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First report on TTX levels of the yellow spotted pufferfish (*Torquigener flavimaculosus*) in the Mediterranean Sea

Ali Rıza Kosker [a, *], Fatih Özogul [a], Mustafa Durmus [a], Yılmaz Ucar [a], Deniz Ayas [b], Vida Šimat [c], Yeşim Özogul [a]

[a] Department of Seafood Processing Technology, Faculty of Fisheries, Çukurova University, Adana, Turkey
[b] Department of Seafood Processing Technology, Faculty of Fisheries, Mersin University, Mersin, Turkey
[c] University of Split, Department of Marine Studies, Split, Croatia

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ABSTRACT

The differences of tetrodotoxin (TTX) levels in various parts of pufferfish (*Torquigener flavimaculosus*) were examined in conjunction with the seasonal and sexual variations. The TTX levels in gonads, liver, intestines, skin and muscle tissue were determined using the Q-TOF LC/MS. Instrumental analysis revealed that all examined tissues from *T. flavimaculosus* contained high TTX concentrations. TTX levels in the gonads, liver, intestines, skin and muscle tissue of pufferfish were within the range of 5.03–100.71, 7.04–106.80, 12.59–86.30, 33.95–139.88 and 15.88–86.07 (μg/g), respectively. It was determined that in all seasons, except for summer, female individuals had higher TTX levels than males. Among all seasons, the highest level of TTX was found in winter and the lowest in autumn. Consequently, *T. flavimaculosus* is a highly toxic pufferfish that is dangerous for human consumption and should not be consumed under any circumstances.

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1. Introduction

Human poisoning by marine toxins has been occurring through human history, and poisoning cases have been recorded since thousands of years (Otero, 2014). For instance, poisonous pufferfish, belonging to the Tetraodontidae family, were documented in the Ancient Chinese Pharmacopoeia compiled in 2800 BCE; thus, the warnings and recommendations associated with handling pufferfish were documented (Otero, 2014). Researches aimed at detecting and discovering unknown toxins have been going on to understand their impact and applicability on human health and ecosystem. Due to lacking toxicological data on unknown toxins, more knowledge about poisonous fish is still important for human health. The natural habitats of poisonous fish are the Pacific and Indian oceans, where tropical marine ecosystems are prominent. Societies living in these regions are familiar with poisonous fish and poisoning cases arising from the consumption of poisonous fish. However, poisonous fish were not an issue of concern in Mediterranean countries until recently. Among many species that entered the Mediterranean Sea and settled into its ecosystem, pufferfish, known for their poisonous characteristics, are of special concern (Kosker et al., 2015). Since pufferfish moved to the Mediterranean region, accidental poisoning by its consumption has happened the Mediterranean region (Eisenman et al., 2008; Bentur et al., 2008; Chamandi et al., 2009; Kheifets et al., 2012).

The majority of alien fish settling in the Mediterranean Sea are known as lessepsian species that migrate from the Red Sea through the Suez Canal. Although these species inhabiting the Mediterranean are economically valuable, some are considered harmful species (Zenetos et al., 2012). Pufferfish are not an economically significant species, and they pressurise economically valuable fish species that are commercially profitable and nutritious for human consumption (Streftaris and Zenetos, 2006; Bentur et al., 2008). Pufferfish stand out among other alien species due to their harmful effects on the economically significant fish species, fishing and public health (Streftaris and Zenetos, 2006). The majority of pufferfish species contain tetrodotoxin (TTX), which is one of the most lethal toxins (Hwang and Noguchi, 2007). Symptoms of TTX poisoning are paralysis, respiratory distress, nausea and muscle coordination disorder (Isbister et al., 2002). TTX acts by blocking the sodium channels in the neurons and has no known antidote

* Corresponding author. Department of Seafood Processing Technology, Faculty of Fisheries, Cukurova University, 01330, Adana, Turkey.
E-mail addresses: akosker@cu.edu.tr (A.R. Kosker), fozogul@cu.edu.tr (F. Özogul).

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(Moczydlowski, 2013). Lethal dose of TTX for humans (MLD_{50}) is around 2 mg (Hwang and Noguchi, 2007).

In recent years, incidents of pufferfish poisoning due to their toxin that causes have become frequent in social and print media in Mediterranean countries. Ten different species of pufferfish belonging to the Tetraodontidae family are present around the Mediterranean Sea (Kosker et al., 2015). Although the most prevalent pufferfish species in the Mediterranean is *Lagocephalus sceleratus,* the population of *Torquigener flavimaculosus* has also increased greatly in recent years, particularly in the eastern Mediterranean Sea. *T. flavimaculosus* is typically found in the western shores of the western Indian Ocean and is particularly widespread in the Red Sea and the shores of Kenya; it has migrated to the Mediterranean Sea in recent years through the Suez Canal (Randall, 1995). *T. flavimaculosus*, also known as the yellow-spotted pufferfish internationally, is also called the dwarf pufferfish in Turkey. It has sometimes been misclassified as *Lagocephalus hypselogenion* or *Amblyrhynchotes hypselogenion* (Randall, 1995) in some studies. Systematic naming of this species present in the western Indian Ocean was specified by Hardy and Randall (1983) as *Torquigener flavimaculosus*.

T. flavimaculosus is a carnivorous pufferfish species that feeds on small benthic crustaceans. It has the ability to quickly inflate its body using water or air like other pufferfish species (Golani et al., 2006). Sabour et al. (2014) indicated that they can reach a maximum length of 16 cm, while Golani et al. (2006) reported a maximum length of 11 cm. Scientific studies on this species, usually in the form of first recordings, are about weight–length relations and burrowing behaviour (Bilecenoğlu, 2005; Corsini-Foka et al., 2006; Sabour et al., 2014). Some other studies focus on toxicity and have reported that pufferfish species present in the Mediterranean Sea, which *L. sceleratus* (Katikou et al., 2009; Rodriguez et al., 2012; Kosker et al., 2016; Kirimer et al., 2016) and *L. lagocephalus* (Saoudi et al., 2008) contain TTX. However, no toxicity studies have been conducted on the TTX contents of *T. flavimaculosus*, either in the Mediterranean Sea or in its natural range of the western shores of the western Indian Ocean, around the Red Sea and Kenyan shores (Randall, 1995). Though this species was reported as harmless to humans by Fishbase (2018), there is no scientific evidence to support or dismiss this claim. To our knowledge, this is the first report on the TTX content of *T. flavimaculosus.* Therefore, in this study, it was determined the changes of the tetrodotoxin (TTX) levels in gonads, liver, intestines, skin and muscle tissue of pufferfish (*Torquigener flavimaculosus*) using Q-TOF LC/MS in conjunction with the variations that may be caused by seasonal aspects and sexual maturity.

2. Materials and methods

2.1. Tetrodotoxin standard

TTX standard was purchased from Abcam Biochemicals (Cambridge, UK). For the instrumental toxin analysis, 1 mg of standard was used. Before instrumental analysis, TTX standards were diluted using methanol containing 0.01 M acetic acid (Merck). Then, 0.05, 0.1, 0.5, 1 and 2 mg/ml standards were prepared by dilution from stock solution for use in Q-TOF LC/MS analysis to draw the standard curve and stored at −20 °C until further use.

2.2. Fish collection, measurements and identification

Pufferfish were caught in the northeastern Mediterranean Sea by commercial trawl fishing from December 2015 to October 2016. The coordinates were between 36°43′31.8″N, 34°54′27.0″E and 36°08′53.6″N, 33°39′40.7″E (Fig. 1). Fish caught from this region were transported to the lab in ice. Size–weight measurements of pufferfish for all seasons were carried out (Table 1) and genders were determined using a microscope. Only sexually mature individuals were used for the study. Among samples, the maximum weight was 37.88 g for females and 31.30 g for males, while the maximum length was 12.80 cm for females and 12.10 cm for males. For each season, 10 male and 10 female *T. flavimaculosus* individuals were selected, and the muscle tissue, gonads, liver, skin and intestines of these individuals were dissected. In each group, tissues from 10 individuals were taken and mixed for toxin extraction. After that all samples were analysed for each parts of fish.

2.3. Preparation of samples and toxin extraction

Fish were dissected to obtain some dorsal muscle (carefully avoiding the gastrointestinal tract), gonads, the entire upper and lateral skin from head to tail, intestines and the liver. Tetrodotoxin extraction from skin, liver, intestines, gonads and muscle tissues was performed according to the method of Silva et al. (2012). Eighty samples from four seasonal groups were analysed in triplicate.

For TTX extraction, 1 g of sample from each tissue of the pufferfish was used. Methanol (3 ml) containing 1% acetic acid was added to the 1 g sample. The mixture was then homogenised using the Ultra Turrax device (IKA T25 Digital Ultra Turrax, Staufen, Germany) at 7200 rpm for 10 min. Thereafter, it was kept in an ultrasonic bath (Bandelin Sonorex RK 100, Berlin, Germany) at 100 Hz for 10 min. The samples were then kept at room temperature for 15 min and centrifuged (Hettich Zentrifugen, Universal 32R, Tuttlingen, Germany) at 4500g for 20 min at 4 °C. After centrifugation, the upper phase was taken away, and 3 ml methanol with 1% acetic acid was added to the residue and the previous steps were repeated. After second centrifugation, the upper phase obtained was combined with the upper phase from the previous stage, and the mixture was completed to 7 ml. One millilitre of the extract was purified by passing it through a 500 mg/3 ml C18 solid-phase extraction (SPE) cartridge (Supelco, Bellefonte, PA, USA). The sample was eluted with 10 ml of 100% methanol and diluted with the same solvent to a final volume of 12 ml. Next, the solution was evaporated till dry, and the residue was mixed with 1 ml methanol (Clarinet, Agela Technologies, Wilmington, USA). Finally, the residue was filtered using 0.45μ membrane filters and transferred to vials for analysis.

2.4. Instrumental tetrodotoxin (TTX) analysis

Instrumental TTX analysis was performed using an Agilent brand 6545 Accurate-Mass Q-TOF LC/MS coupled with an Agilent 1260 HPLC (Agilent Technologies, Inc., Santa Clara, CA, ABD) device.

Before analysis, a standard calibration curve was created using a TTX standard. R^2 value was determined as 0.9992. Poroshell 120 HILIC (3.0 × 50 mm; 2.7 μm) column (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for analysis. The toxin was separated in the column using two different mobile phases. Mobile phase A was 20-mM ammonium acetate in distilled water (Sigma-Aldrich), and mobile phase B was 20-mM ammonium acetate in acetonitrile (Sigma-Aldrich). Analysis was completed in 8 min. TTX molecule was observed at 3.9 min. A gradient program was established as 3% mobile phase A and 97% mobile phase B in the first 2.5 min, followed by 30% mobile phase A and 70% mobile phase B for 2 min and again 3% mobile phase A and 97% mobile phase B. The column temperature was 20 °C and injection volume was 10 μl. The LC system was operated in the positive ion mode with an ESI (electrospray ionisation) interface using the parameters below: collision-activated dissociation gas, 6 psi; gas flow, 12 l/min; ion spray voltage, 3500 V; temperature, 400 °C and nebuliser pressure,

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