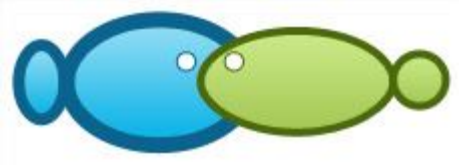

Toxicity pattern of pufferfish *Lagocephalus sceleratus* (Gmelin, 1789), Mediterranean Sea, Egypt: awareness and food safety

Mahmoud M. S. Farrag

Zoology Department, Faculty of Science, Al-Azhar University, Assiut, Egypt.
Corresponding author: M. M. S. Farrag, m_mahrousfarrag@yahoo.com; mahmoudfarrag42@azhar.edu.eg

Abstract. Toxicity patterns of 112 pufferfish (*Lagocephalus sceleratus*) specimens from the Egyptian Mediterranean coast have been studied using mice bioassay for muscles, livers and gonads. Total toxic samples of different organs were amounted 42.99%, while the non-toxic amounted to 57.01%. The toxicity concentration was found in two levels (weak level with 31.67%; 39.07±24.19 MU g^{-1}, and strong level with 11.31%; 265.86±150.7 MU g^{-1}). Gonads have the highest toxic abundance (49.15%), followed by livers (41.03%), then muscles (40.48%). While the livers ranked the first toxicity Conc. (MU g^{-1}). Winter and summer exhibited the most toxic abundance (66.67%) for all organs. Muscles exhibited the highest toxic abundance in summer (75%), while the lowest was in autumn (17.39%), it was the lowest toxic organ in males (40.19±34.45) and females (46.54±36.37 MU g^{-1}). According to length, toxic samples abundance (41.94 and 41.41%) for groups < 50 cm and > 50 cm, respectively for all, except muscles exhibited an increment in the toxicity (%) with the increase in the fish length. In regard to the toxicity per MU g^{-1}, it has increased as increase of length, where the strong toxicity was dominant in fish > 50 cm, while the weak toxicity was dominant in fish < 50 cm. The freezing does not remove toxicity, muscles and gonads showed elevated toxicity versus livers. The muscles of the Mediterranean specimens exhibited higher toxicity occurrence (%) than specimens from the Red Sea. However, the toxicity per MU g^{-1} was lower than that of their Red Sea counterparts. This may explain the intoxications along the coast of Egypt, with lower death rate. In conclusion, *L. sceleratus* (fresh and frozen) is not safe for human consumption and its marketing should be avoided with efforts to be utilized in industrial applications.
Key Words: puffer fish toxicity, *L. sceleratus*, Mediterranean Sea, Egypt, awareness, food safety.

Introduction. Several toxic and nontoxic species have been introduced to the Egyptian coast of the Mediterranean Sea via the Suez Canal (Farrag 2014; Farrag et al 2016). Among them, the Lessepsian pufferfish *Lagocephalus sceleratus* that has spread quickly to become the most common invasive species in the Mediterranean basin when compared with other pufferfish species (Akyol et al 2005; Farrag et al 2016). Despite the fishing of these species is being prohibited by the Egyptian government, there are still illegal fishing operations that provide large quantities of this fish due to the increase in the catch per unit effort (CPUE) of fishing gears (El-Haweet et al 2016; Farrag et al 2016). After fishing, they are transferred to food markets for human consumption in different form such as fillet to hide the muscles/flesh's original source. Consequently, numerous cases of poisoning have appeared due to its toxicity. Many people think that this species might be safe to eat, especially if it is cleaned well, whereas others believe that the fish muscle is free of toxin.

L. sceleratus is an Indo-Pacific pufferfish. It is known as the silver-stripe blaasop, and it has the potential to cause food poisoning through tetrodotoxin (TTX), a toxin found in its organs, including the muscle, intestine, liver, gonads, and skin (Aydın 2011). Intoxications by this fish have been reported from the eastern Mediterranean Sea (Bentur et al 2008) and from the Egyptian Mediterranean coast (Grey reports from Al-Ameri hospital in Alexandria, during 2011-2018) (personal communication of the author: this study). TTX can cause death by muscular paralysis, respiratory depression, and circulatory failure (Bilecenoglu et al 2006). It was previously believed to be a strong

neurotoxin specific to pufferfish, but it has been found in a wide range of amphibians and micro- and macro-aquatic organisms (Asakawa et al 2006; Tsai et al 2006; Yu et al 2011). The majority of human intoxications have been reported in Southern Asia, including Malaysia, Taiwan, Hong Kong, and Korea (Yang et al 1996; Kanchanapongku 2001). One of the recent works on the impact of TTX on human health was done by Guardone et al (2020) in regard to global retrospective study on human cases of TTX poisoning after seafood consumption.

In Egypt, thirteen species of pufferfish inhabit the Egyptian Red Sea, Suez Canal, River Nile, and Mediterranean Sea, constituting 13% of the species reported in the world (Mohamed 2003). Before its migration to the Mediterranean Sea, the silver-stripe blaasop *L. sceleratus* was a common species known well in the Red Sea where numerous intoxications have been reported (Ali 1996; Zaki 2004). Several studies focused on this species in the Red Sea, revealing its toxicity that varied in liver, gonad, intestine, skin, and muscles combined (Kotb 1998; Mohamed 2003; Sabrah et al 2006; Ali et al 2014).

In the Mediterranean Sea, particularly during the two last decades, *L. sceleratus* ranked as the first and most abundant of its kind (Farrag et al 2016). Moreover, the number of pufferfish species has increased due to the new incidents of entry of other species from the Atlantic Ocean that reached up to six new species. Therefore, its effect extended to the ecosystem beside the public health due to its toxic effect. Since its appearance, quick spreading and establishment in the Mediterranean Sea, the intoxication cases started to emerge in the Eastern Basin since 2008 (Bentur et al 2008). This was followed by various studies on their toxicity outside Egypt such as Katikou et al (2009) from the Aegean Sea and Kheifets et al (2012) who studied how the intoxication could be treated with a cholinesterase inhibitor. Other studies by Rodríguez et al (2012) from the European waters; Kara et al (2015); Kosker et al (2015) from Mersin Bay; Katikou & Vlamis (2017) who studied the methods of analyzing TTX; Rambla-Alegre et al (2017) from Spain; Acar et al (2017) from Turkey; Guardone et al (2018) from the Italian coast, then others have identified the toxic species and their effects on public health (Giusti et al 2019; Guardone et al 2020). In spite of the above studies, there are no studies concerned with the toxicity of pufferfish on the Egyptian coast of the Mediterranean Sea, which is the closest to the Suez Canal. The interest towards *L. sceleratus* was mainly in the field of fisheries and biological studies, distribution, and taxonomy (Farrag et al 2015a, b; El-Haweet et al 2016; Farrag et al 2016, 2019). Moreover, the conflict in knowledge about this species and its toxicity has constructed some misconception in public awareness towards its toxicity pattern and the presence/absence of toxic specimens. Therefore, this study aimed to assess the toxicity pattern of the common pufferfish species *L. sceleratus* in different forms throughout the year with a comparison between its toxicity in frozen and fresh specimens to elucidate the misunderstanding and lack of awareness among people and fishers towards its consumption despite cases of poisoning being reported linked to these fishes.

Material and Method. A total of 112 specimens of *L. sceleratus* from the Egyptian Mediterranean coast were collected randomly throughout the year (2017) in order to detect the toxicity pattern of this species for the most length ranges, different sexes, and different maturity stages. After transport to the lab, each specimen was measured to the nearest centimeter and weighed to the nearest gram. Then, dissection was conducted to detect the sex and stage of maturity. The fish varied in length from to 26.9 to 76 cm total length (TL), and weight from 245 to 3956 g respectively. The specimens were sexed to 52 (males) and 50 (females), while the other 10 specimens were unsexed; the specimens were also divided to length groups (less than 50 cm and more than 50 cm) which were confirmed as mature and the fact that they were more attractive for illegal trading.

Three organs, muscles (n = 104), livers (n = 98), and gonads (n = 89), were analyzed as frozen. The number of each investigated organ is different and lower than the total fish samples because some samples hadn't enough tissue of a certain organ, which may be immature gonads or very small liver or damaged organs. The chosen organs were extracted and preserved in plastic bags at -20°C for toxicological examinations, according to the Japanese Official Mouse Bioassay (Kawabata 1987). The

other experimental part was the comparative study between fresh and frozen specimens using ten individuals during July to detect the effect of freezing and preservation on toxicity. The comparison was conducted during July to ensure the presence of toxicity as mentioned in other works, and known as the time of maturity. The same toxicity test conditions were applied on both specimens while avoiding the extra moisture in frozen samples.

Toxin extraction. The tetroadotoxin (TTX) was extracted from the organs' tissues of *L. sceleratus* according to Kawabata (1987). The extraction filtrate solution or supernatant (1 mL) was injected into the intraperitoneal cavity (IP) of each of the three Albino Swiss male mice weighing 18-22 g. The symptoms were observed, while the death time was recorded and used to calculate the mouse units (MU), where 1 MU was defined as the amount of toxin required to kill a 20 g male mouse in 30 min after IP injection Kawabata (1987). The scales of toxicity were as values (less than 10 MU g^{-1} are nontoxic; from 10 to 99 MU g^{-1} are weakly toxic from 100 to 999 MU g^{-1} are strongly toxic and toxicity values of 1000 MU g^{-1} or more are considered extremely toxic). All animal manipulations were conducted in accordance with the European Council Directive 86/609/EEC (1986) under an official license from the Prefectural Veterinary Service of Thessaloniki, Greece.

Statistical analysis. Multivariate analysis of variance on SPSS (V 20) software was applied. Mann-Whitney was applied to test the current nonparametric data data as they were not normally distributed, followed by Wilcoxon W. The Multiple Range Comparisons (Least Significant Difference; LSD, Mann-Whitney) were selected from Post Hoc window on the same statistical package to detect the significant difference between season and organ toxins. Paired sample test was applied based on Wilcoxon assumption to evaluate the significant differences between means of some paired data. Probability for significance was as the values > 0.05 were considered non–significant, while between 0.05 and 0.01 (both are included) were evaluated as significant. Statistically non-significant, significant and highly significant outputs were accompanied by symbols NS, * and ** respectively.

Results

General description of toxicity pattern and symptomes. Toxicity was investigated as the percentage of toxic samples and the concentration of toxicity per MU g^{-1} (Tables 1, 2 and Figures 1, 2). Toxic samples represented 42.99%, while the non-toxic were 57.01%. Toxic samples had toxicities of 11-633 MU g^{-1}, with an average of 96.85±13.26 MU g^{-1}. The toxicities were found in two levels (weak and strong levels), while no extremely high toxicity was observed. Weak toxicity was 31.67% with an average of 39.07±24.19 MU g^{-1}, while the strong toxicity recorded 11.31% with an average of 265.86±150.7 MU g^{-1}.

The gonads showed the highest occurrence of toxicity (49.15%), followed by the livers (41.03%) and muscles (40.48%). From the opposite side, livers showed the highest average of toxicity (121.68±143.39 MU g^{-1}) including weakly toxic samples (25.64%) with an average of 33.00±19.60 MU g^{-1}, and strongly toxic samples (15.38%) with an average of 266.65±143.40 MU g^{-1}. Gonads showed their toxicity average (110.37±119.94 MU g^{-1}), and their weakly toxic samples amounted to 33.90% with an average of 46.46±27.62 MU g^{-1}, whereas their strongly toxic samples amounted to 15.25% with an average of 270.29±120.71 MU g^{-1}.

The muscle has ranked third, with an average of 61.92±106.75 MU g^{-1}. The weakly toxic samples had an average of 36.98±23.49 MU g^{-1}, whereas their strongly toxic samples amounted to 3.57% with an average of 303.22±287.43 MU g^{-1}. The injected mice exhibited symptoms by direct observation due to TTX intoxication, including neuromuscular changes, respiratory distress, fatigue, fast heartbeat, cramps, weak hind limbs, weak breathing, trotting and jogging excessively, jumping, and death. Statistically, the multivariate analysis has revealed the presence of significant differences ($0.05 > p = 0.01$). Probability values between 0.05 and 0.01 were both included (Table 3).

Table 1

General pattern of the toxic and non toxic sample of the *L. sceleratus* from the Mediterranean Sea, Egypt

Organ	General pattern (whole year for all samples)			According to sex				According to lenght			
				Males		Females		Less than 50 cm		More than 50 cm	
	No.	Non-toxic (%)	Toxic (%)	Non-toxic (%)	Toxic (%)	Non-toxic (%)	Toxic (%)	Non-toxic (%)	Toxic (%)	Non-toxic (%)	Toxic (%)
Total organs	221	57.01	42.99	75.86	24.14	36.79	63.21	58.06	41.94	58.59	41.41
Muscles	84	59.52	40.48	77.78	22.22	40	60	61.54	38.46	58.82	41.18
Livers	78	58.97	41.03	71.05	28.947	47.5	52.5	56.82	43.18	61.76	38.24
Gonads	59	50.85	49.15	78.79	21.21	15.385	84.62	46.43	53.57	54.84	45.16

	According to season							
	Winter		Spring		Summer		Autumn	
Organ	Non-toxic (%)	Toxic (%)	Non-toxic (%)	Toxic (%)	Non-toxic (%)	Toxic (%)	Non-toxic (%)	Toxic (%)
Total organs	33.33	66.67	61.76	38.24	33.33	66.67	75.00	25.00
Muscles	46.67	53.33	66.67	33.33	25.00	75.00	82.61	17.39
Livers	0.00	100.00	58.33	41.67	50.00	50.00	85.00	15.00
Gonads	33.33	66.67	61.11	38.89	14.29	85.71	46.15	53.85

Table 2

General pattern of the toxicity by percentage (%) and by mouse unit per gram (MU g^{-1}) for all samples and based on sex and length

Organ	Whole year (all samples)			
	Range (MU g^{-1})	Av.±SD	Weakly (%)	Strong (%)
Total organs	11- 633	96.85±13.26	31.67	11.31
Muscles	11- 633	61.92±106.75	36.90	3.57
Livers	11.9-617	121.68±143.39	25.64	15.38
Gonads	11-407	110.37±119.94	33.90	15.25

	According to sex							
	Males				Females			
Organ	Range (MU g^{-1})	Av.±SD	Weakly (%)	Strong (%)	Range (MU g^{-1})	Av.±SD	Weakly (%)	Strong (%)
Total organs	10.22-366	52.04±70	20.69	3.45	10-633	115.91± 139.67	42.45	20.75
Muscles	10-105	40.19±34.5	20.0	2.22	10-633	46.54± 36.37	55.0	5.0
Livers	12-366	70.06± 103.8	23.68	5.26	15-617	148.73± 155.72	27.5	25.0
Gonads	10-123	40.64± 41.3	18.18	3.03	11-407	132.56± 128.68	53.85	38.46

	According to lenght							
	Less than 50 cm				More than 50 cm			
Organ	Range (MU g^{-1})	Av.±SD	Weakly (%)	Strong (%)	Range (MU g^{-1})	Av. ±SD	Weakly (%)	Strong (%)
Total organs	47	5	10 -368	55.77 ±64.6	25	16	10 - 618	147.11 ±162.3
Muscles	19	1	10-106	39.61 ±30.2	13	1	10-171	94.05± 160.5
Livers	15	4	12-368	75.07 ±98.3	5	8	19-617	189.82 ±173.7
Gonads	13	2	10-123	53.11 ±35.8	7	7	11-407	171.72 ±147.5

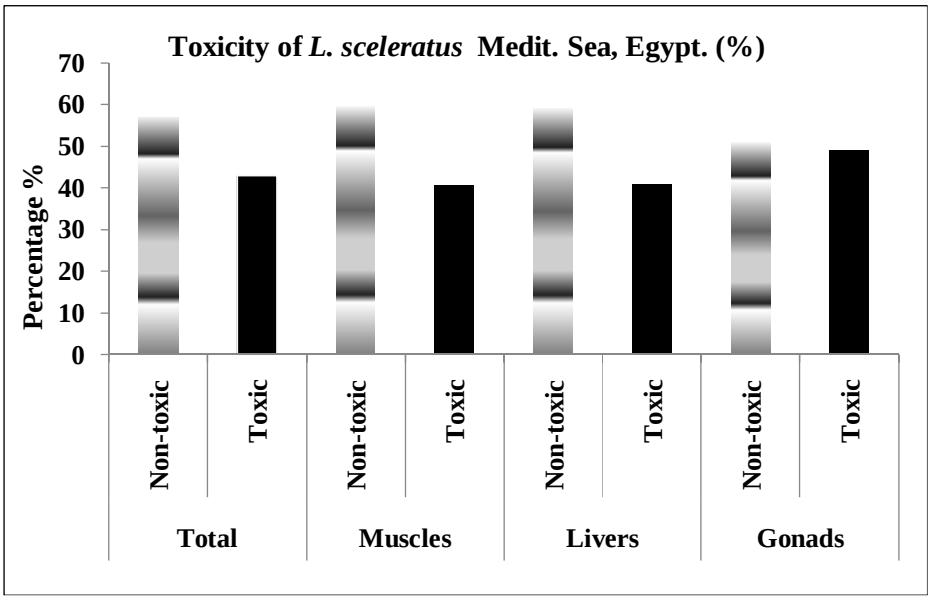

Figure 1. Toxic and non-toxic samples according to the population percentage (%).

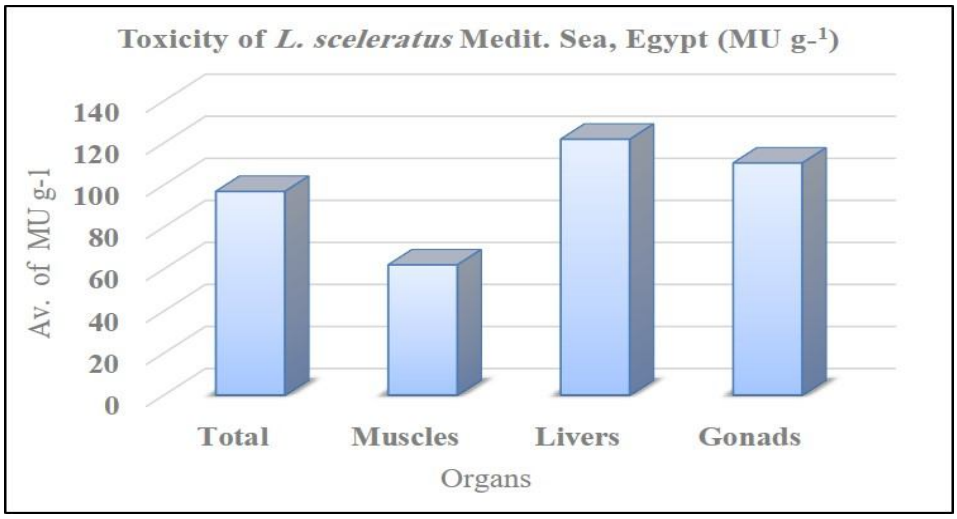

Figure 2. Variations in toxicity according to mouse unit per gram tissue (MU g^{-1}).

Seasonal variations in toxicity pattern. Seasonal variations (Tables 1, 3; Figure 3) revealed the highest frequencies of toxic samples during winter and summer (66.67%), followed by 38.24% and 25.00% during spring and autumn, respectively. Livers were most toxic during winter (100.00%) and summer (50%) and the least toxic in autumn (15%). However, the gonads were most toxic during summer (85.71%), followed by winter (66.67%), and were the least toxic during spring. Muscles were most toxic during summer (75%) and the least toxic in autumn (17.39%).

Regarding the magnitude of toxicity, the gonads exhibited the highest toxicity (80.98±154.98 MU g^{-1}) during spring, followed by summer (201.08±158.30 MU g^{-1}). An inverse trend was observed in liver as toxicity showed its lowest average (MU g^{-1}) during spring (60.46±51.56). Subsequently, toxicity reached its highest value during summer (294.24±213.55 MU g^{-1}). Livers gave a high percentage of very toxic samples. For muscles, the highest level of MU was observed during winter (118.94±248.29 MU g^{-1}, with a range of 11.5-633 MU g^{-1}), whereas the lowest value was recorded during spring (17.045±9.33 MU g^{-1}, with a range of 10.6-170.72 MU g^{-1}). Only a few of the toxic specimens of muscles had high toxicities during winter and spring. Low toxicity of muscles were found in all seasons. Unlike muscles, highly toxic specimens of livers and gonads were found during all seasons, with the highest percentage (43.15% and 71.43%) being observed during summer for livers and gonads, respectively.

Table 3

General pattern of the toxicity by percentage (%) and by mouse unit per gram (MU g^{-1}) according to season

Organ	According to season															
	Winter				Spring				Summer				Autumn			
	Range (MU g^{-1})	Av. ±SD	Weakly (%)	Strong (%)	Range (MU g^{-1})	Av. ±SD	Weakly (%)	Strong (%)	Range (MU g^{-1})	Av. ±SD	Weakly (%)	Strong (%)	Range (MU g^{-1})	Av. ±SD	Weakly (%)	Strong (%)
Total	12-633	100.64± 159.1	50.0	16.67	11-381	66.46± 89.5	32.35	5.88	12-618	137.34± 149.8	35.90	30.77	10.18-305	77.75± 90.6	19.64	5.36
Muscles	12-633	118.94± 248.3	40.00	13.33	11-171	17.045± 9.3	30.0	3.33	12-83	49.34± 26.0	75.00	00	10-60	34.47± 36.2	17.39	0000
Livers	12-366	80.87± 140.1	83.33	16.67	14-381	60.46± 51.6	36.11	5.56	58-617	294.24± 213.6	6.25	43.75	22-233	136.25± 106.5	5.00	10.00
Gonads	50-123	86.59± 51.2	33.33	33.33	10.22-109	80.98± 155.0	30.57	3 (8.33)	41-407	201.08± 158.3	14.29	71.43	10.18-305.4	91.13± 107.3	46.15	7.69

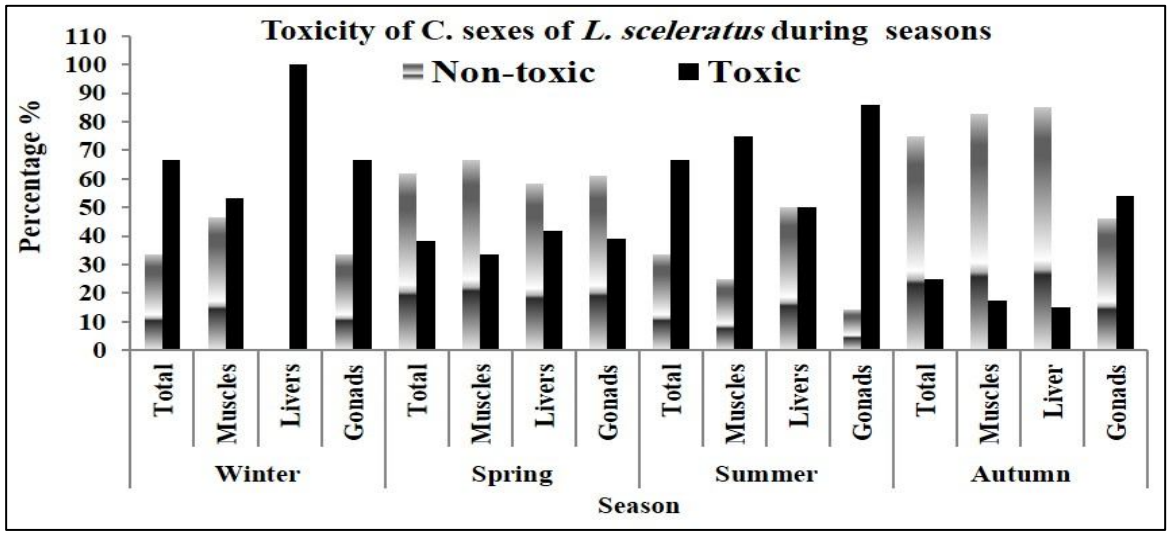

Figure 3. Seasonal variations in toxicity of *L. sceleratus* (C. sexes = combined sexes).

Statistically, during the warm seasons (summer-autumn), gonads and muscles gave remarkable significant differences ($0.05 > p < 0.01$) in their resulted content of toxin (MU g^{-1}). The interactions between seasons and target organ relevant to toxicity (MU g^{-1}), was applied by Mann-Whitney test (Table 4). The average of toxin (MU g^{-1}) of muscles was significantly different between winter and autumn ($p < 0.05$). While between spring and summer, the toxin (MU g^{-1}) exhibited different levels of significance $p < 0.05$, $p < 0.01$ and $p = 0.01$ in the case of muscles, livers and gonads respectively. In addition, the toxic averages of showed a significant difference at $p < 0.05$ when compared between spring and autumn, with no significant differences between spring and autumn ($p > 0.05$) for the toxin processed from liver and gonads.

Table 4

General multivariate model testing the variances between the means of organ-toxin extracted (MU g^{-1}) during the different seasons

Statistics	*Organ*		
	Muscles	*Livers*	*Gonads*
Kruskal-Wallis H	11.195	8.083	7.848
df	3	3	3
Sig.	0.011**	0.044*	0.049*

Note: (* = significant; ** = highly significant).

Toxicity pattern according to sex. An inverse trend was observed between sexes (Tables 1, 2; Figures 4, 5). For males, the nontoxic samples were dominated all tested organs. Toxic samples abundance amounted 24.14%, 22.22%, 28.95%, and 21.21% of the total samples, muscles, livers, and gonads, respectively. Livers of males were the most toxic organ (70.06±103.80; 11.9 to 366.24 MU g^{-1}), whereas the muscles ranked the lowest percentage (40.19±34.45; 10.6-105.95 MU g^{-1}). The highest average for males was observed during winter (87.90±111.68 MU g^{-1}), whereas the lowest value was observed during spring (33.50±38.72 MU g^{-1}) with the exception of one liver sample that was highly toxic. Liver samples in males exhibited the highest value during spring (109.45± 171.47 MU g^{-1}), whereas the lowest toxicity was observed in summer (21.6 MU g^{-1}).

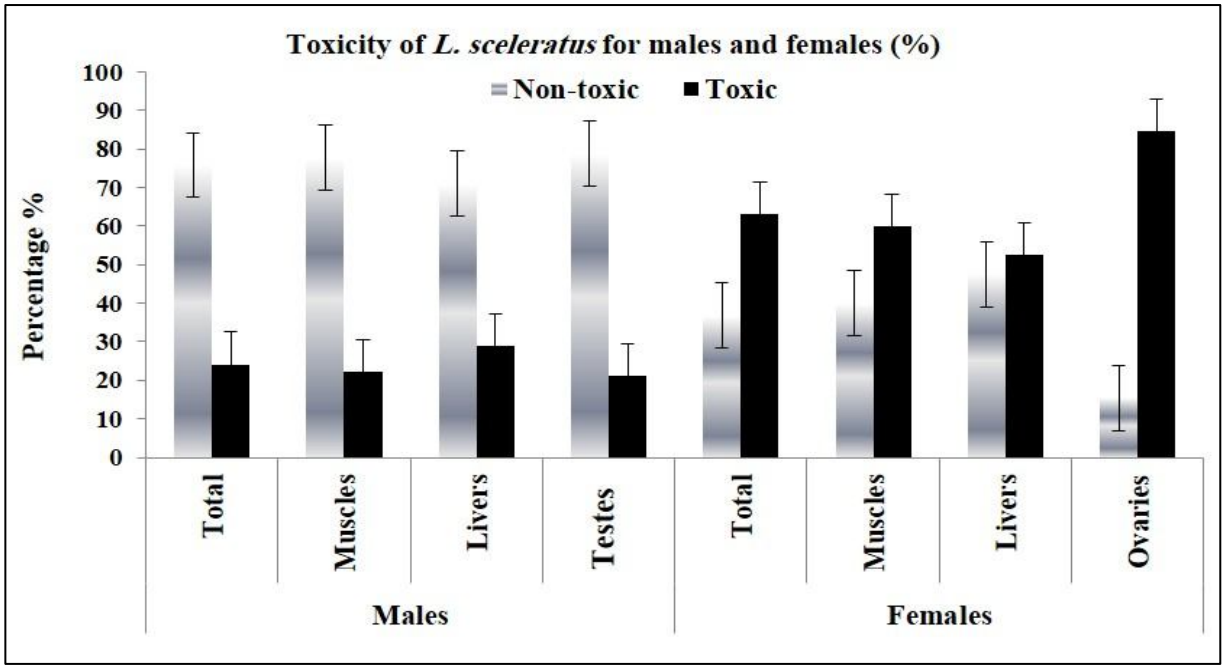

Figure 4. Variations in toxicity (%) of *L. sceleratus* according to different sexes.

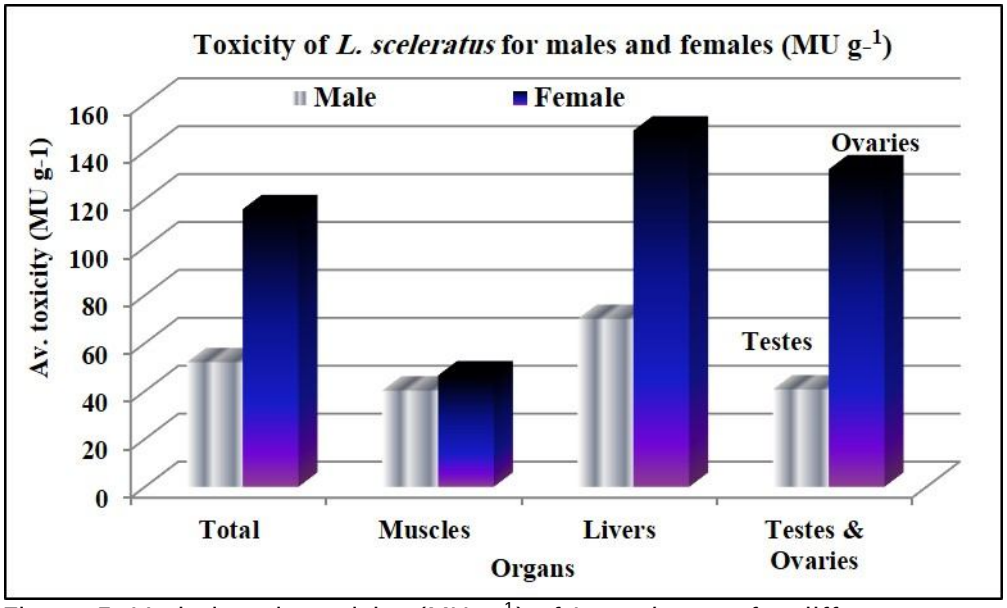

Figure 5. Variations in toxicity (MU g^{-1}) of *L. sceleratus* for different sexes.

For females, the highest average toxicity (193.98±185.98 MU g^{-1}) was observed during summer, whereas the lowest toxicity (84.54±96.47 MU g^{-1}) was observed during autumn. The ovaries were most toxic during spring (132.30±133.99 MU g^{-1} with a range of 27.98-368.74 MU g^{-1}) and ranked second to livers during other seasons. The livers exhibited the lowest toxicity during winter (23.71±11.01 MU g^{-1}) and reached their highest toxic value during summer (316.76±197.92 MU g^{-1}). Muscles exhibited the highest average toxicity during winter (224.49±390.32 MU g^{-1}), whereas the lowest toxicity was recorded during autumn (23.59±24.32 MU g^{-1}). In general, the investigated fish-toxin was sex-dependent (Table 5; Figures 6, 7, and 8), in particular in the case of liver and gonads at $p < 0.05$.

Table 5

General Mann-Whitney test sorting the site – organ toxicity dependence of toxin (MU g^{-1})

Statistics	*Winter & Spring*			*Winter & Summer*		
	Muscles	*Livers*	*Gonads*	*Muscles*	*Livers*	*Gonads*
Mann-Whitney U	54.000	130.000	9.000	52.000	66.000	4.000
Wilcoxon W	145.000	208.000	180.000	107.000	144.000	7.000
Z	-0.682-	-1.141-	-1.134-	-0.528-	-1.226-	-0.667-
Sig.	0.495^{NS}	0.254^{NS}	0.257^{NS}	0.598^{NS}	0.220^{NS}	0.505^{NS}
	Winter & Autumn			*Spring & Summer*		
	Muscles	*Livers*	*Gonads*	*Muscles*	*Liver*	*Gonad*
Mann-Whitney U	18.000	12.000	5.000	43.000	31.000	16.000
Wilcoxon W	63.000	33.000	33.000	134.000	262.000	187.000
Z	-2.205-	-0.961-	-0.586-	-1.904-	-2.874-	-2.533-
Sig.	0.027*	0.337^{NS}	0.558^{NS}	0.057*	0.004**	0.011**
	Spring & Autumn			*Summer & Autumn*		
	Muscles	*Livers*	*Gonads*	*Muscles*	*Livers*	*Gonads*
Mann-Whitney U	28.000	124.500	49.000	11.000	71.000	7.000
Wilcoxon W	73.000	215.500	220.000	56.000	162.000	35.000
Z	-2.037-	-1.644-	-0.847-	-3.057-	-1.286-	-2.000-
Sig.	0.042*	0.100^{NS}	0.397^{NS}	0.002**	0.198^{NS}	0.046*

Note: NS = non-significant; * = significant; ** = highly significant.

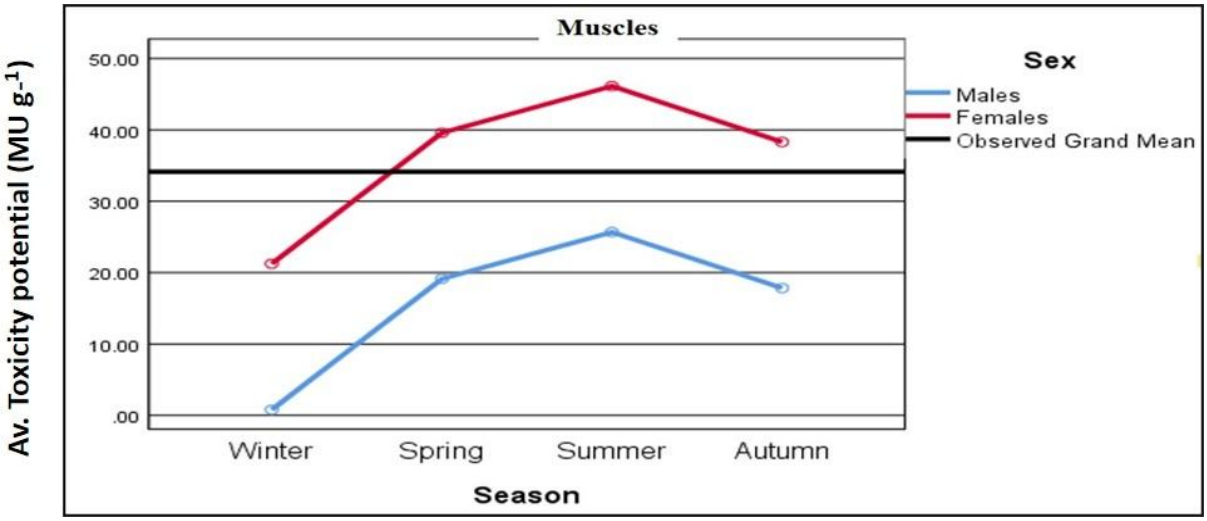

Figure 6. The average toxin extracted from muscles (MU g^{-1}) between males and females during the different seasons.

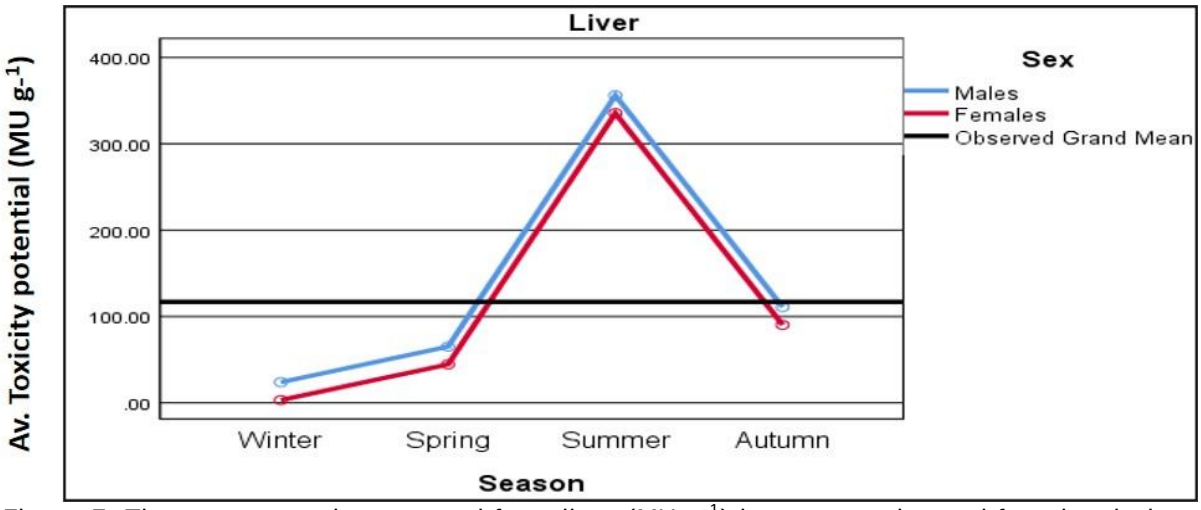

Figure 7. The average toxin extracted from liver (MU g^{-1}) between males and females during the different seasons.

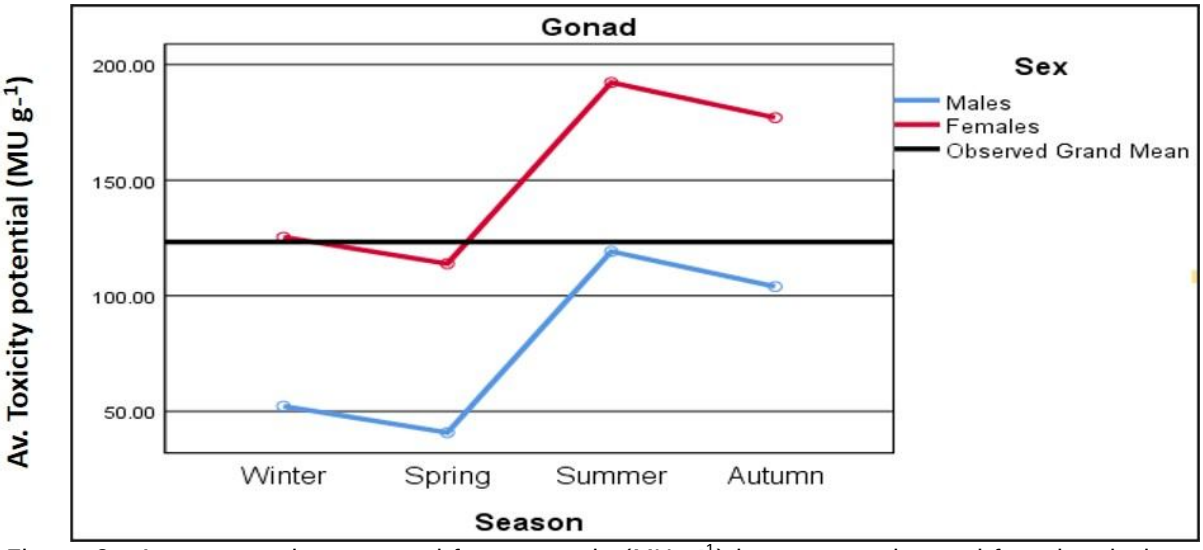

Figure 8. Average toxin extracted from gonads (MU g^{-1}) between males and females during the different seasons.

Toxicity according to length. Toxicity pattern according to length is presented in Tables 1 and 2 and Figures 9A, 9B, 9C, 10, and 11.

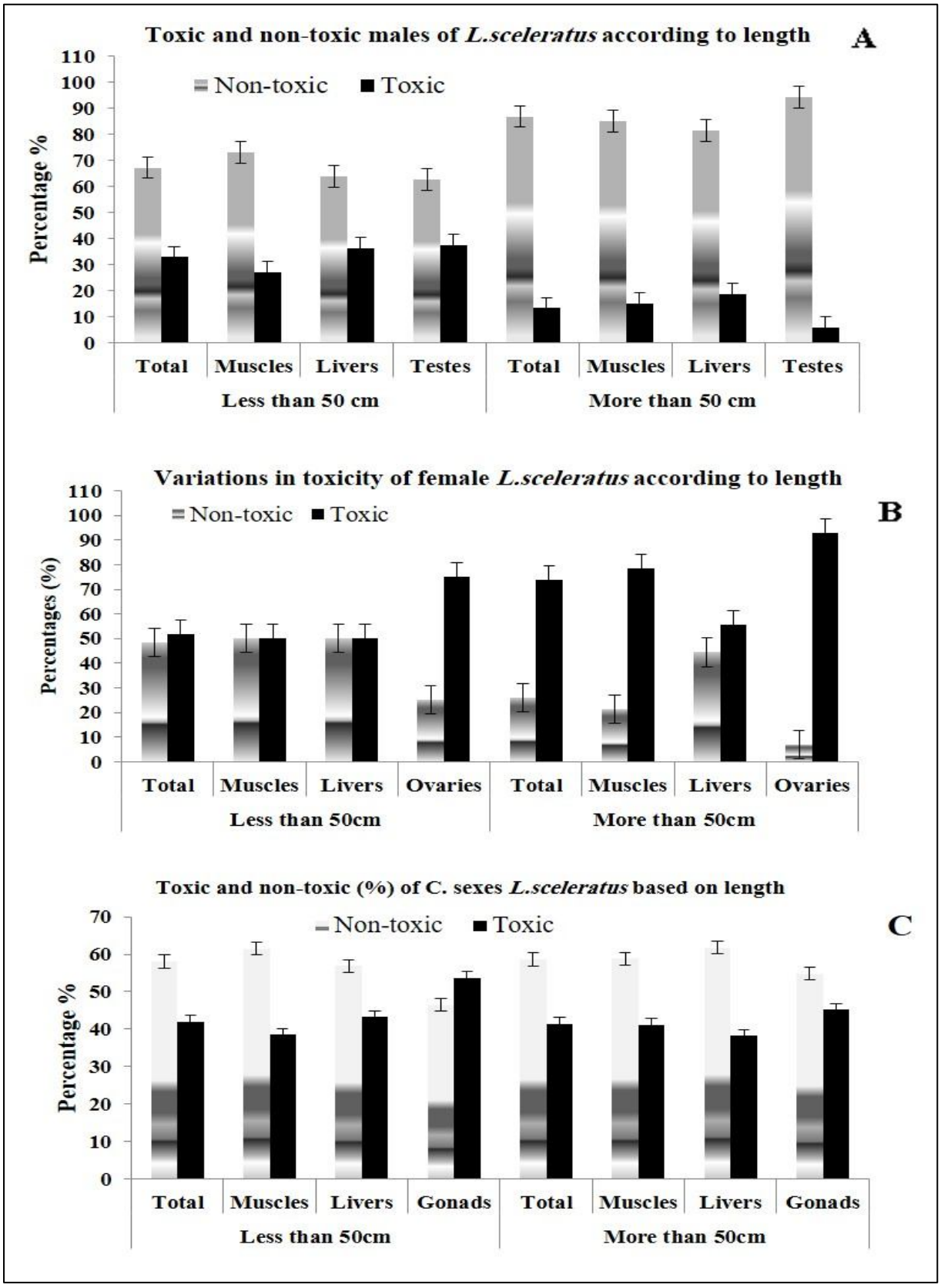

Figure 9. (A, B, C). Toxic and non-toxic samples in percentages (%)/ abundance according to length (A: Males, B: Females, C: combined sexes).

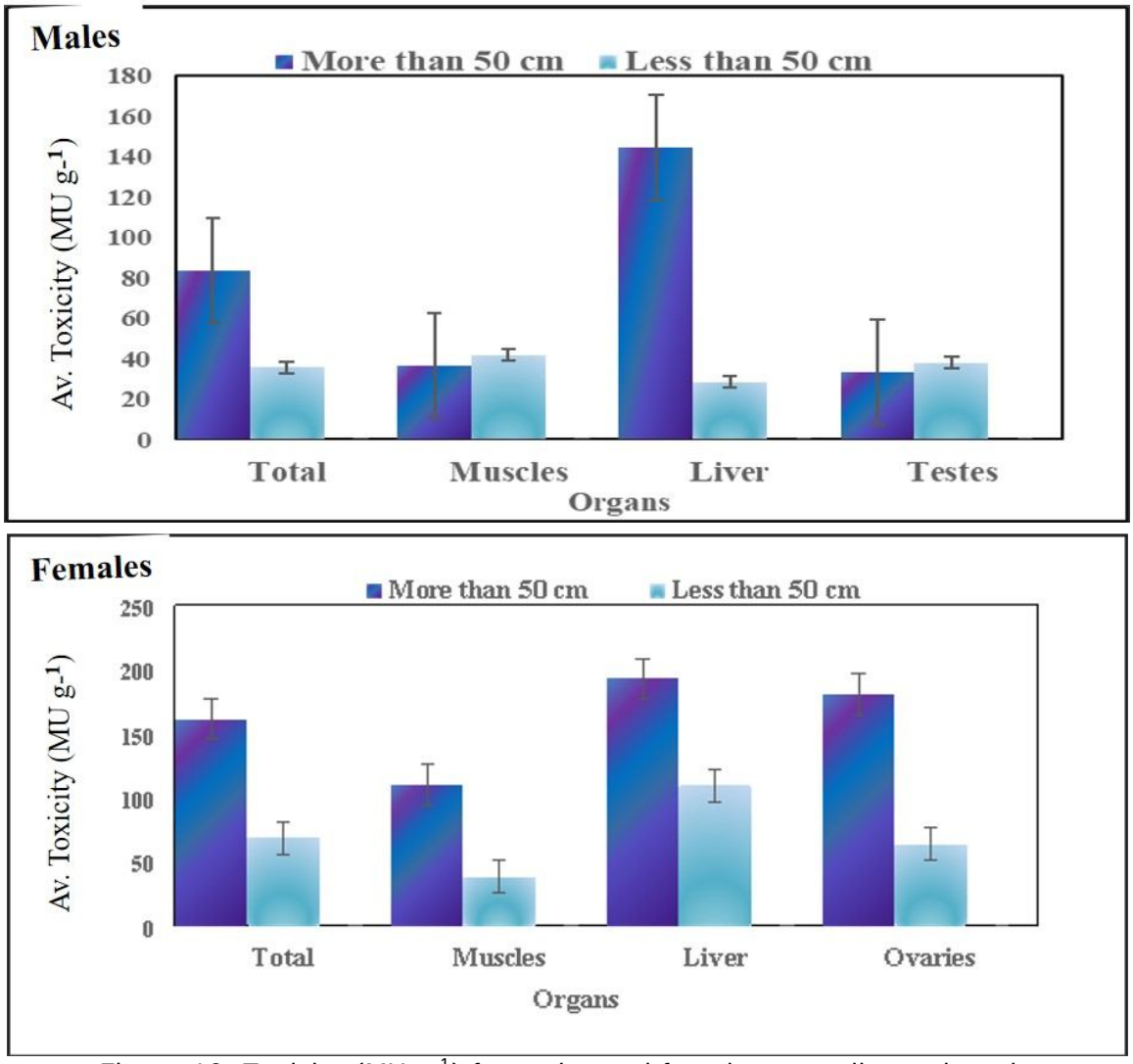

Figure 10. Toxicity (MU g^{-1}) for males and females according to length.

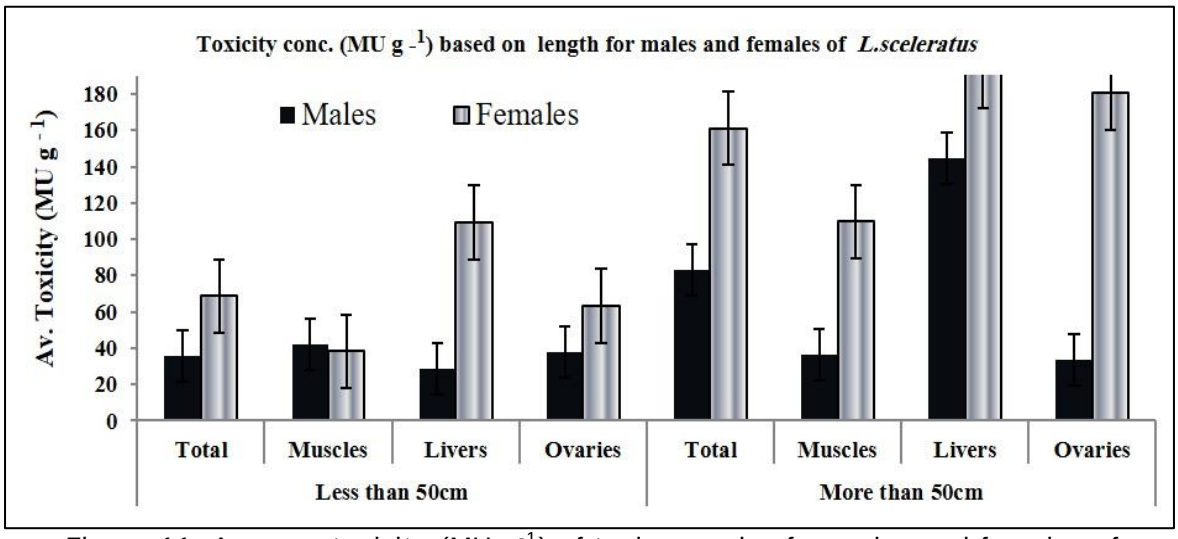

Figure 11. Average toxicity (MU g^{-1}) of toxic samples for males and females of *L. sceleratus* according to length.

The samples were sorted into two groups (up to 50 cm and more than 50 cm), and the total percentages of toxic samples (41.94% and 41.41%, respectively) for both length groups. However, the number of weakly toxic samples for fish < 50 cm (n = 47) was

approximately twice that of fish > 50 cm (n = 25). In addition, the total number of highly toxic fish > 50 cm (n = 16) was approximately triple that of fish < 50 cm (n = 5). Both livers and gonads exhibited a decrease in toxicity when length increased more than 50 cm, whereas the muscles had a higher percentage of toxicity than the length group less than 50 cm.

The toxicity per MU g^{-1} increased with the increase in length for the whole organs. It increased from 55.77±64.55 MU g^{-1} for the length group of up to 50 cm to 147.11± 162.26 MU g^{-1} for the length group > 50 cm. Muscle toxicity increased from 39.61±30.24 MU g^{-1} for the length group of up to 50 cm to 94.05±160.54 MU g^{-1} for the length group > 50 cm. Toxicity of livers increased from 75.07±98.29 MU g^{-1} in length group of up to 50 cm to 189.82±173.65 MU g^{-1} for the length group > 50 cm. For the gonads, toxicity increased from 53.11±35.83 MU g^{-1} for length group < 50 to 171.72±147.53 MU g^{-1} for the length group > 50 cm. This increase was combined with the increase of highly toxic samples for liver and gonads. Statistically, the average length (as age variable) of the tested fish had played an obvious role governing the relation between length and the given amount of toxins. This relation could be verified by the correlation analysis (Table 6). A strong and positive relation was found between organ-collected toxin and the average of measured length ($p \leq 0.01$).

Table 6

Rank means of toxin extracts from the two sexes of fish species

	Statistics between two sexes		
	Muscles	*Livers*	*Gonads*
Kruskal-Wallis H	2.593	4.453	3.672
df	1	1	1
Sig.	0.107^{NS}	0.035^{*}	0.055^{*}

Note: NS = non-significant; * = significant; ** = highly significant.

Comparative toxicity of fresh and frozen samples. This trial was conducted to determine the effect of freezing and preservation on the toxicity of *L. sceleratus* (Table 7), in fresh samples and samples frozen for more than 3 months. The frozen muscles and gonads showed higher values than the fresh samples, whereas the other samples exhibited the inverse trend. However, both kinds of samples showed low levels of toxicity for muscles, while gonads showed different levels of toxicity. The liver was unlike the other organs. It showed lower values for frozen samples than those for fresh samples, and both kinds of samples exhibited different levels of toxicity. Therefore, freezing and preservation did not remove the toxicity as no evident changes were observed after freezing. Statistically, Wilcoxon rank test was applied at significance level $p < 0.05$. These data were tabulated (Table 8) and graphically presented (Figure 4). There was no significance between the average differences, and this assumption is in accordance with the case of muscles and gonads ($p < 0.05$). In the case of liver, the highest significant difference was achieved between the average records of liver (fresh-frozen) data ($p < 0.01$). Therefore, we reject the null hypothesis in the case of liver ($p = 0.009$). The differences were insignificant despite the average assigned % of the amount of toxin extracted from muscles and gonads, between fresh and frozen, was 10% and 18% respectively (Figure 12; Table 9).

Table 7

Correlation coefficients between length and the toxic potential in relevant to the selected organs

Organ	*Total length*	
	r Significance	*P value*
Muscles	0.33**	0.016
Livers	0.48**	0.001
Gonads	0.40**	0.010

Note: NS = non-significant; * = significant; ** = highly significant.

Table 8

Comparative investigation for toxicity of fresh and frozen samples of *L. sceleratus*

		Muscles (MU g^{-1})		*Liver (MU g^{-1})*		*Gonads (MU g^{-1}) (ovaries and testes)*	
TL (cm)	*Sex*	*Fresh*	*Frozen*	*Fresh*	*Frozen*	*Fresh*	*Frozen*
37.2	♂	39	36	190.19	168.33	495.75	420
38.4	♂	12	12.5	19	21.6	12.2	26
40	♀	45.76	56.64	273.13	197	1427.55	630.5
40.5	♂	48.27	28.39	47.43	42.3	< 5	< 5
42.2	♀	40.734	48.6	212	211.96	255	275.3
47.3	♀	71.775	98	762.75	264.6	420	408
47.5	♀	50.76	97	1068.75	433.38	465.4	386
55	♀	38.79	63.612	270.4	240.35	584.25	133.3
61	♀	63.8	54.18	191.34	172.98	223.51	241.33
68.8	♀	36.14	62.23	1225	617	268.77	391
Av.±SD		44.7±16.2	55.7±27.2	426.0±431.8	236.9±176.1	461.4±401.5	323.5±177.8

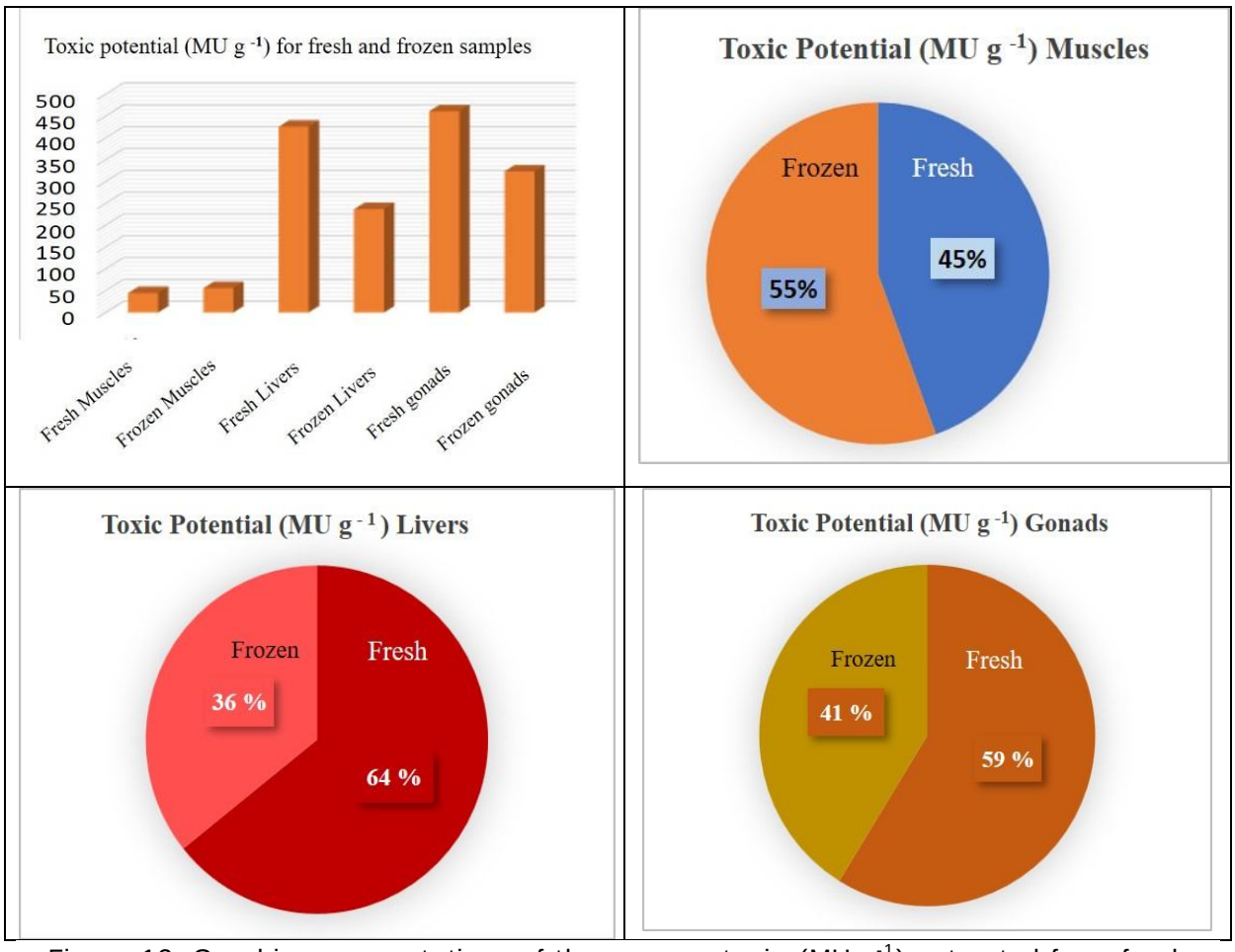

Figure 12. Graphic representations of the average toxin (MU g^{-1}) extracted from fresh and frozen organs of the investigated fish.

Table 9

Pair test testing the null hypothesis between the average-organ toxicity toxic potential (MU g^{-1})

Paired variables	*N*	*Test statistics*	*Std. error*	*St test statistics*	*Sig*
Fresh muscles – frozen muscles	10	43	9.811	1.580	0.114
Fresh livers – frozen livers	10	2	9.811	2.599	0.009
Fresh gonads – frozen gonads	9	16	8.441	0.770	0.441

Discussion. Our results have introduced new detailed knowledge on the toxicity pattern of the common pufferfish *L. sceleratus* from the Egyptian Mediterranean waters. The general results of the mouse bioassay for the TTX revealed a positive responses showing toxic symptoms that appeared on the injected mices such as the effects on the heart muscle and some signs of intoxication (hypotension, conduction disorder, and bradycardia, absence of tendon reflexes, fasciculation, lethargy, ascending progressive paralysis, and breathlessness) in different grades. Similar symptoms have been observed by different authors (Noguchi et al 1985; Ali et al 1995; Zimmer 2010). These symptoms may be the result of the intoxication with TTX, which causes blocking of the sodium channels and inhibits the production and propagation of action potentials, primarily in the skeletal muscles, neurons, and nerve fibers.

Chowdhury et al (2007) stated the same effect of symptoms on human as a result of food consumption for the pufferfish. The death in humans may happen due to the complex action of TTX on the respiratory system through its impact on the phrenic nerve, diaphragm, and neurons in the central respiratory network. The toxin is eliminated faster from the serum *via* excretion in the kidney and an efficient binding to the TTX receptors. Low levels in the brain suggest the limited ability of TTX to cross the human blood-brain barrier (Chowdhury et al 2007).

The present percentage of toxic samples was more than 40% of the total samples regardless of its toxicity power (MU g^{-1}), reflecting the favorable condition in the Mediterranean Sea and variations between different habitats with the toxicity being low in the Red Sea. The present pattern showed the highest percentage of toxic individuals in gonads, followed by the liver, and the lowest toxicity was observed in muscles. The present results for muscles agreed with those of Ali et al (1995) and Sabrah et al (2006) from the Red Sea, Egypt, and also with Noguchi & Arakawa (2008) from Japan, Dao et al (2012) in Vietnam, and Katikou et al (2009) from the Aegean Sea (Greece). However, the toxicity of muscles did not agree with the findings of Simon et al (2009), Monaliza & Samsur (2011), and Kosker et al (2016), as these authors reported that muscle extracts did not kill mice.

Unlike gonads and muscles, the liver was ranked the most toxic organ starting from winter and declining towards the spawning season to rank the lowest during summer and autumn. It is worth mentioning that winter season is considered a preparatory time for pre-vitellogenin and the preparation of stored food, which is allocated to the spawning seasons. Many scientists mentioned that the highest toxicity was sometimes recorded for skin of different pufferfish species (Shiomi et al 1985b; Ali et al 2014; Farrag et al 2022). The toxicity of skin of pufferfish may be due to the presence of parasitic bacteria, which is responsible for producing TTX or may be due to the presence of TTX secretory glands on the skin (Tanu et al 2002). The skin has not been tested in the present work because the human food consumption and fishermen as well as sellers remove the skin and discard it as waste in Egypt. Nevertheless, it should be avoided as stated by the above authors.

Seasonal variation is a characteristic feature in most animals with TTX and other marine toxins (Noguchi et al 1985; Ali et al 1995). High percentages of toxic samples were during summer and winter, while the lowest values were during autumn. The highest toxicity scores of *L. sceleratus* from the Red Sea were recorded earlier during April, May, and June, which coincide with the spawning season (Ali et al 1995; Kotb 1998; Sabrah et al 2006). The spawning season in the Red Sea is earlier than that in the Mediterranean and finishes faster due to the higher temperature. Therefore, this may be the reason for the extended toxic percentages during summer and autumn, which coincided with its spawning season in the Mediterranean Sea (Farrag et al 2019). The latter authors reported the presence of ova in gonads in certain individuals via histological investigation of *L. sceleratus* after the end of the spawning season, providing an explanation for the presence of toxic samples during the autumn. Gonads and muscles exhibited their highest abundant of toxic samples during summer (85.71% and 75%, respectively).

With respect to sex, many investigators have reported that the gonads, especially the ovaries, have the highest toxicity compared with the other tested organs (Ali et al

1995; Kotb 1998; Sabrah et al 2006; Ikeda et al 2010). Moreover, Ali et al (2011) tested the toxicity of two species, *L. sceleratus* and *A. hypeslogenion*. They illustrated that the most toxic organ was the ovary (666.9 MU g^{-1}). This finding disagrees with the present results, which indicated that ovaries (132.56±128.68 MU g^{-1}) ranked second for toxicity following livers (148.73±155.72 MU g^{-1} with a range of 15-617 MU g^{-1}). Kosker et al (2016) revealed that TTX in gonads ranged from 14.82 MU g^{-1} in spring to 146.12 MU g^{-1} in winter showing a slight agreement with the present criteria. The TTX in the liver was found to be > 10 MU g^{-1} in female fish during summer and winter. Katikou et al (2009) reported that TTX in the gonads and livers of *L. sceleratus* from the Aegean Sea ranged from 77.5 to 1090 MU g^{-1} and from 23.1 to 398 MU g^{-1}, respectively. These findings indicate the variation in the toxicity of gonads and that their toxicity falls within the risky range. The present toxicity was higher in the larger-sized group (more than 50 cm) than in the smaller group. This finding is in agreement with Sabrah et al (2006), who stated that this species should not be consumed, specifically the larger fish of more than 40 cm, which have reached maturity, as they sometimes have moderately toxic muscles (more than 100 MU g^{-1}), especially during spring and summer. The variations in the length of this species need to be considered, as in the Mediterranean Sea it has been found to reach more than 80 cm (Farrag et al 2016).

Knowledge of the toxicity patterns, awareness of neurotoxins and symptoms are very important to avoid cases of intoxication. In marine pufferfish species, TTX is generally high in the liver and ovary, which agrees with the present results, whereas in freshwater species, toxicity is higher in the skin (Farrag et al 2022). The symptoms of TTX poisoning due to pufferfish consumption include slight numbness of the lips and tongue with purplish discoloration of the lips and skin, a tingling sensation in the face and extremities, headache, abdominal pain, nausea, diarrhea, vomiting, difficulty in walking, paralysis, respiratory distress, difficulty in speaking, shortness of breath, low blood pressure, mental impairment, irregular heartbeat, and death (Noguchi & Ebesu 2001; Cho et al 2012; Kheifets et al 2012). More or less, the human symptoms are like the symptoms that appeared on the injected mice mentioned above. Moreover, the same symptoms appeared on the intoxicated cases or persons who eat the pufferfish muscles from the Egyptian Mediterranean waters (Grey reports from Al-Ameri Hospital in Alexandria since 2011 until 2018, by personal communication of author and the head of the department). Symptoms usually start between 30 min and 3 h after consumption and may last for 20 min to 8 h. Death may occur within 4 to 6 h if respiratory aid is not provided (Cho et al 2012; Kheifets et al 2012). Other authors have mentioned that the symptoms began in 10-45 min and appeared due to blocking of sodium channels and the effect on neuronal transmission in the skeletal muscles (Mak & Ho 2006), they stated also, the intoxication can be rapidly fatal, and no antidotes is known.

Other pufferfish species such as *L. lagocephalus* is endemic to the Mediterranean Sea. However, there have been no reported cases of toxicity by this species. This may cause confusion whether this species is toxic or not. The endemic species emerged in very few samples in accordance with Farrag et al (2016) in Egypt. Hence, it does not cause an ecological or health problem like *L. sceleratus* despite the lack of evidence of its safety. Saoudi et al (2008) mentioned that the toxicity of liver was higher than that of muscle, with a decrease in antioxidant enzyme activities for *L. lagocephalus* from the Mediterranean Sea. This suggests that symptoms may vary with species variations. However, these effects are not specific, as they are reported in numerous toxicity studies (Ding et al 1998; Li et al 2003; Sicinska et al 2006). Recently, Guardone et al (2018) studied three species, *L. sceleratus*, *L. lagocephalus* and *Sphoeroides pachygaste*r from the Italian coast. They suggested that the presence of *L. sceleratus* should be strictly monitored.

From another point of view, Poletti et al (2003) mentioned that TTX, brevetoxin (neurotoxic shellfish poisoning, NSP), and ciguatoxin (ciguatera fish poisoning, CFP) have not been found in the Mediterranean at least until 2003. It is worth mentioning that the pufferfish flourished in the Mediterranean Sea, especially the Lessepsian pufferfish, with its first appearance in the Mediterranean after 2003. Contrarily, TTX was known in other parts of Egypt such as the Red Sea (Zaki et al 2001; El-Sayed et al 2003), where

numerous species of pufferfish were known. The toxicity in pufferfish may vary according to species, habitats, and culture of people who eat it. Thus, muscles of numerous toxic species are regarded as edible by the Japanese Ministry of Health and Welfare (Mahmud et al 2000), and they are of considerable interest for food in many countries (Isbister et al 2002). The consumption of these pufferfish has occasionally caused food poisoning, including fatal cases (Mahmud et al 2000; Kodama & Sato 2005). The recent review made by Guardone et al (2020) was about a global retrospective study on human cases of TTX poisoning after seafood consumption (fish, gastropods, cephalopods, and arthropods). Over half of the cases were attributed to fish, followed by gastropods, arthropods and cephalopods. Recent intoxications in Spain, Middle East and North Africa were caused by locally caught products, confirming the occurrence of TTX even in areas previously not affected by this public health issue.

Regarding the origin of TTX, many scientists suggest that bacteria and other microbes are the primary sources of TTX. Moreover, pufferfish is considered as a TTX-bearing organism *via* the food chain (Tsai et al 2006) similar to *Vibrio* sp., which is isolated from the xanthid crab, *Amphicteis floridus* (Wang & Fan 2010), *Shewanella algae* and *Alteromonas tetraodonis* isolated from the red calcareous alga *Jania* sp. (Lee et al 2007), and strains of Vibrionaceae extracted from the pufferfish *Takifugu rubripes* (Noguchi et al 2006). This postulation suggests that all TTX-bearing organisms are infected by TTX-producing microorganisms living symbiotically within their bodies, which is later confirmed by the isolation of TTX-producing bacteria from various TTX-bearing animals (Lee et al 2007). However, few reports mentioned that TTX may be synthesized in pufferfish themselves as *in vivo*-cultured TTX-producing bacteria do not produce TTX in sufficient quantities, which explains the intoxication after the consumption of TTX-bearing animals (Wu et al 2005).

In general, these different opinions do not guarantee the safety of eating pufferfish with or free of microbes/bacteria. The same goes for the toxic freshwater pufferfish in the absence of marine bacteria. Many scientists studied the freshwater pufferfish and they reported its toxicity in different levels particularly in the skin (Ali et al 2014; Farrag et al 2022). In short, the information on the absolute safety of pufferfish consumption is scarce (Ghosh et al 2004; Hwang & Noguchi 2007; Saoudi et al 2008).

Saoudi et al (2008) indicated that the muscles of *L. lagocephalus* contained 10.28 MU g^{-1} of TTX. Previous data of El-Sayed et al (2003) indicated that 3000 MU g^{-1} can be fatal to humans. Thus, about 300 g of *L. lagocephalus* flesh is enough to kill a human adult. This reflects the danger of consuming this part of the fish. The minimum lethal dose of TTX in humans is approximately 2 mg (Cohen et al 2009). Others such as Hajeb et al (2012) stated that the yellow pufferfish in Malaysia is safe for consumption due to well handling and cleaning, and no cases of poisoning following its consumption were recorded. However, several cases of TTX intoxication and deaths have been reported from different countries, such as the USA, Brazil, Japan, Thailand, and Hong Kong (Hajeb et al 2012). As mentioned before, it is impossible to ensure safe consumption of specific organs or the handling of the pufferfish. Whereas, in most cases the muscles are eaten after appropriate handling from professionals who are fully aware of the toxicity of this species. Others thought that this species has a certain gland and that it is safe to eat if the gland is removed, this gland is known as gall bladder by the simple persons and fishermen. This thinking was in agreement with other scientific mention by Arakawa et al (2010) who mentioned that there is a gland under skin which is responsible for the contamination with TTX. However, the later author concluded that the pufferfish is an exogenous for TTX and much affected by food chain. In addition, a lot of people with awkward behavior insist on eating the fish muscles, especially during the spawning season, to get a harmonious effect on their tongue and brain (author observation and communication). According to the current European legislative requirements (Regulation (EC) No. 853/2004; Regulation (EC) No. 854/2004), the poisonous fish of the family Tetraodontidae and products derived from them must not be displayed on the market. Nevertheless, some countries, such as Japan or Korea give license to certain cookers who have ability to deal with such toxic fishes based on their thought that the presence of

specific toxic organs and the cooker may remove these organs. Also, there is no absolutely guarantee to its safe consumption.

The present study aimed also to find some differences between the toxicity of fresh and frozen samples of *L. sceleratus* after freezing and preservation. Most frozen muscles and gonads exhibited values higher than those in fresh sample. The liver was unlike the other organs, the frozen samples showed toxicity higher than fresh samples. Thus, freezing and preservation did not remove toxicity; with no evident positive changes confirming that the fresh and preserved pufferfish are unsafe for consumption with no exception for certain organs. Different studies have been done to evaluate the effect of freezing on pufferfish toxicity (Shiomi et al 1984, 1985a). Yamamoto et al (2005) investigated the quality of frozen tiger puffer meat in comparison with frozen yellowtail. Their thawing was applied by two methods (running water at 17°C and in air at 4°C), and no significant difference in breaking the toxin was observed for the tiger puffer muscles accompanied with less histological damage in their cells than in the yellowtail. Moreover, Noguchi et al (1997) studied alive and frozen muscles of pufferfish *Takifugu vermicularis* from Japan and the Korean seas and they stated that the muscles of alive fish were non-toxic while the skin was toxic. Later the frozen muscles became toxic and this might be due to the contamination from toxic skin. In the present investigation, the precautions to separate different organs were taken before freezing to prevent contaminating each other, and after thawing the extra water or crystals were removed and dried completely regardless the effect on muscle structure. However, the present results were in disagreement with Noguchi et al (1997) in spite of the presence of their results of frozen samples in the weak level of toxicity, which was slightly a similar trend in the present frozen muscles. The later author recommended the alive meat of pufferfish for human consumption under the control of professionals unlike the present status in Egypt. This opinion may meet a specific species, location and season, while it is different for other species of pufferfish especially *L. sceleratus,* which causes many intoxications in both fresh and frozen. So, it will be better to highlights its importance in industrial applications and pharmaceutical as it has an analgesic activity and highly selective sodium channel blocker in agreement with Hagen et al (2008).

Conclusions. The present results introduced detailed data regarding toxicity patterns of *L. sceleratus* in the Mediterranean Sea, Egypt, and how the opinions varied among different countries and from species to species. The avoidance of these fish is recommended for both fresh and frozen specimens, and other applications of its TTX are encouraged for industry and pharmaceutical uses. Moreover, the awareness towards these fishes needed in scientific and social scale for food safety and human consumption.

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Conflict of interest. The authors declare that there is no conflict of interest.

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Author:
Mahmoud M. S. Farrag, Marine Science and Fishes, Zoology Department, Faculty of Science, Al-Azhar University, Assiut, Egypt, e-mail: m_mahrousfarrag@yahoo.com; mahmoudfarrag42@azhar.edu.eg

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