration of tubules and infiltration of haemocytes. By the end of 4 weeks, there were histological changes in the hepatopancreatic tissues in all the shrimp fed over 50 ppb AFB1. In the shrimps given the doses of 150 and 500 ppb, the distinct changes were necrotic cells in the lumen, loss of architecture of cells and tubules, extensive necrosis and an inflammatory reaction in between tubules. In the 1000 and 2000 ppb groups, cyst-like and papillomatous growth was observed, along with severe necrosis, cell elongation, inflammatory cells, cystic hyperplasia, haemocytic infiltration, complete loss of architecture of tubules, rounding of cells or apoptosis, desquamation and a cellular inflammatory response. Lightner et al. (1982) reported that the smallest dosage of 2 ppm in P. stylirostris and P. vannamei resulted in a low detectable hepatopancreatic lesion and doses higher than 2 ppm (2000 ppb) resulted in distinctive histopathologic alterations in the hepatopancreas and mandibular organs.

In P. monodon fed 26.5–202.8 ppb AFB1 for 60 days, the first response was in the hepatopancreas and atrophy of R cells (Lavilla-Pitogo et al. 1994). Penaeid shrimps fed 50–300 ppm AFB1 showed primary lesions in the hepatopancreas, mandibular organ and haemopoietic organs (Wiseman et al. 1982). In the present study, the doses ranging from 50 to 2000 ppm were much lower than the LD_{50} value of AFB1 reported for P. stylirostris and P. vannamei (Lightner et al. 1982); hence, the histological changes were targeted mainly on the hepatopancreas, while mild necrosis was noticed in the mandibular organ, lymphoid organ, antennal gland and haemopoietic organ.

Aflatoxin-related histopathologies were apparent in the hepatopancreas and antennal gland, when experimental diets containing 0–15 ppm AFB1 were given to juvenile P. vannamei for 8 weeks (Ostrowski-Meissner et al. 1995). In contrast to the findings of Boonyaratpalin et al. (2001) that no histological changes were noted in P. monodon fed 50 ppb for 4 weeks, the present study, shrimps fed the 50 ppb AFB1 diet at 4 weeks showed mild necrosis and a change in the structure of cells and tubules. The general histopathological changes observed in this study are consistent with the previous findings in penaeids (Lightner et al. 1982; Wiseman et al. 1982; Lavilla-Pitogo et al. 1994; Ostrowski-Meissner et al. 1995; Boonyaratpalin et al. 2001). Jantrarotai and Lovell (1990) and Jantrarotai, Lovell & Grizzle (1990) have reported necrotic foci in livers of channel catfish due to acute and subacute aflatoxicosis. Aflatoxin resulted in fatty liver, nuclear hypertrophy, cellular atrophy and leucocytic infiltration in the liver of Oreochromis niloticus (Chavez, Palacios & Moreno 1994).

The level of aflatoxin B1 above 50 ppb caused significant damage to the hepatopancreas at the histological level. Histological changes were directly related to a corresponding increase in AFB1 in different groups. The important changes in the hepatopancreas were a reduction in the number of R cells, B cells and F cells, loss of structure of cells and tubules, desquamation in the tubules, fibrosis, necrosis, cellular inflammation, haemocytic nodule formation and haemocytic infiltration. Other organs affected were the gills, lymphoid organ and antennal gland. Thus, the histological analysis clearly showed the disruption of the digestive functions of the hepatopancreas by AFB1. The disruptions would upset the function of absorption and storage of nutrients due to the reduced number of R cells, production of digestive enzymes by F cells and secretion of enzymes by the B cell culminating in the disruption of the digestive, metabolic and detoxification functions of the hepatopancreas.

To conclude, the present study has important implications in practical feeding programmes of P. monodon culture. It demonstrates how low levels of AFB1 (50 ppb) could affect the functions and architecture of the hepatopancreas without affecting the survival. Histologically, aflatoxin directly attacks the hepatopancreas, the main organ for detoxification of xenobiotics, and several categories of hepatocellular pathology are now regarded as reliable biomarkers of toxic injury and representative of a
biological endpoint of contaminant exposure. Consequently, the hepatopancreas has attracted the most attention as a target organ for biological effects monitoring programmes since pathological alterations occur at a very early stage of exposure. We recommend the enforcement of strict regulation of 20 ppb AFB$_1$ in feeds and foods. This calls for the need for safe storage of feeds.

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References


Spensley P.C. (1963) Aflatoxins, the active principle in turkey "X" disease. Endavour 22, 75–79.