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-RESEARCH ARTICLE-

Maturation and Gonad Development of Yellowspotted Puffer *Torquigener flavimaculosus* (Osteichthyes: Tetraodontidae) from Iskenderun Bay, North-eastern Mediterranean

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Abstract
Marine pufferfish of the family Tetraodontidae accumulate the highest levels of tetrodotoxin (TTX) in the ovary. The level of TTX accumulation is reported to differ between males and females and fluctuate through gonadal development and maturation stages. Therefore, in the present work, maturation and gonad development of Yellowspotted Puffer (Torquigener flavimaculosus) from Iskenderun Bay, North-eastern Mediterranean were investigated histologically and morphologically. Mean length and weight of specimens were 12.1±0.58 cm and 20.25±0.17 g respectively. Histological examination of the gonads showed that maturation occurs in every single male T. flavimaculosus collected in summer 2017. Moreover, vitellogenic and matured oocytes were also consistently found in every female collected during the summer months. These data suggest that both sexes are reproductively active at the same time of the year. Where the spawning season for both males and females was detected in summer. T. flavimaculosus was found to be dioecious. Five and six developmental stages were indicated for testis and ovaries, respectively. The developmental pattern of ovaries was categorized as the asynchronous or group synchronous type.

Keywords:
Yellowspotted Puffer, Gametogenesis, Histology, Iskenderun Bay

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Introduction

Pufferfish are among the most poisonous animals on earth. Tetrodotoxin (TTX) is known to be the substance of pufferfish poison. TTX is 100 times more toxic than cyanide and it has been suggested that TTX in pufferfish is a result of accumulation through food chain, which starts from marine bacteria *Vibrio alginolyticus, Shewanella sp., S. putrefaciens, Alteromonas tetraodonis* (Nouguchi & Arakawa, 2008). Itoi et al., (2015), suggested that *Takifugu niphobles* ingested the toxic eggs of another pufferfish *Takifugu pardalis* to toxify themselves more efficiently via a TTX loop consisting of TTX bearing organisms at a higher trophic level in the food chain. The origin of TTX was suggested to be exogenous by Jal & Khora (2015). Fouad (2005) and El-Dayem (2013) have proved anti-cancer activity of TTX. In their study, the anti-tumor activity of the TTX increased by 46%, in addition to decreasing the number of tumor cells. Biomedical and pharmacological potential of TTX-producing bacteria was isolated from a marine pufferfish by Bragadeeswaran et al. (2010). China and Canada have performed clinical trials for using TTX as an analgesic for reducing the pain in the cancer patients (Alonso et al., 2003). Recently, antimicrobial, hemolytic activity and cytotoxicity of pufferfish have also been detected (Priya & Khora, 2013).

Yellow-spotted puffer fish (*Torquigener flavimaculosus*) belongs to the Tetraodontidae family. The species belonging to the tetraodontidae contain high amount of TTX (Hardy, 1983; Ha & Sato, 2013; Azman et al., 2014). *T. flavimaculosus* is distributed in tropical and temperate regions of the east Africa, India, and Persian Gulf. Recently, it has been recorded in Turkey (Hardy & Randall, 1983; Bilecenoğlu, 2003;2005; Ergüden et al., 2015; Engin & Seyhan, 2017), in the northern red sea (Golani & Lerner, 2007), in the eastern Mediterranean Egyptian coast (Farrag et al., 2016), in the Aegean sea (Corsini-Foka et al., 2006). It is a reef-associated fish dwelling at depths of 3 to 57 m. It is known as Yellowspotted Puffer in Turkey and cannot be used commercially for human consumption (Turan et al., 2007; Turan, 2010). Studies on the *T. flavimaculosus* are very limited and most of the studies are related to the distribution of it (Golani, 1987; Bilecenoglu, 2003; 2005; Ergüden & Gürlek, 2010; Turan, 2010; Froese and Pauly, 2013; Sabour et al., 2014; Farrag et al., 2016). The other solely published study was related the burrowing behavior of the *T. flavimaculosus* which was investigated by Bilecenoglu (2005). The author suggested that burrowing behavior was an anti-predator adaptation by this species.

TTX is the most spectacular substance of pharmacological importance extracted from pufferfish. Thattiyaphong et al., (2014), performed a comprehensive study on pufferfish TTX extraction. They found out that the highest concentrations of TTX accumulated in the gonads followed by the liver of the pufferfish. Acar et al. (2017) were also detected the highest concentration of TTX in the ovary of a pufferfish. TTX concentration was fluctuated regarding to the spawning season. It has also been suggested that maximum TTX content of the pufferfish might be affected by the gonadal development (Ikeda et al., 2010; Itoi et al., 2012; 2015). Recently, Itoi et al. (2016) found out that TTX content was significantly higher during the oocytes maturation and spawning period. Study was performed on pufferfish *Takifugu niphobles.* Male and female *T. niphobles* deposited TTX differently during the spawning period (Itoi et al. 2016). Yin et al. (2017) revealed a novel function of the vitellogenin subdomain as binding with TTX, and its involvement in the toxification of the pufferfish ovary. In their study, TTX was suggested to transfer from the liver to the ovary in female pufferfish during maturation. TTX was also suggested to act as a sexual pheromone to attract male pufferfish (Yin et al., 2017).

In order to extract TTX from the gonads and liver of the *T. flavimaculosus*, spawning season, maturation stages, and reproductive pattern of fish should be known. Currently there is no studies on the developmental pattern, developmental stages of ovaries and spawning season. In the present study, maturation stages, spawning season, formation of oocytes and sperm were examined.

Material and Methods

Specimen Collection

Eight specimens of *T. flavimaculosus* was caught by gill nets from the Iskenderun Bay southeast coast of Turkey, and immediately brought fresh to the laboratory (Fig. 1A and 1B). Fish were preliminary identified by morphological characteristics as previously reported (Golani, 1987; Ergüden & Gürlek, 2010; Turan et al., 2010). They were sexed and their body weight and length were recorded.

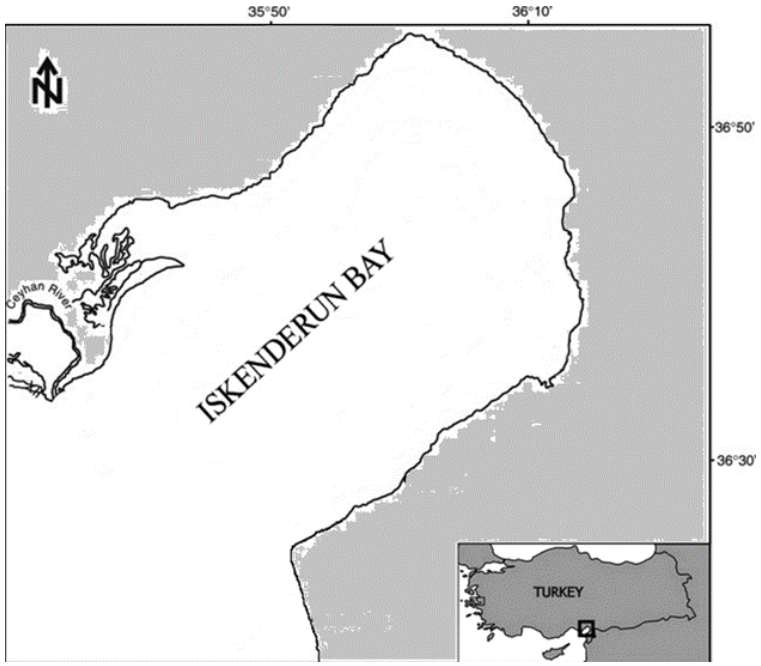


Figure 1. A). Picture of the yellowspotted puffer, *Torquigener flavimaculosus*, Hardy and Randall, 1983. Photograph taken by Servet A. Doğdu. B). Capture locality of *T. flavimaculosus* in the Iskenderun Bay (Indicated by an asterisk).

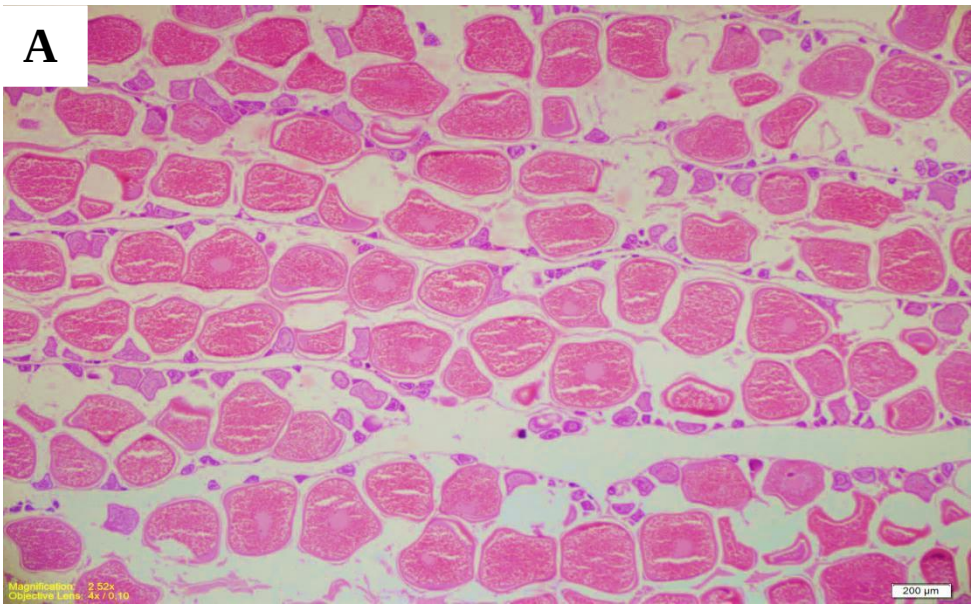
Histological Procedures

The gonads were directly fixed in 10% neutral buffered formalin (prepared in neutral buffered saline modified for use with teleost tissue, 4 g NaH_2PO_4, 6.5 g $NaHPO_4$, 100 ml formaldehyde and 900 ml distilled water). A cross section from the center of each ovary and testis was fixed in formalin, dehydrated in graded ethanol, embedded in paraffin, sectioned at 5µm thickness by microtome and sticked on slides (sizes) before stained with haematoxylin and eosin (MERCK, Germany) for histological examination (Çek, 2006; Turan et al. 2006; Çek & Turan, 2007; Çek & Yılmaz, 2007). After histological work, all slides were examined under a light microscope (CH-2 Olympus, Japan). Photomicrographs were taken to illustrate developmental stages of ovary and testis. Spermatozoa classification was based on the histological criteria adapted from Grier (1981). Oocytes were classified by developmental stages modified from Çek et al. (2001). Maturation stages were detected.

Results

Sex Ratio

During the study, mean length (ort.±S.E) and weight (ort.±S.E.) of specimens were 12.1±0.58cm and 20.25±0.17g respectively. Based on morphological and histological examination of the gonads, the population seems to consist of gonochoristic individuals (Fig. 2A and 2B). Of the 2017, Iskenderun Bay 8 T. flavimaculosus examined five (62.5%) were females and three (37.5%) were males. Because of the small number of specimens caught, the annual sex ratio could not be calculated using a Chi-square test. Nevertheless, a female biased sex ratio was recorded (5♀, 3♂).



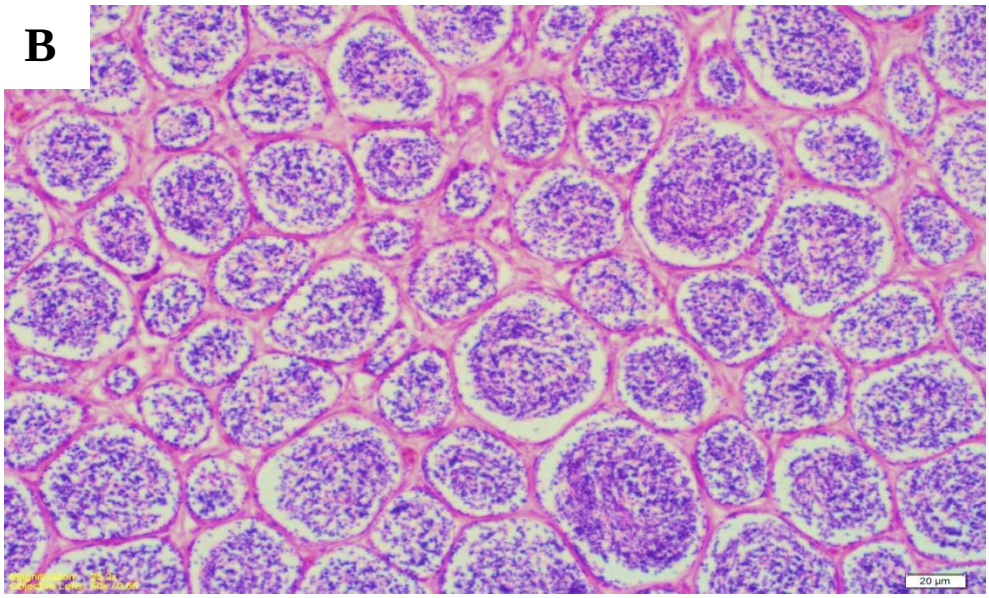


Figure 2. A). A portion of the *Torquigener flavimaculosus* ovary. Scale bars= 200µm. B) A portion of the *Torquigener flavimaculosus* testis. Scale bars= 20µm. All sections stained with Haematoxylin and Eosin (H&E).

Phases of Oocyte Development in T. flavimaculosus

The phases of oogenesis were classified with respect to the appearance of nuclei and nucleoli and distribution of ooplasmic inclusions. Based on histological examination, oocytes development in *T. flavimaculosus* was divided into three stages: Primary growth phase (PGP), Secondary growth phase (SGP), Maturation and hydration phase (MHP). Primary growth phase was subdivided into two stages as chromatin nucleolar and perinucleolar stages. Chromatin nucleolar stage was characterized by a large nucleus in the central position surrounded by a narrow cytoplasm. In perinucleolar stage, the nucleus increased in size and multiple nucleoli appeared. These were generally arranged at the periphery of the germinal vesicle (Fig. 3A). Secondary growth phase was subdivided into three stages as oocytes at stage three, four and five. This stage comprises endogenous and exogenous vitellogenesis and was detected in all collected samples (Fig. 3B). At stage 3 oocyte, cortical vesicles were detected for the first time. These usually spherical structures appeared at random at various depths in the ooplasm. They provided the first evidence for initiation of the secondary growth phase and appeared as empty unstained vacuoles (Fig. 3B). At stage 4 oocyte, cortical alveoli increased in size and number. The germinal vesicle consists of many nucleoli. At stage five oocyte, yolk granules were first detectable between vacuoles. At the end of this stage, the nucleus showed a significant number of projections into the cytoplasm (Fig. 3B). Final oocyte developmental stage was Maturation and hydration phase, which was sub-divided into stage six and stage seven oocyte. Stage six was distinguished by migration of the nucleus to the animal pole (Fig. 3C). At stage seven oocytes, the yolk granules enlarged and there were no sing of nucleus. After the germinal vesicle breakdown at stage 6, the oocytes ovulated into the ovarian lumen at the end of the stage seven. These two stages were detected from June to October.

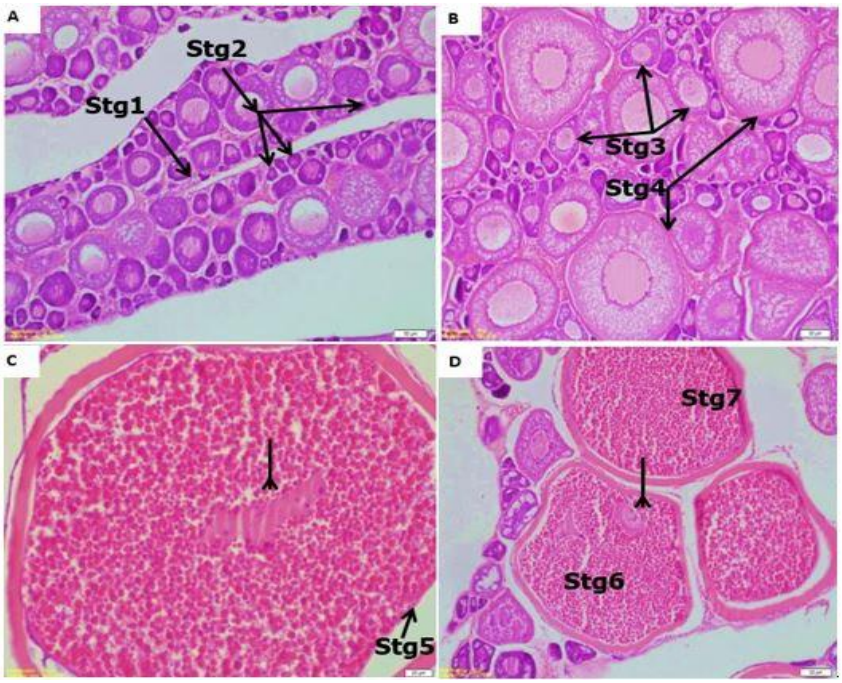


Figure 3. Photomicrograph of T. flavimaculosus ovaries in different stages of oogenesis, recorded from June to October 2017. A) Arrows show oocyte at stage 1 and stage 2. Scale bar=50µm. B) Arrows show oocyte at stage 3 and 4. Scale bar=50µm. C) Arrow shows oocytes at stage 5. Notched arrow shows the projection of nucleus into the cytoplasm. Scale bar=20µm.D) Oocyte at maturation stage is shown (stage 6) and at the hydration phase (stage 7) is shown. Notched arrow shows the germinal vesicle break down and migration. All section stained with Haematoxilen and Eosin.

Phases of Sperm Development in T. flavimaculosus

Phases of spermatogenesis were distinguishable based on their characteristic nuclear and cytoplasmic morphologies. Formation of spermatozoa in *T. flavimaculosus* was divided into five stages: Spermatogonia (Stg1), primary spermatocytes (Stg2), secondary spermatocytes (Stg3), spermatitds (Stg4) and spermatozoa (Stg5), (Fig. 3A, 3B and 3C). Spermatogonia cells were present in the testis of *T. flavimaculosus* from June to October. The spermatogonia were the largest cells in the germinal tissue of the testis. These cells divided mitotically and formed primary spermatocytes. At stage two, primary spermatocytes were detected. They were smaller than the spermatogonial cells and larger than secondary spermatocytes. The nuclear membrane was not clear and the chromatin material occupied most of the cell. They divided meiotically and produced secondary spermatocytes (Fig. 3A). At stage three, the secondary spermatocytes were clearly detected. The secondary spermatocytes were smaller than the primary spermatocytes and were more basophilic. In this stage, the nuclear membrane was also not visible. These two stages (Stg2 and 3) were also detectable from June to October. At stage four, the secondary spermatocytes continued meiotic division and produced spermatids. Spermatids were smaller than the secondary spermatocytes, irregular in shape and very strongly basophilic (Fig.3B). At stage five, no cell division was recorded. Spermatozoa were smaller than the spermatids. Transformation of spermatids into mature spermatozoa consisted of reorganization of the nucleus and ooplasm, together with the development of a flagellum. At the end, the lobular wall breaks down, and the

sperm become unattached and lie free in the lumen. Spermatozoa were detected from June to October (Fig. 3C).

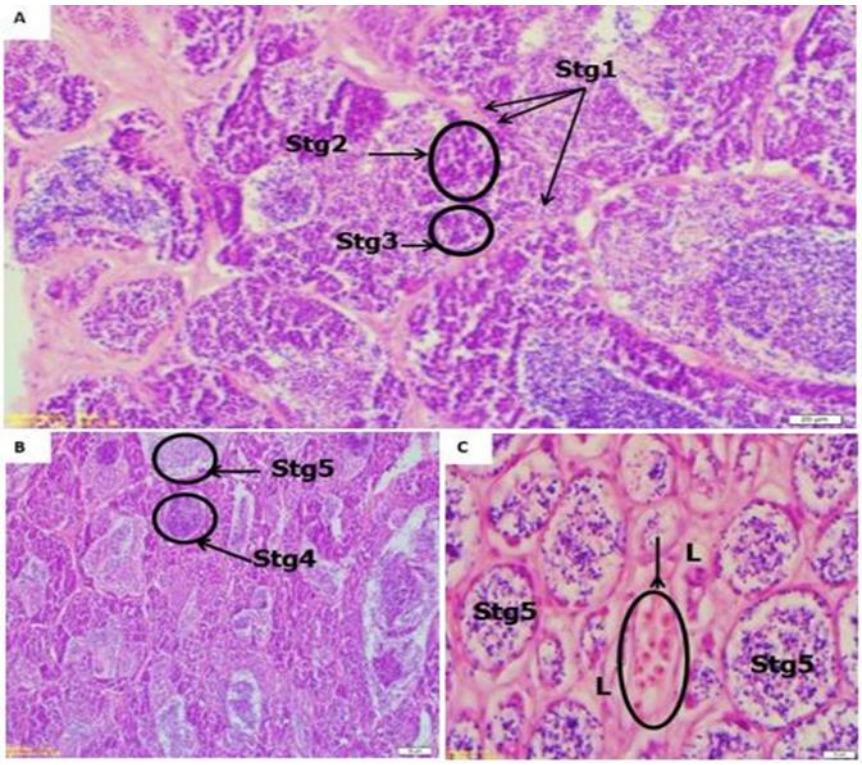


Figure 3. Photomicrograph of *T. flavimaculosus* testis in different stages of spermatogenesis, recorded from June to October 2017. A) Arrows show stage 1, stage 2 and 3. Scale bar=20µm. B) Arrows show stages 4 and 5. Scale bar=50µm. C) After spermiation, stage 5 is shown, L: lumen. Notched arrow shows blood vessel. Scale bar=10µm. All section stained with Haematoxilen and Eosin.

Discussion

Our results showed that the sex ratio of *T. flavimaculosus* was 62.5% female and 37.5% male. Sex ratio in Silver-cheeked toadfish , *Lagocephalus sceleratus* was investigated by Sabrah et al. (2006) investigated sex ratio of Silver-cheeked toadfish , *Lagocephalus sceleratus* and the overall sex ratio of males to females was found to be 1:1.3, which was not significantly different from 1:1 ($p>0.05$). Aydın (2011) studied sex ratio on the same species and found out no statistical differences on the sex ratio of males to females. Similar to the present study, in both studies sex were separated and population consisted of dioecious specimens. In a study by Matsunaga et al. (2014), the sex ratio of tiger pufferfish was highly skewed towards males. While 77 fish had ovaries, 201 fish contained testes. Moreover, 15 fish possessed both testicular and ovarian tissue (Matsunaga et al., 2014). Currently, there is no available data on the sex ratio of *T. flavimaculosus*. In the present study, only eight specimens were caught and this number is not enough for comparison or for making a certain conclusion. Research on sex ratio of *T. flavimaculosus* is going on.

Our study briefly described the light microscopical characteristics of oocytes development in *T. flavimaculosus*, which was divided into 3 phases: PGP, SGP and MHP. Oogonia of *T. flavimaculosus* divided and turned into prenucleolus stage, which subsequently grow within follicles, formed cortical alveoli, entered vitellogenesis underwent maturation and finally ovulated. The changes that occurred during these phases were similar to those reported for other pufferfish (Hamasaki et al., 2013; Matsunaga et al., 2014). Based on histological observations, maturation in *T. flavimaculosus* occurred from June to October, which was suggested as a spawning season. Oocytes at all stages of maturity were detected in the ovary of *T. flavimaculosus*. Although, no dominant oocytes populations were observed in the ovary, there seemed to be protracted and continuous ovulation. The oocytes at stage 7 were detected in June, July, August, September and October. Therefore, *T. flavimaculosus* might be classified as group synchronous or asynchronous type. In order to make a clear conclusion regarding to reproductive pattern of *T. flavimaculosus*, oocyte size frequency analysis will be performed.

In the male *T. flavimaculosus* formation of spermatozoa was subdivided into five developmental stages. Stage 1 testes only contained spermatogonia. Stage 2 testes contained primary spermatocytes and meiotic germ cells. Stage 3 testes contained spermatogonia, primary and secondary spermatocytes and meiotic germ cells. Stage 4 testes contained spermatids. In stage five testes, all germ cell stages, including spermatozoa were present. These all five stages were detected from June to October and often spermiation was clearly observed. Maturation season for male *T. flavimaculosus* were also summer. Maturation in males was concomitant with maturation in females. These findings were similar to those reported by Hamasaki et al. (2013), Matsunaga et al. (2014).

In the ovary, the level of tetrodotoxin was reported to be highest (Thattiyaphong et al., 2014; Itoi et al., 2017; Acar et al., 2017). TTX content was fluctuated regarding to developmental stages in ovary and testis of pufferfish. The level of toxin was different between ovary and testis during the spawning season (Itoi et al., 2015; 2016). It was suggested that TTX only transferred from the liver to the ovary. Is TTX only transferred from the liver to the ovary needs to be elucidated. It has been suggested that the toxin is protective against predators and secreted to attract male pufferfish (Itoi et al., 2012, 2015, and 2016). Recently, Kiriake et al., (2016) suggested candidate genes related to TTX toxification in the liver of tiger pufferfish, *Takifugu rubripes*. Intramuscular injection of TTX to the nontoxic juvenile pufferfish to observe short-term toxin behavior revealed that the administered TTX is transferred first to the liver and then to the skin via blood circulation, however toxin transfer to the gonads differs greatly between males and females (Ikeda et al., 2010). TTX was only accumulated in the ovary (Ikeda et al., 2010). Further studies are need to examine how TTX level changes with ovary and testis developmental stages and maturation.

Although TTX is extremely poisonous to humans and other living creatures, it has been extensively used for human benefits (Alonso et al., 2003; Fouda, 2005; El-Dayem, 2013; Bragadeeswaran et al. 2010; Priya & Khora, 2013). The physiological function of tetrodotoxin in pufferfish ovary and testes seems obscure and deserves much more attention and if TTX is going to be extracted from the gonads and used for human benefits, more studies on gonadal development and maturation stages of *T. flavimaculosus* should be conducted.

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