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Comparative histopathological studies on kidney and liver of rat treated by tetrodotoxin (TTX) extracted from gonads and muscles of porcupine fish species

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Abstract
The present work aimed to investigate the acute histopathological effects of tetrodotoxins (TTX) extracts of gonads (ovary) and muscles of the porcupine fish, *Diodon hystrix* on the kidney and liver of male albino rats. The porcupine fishes were collected from the Egyptian Red Sea coast at Hurgada. A sub-lethal dose of each tetrodotoxin extract was intraperitoneally injected to male albino rats, and samples of liver and kidney were obtained 2 and 4 hours after injection. The results showed that severe histopathological effects recorded in both kidney and liver of male rat injected with TTX which extracted from ovary and muscles, represented by shrinkage of glomeruli, glomerular distortion, necrotic renal tubules, severe congestion and atrophy of renal tubules, necrotic hepatocytes, and congestion in central vein. While TTX which extracted from muscles, showed that moderate histopathological effect in both kidney and liver represented by shrinkage of glomeruli, increased of bowman space, fatty change liver and congestion in central vein. Conclusively, this study proves that, the gonads, particularly ovaries of puffer fish, are more toxic than muscle and TTX from these marine can produce surplus detrimental effects when it enters directly in the blood tissues of living organisms and the degree of damage varies in intensity according with the duration of exposure.

Keywords: Tetrodotoxins, Porcupine fish, *Diodon hystrix*, histopathology, liver, kidney, albino rat

1. Introduction
Puffer fish, intoxication is the best known of all types of fish intoxications and has been recognized from ancient times. Many species of marine puffer fishes possess the potent neurotoxin, tetrodotoxin (TTX) in its body organelles like liver, gonads, skin and muscles [1].
There are 29 genera of tetraodontidae has been recognized [2]. And about 120 species of puffer fish reported from the tropical seas [3]. Only 20 puffer fish species belonging to the family tetraodontidae has been reported from the waters around Andaman and Nicobar Islands [4].
Tetrodotoxin (TTX) is a naturally occurring toxin that has been responsible for human intoxications and fatalities [5]. TTX was believed to be confined to regions of South East Asia, but recent studies have demonstrated that the toxin has spread to [6]. The toxin was first discovered in 1909 by Dr. Yoshizumi Tahara from the ovaries of globefish [7]. But puffer fish have been known to be toxic to humans for a long time. TTX is a very potent neurotoxin that is found in a variety of marine organisms [8, 9]. And also in some terrestrial ones [10]. Its toxicity is often emphasized by referring to the fact that it is over a thousand times more toxic to humans than cyanide; TTX has no known antidote [11, 12]. The mechanism of TTX toxicity has been investigated in animal models [13, 14]. It is a sodium channel blocker. The toxin binds to the sodium channels of the excitable tissues of the victim (muscles and nerves); the inhibition of sodium ions through the channels effectively immobilizes these tissues [15]. In humans the onset and severity of the symptoms of TTX poisoning after ingestion is dose dependent [16]. Initial symptoms include tingling (paresthesias) of the tongue and lips, followed by or concurrent with headache and vomiting, which may progress to muscle weakness and ataxia. In severe cases death may occur due to respiratory and/or heart failure [17]. The only treatment for TTX intoxication is observation and appropriate supportive care [18]. Accordingly, the current work was focused on the histopathological effects recorded in kidney and liver of male rat injected with TTX extracted from the different tissues (gonads and Muscles) of porcupine fish, *Diodon hystrix* (Family: Tetraododontidae).

2. Materials and Methods

A total of 7 specimens of porcupine fish, *Diodon hystrix*, were collected from the Egyptian Red Sea coast at Hurgada. Each fish was kept in a separate plastic bag and immediately frozen at -20 ºC. Then fish were defrosted, then dissected, both ovary and muscles were removed and kept in separate plastic bags then frozen till the time of extraction. The extraction of the tetrodotoxins (TTX) from ovary and muscles was performed according to the method of [19]. The tissues of fish were pooled and one volume of the pool was homogenized in three volumes of 1% acetic acid in methanol. The homogenate was then heated in boiling water bath for 10 min. The samples were then cooled, centrifuged at 1000 rpm for 3 min. and the supernatant containing the TTX was kept at 4 ºC till the time of experimentation. For determination of the amount of crude TTX within the different extracts, the methanolic extracts were placed in clean glass weighed Petri dishes. After dryness or evaporation of the methanol, the dishes were reweighed and the differences were calculated to the nearest mg. A back calculation was made in order to determine the amount of crude toxin per gram of the extracted tissue. The toxicity of the extracted tetrodotoxins was determined (in mouse units) according to the method described by [19]. A series of concentrations were injected intra-peritoneally into 20 g male albino rat obtained from the animal house of Theodor Belharis Institute. One mouse unit is defined as the amount of toxin required to kill a 20 g rat within 30 min. The LD_{50} of the extracted tetrodotoxins was also determined, after 96 h, according to the method of [20]. 50 healthy male albino rats (100-110gm in body weight) were used. The animals were kept under the normal laboratory conditions in wire cages throughout the experimental period. They were divided into 5 groups (each of 10 rats). Rats of the first group served as control and they were intra-peritoneally injected with 1 ml of saline solution. Rats of the 2nd and 3rd groups were intra-peritoneally injected with 1 ml of saline solution containing a sublethal dose (1/10 of LD_{50} /rat) from ovary crude TTX extracts. Rats of the 4th and 5th groups were intra-peritoneally injected with 1 ml of saline solution containing a sublethal dose (1/10 of LD_{50} /rat) from skin crude TTX extracts. The control and treated rats were decapitated after 2 and 4 hours of injection, and then their liver and kidney were excised out and immediately fixed in alcoholic Bouin's solution for 24 hours. These tissues were dehydrated in ascending concentrations of ethyl alcohol, cleared in xylol and embedded in paraffin wax. Transverse sections were cut at 5µ, and stained with Harri's haematoxylin and subsequently counter stained with eosin. Finally, the slides were microscopically examined and photographed using camera mounted on light microscope and described.

3. Results

3.1. Toxicity of TTX extracts

The results of toxicity experiment showed that 1.8 mg of ovary extract and 8.8 mg of muscle extract were sufficient to cause death of a 20-g mouse in 30 min (Mouse Unit). The results showed that LD_{50} were 0.15±0.09 mg and 2.05±0.27 mg for ovary extract and muscles extract respectively. Accordingly, the doses of 1/10 LD_{50} used in the experiments were 0.015 and 0.205 mg/100 gm body weight, for ovary extract and muscles extracts respectively.

3.2 Effect of TTX extracts on rat liver

The liver of control rat appeared to be formed of the classical hepatic lobules. Each lobule showed radial arranged hepatocytes, forming cords around the central veins. Hepatocytes appeared polygonal in shape with rounded central vesicular nuclei. Blood sinusoids were seen separating cords of hepatocytes and lined by flattened endothelial cells and Von Kupffer cells (Fig. 1). After 2 hours of injection by TTX extracted from muscles of porcupine fish, *D. hystrix*, the microscopic observations revealed moderate histopathological effect on liver tissue represented by congestion in central vein, pyknotic nuclei in some hepatocytes and probably necrosis or vaculation and mild infiltrate of polymorph leukocytes is present (Fig.2). While, injection by TTX extracted from ovaries of porcupine fish, *D. hystrix* showing that the trabecular structure of the liver is blurred; cell size was enlarged, nuclear chromatin was more compact, slightly smaller nucleoli were not conspicuous. Necrosis of single hepatocytes; nuclei were contracted, pyknotic nuclei with condensed chromatin, strongly acidophilic cytoplasm was appeared (Fig. 3). After 4 hours of exposure to TTX, extracted from muscles of porcupine fish, *D. hystrix* showing abnormal architectural organization of hepatic tissue, Blood vessel congestion with disturbed epithelium, the hepatocyte cytoplasm is light, foamy and filled with vacuoles or fatty change liver, and multi pyknotic nuclei were also recorded (Fig. 4). While injection by TTX extracted from gonads of porcupine fish, *Diodon hystrix* showing severe degenerated hepatic cells represented by abnormal hepatic cells, large hyper chromatic nuclei, and severe dilated congested blood vessels in central vein, pyknotic cells, vacuolation, and necrotic area were observed (Fig. 5).

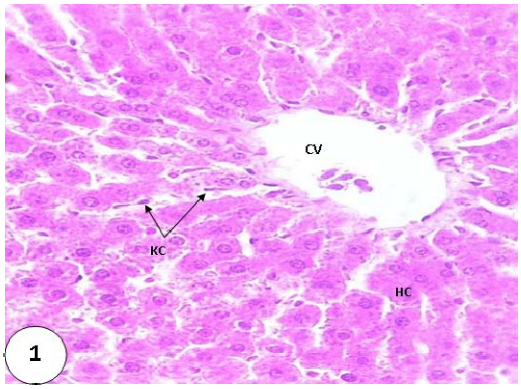

Fig 1: A photomicrograph of the normal liver structure of adult albino rat showing hepatic polygonal cells (HC) with round nucleus, normal central vein (CV) and Kuppfer cells (KC) (Hx. & E., x400).

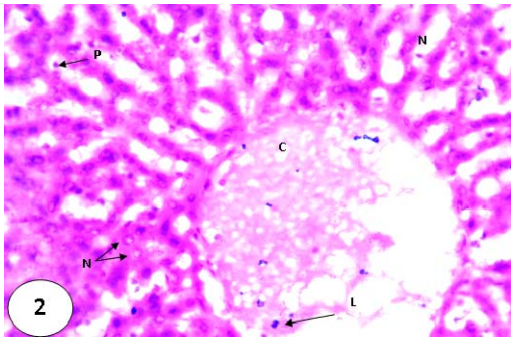

Fig 2: Enlarged section of liver structure in adult albino rat after 2hours of exposure to TTX, extracted from muscles of porcupine fish, *Diodon hystrix* showing moderate abnormal hepatic central vein as, congestion in central vein, some hepatocytes have pyknotic nuclei(P) and probably necrotic (N) or vaculation and mild infiltrate of polymorph leukocytes (L) is present (H&E x400).

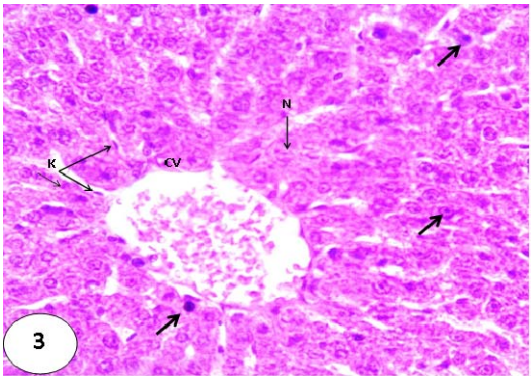

Fig 3: Enlarged section of liver structure in adult albino rat after 2hours of exposure to TTX, extracted from gonads of porcupine fish, *Diodon hystrix* showing the trabecular structure of the liver is blurred. Cell sizes are enlarged, nuclear chromatin is more compact, slightly smaller nucleoli are not conspicuous. Necrosis (N) of single hepatocytes, nuclei are contracted, pyknotic with condensed chromatin (arrow head), cytoplasm is strongly acidophilic. Accumulation of mononuclear cells in the vicinity of sinusoids. The sinusoid walls show numerous Kupffer cells (K). (Hx. & E., x400).

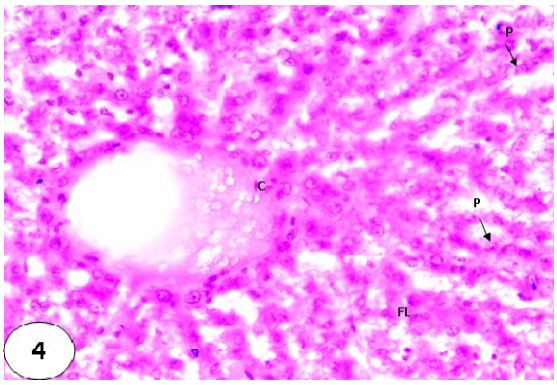

Fig 4: Enlarged transverse section of liver of adult albino after 4hours of exposure to TTX, extracted from muscles of porcupine fish, *Diodon hystrix* showing abnormal architectural organization of hepatic tissue, Blood vessel congestion (C) with disturbed epithelium, the hepatocyte cytoplasm is light, foamy and filled with vacuoles or fatty change liver (FL), and multi pyknotic nuclei (P) were also recorded (Hx. & E., x400).

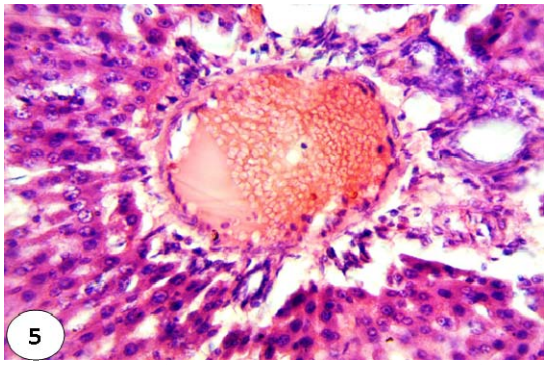

Fig 5: Enlarged section of liver structure in adult albino rat after 4hours of exposure to TTX, extracted from gonads of porcupine fish, *Diodon hystrix* showing severe degenerated hepatic cells represented by abnormal hepatic cells, large hyper chromatic nuclei, and severe dilated congested blood vessels in central vein, pyknotic cells, vacuolation, and necrotic area were recorded (Hx. & E., x400).

3.3 Effect of TTX extracts on rat kidney

Histological observations of control kidney of albino rat revealed that, kidney is mainly composed of renal tubules and renal corpuscles. The renal corpuscles were formed of lobulated glomeruli surrounded by Bowman's spaces. The proximal convoluted tubules appeared to be lined by a single layer of cuboidal epithelium enclosing a narrow lumen. The distal convoluted tubules were lined by cubical cells surrounding wider lumina (Fig. 6). After 2 hours of injection by muscles TTX extract, the microscopic observation revealed a moderate histopathological effect represented by shrinkage with lobulation or fragmentaion in the glomeruli leading to increase of bowman's space (BS), but the most histological structures of kidney still normal like in the control group (Fig. 7). While when after injection by gonads TTX extract, examination of kidney sections showed severe degeneration and deformation in all renal cells represented by necrotic areas encapsulated by fibrous tissue and leukocytes forming neoplasia cyst, severe blood congestion in renal cells stagnation fluid of some renal tubules and melanomacrophage cells were appeared with dense blue colure (Fig. 8). After 4hours of injection by muscles TTX extract, the abnormal renal tubules showing moderate pathological effect in renal cells as shrinkage with fragmentation of glomeruli leading to increase of bowman's space, but the most histological structures of kidney still normal like in the control group (Fig. 9). While when injection by TTX extracted from gonads, abnormal kidney structure showing severe damage of renal tubules, as shrinkage with glomerular distortion, and atrophy of renal tubules with multi necrotic tubular cells, or Parenchymatous degeneration of cells of renal tubules (Fig. 10).

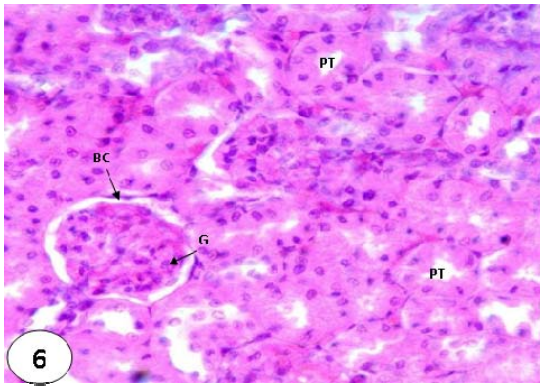

Fig 6: A photomicrograph of the normal kidney structure of adult albino rat showing normal Bowman's capsule (BC), glomeruli (G), and proximal tubules (PT) (H&E x400).

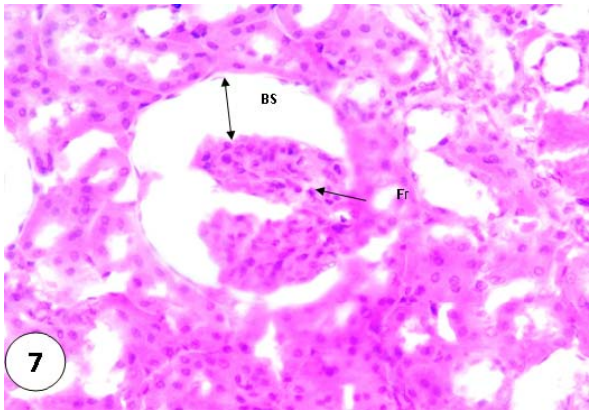

Fig 7: Enlarged section of kidney structure in adult albino rat after 2hours of exposure to TTX, extracted from muscles of porcupine fish, *Diodon hystrix* showing moderate pathological effect represented by shrinkage with lobulation or fragmentaion (Fr) in the glomeruli leading to increase of bowman's space (BS), but the most histological structures of kidney still normal like in the control group (H&E x400).

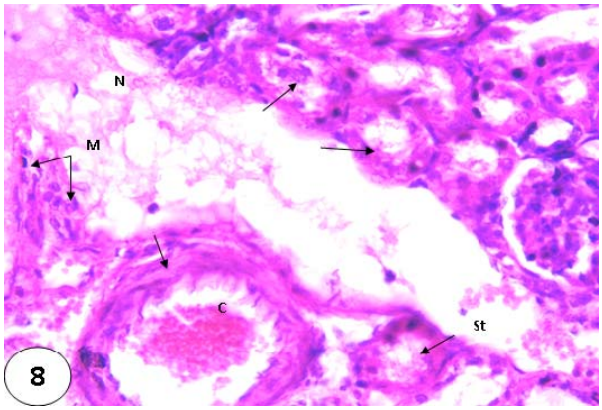

Fig 8: Enlarged section of kidney structure in adult albino after 2hours of exposure to TTX, extracted from gonads of porcupine fish, *Diodon hystrix* showing abnormal renal cells represented by necrotic areas encapsulated by fibrous tissue and leukocytes forming neoplasia cyst (arrow head), severe blood congestion in renal cells (C), stagnation fluid of some renal tubules (St) and melanomacrophage cells (M) were recorded with dense blue colure. (Hx. & E., x400).

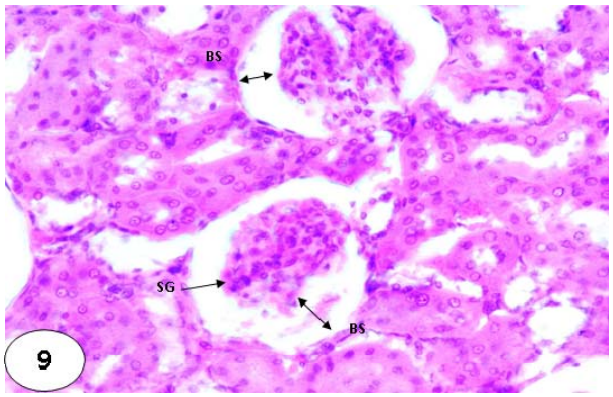

Fig 9: Enlarged section of kidney structure in adult albino after 4hours of exposure to TTX, extracted from muscles of porcupine fish, *Diodon hystrix* moderate pathological effect in renal cells represented as shrinkage (SG) with lobulation or fragmentation of glomeruli leading to increase of bowman's space (BS), but the most histological structures of kidney still normal like in the control group (H&E x400).

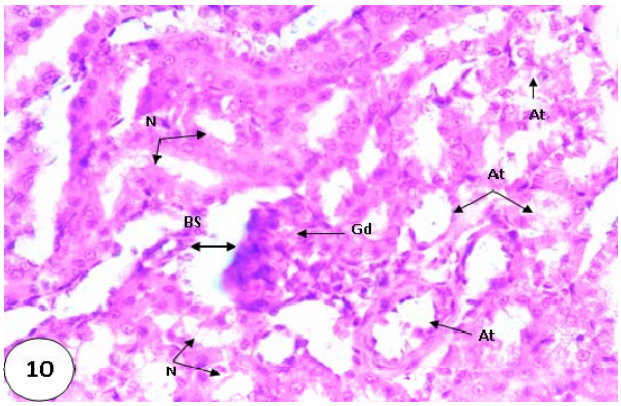

Fig 10: Enlarged section of kidney structure in adult albino after 4hours of exposure to TTX, extracted from gonads of porcupine fish, *Diodon hystrix* showing severe damage of renal tubules, as shrinkage with glomerular distortion (Gd), and atrophy (At) of renal tubules with multi necrotic (N) tubular cells, or Parenchymatous degeneration of cells of renal tubules(H&E x400).

4. Discussion

Liver and kidney are important organs of metabolism, detoxification, storage and excretion of xenobiotics and their metabolites, and are especially vulnerable to damage [21]. The liver is the largest gland in the body and is characterized by a multiplicity of complex functions; excretion (waste products), secretion (bile), synthesis (fibrinogen, globulins, albumin and clotting factors), storage (lipids, vitamins A&B and glycogen), phagocytosis (foreign particular matter), detoxification (lipid-soluble and drugs), conjugation (toxic substances, steroid hormones), esterification (free fatty acids to triglycerides), metabolism (proteins, carbohydrates, lipids, hemoglobin and drugs) and hemopoiesis in the embryo and potentially in the adult [22]. The liver tissue consists of hepatocytes that aggregate in masses, separated from each other by blood sinusoids, and arranged in anastomosing laminae and in rings around a central vein, and is light brown in herbivores animals [23].

Damage to the liver is the most frequently reported histopathological response to organic compounds, the importance of the liver as a marker for pathological change reflects the central role of mammalian hepatic tissue in nutrition, lipid and carbohydrate storage, synthesis of protein and enzymes, fatty acid metabolism, and biotransformation and elimination of wastes [24].

Vacuoles in the cytoplasm of the hepatocytes can contain lipids and glycogen, which are related to the normal metabolic function of the liver [25]. The vacuolization of hepatocytes might indicate an imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of their release into the systemic circulation [26]. Whereas, the increased vacuolization of the hepatocytes as a signal of degenerative process that suggests metabolic damage, possibly related to exposure to contaminated food [27]. Also, vacuole formation was considered as a cellular defense mechanism against injurious substances to hepatocytes and this mechanism responsible for collecting the injurious elements and preventing them from interfering with the biological activities of these cells [28].The occurrence of fatty changes suggested inhibition of some lipid metabolic enzymes, causing thereby, a disturbance in metabolic activity required for maintenance of tissue. Prominent fatty changes with necrosis in portal areas indicated that some toxic metabolites may be transported from intestine to liver,

resulting in these changes. The presence of definite necrosis indicated capability of the toxic metabolites causing cell death [29]. Vacuolar degeneration and necrosis of liver cells were illustrated in many investigations. It may be due to a direct effect of toxic materials on the cell membranes [27]. Another explanation for the observed necrosis in liver cells exposed to Tetrodotoxin (TTX) could be higher metabolic activity; in highly active cells, the nuclear material is dispersed feature [30]. Melanomacrophage centers, also known as macrophage aggregates, are distinctive groupings of pigment-containing cells located in the stroma of the haemopoietic tissue of the spleen and the kidney fishes [31]. Several authors have suggested that, the involvement of melano-macrophage centers in various disease processes and the changes brought about in them by chemical exposure [32]. The present results showed that, the kidney of albino rat treated to TTX extracted from liver and skin of the porcupine fish, *Diodon hystrix* showed different signs of histopathological deformations, which varies in intensity according to the duration of exposure. It represented by shrinkage of glomeruli, glomerular distortion, necrotic renal tubules, severe congestion and atrophy of renal tubules, or the ovary extract was found to be more toxic in comparison to muscles extract. These results are agreed with [1]. Histopathological studies in Kidney revealed the acute cellular degeneration or atrophy in the epithetical lining of renal tubules is due to toxic effect of TTX which may be caused by cell membrane injury or effect on mitochondria, this effect lead to depletion Adenosine Triphosphate (ATP) or defect in sodium-potassium pump, this lead to fluid disturbance in and outside the cell [33]. This lesion may be return to cellular degeneration as well as increasing the amount of edematous fluid in the interstitial substance [34]. The atrophy of renal tubule may be interpreted as because of water reabsorption taking place in the distal tubules, relatively high concentrations of toxins may have an effect on renal cells [35]. The interpretation of neoplesia appears due to the encapsulation of necrotic areas by fibrocytes and leukocytes [36]. Most of the work on puffer fish showed the liver to be the most toxic part of puffer fish and the muscle being the least toxic [37]. On the contrary, this study shows that the ovaries can be more toxic than liver because toxin transfer to the skin decreases somewhat on the onset of spawning season and most of the TTX taken up into the liver would be transported to the ovary, presumably with the precursor of yolk proteins that are synthesized in the liver [38].

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