Accidental discovery of a Tetraodontidae (*Sphoeroides marmoratus*) within a cuttlefish (*Sepia officinalis*) bought in a fish shop in Italy: risk assessment associated with the presence of Tetrodotoxin

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Abstract

The discovery of a pufferfish specimen (Tetraodontidae) inside a frozen cuttlefish, purchased by a fishmonger, and caught in the Eastern Central Atlantic (FAO 34) is reported. The consumer, who reported this case to FishLab (Department of Veterinary Sciences, University of Pisa) for investigation, was a student of Veterinary Medicine at the University of Pisa. He recognized the Tetraodontidae because he attended practical lessons on fish morphological identification during the course of food inspection and was aware of the risks to human health linked to the Tetrodotoxin (TTX). In this study, the pufferfish was identified morphologically, using the FAO morphological keys, and molecularly, analyzing two markers, the cytochrome oxidase I (*COI*) and the cytochrome b genes, by DNA barcoding. The pufferfish was identified morphologically as *Sphoeroides* spp., and molecularly as *Sphoeroides marmoratus* using the *COI* gene (99-100% identity values). Literature reports that *S. marmoratus* from the Eastern Atlantic contains high concentrations of TTX in the gonads and the digestive tract. However, the possible passage of TTX from fish to other organisms linked to contact or ingestion has never been reported. This represents the first case of a potentially toxic pufferfish entering the market inside another organism. The fact that a student observed this occurrence highlights the key role of citizen science in the management of emerging risks.

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Introduction

Tetrodotoxin (TTX) is a generic term referring to a group of naturally-occurring marine neurotoxins (EFSA, 2017). TTX was first isolated from fish of the Tetraodontidae family, including pufferfish, from which it takes its name (Suehiro, 1994). TTX poisoning is among the main intoxications characterized by neurological disorders that are caused by a marine toxin (Isbister and Kiernan, 2005). Human ingestion of potentially lethal TTX-contaminated products causes severe neurological symptoms which develop between 10 minutes and 15 hours later. TTX can block voltage-dependent Na+ channels by preventing the conduction of these cations across the membrane (Denac *et al*., 2000) and, when this occurs at the level of excitable cells, such as neurons and muscle cells, it leads to paralysis. In the early stages, clinical signs are paranesthesia of lips and tongue, headache, and gastrointestinal symptoms until ataxia, seizures, cardiac arrhythmias, and respiratory failure, leading to death (Isbister and Kiernan, 2005).

Despite being mainly associated with Tetraodontidae pufferfish, TTX has also been found in other marine species belonging to different taxa such as mollusca (gastropods, bivalves, and cephalopods), arthropods, echinodermata, chordata, platyhelminthes, annelida and algae (Noguchi and Arakawa, 2008) and from a single class of terrestrial vertebrates (amphibia) (Hanifin, 2010). It was observed that pufferfish fed with a controlled diet in a bacteria and TTX-free environment became non-toxic, while non-toxic pufferfish fed with a TTX-rich diet became toxic (Kono *et al*., 2008; Noguchi and Arakawa, 2008; Ikeda *et al*., 2009) This evidence led to the assumption that TTX is not produced by pufferfish but accumulated along the trophic chain from marine bacteria (Noguchi and Arakawa, 2008). To date, 31 genera of bacteria are recognized as TTX producers with *Vibrio* sp. as the most common (Tonon *et al*., 2020).

TTX are potentially accumulated in all fish tissues and in higher concentrations in liver and gonads. However, its concentration can vary according to sex, marine habitat, environmental changes, season, and geographic area (Bane *et al*., 2014). Usually, female specimens presented higher toxicity than males, while, in terms of geographical area, TTX poisonous pufferfish especially inhabit tropical and subtropical Atlantic regions (Tonon *et al*., 2020). Despite restrictions on consumption, pufferfish (*fugu*) is a traditional delicacy consumed in southeast Asian and Asian countries like Japan, Taiwan, and Bangladesh (Guardone *et al*., 2020). On the contrary, the selling of fish belonging to the Tetraodontidae families, as well as the Molidae, Diodontidae, and Canthigasteridae families, is banned in the European Union (EU) (European Commission, 2004).

In the EU, the risk associated with TTX intoxication was historically related to the consumption of illegally imported products (Guardone *et al*., 2020). Although no intoxication has been reported so far, cases of species substitution recently occurred in Italy, testifying to the possible presence of toxic species on the EU market (Armani *et al*., 2015). Currently, the risk of ingestion of toxic pufferfish is even higher due to the entry of toxic species such as *Lagocephalus sceleratus* into the Mediterranean Sea (Rambla-Alegre *et al*., 2017; Kosker *et al*., 2019). During the last 2 decades, poisoning cases have been reported in the Mediterranean basin along the coasts of Egypt, Israel, Lebanon, and Turkey but also in Greece (cases reviewed by Guardone *et al*., 2020). Rambla-Alegre *et al*. (2017) reported the first evidence of high amounts of TTXs also in a *L. sceleratus* specimens caught in the western Mediterranean Sea (Rambla-Alegre *et al*., 2017). Besides *L. sceleratus*, *L. lagocephalus* (oceanic pufferfish) and *S. pachygaster* (blunthead pufferfish) have also been found along the Italian coasts, even though the presence of TTX in these species is not reported to date (Guardone *et al*., 2018).

Therefore, toxic pufferfish represent an emerging public health risk, for which public informative campaigns were undertaken to raise awareness of this issue among EU citizens (ISPRA, 2016; Directorate-general for maritime affairs and fisheries, 2022). In Italy, we organized meetings with fishermen to avoid the accidental entry of pufferfish into the supply chain. These meetings were aimed at receiving information directly from the fishermen to improve data collection for a proper risk assessment associated with the spread of toxic species in the Mediterranean Sea. Collected specimens were used for practical teaching practical teaching activities activities addressed to the fourth year students of Veterinary Medicine degree, at the Department of Veterinary Sciences of the University of Pisa, within the course of food inspection. This type of approach, which makes the population an active part of the process, is called *citizen science* (CS). Through CS, not only do citizens acquire knowledge and skills (as in the case of toxic species recognition), but they also actively contribute to research (Vohland *et al*., 2021).

In this respect, on 9 June 2022, a student of Veterinary Medicine at the University of Pisa discovered a suspected pufferfish inside the stomach of frozen cuttlefish caught in the Eastern Central Atlantic (FAO 34) and purchased by a fishmonger in Italy. The student, being aware of the risks to human health linked to the presence of TTX, brought the specimens to FishLab (Department of Veterinary Sciences, University of Pisa). This study aimed to perform a morphological and molecular confirmation of the pufferfish specimen for a proper risk assessment due to the presence of TTX.

Materials and Methods

Morphological identification

The following parameters were measured from both whole specimens [the suspected pufferfish and the cuttlefish (Figure 1)]: weight, total length, length at peduncle (for pufferfish), and mantle length (for the cuttlefish). The morphological identification was then achieved through the application of FAO morphological keys (Colapisci, 2022; FishBase, 2022; SealifeBase, 2022). The information reported on the label was recorded.

Molecular identification

Total DNA extraction from 50-100 mg of tissue was performed according to Armani *et al.* (2015). Final DNA concentration and quality were checked with a Nanodrop ND-1000 spectrophotometer (Thermofisher Scientific Inc.). Two mitochondrial genes, Cytochrome C oxidase subunit I (*COI*) and cytochrome b (*cytb*) were selected as targets for species identification. A fragment of 655 pb of the *COI* gene was amplified from both the pufferfish and cuttlefish using the protocol described in Tinacci *et al*., (2018). Additionally, the whole *cytb* gene was amplified from the pufferfish, according to Tinacci *et al*. (2018). Polymerase chain reaction (PCR) products were purified with EUROSAP kit (Euroclone SPA Pero, Milano, Italy). Purified PCR products were sent for sequencing. The sequences obtained were edited using Bioedit version 7.1 (Hall, 1999), and then subjected to BLAST analysis on GenBank (www.ncbi.nlm.nih.gov) and, for the *COI* gene, also IDS analysis on BOLD (www.boldsystems.org). A top match identity score >98% was applied for species allocation (Tinacci *et al*., 2018).

Results

Morphological and molecular identification

According to the morphological analysis, the cuttlefish was identified as *Sepia officinalis* confirming the species reported on the label. As for the suspected pufferfish found in the cuttlefish's stomach, it was completely intact, testifying that it had plausibly been ingested shortly before capture. Thus, thanks to the absence of defects and the preservation of anatomical features, the morphological identification of pufferfish was also successfully performed (Figure 1). The comparative analysis between the morphological features collected (Table 1) and the morphological keys available on FAO catalogs allowed a first allocation of the specimen to *Sphoeroides* sp. Further comparative analysis with the morphological sheet available on colapisci.it oriented the identification to the species *Sphoeroides marmoratus*, whose natural habitat is concentrated almost exclusively in eastern central Atlantic - FAO 34 (FishBase, 2022) corresponding to the fishing area declared on the label. The morphological and morphometric data collected for the two analyzed specimens are summarized in Table 1.

The molecular analysis of the *COI* gene confirmed the results of the morphological identification. The final species allocation was only reached on BOLD database, thanks to the presence of 6 private reference sequences, which returned a matching ID of 100-99.38% against the sequence obtained in the study. Inversely, neither *COI* nor *cytb* sequences of *S. marmoratus* were available on GenBank.

Discussion

Habitat and toxicity of *Sphoeroides marmoratus*

In this study, the molecular analysis allows us to identify the pufferfish at the species level as *S. marmoratus,* although the molecular identification highlighted criticisms linked to the absence of reference sequences in public databases. In particular, the need to improve databases with reliable sequences from properly identified pufferfish specimens was already reported by Giusti *et al*. (2019).

The pufferfish *S. marmoratus* belongs to the Tetraodontidae family and it is distributed in the Northeast Atlantic Ocean from Portugal to Angola (FishBase, 2022). Even though on Fishbase it is not reported as a toxic species, Pinto *et al*. (2019) found a large amount of TTX and analogs in several tissues (gonads, liver, digestive tract, and muscle) of 4 specimens caught over the southern coast of Madeira Island (Portugal), caught in the FAO area 34 (Eastern Central Atlantic Ocean), the same from which the specimen of this study was caught. Also, in the study conducted by Silva *et al*. (2019), TTX was found in several tissues of a female specimen of *S. marmoratus* caught in the Azorean Archipelago (Northeast Atlantic Ocean, FAO area 27) bordering the FAO area 34 (Silva *et al*., 2019). A specimen of *S. marmoratus*, initially wrongly identified as *L. lagocephalus* (Vacchi *et al*., 2007), was found in the waters of Gallipoli (Apulia) in the late 1970s demonstrating how this species could enter the Mediterranean Sea through the Strait of Gibraltar (FAO area 37.1.1) as already reported for *L. lagocephalus*. Moreover, once it has entered, it is plausible to think that this species may eventually settle in the Mediterranean Sea as it happened for *S. pachygaster* (Guardone *et al.* 2018). Therefore, the risk of finding toxic species on the market may be linked to their introduction through both importation from the geographical area in which TTX is reported and fishing in Mediterranean waters.

Citizen science and risk management

CS has been defined by the Oxford English Dictionary, as *scientific work undertaken by members of the general public, often in collaboration with or under the direction of professional scientists and scientific institutions* (OED, 2022). CS can benefit participants in several ways: by enhancing their overall knowledge of the topic, enhancing their understanding of the scientific process and method, improving the access to scientific information, and the ability to interpret scientific information (Den Broeder *et al.*, 2018). Factually, CS is normally applied as a dissemination method within universities, through the Third Mission, where dedicated committees organize meetings or participate in events to inform the population about its research activities. To date, specific projects of CS have been developed especially in ecology and environmental sciences, while they are still rarely applied for public health purposes. However, the CS approach should be used more for public health management, and extended to various categories of people, institutions, and companies. Moreover, in light of the benefits that CS brings to both science and citizens, cooperation between universities and the population should be implemented and strengthened. At an international, EU, and Italian level, invasive species monitoring campaigns were conducted to collect data on the presence of alien species over vast geographical areas with the help of citizen reports submitted online. Within the IZSLT (2014) project, in which FishLab also participated (Guardone *et al.* 2018), CS was applied as a training tool in public health since cat-

Figure 1. Cuttlefish and pufferfish specimens analyzed in this study.

Table 1. The morphological and morphometric data collected for the two analyzed specimens.

Data	Cuttlefish	Pufferfish
Total weight	425 g 43 g	
Total length	24 cm	13 cm
Mantle length/length at caudal peduncle	14.5 cm	12 cm
Mantle/skin color	Greyish with long vinelike bands.	Dorsal part: marbled color, brownish-grey background with greenish highlights, darker dorsally and lighter on the flanks, presence of large dark spots. Ventral part: pearly whitish ground, presence of a fine dark grey punctuation.
Descriptive analysis	Oval body, rounded posteriorly, bordered throughout its length by a narrow fin. The mouth is surrounded by eight non-retractile arms and two long, retractile, tentacles that are inserted laterally.	Elongated body, anteriorly higher, covered with numerous small spines more evident ventrally, lacking scales. The head is large, and squat with a straight diagonal profile. Small mouth, with four sharp teeth forming a beak-like shape. Peculiar eye with reddish-orange cornea.
Fins	Two long fins running the length of the mantle not fused at the apex, lateral insertion with a narrow base.	Large pectoral fins, with convex margin, ventral fins absent. Dorsal fin and caudal fin inserted posteriorly. Dorsal fin, with 8 soft, segmented rays, spiny rays absent. Anal fin opposite and in line with the dorsal, with 6 soft rays, spiny rays absent. Caudal fin slightly convex.

egories operating along the supply chain (fishermen) up to the final consumer (including students) were especially involved. Specifically, fishermen and citizens were trained in the recognition of toxic species and risks associated with their consumption, and at the same time, they were encouraged to provide information about sightings and catches of toxic specimens so that they could be collected. The active participation of fishermen was crucial for the collection of pufferfish specimens that were used for the practical teaching of students. The present study demonstrates how adeguately trained citizens, in this case represented by a student can play an instrumental role in managing risk and preventing the consumption of potentially toxic seafood products.

Conclusions

This case represents the first report of potentially toxic pufferfish specimens entering the market inside a cephalopod. Therefore, it suggests a new route through which TTX can reach the final consumers. While in all the cases described in the literature, intoxications are related to the direct consumption of pufferfish specimens (Guardone *et al*. 2020), in this case, TTX migration from pufferfish to cuttlefish should be taken into consideration. Therefore, both specimens were sent to the Marine Research Centre, National Reference Laboratory on Marine Biotoxins, for TTX detection to conduct an appropriate risk assessment, also in light of the origin of the specimen. This new potential hazard should be considered by veterinary hygienists and inspectors, as well as by fish operators. Finally, this study also demonstrates how CS can be applied to public health and improve the management of emerging risks through informed and aware citizens.

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