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First report on toxicity assessment of the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean Sea, Greece)

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ABSTRACT

According to the current European Union legislative requirements (Regulation 853/2004/EC; Regulation 854/2004/EC, poisonous fish of the family Tetraodontidae and products derived from them must not be placed on the European markets. Following the increased publicity regarding the presence of the pufferfish species *Lagocephalus sceleratus* in Greek waters, this study was undertaken in order to confirm its toxicity and assess the risk of poisoning in case of accidental consumption. Acidic extracts from tissues of *L. sceleratus* specimens of different sizes were examined by means of the official mouse bioassay for tetrodotoxin, while some of the extracts were also tested for the presence of Paralytic Shellfish Poisoning (PSP) toxins with a commercial ELISA kit. Toxicity in mice, with symptomatology indicative of tetrodotoxin, was confirmed in a number of samples and indicated a correlation with fish size. Toxicity of certain tissues (liver, gonads, gastrointestinal tract) in larger individuals, expressed as μg/g tetrodotoxin equivalents, was largely above levels required to cause death in human adults. On the other hand, all tested extracts provided a positive reaction in the ELISA test for PSP toxins. This constitutes the first report for presence of toxicity in *L. sceleratus* caught in European coastal waters.

1. Introduction

With the opening of the Suez Canal in 1869, two markedly different zoogeographical areas were joined: the subtropical Mediterranean Sea, which connects with the Atlantic, and the tropical Red Sea, the most northern extension of the Indian Ocean. In order to pass between these areas, organisms must be able to bridge the difference in adaptive requirements, and also withstand the extreme conditions in the Canal itself (Papaconstantinou, 1990). In this context, the term "Lessepsian migration" has been introduced to characterize a new phenomenon of unidirectional and successful biotic advance from the Red Sea to the Eastern Mediterranean, while the term "Lessepsian migrant" refers to the Red Sea species that have passed through the Suez Canal and settled in the Eastern Mediterranean (Por, 1978).

The Lessepsian migrant *Lagocephalus sceleratus* (Gmelin, 1789), also known as silverstripe blaasop, is an Indo-Pacific

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originated pufferfish of the family Tetraodontidae. Similar to its congeneric tropical species, *L. sceleratus* maybe a source of food poisoning with a high associated risk of mortality, as it commonly contains tetrodotoxin (TTX), a toxin which can cause death by muscular paralysis, respiratory depression and circulatory failure (Field, 1998; Bilecenoglu et al., 2006). The minimum lethal dose and minimum acute dose of TTX to human (wt. 50 kg) are estimated to be around 2 mg and 0.2 mg, respectively. Depending upon the amount of the toxin ingested, symptoms usually appear within 10–45 min of exposure, though some cases have reported being asymptomatic until as much as 3–6 h after exposure. Oral paresthesia is usually the initial symptom, which gradually spreads to the extremities and trunk. Other early symptoms include taste disturbance, dizziness, headache, diaphoresis, and pupillary constriction. These may or may not be accompanied by gastrointestinal symptoms of salivation, hypersalivation, nausea, vomiting, hyperemesis, hematemesis, hypermotility, diarrhea, and abdominal pain (Noguchi and Ebesu, 2001). On the other hand, several species of pufferfish, mostly freshwater ones, have been reported to also contain Paralytic Shellfish Poisoning (PSP) toxins and mainly saxitoxin (Nakamura et al., 1984; Sato et al., 2000), which cause to humans a very similar symptomatology to that of TTX.

L. sceleratus was first collected in the Mediterranean Sea on February 2003 from Gökova Bay (southern Aegean Sea, Turkey) (Akyol et al., 2005), and on November 2004 from Jaffa along the Israeli coast (Golani and Levy, 2005). In Greek waters, *L. sceleratus* was first recorded from the Cretan Sea (Aegean Sea) in July 2005 (Kasapidis et al., 2007). Since then, *L. sceleratus* has been recorded with an increasing frequency, in many areas of Aegean Sea, Greece and for this reason it is considered as one of the faster expanding Lessepsian immigrants. This fast expansion rate indicates a better ability to adapt to different environmental conditions and may also affect diversity and/or abundance of native species in the near future (Peristeraki et al., 2006). On the other hand, this increased reporting could also be attributed to more public awareness and provision of relevant information by the competent authorities, as *L. sceleratus* occurrence has been a major issue in the Greek press since the beginning of 2007. The collection of numerous juvenile *L. sceleratus* fish, together with picarel, bogue and smelt in islands of the Southeast Aegean Sea, which resulted in confusion to both fishermen and consumers was one of the most important incidents reported in the press in October 2007. This increased publicity has resulted in familiarization of the fishermen with the species' characteristics, and subsequent contribution to over-reporting, since all specimens are being delivered to local authorities. It is also interesting to point out the fact that a wide range of sizes of *L. sceleratus* fish are caught, which is evidence that the species is now well established and reproducing in the Aegean Sea.

According to the current European legislative requirements (Regulation 853/2004/EC; Regulation 854/2004/EC), poisonous fish of the family Tetraodontidae and products derived from them must not be placed on the market. Despite this fact, however, one cannot exclude the possibility for accidental consumption of the species, as in a recent similar case in the Eastern Mediterranean (Bentur et al., 2008). The increased incidence of *L. sceleratus* in Greek waters together with the publicity and consumer concern related to this species, triggered off the investigation with regard to toxicity of the species, taking also into consideration the size of the fish, in order to assess the potential risk of poisoning in case the species was accidentally consumed. To the best of our knowledge, this is the first report of *L. sceleratus* toxicity investigation in European waters.

2. Materials and methods

2.1. Fish collection, identification and measurements

Specimens no. 1–4 and 6 (see Table 1) of the pufferfish *L. sceleratus* were collected by trawl fishing in the Southeast Aegean Sea, near the island of Rhodes, in depths between 27 and 36 m during October–December 2007. Specimen no. 5 was caught in the North West part of Aegean Sea in the area of Horeyto, Pelion in June 2007. All fish were submitted by fishermen to the relevant authorities for identification. Fish were identified as *L. sceleratus* by an expert marine biologist according to the characteristics described in the FAO Species Identification Sheets for Fishery Purposes (FAO, 1984), the morphological characteristics reported by Akyol et al. (2005) and with the aid of fish guide pictures. Measurements of total length and weight, or average weight in the case of group samples, were also conducted. After identification and conduction of measurements, fish were frozen and transferred to the National Reference Laboratory on Marine Biotoxins (NRLMB). Samples were kept frozen at −70 °C until analysed.

2.2. Sample preparation – toxin extraction

Toxicity of the different organs obtained from individual fish was determined according to the Japanese official method for tetrodotoxin in puffer (Kawabata, 1978b). Each specimen (individual or group of fish) was partially thawed, in order to avoid migration of toxin between tissues, and subsequently dissected into the muscle, liver, skin, gonads (when available) and gastrointestinal tract. A portion of 10 g from each organ was extracted individually with 25 ml of 0.1% acetic acid (Riedel de Haen, Sigma–Aldrich, Seelze, Germany) by heating for 10 min in a boiling water bath with occasional stirring. The mixture was cooled down and then filtered. The residue on filter paper was washed with portions of 0.1% acetic acid and the filtrate and washings were combined and made up to 50 ml with the same

Table 1
Sampling details and weight and length measurements of the experimental fish.

Specimen	Collection depth (m)	Collection date	No. of individuals	Average weight (g)	Length range (cm)
1	30	10/10/2007	15	5	5–10
2	36	3/12/2007	14	40	12–16.4
3	27	22/11/2007	11	78	16–20
4	27	22/11/2007	1	1060	43
5	nr	20/6/2007	1	1200	49.5
6	36	3/12/2007	1	3786	66

nr = Not reported.

solution in a volumetric flask. In case that sample quantities lower than 10 g were available, all volumes were relatively adjusted. The resulting solution, of which 1 ml was equivalent to 0.2 g of tissue, was used for conduction of the mouse bioassay and also for testing with the ELISA kit. In the latter case, pH value of the extracts (initially between 5.0 and 5.5) was adjusted to <4.0 with 0.1 N HCl in order to comply with the instructions of the ELISA kit and the extracts were further diluted 1:10 with the dilution buffer included in the kit.

2.3. Conduction of mouse bioassay

One millilitre of the prepared solution intraperitoneally administered into each of three Albino Swiss male mice and mice were observed for symptoms and times of death. The median death time (period between injection and death) was used to calculate the number of mouse units (MU). Toxicity level of the sample (expressed in MU/g) was calculated from the dose–death time relationship table of Kawabata (1978b) where 1 MU (mouse unit) was defined as the amount of toxin required to kill a 20 g male mouse by 30 min after intraperitoneal injection. Results were subsequently converted to μg tetrodotoxin equivalents/g of tissue, taking into account that 1 MU is equivalent to 0.22 μg of tetrodotoxin.

The mice were allowed laboratory chow and water ad libitum throughout the observation period. All animal manipulations were performed in accordance to the EU Directive 86/609/EEC (1986), under official license from the Prefectural Veterinary Service of Thessaloniki, Greece.

2.4. PSP ELISA test

A commercially available ELISA kit (Fast Saxitoxin, Ridascreen®, Darmstadt, Germany), based on the method of Usleber et al. (1991), was employed for testing some of the extracts for Paralytic Shellfish Poisoning (PSP) toxins content. Specifically, the extracts from specimens no. 3 (liver, gastrointestinal tract, muscle and skin) and no. 4 (liver, gonads, gastrointestinal tract, muscle and skin) were chosen as they were the smallest in size exhibiting some toxicity in the mouse bioassay. The entire testing procedure is described in the manufacturer's manual. Briefly, 50 μl of saxitoxin (STX) standards (included in the kit) or diluted sample extracts obtained during the previous step was allowed to react with the coated antibodies in competition with 50 μl of STX-enzyme complex solution for 60 min at room temperature in the wells of microtiter strips. After a thorough wash of the wells, 50 μl aliquots of substrate and chromogen solution were added and incubation was continued for a further 30 min at room temperature in the dark. The reaction was stopped by adding 100 μl of stop reagent, and OD_{450} was measured for each well in a UV–vis spectrophotometer (Versa Max Tunable Microplate Reader, Molecular Devices, Sunnyvale, CA, U.S.A.). The absorption read is inversely proportional to the saxitoxin concentration of the sample, which is calculated by means of a 6-point calibration curve corresponding to saxitoxin concentrations of 2.5–40 ng/ml of standard, or 0.125–2 μg/g in our samples, according to the preparation protocol applied.

3. Results and discussion

3.1. Fish identification and measurements

All fish were dark grey-brownish in colour with black, regularly distributed spots of equal size dorsally, with a wide silver band present on the lower parts of the flanks, from the mouth to the caudal fin, a silvery blotch in front of the eyes, while the pectoral fin base was black and the belly was white (Fig. 1), exactly as described by Akyol et al. (2005). Data regarding average weight and length range of the fish used in our study, together with details regarding their collection, are summarised in Table 1. Fish were captured in sea depths of 27–36 m, which is within the depth range of 18–100 m reported by Randall (1995) for *L. sceleratus*. Length of all fish tested was below the maximum values of 110 cm reported in Japan (Masuda et al., 1984), 78.5 cm in the Suez Canal (Sabrah et al., 2006) or 71.5 cm in New Caledonia (Letourneur et al., 1998), while maximum weight of the tested fish was also below the maximum value of 7000 g reported by Smith and Heemstra (1986). An allometric length–weight relationship was also indicated by the data, which is in agreement with other studies on *L. sceleratus* (Letourneur et al., 1998; Sabrah et al., 2006).

3.2. Toxicity by MBA

Results of the toxicological analyses by MBA are presented in Table 2. The limit of detection of the MBA method was 5 MU TTX eq/g (or 1.10 μg TTX eq/g). A tendency for toxicity to be higher in fish of larger sizes was observed, which was more evident in fish sampled in the same area in a short time period (specimens 1–4 and 6). In all toxic specimens, gonads, gastrointestinal tract and liver were the tissues possessing the highest toxicities, while toxicity was also present in muscle and skin but was much lower, without exceeding 46.20 MU TTX eq/g (10.16 μg TTX eq/g). Similarly, internal organs were frequently the most toxic in other studies with *L. sceleratus* (Kanoh et al., 1984; El-Sayed et al., 2003; Noguchi et al., 2006; Hoang, 2008). Despite the fact that distribution of toxicity is reported to be species-specific (Noguchi and Arakawa, 2008), seasonal, individual and local variations of toxicity and toxin composition in pufferfish are occasionally observed, even within the same species (Yu and Yu, 2002). Similar toxicity distribution with internal organs possessing the highest levels has also been reported for other species among which *Fugu vermicularis vermicularis* (Harada and Uchida, 1996), *Sphoeroides lispus* (Nuñez-Vázquez et al., 2000), as well as several species of the genera *Takifugu* (Noguchi and Arakawa, 2008), *Fugu* and *Lagocephalus* (Hwang et al., 1992).

Toxicity was not detected in any of the tested tissues in the two smallest fish specimens (no. 1 and 2). Although the number of samples of the present study is limited to be representative of the species, this result is quite encouraging, because fish of this size are easier to confuse with other – non toxic – edible species, such as picarel, bogue and smelt. A similar result derived solely by determination of toxicity in gonads, was reported by Sabrah et al. (2006) for immature fish of small sizes. On the contrary, in the

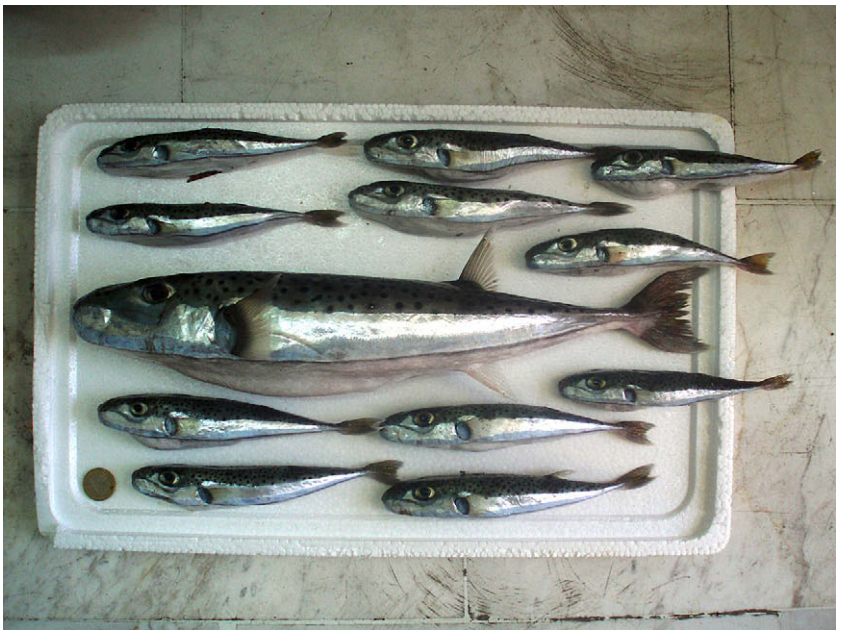

Fig. 1. *Lagocephalus sceleratus* fish of our survey; specimens 3 (11 individuals) and 4 (1 individual in the middle).

largest specimen (no. 6) toxicity reached 1087.80 MU TTX eq/g (239.32 μg TTX eq/g) in the gonads and 397.88 MU TTX eq/g (87.53 μg TTX eq/g) in the liver. The minimum lethal dose of TTX for a 50 kg human has been reported to be 10,000 MU or 2 mg (Tani, 1945; Noguchi and Ebesu, 2001), while in another study a value as low as 3000 MU is indicated as a lethal dose (Halstead, 1965), which means that consumption of less than 10 g of gonads or around 25 g of liver of this fish (no. 6) would be enough to cause death in humans. Taking into account that gonads and liver of this individual fish weighed 60 g and 279 g, respectively, these two organs alone could contain at least 17 lethal doses of TTX. Consumption of fish liver and gonads is quite common and cases of poisoning due to ingestion of liver and gonads of *L. sceleratus* were recently recorded in the Eastern Mediterranean (Bentur et al., 2008).

It is interesting to point out that muscle toxicity of various – sometimes high – levels was reported in practically all studies involving *L. sceleratus* (Kanoh et al., 1984; Khora et al., 1991; Hwang et al., 1992; El-Sayed et al., 2003; Teruya et al., 2006; Hoang, 2008; present study). A toxin content of 10 MU TTX eq/g or 2.2 μg TTX eq/g of flesh is regarded as a criterion to judge the acceptability of puffers as food in Japan (Tani, 1945; Kawabata, 1978a). In this context, muscle toxicity of our largest specimen (no. 6) indicated that consumption of approximately 200 g of flesh could result in human death. All these data justify classification of this species as inedible, even after removal of the internal organs and skin according to the Japanese "sashimi" processing method (Noguchi and Ebesu, 2001), and the reason why it is not included in the list of edible pufferfish in Japan (Noguchi and Arakawa, 2008).

3.3. PSP toxins by ELISA

All tested extracts which had also shown toxicity with the MBA, provided a positive result in the ELISA assay, with all concentrations being higher than that of the highest STX standard, i.e. containing more than 2 μg/g STX equivalents (data not shown). This concentration would be 2.5 times higher than the current European regulatory limit for PSP toxins in shellfish (0.8 μg/g STX equivalents) as there is no respective limit for fish (Regulation 853/2004/EC). Unfortunately, it was not possible to carry out further ELISA

Table 2
Toxicological results (TTX equivalents expressed as MU/g or μg/g) obtained by mouse bioassay analysis of the *Lagocephalus sceleratus* specimens.

Specimen	Liver		Gonads		Gastrointestinal tract		Muscle		Skin	
	MU/g	μg/g	MU/g	μg/g	MU/g	μg/g	MU/g	μg/g	MU/g	μg/g
1	<5.00	<1.10	N/A		<5.00	<1.10	<5.00	<1.10	<5.00	<1.10
2	<5.00	<1.10	N/A		<5.00	<1.10	<5.00	<1.10	<5.00	<1.10
3	23.09	5.08	N/A		277.50	61.05	7.69	1.69	10.63	2.34
4	73.26	16.12	77.52	17.05	258.08	56.78	11.47	2.52	10.99	2.42
5	49.25	10.84	6.76	1.49	28.67	6.31	<5.00	<1.10	<5.00	<1.10
6	397.88	87.53	1087.80	239.32	807.52	177.65	46.20	10.16	30.15	6.63

N/A = Not available (juvenile or immature fish).

analyses with diluted extracts in order to reach a more precise determination of the PSP toxin content of the extract. It should be noted however, that the high concentrations of PSP toxins obtained in our samples are only indicative, as the PSP ELISA test used in this study has been reported to sometimes significantly overestimate concentration of saxitoxin and generally PSP toxins when present at high levels (van Egmond et al., 1994; Kasuda et al., 1996; Usleber et al., 1997), compared to the MBA, at least where shellfish tissues are concerned. On the other hand, there are also reports for underestimation of PSP toxins content by the ELISA kit, especially when toxin profile comprised mostly of the gonyautoxins (GTX) group (Kasuda et al., 1996; van Dolah and Ramsdell, 2001).

The observed positive reaction in the ELISA test for PSP toxins was a quite unexpected result, as the majority of available studies on toxicity and analytical determinations in *L. sceleratus* reported only the detection of TTX itself or TTX analogues in this species (Field, 1998; Miyazawa and Noguchi, 2001; El-Sayed et al., 2003). It could be assumed though that the occurrence of PSP toxins has not been reported until now in *L. sceleratus*, most probably because these toxins were not suspected *per se* to be present, although saxitoxin and in general PSPs have been frequently reported in other pufferfish species (Kodama et al., 1983; Nakamura et al., 1984; Sato et al., 2000). It is therefore possible that *L. sceleratus* indeed does contain saxitoxin and/or other PSP toxins, as the ELISA kit employed in this study shows 100% specificity for saxitoxin, but also presents a more limited cross-reactivity with other toxins of the PSP complex, which are 20% for decarbamoyl-saxitoxin (dc-STX), 70% for gonyautoxins 2 and 3 (GTX-2 and -3) and 12% for neosaxitoxin (neo-STX). This hypothesis is further supported by a recent report from Vietnam, in which TTX and TTX analogues together with limited concentrations of PSP toxins have been detected in *L. sceleratus* by HPLC. In that study, TTXs (TTX, 4-epi TTX and 4,9-anhydro TTX) accounted for 95.87% while PSPs (STX, neo-STX and dc-STX) accounted for only 4.13% of total toxins detected (Hoang, 2008).

In contrast to this, cross-reactivity of the anti-STX antibodies contained in the ELISA kit used in this study has not been investigated with regard to response towards TTX or TTX analogues. The common mode of action and receptor binding site of STX and TTX, together with their structural similarity, recognised already from early days (Kao, 1966; Dettbarn, 1971), indicate that a possibility of cross-reactivity of anti-STX antibodies with TTX or TTX analogues cannot be excluded. This might actually be the underlying reason for the positive reaction observed in the tested *L. sceleratus* extracts in the ELISA PSP test, and is therefore a matter pending investigation.

4. Conclusions

Tetrodotoxin and/or possibly PSP toxins from the pufferfish *L. sceleratus* could be a new emerging risk in the European fisheries sector. Additionally, the recent report for the presence of tetrodotoxin in the marine gastropod *Charonia lampas lampas* (Rodriguez et al., 2008), confirms that occurrence of tetrodotoxin is a matter of concern, since a potential reason beneath this phenomenon could be an ecological change due to the global warming effect. There is a definite need for further research with regard to the specific toxin profile (tetrodotoxin and analogues and/or PSP toxins) by analytical methodology, as well as for investigating the relation of toxicity with size, season and maturity stage of the *L. sceleratus* caught in our region. Such research is currently underway and will be reported in the near future.

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Conflicts of interest

None declared.

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