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Full Length Research Paper

Effect of Tetrodotoxin of Puffer fish *Arothron immaculatus* on *Oreochromis mossambica* from South Andaman, Indian EEZ

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Abstract

Tetradotoxin poisoning from Puffer fishes (Family Tetreaodontidae) is very common in coastal regions of Asian countries. In the present study, bioassays were conducted to investigate the toxicity of liver and ovaries of puffer fish *Arothron immaculatus* on *Oreochromis mossambica* collected from South Andaman. Various concentrations of tissue extract (0.1, 0.2 and 0.3 ml/g body weight) were introduced into the body of the fish by integrating the same in artificial pellet feed as well as through intra-peritoneal injection. The behavioral and morphological changes in fishes were recorded continuously after the introduction of extract till the time of mortality. The tissue extract produced no lethal change on *O. mossambica* during the feeding experiment with no mortality. The same concentration of toxin when injected intra-peritoneally was proved to be lethal for the experimental organisms triggering a sudden body color change, paralysis followed by death. The introduction of ovary extract was more fatal and the average time for color change was observed at 5.4, 3.1, 1.4 seconds and the average time of death at 93, 72, 70 seconds respectively in three concentrations. Whereas, the intra-peritoneal introduction of liver extract was more lethal as the average time of color change in 0.1, 0.2 and 0.3 ml/g was found to be 77, 87 and 59 seconds and average time of death occurred after 10.22, 4.03, 3.18 minutes respectively. The study suggests that puffer fish, *Arothron immaculatus* collected from the coast of South Andaman is showing more toxicity in the ovary tissue.

Keywords: Tetrodotoxin, *Arothron immaculatus*, Andaman, *O. mossambica.*

INTRODUCTION

Toxicity is widespread among marine organisms and has been reported for algae, jellyfish, ascidians, bryozoans, sea anemones, corals, sponges, echinoderms, mollusks, crustaceans and fishes (Halstead, 1967; Cameron, 1974; Bakus, 1981; Bakus et al., 1986; Hay, 1996). 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. Tetrodotoxin (TTX) blocks sodium ion channel of the nerve cell membrane resulting in paresthesias, ataxia, diarrhea, vomiting, respiratory insufficiency, paralysis and even rapid death in seriously intoxicated human (Narahashi, 2001). It is been reported that the main source of TTX is some species of marine bacteria(Noguchi et al., 1986; Simidu et al., 1987; Hashimoto et al., 1990; Kono et al., 2008) and is bio-concentrated through the marine food chain, symbiosis and/or parasitism (Arakawa et al., 2010; Noguchi and Arakawa, 2008; Hwang and Noguchi, 2007) as it is present in many marine organisms along with puffer fish including many invertebrates (e.g. blue ringed octopus, starfish, xanthid crab, gastropods) as well as some vertebrates (e.g. atelopids frogs, gobies, newts). In most

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of the puffers, high concentrations of TTX were found in livers and ovaries/eggs, especially during spawning. The toxin is exogenous, and toxicity may be greatly affected by a change in the marine environment, such as elevations in water temperature due to global warming and in general, puffer fish shows size, regional and seasonal variations in toxicity and an individual of highly toxic species may not necessarily be toxic at a given time and in most of the cases of puffer fish intoxications ovary and liver were the tissues which leads to a frequent occurrence of human poisonings (Arakawa et al., 2010).

There are 29 genera of tetraodontidae has been recognized by Froese and Pauly (2008) and about 120 species of puffer fish reported from the tropical seas (Sabrah et al., 2006). Only 20 puffer fish species belonging to the family tetraodontidae has been reported from the waters around Andaman and Nicobar Islands (Rajan et al., 2013). Toxicological investigations on puffer revealed that the ovaries showed maximum lethal potency in their spawning season when ovaries are full of eggs (Ghosh et al., 2004). There is a Gonado-Somatic Index (GSI) suggesting that the increased toxin is the product of embryos as postulated by Matsumura (1998).

It is probably the most common fish intoxication along the coasts of Asia (Ahasan et al., 2004; Hwang et al., 2002; Wu et al., 2005). Puffer fish from the South Indian coast have not been studied yet *Lagocephalus lunaris* popularly known the green toadfish is the most commonly available species in South Indian coast. This species is reported to be toxic elsewhere (Berry and Hassan, 1973; Monaliza and Samsur, 2011; Shiomi et al., 1985) but it is consumed by the locals of South India (Mandal et al., 2013). This is because the toxicity of puffer fish changes from the place they are collected from (Mandal et al., 2013).

To understand the nature of TTX, it explicitly demands toxicity studies based on bioassays to recognize the behavioral and morphological variations in the test organism *(Oreochromis mossambica).* In the present study, TTX was introduced in the test organisms and various changes in the behavioral activity has been observed and the values for GSI were compared to throw light on the intensity of toxin production on the onset of spawning period in *A. immaculatus*.

MATERIAL AND METHODS

Sample Collection

A total of 20 specimens of puffer fish *A. immaculatus* (Bloch and Schneider, 1801) were collected from the Marina Park, South Andaman coast, Andaman Islands (Figure 1), during the month of December 2013 to March 2014 and transported to the laboratory in dry ice. Samples were frozen at -20˚C until use. The specimens were identified by the assistance of Reef Fish Identification keys (Allen et al., 2003).

Gut Content Analysis (GCA) and Ganado Somatic Indices(GSI) analysis of *A. immaculatus*

For Gut Content analysis, the gut contents were removed by cutting the pylorus and the anus. The contents were taken out and analyzed quantitively and qualitatively by quantifying the specific items in the gut and identifying the undigested food items in the gut content. The gut content analyses were calculated numerically as per Jimmy et al., 2003.

To analyze the Ganado Somatic indices, the gonads were removed and weighed. The GSI was determined as

$$GSI = \frac{\text{Weight of gonad}}{\text{Weight of body}} \times 100$$ (Welcomme, 2001)

Crude Toxin Preparation

The extraction was done according to the methodology of Khora (1991). In this method all samples were partially thawed and liver and ovary tissues were dissected separate. The total weight of each tissue was recorded before being minced into small portion at about 10g. To each minced tissue 2.5 volumes of 0.1% acetic acid was added and heated it in a boiling water bath for 10 minutes. The slurry was centrifuged at about 3000 rpm for 10 minutes to obtain the supernatant. This procedure was repeated for three times to obtain 5 volumes of TTX from the sample. Then all the three supernatants were mixed together.

Animals

For the study of toxicity assay, *Oreochromis mossambica* (Tilapia) a brackish water fish were collected from the mangrove area of Sipighat, South Andaman. A total of189 fishes that were 8 grams in weight and 10 cm in size were brought to the laboratory one week prior to the performance of experiment for acclimatization. They were housed in 30 x 15cm aquarium tanks with aerator in each of the tanks with three fishes in each with a salinity of 10 psm. They were fed with artificial pellets (Skretting, Australia).

Experimental Design

After acclimatizing *O. mossambica* to the laboratory conditions for one week, 3 individuals each with an

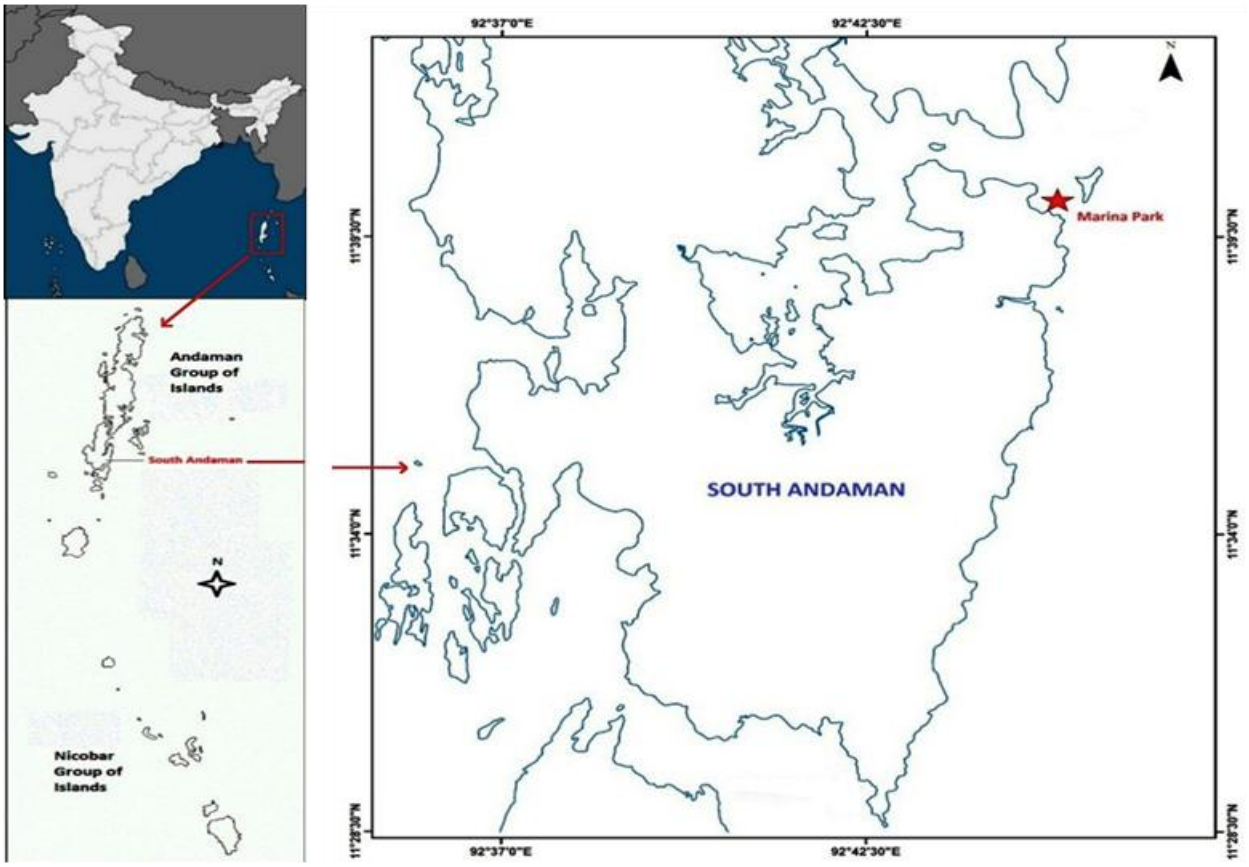

Figure 1. Map showing the location of South Andaman and the study station

average size of 10 cm and weight 8 grams were introduced into aquarium tanks filled with equal volume of water (4410cm^3) with a salinity of 10 psm.

The tanks were grouped into three based on the three concentrations 0.1, 0.2 and 0.3 ml extract/gram body weight of fish. A set of controls were also maintained at a concentration of 0.1, 0.2 and 0.3 ml saline/gram body weight for each set of experiment. The liver and ovary extracts of *A. immaculatus* as well as the saline solution were injected intra-peritoneally to *O. mossambica*. Fishes in the control tanks were injected with 0.1, 0.2 and 0.3 ml of saline solution (0.9% NaCl)/g body weight (BW) respectively. Then, the fishes were injected with 0.1, 0.2 and 0.3 ml of liver extract/g BW in the experimental tanks (in triplicate). Similarly the three different concentrations of ovary extract were also injected (in triplicate) and were monitored continuously.

In the second set of experiment, the liver and ovary extracts of *A. immaculatus* were amalgamated discretely with the feed of *O. mossambica* in the same concentration of 0.1, 0.2 and 0.3 ml/g of BW and controls with normal feed. The formulated pelleted commercial feed (Skretting, Australia) was incorporated with the liver/ovary extracts by means of absorbing the fish feed pellets with the liver and ovary extract separately at different concentrations (0.06ml/g, 0.09 ml/g, 0.1 ml/g respectively) and as the tissue extract get absorbed, the feed was coated with a layer of cod liver oil to conceal the odor of toxin and was kept to get immersed immaculately. Fishes were kept individually in segregate tanks including the controls in order to make sure that the feed introduced was consumed by the same individual. Each fish was fed with amalgamated feed in the quantity of 3 % of its BW (in grams), per day and were observed periodically to record any behavioral and morphological changes (body color change; abnormal behavior like whirling movement in response to stress caused by TTX)

RESULTS AND DISCUSSION

GCA depicts the result that the fish mainly prefers to feed on benthic organisms and detritus (Figure 2). It also indicates that maximum available contents in the gut were that of molluscan shells followed by shrimps and crab appendages and the least were that of sponge spicules. A similar result was reported in the milk spotted puffer *chelonodon potaca* near Townsville (Australia) by Beumer (1978). GCA of the present study also

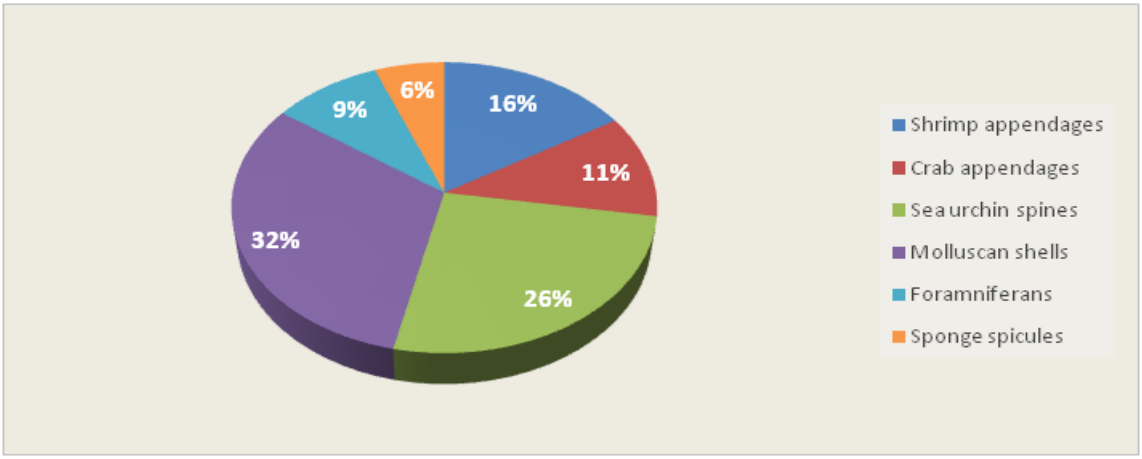

Figure 2. Gut Content Analyses of *A. immaculatus* displaying the percentage value of different food items.

demonstrate that the species mainly feed on hard shelled organisms including foramniferans which were found in considerable proportion indicating the carnivorous nature of fish and the same trend has been observed in *A. immaculatus* by Krumme et al. (2007). Studies by Ikeda et al. (2010) on the seasonal changes in the GSI suggest that maturation of female puffer *T. poecilonotus* inhabiting the Ariake Sea occurs during December-March and that of males occur during November-March and spawning occurs during March-April. The puffer fish *Takifugu rubripes* that also live in the Ariake Sea as their spawning ground also spawn from the second half of March to May at the entrance of the sea (Takita and Intong, 1991).

In present study, the peak value of GSI in *A. immaculatus* was found to be 5.87 and the remaining ranges from 0.83 to 4.89 (Figure 3) and it depicts that most of the fishes have a more or less high GSI which indicates they are approaching their spawning season and hence the intensity of toxicity of gonads will also be high as it is already have been reported that puffer fishes uses TTX as a biological defense weapon to protect eggs or themselves from the predator (Arakawa et al., 2010).

Generally, TTX is accumulated in the liver, gonads, intestine, muscle and skin of the puffer fish (Fuchi et al., 1991; Mahmud et al., 2000; Panichpisal et al., 2003). Mostly in marine species of puffer fish, liver and ovary showed the highest toxicity (more than 1000 MU/g), followed by intestine and skin (Noguchi et al., 2006). During the present study, the feeding experiment with liver and ovary tissue extracts produced no lethal change on *O. mossambica* and no mortality was observed. In the feeding experiment with liver extract, 33% fishes from 0.2 ml/g and 44% fishes from 0.3 ml/g have shown color change after 4 days and no change were observed in 0.1 ml/g. (Table 1) and when fed with ovary extract, 77.77% of the fishes from 0.1 ml/g concentration have shown color change and all the fishes from the concentration of 0.2 and 0.3 ml/g showed color change. But when the feed merged with ovary and liver extract and fed to separate group of fishes, 33% in 0.1ml/g, 44% in 0.2ml/g and 66% in 0.3ml/g have shown color change after 3 days, (from normal to black color) this color change was reverted to normal automatically after 3 days without any behavioral changes. This negligible effect can be attributed to the reduced absorption of toxin through the digestive system and thereafter acclimatization of the fishes to the less concentration of the same in their body tissue. On the contrary, while injecting liver and ovary tissue extract of *A. immaculatus,* in 0.1, 0.2 and 0.3 ml concentration per gram of BW, *O. mossambica* exhibited symptoms like body color change, edginess, paralysis and ultimately death in all the cases at different intervals of time.

Liver and ovaries are mainly tested as these are the organs where the toxin is concentrated more in most cases and mainly responsible for clinical poisoning while ingested (Hwang et al., 1989). Similarly in this study at case of liver extract, the average rate of color changes, paralysis, and complete death upsurges with the proliferation in the concentration of the liver extract and the same trend was followed while injecting the ovary extract, but the ovary extract was found to be more toxic in comparison to liver extract, as the average rate of body color change in tilapia was so quick i.e. at 5 and 1 seconds and the average rate of paralysis in *O. mossambica* was found to be 33 and 20 seconds while the average time of death stretches up to 93 and 70 seconds at 0.1 and 0.3 ml/g of concentrations respectively(Figure 5). The average rate of body color change while introducing liver extract intra-peritoneally to test organism, was observed at 77 and 35 seconds while average rate of paralysis was perceived at 111 and 66 seconds and the mortality was witnessed at 548 and 198 seconds at 0.1 and 0.3 ml/g of concentrations

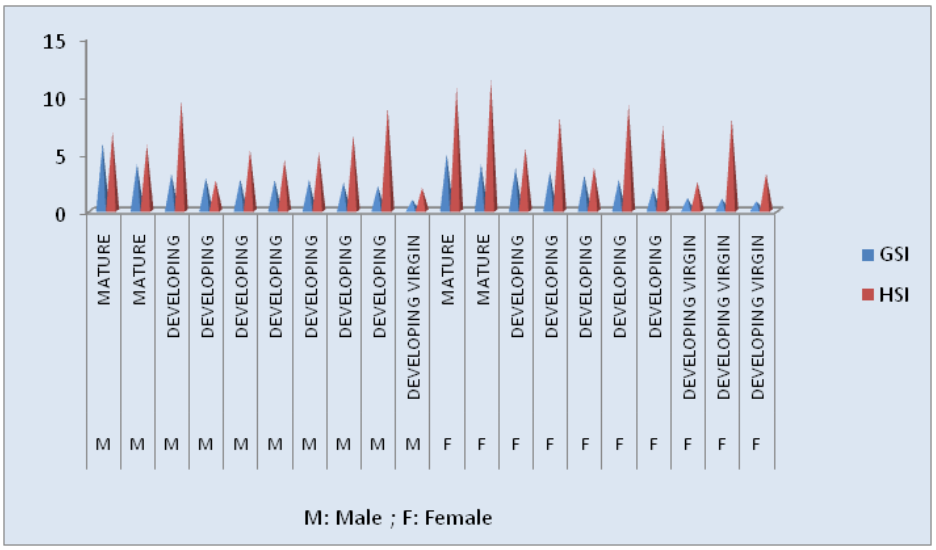

Figure 3. Sex wise Hepato-Somatic and Gonado-Somatic Indices indicating the reproductive maturity level of *Arothron immaculatus*

Table 1. Concentration wise color change exhibited by Tilapia(in %) along with time duration.

CONCENTRATION	TISSUE	FISHES SHOWING COLOURCHANGE (%)	TIME TAKEN
0.1	LIVER	-	4 DAYS
0.2	LIVER	33.33%	4 DAYS
0.3	LIVER	44.44%	4 DAYS
0.1	OVARY	77.77	2 DAYS
0.2	OVARY	100 %	2 DAYS
0.3	OVARY	100 %	2 DAYS
0.1	LIVER+ OVARY	33.33%	3 DAYS
0.2	LIVER+ OVARY	44.44%	3 DAYS
0.3	LIVER+ OVARY	66.66%	3 DAYS

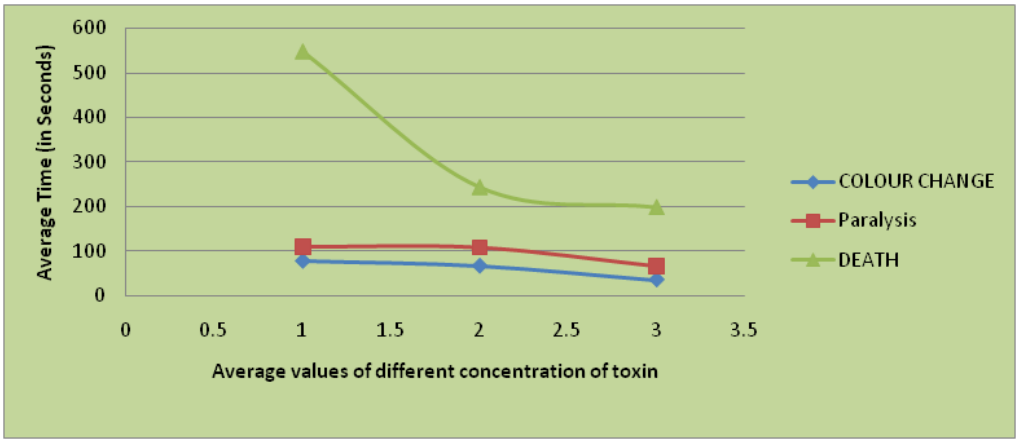

Figure 4. Graph showing the average time period at which different morphological and behavioral changes were observed in treated test organism (*O. mossambica)* after the injection of liver extract of puffer fish (*A. immaculatus)* at different concentrations(v/w).

respectively(Figure 4). 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 (Hashimoto, 1976; Matsui et al., 1981; Nagashima 1999; Saoudi et al.,

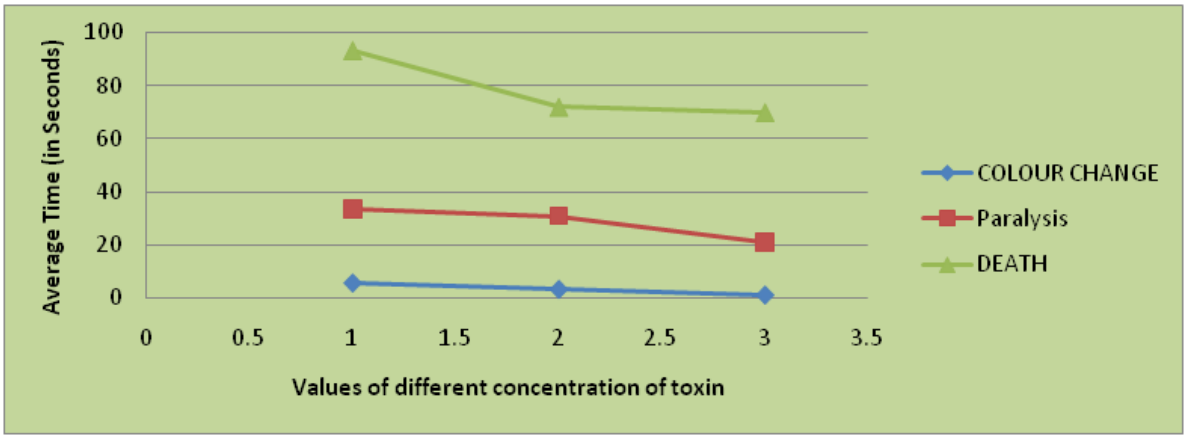

Figure 5. Graph showing the average time period at which different morphological and behavioral changes were observed in treated test organism (*O. mossambica)* after the injection of ovary extract of puffer fish (*A. imaculatus)* at different concentrations(v/w).

2008). 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 (Wallace 1985; Specker and Sullivan, 1994). A Cumulative drift has been observed in all the behavioral and morphological features while intensifying the concentrations of liver and ovary tissue extracts, but the changes observed after introduction of ovary extracts was so abrupt that it may be attributed to the commencement of spawning season of *A. immaculatus* as it is previously indicated in the GSI indices.

Conclusively, this study proves that the gonads, particularly ovaries of puffer fish, A. *immaculatus* from Southern coast of Andaman are more toxic on the onset of spawning season and TTX from these marine can produce surplus detrimental effects when it enters directly in the blood tissues of living organisms. Further in depth research is required for understanding more intricacies of the effect of TTX produced by *Arothron immaculatus*.

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