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Original Article

Ecotoxicological responses of two *Planorbarius corneus s. lato* (Mollusca, Gastropoda) allospecies to exposure of heavy metals

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Abstract: *Planorbarius corneus* (Linnaeus, 1758) is the most widespread and numerous gastropods in Central-European waters, which range covers from the Atlantic to the Ural and outside the latter to the Ob river basin. Before the beginning of 21 century, malacologists had no doubts about its species status. This situation changed when genetic labeling showed that *P. corneus* is not a species, but a superspecies complex, *Planorbarius* (superspecies) *corneus* s. lato, according to the centromere indices of the 12th pair chromosome. This complex consists of two vicarious genetic allospecies, western and eastern one, which ranges are separated by a narrow (up to 100 km) zone of the introgressive hybridization lying just in Ukraine. Ecotoxicological features of *P. corneus* s. lato allospecies under the influence of any pollutants have not been studied yet. Our research focused on how different concentrations (0,001–1000 mg L^{-1}) of some heavy metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}) in the water environment affected the main ecotoxicological parameters of each vicarious allospecies, and on the limits of toxic effects for studied pollutants. We found the duration of the latency period, time-to-death, and mean time-to-death, as well as the coefficients of persistence and adaptation for each allospecies. According to our results, eastern allospecies are more sensitive to the heavy metals in the aquatic environment than western one. That creates a significant threat to the eastern allospecies populations, because the concentrations of these pollutants in the Ukrainian waters remain rather high, despite some positive downward trends.

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Introduction

Heavy metals are ecologically toxic substances that are found in Ukrainian water bodies. This type of pollution is one of the limiting factors for the functioning and bioproductivity of aquatic ecosystems. Heavy metals participate in hydroecosystem biogeochemical cycles and persist for a long time playing a key role in energy metabolism processes that provide the viability of aquatic organisms (Afanasyev and Grodzinsky, 2004; Grubinko et al., 2012). Their concentrations in the Ukrainian waters are reported to be rather high, despite some positive downward trends (Giriy et al., 2011). As microelements, heavy metals (Cu, Zn, Ni, Mn, etc.) are included in many organic compounds that are biologically important affecting basic biochemical processes of aquatic organisms (Hochachka and Somero, 1988).

Zn^{2+} is an essential component of the carbonic anhydrase enzyme that catalyzes the reaction of carbonic acid decomposition and the reverse reaction of its formation. It provides normal growth and development of the mollusks by regulating the reactions that mitigate the effects of haemolymph stability disruption (Kyrychuk, 2010). Cu^{2+} is also an essential component of their respiratory pigments, Mn^{2+} affects their growth and development, and Ni^{2+} influences the processes of haematopoiesis in mollusks (Romanenko, 2001). However, these pollutants may accumulate in aquatic animals and are highly toxic at relatively low concentrations. There are several factors that determine the accumulation of heavy metal ions: hydrochemical conditions of the environment, bioavailability for animals, type of

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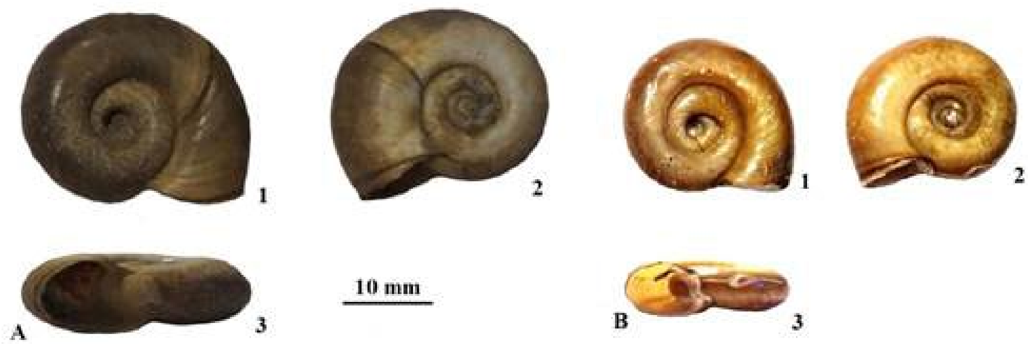

Figure 1. The shells of *Planorbarius corneus s. l.* allospecies: A – «western»; B – «eastern»; 1 – top view; 2 – bottom view; 3 – side view.

nutrition and nutrient digestibility level, genetic differences, and physiological status of affected species (Oros and Gomoiu, 2010). When heavy metal concentrations in the water bodies exceed the threshold limits, the boundary between their physiological and toxic effects blurs (Gorovaya and Stolyarova, 1987) with the detrimental consequences for aquatic organisms due to pathogenic (toxic or cancerogenic) effects.

Planorbarius corneus (Linnaeus, 1758) is a widespread and often numerous mollusk (Didukh, 2012) as only representative of Bulinidae family in European waters. Genetic labeling has recently proved (Mezhzherin et al., 2005) that *P. corneus* is not a species but a superspecies complex that consists of two spatially separated vicarious allospecies, "western" and "eastern" one. They significantly differ genetically and by some conchological, anatomical and ecological characteristics (Mezhzherin et al., 2006; Garbar and Garbar, 2007; Garbar, 2009; Stadnychenko et al., 2020). These taxa are well-differentiated by gene frequency distribution peculiarities, namely by the centromere index of the 12th chromosome pair (Mezhzherin et al., 2006; Garbar and Garbar, 2007; Garbar, 2009; Stadnychenko et al., 2020). Western allospecies are marked by slightly larger shell size and its dark brown or almost black color. Eastern allospecies have a smaller and lighter shells. These allospecies also significantly differ by having six numerical indices that characterize the whorl expansion rate of their shells and the relative size of apertures. The conchological parameters of both allospecies have significant geographical variability (Garbar, 2009): their shell sizes decrease in the west-east direction and increase in the north-south one. The allospecies of *P. corneus s. lato* also significantly differ by several linear parameters of their reproductive system (8 out of 10 usually measured ones) and by some dimensional anatomical parameters, namely the size of the vagina, sperm reservoir, and its ducts (Mezhzherin et al., 2005).

The ranges of both allospecies are spatially separated. Western allospecies are common in the western and central part of the Right Bank Ukraine, and eastern allospecies inhabit the northeast and east of Left Bank and the extreme south of Ukraine from the Severskiy Donets to the Danube inclusive. Between their habitats lies a zone of introgressive hybridization (about 100 km wide). The chorological separation of these allospecies is due to the different environmental conditions in their habitats, first of all, the different rates and duration of summer droughts in these areas. The purpose of this research is to find out the differences in the ecotoxicological characteristics of "western" and "eastern" *P. corneus s. lato* allospecies affected by various Cu^{2+}, Zn^{2+}, Ni^{2+}, and Mn^{2+} concentrations.

Materials and Methods

A total of 1480 specimens of *P. corneus* s. lato (748 specimens of western allospecies and 732 specimens of eastern allospecies) were collected in summer 2020 (Fig. 1). The first allospecies was collected from Miropol vicinity (49°40'17"N, 33°45'39"E) in Sapohivka River, the latter one from Bilotserkivka

Table 1. The main toxicological characteristics of *Planorbarius corneus s. lato* allospecies under the exposure of heavy metal ions (48 hours).

Indicator, mg L^{-1}	"Western" allospecies				"Eastern" allospecies			
	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}
Threshold concentration	0.0001	0.001	0.01	0.1	0.00001	0.0001	0.001	0.01
LC_0	0.001	0.05	0.1	50	0.0001	0.01	0.05	40
LC_{50}*	0.05	0.4	20	250	0.005	0.2	2	150
LC_{100}	0.01	1	50	1000	0.001	1	10	1000
Toxicity rate	0.05	0.4	20	250	0.005	0.2	2	150

*Graphically defined.

Figure 2. Map showing the type localities of *Planorbarius corneus s.l.* allospecies: black circle – «weastern»; black diamond – «eastern».

(50°6'27"N, 27°41'45"E) in Psel River (Fig. 2). Identification of allospecies was performed by their conchological peculiarities (Fig. 3) (Garbar, 2009).

In each river, the material was collected in the biotopes of four types: (1) coastal shallow waters, covered with clusters of filamentous algae (*Cladophora* sp.), with the almost imperceptible flow and depth up to 5-7 cm, (2) coastal thickets of hard semi-submerged aquatic vegetation (*Scirpus* sp. and *Typha* sp.), overgrown with periphyton, that was concentrated in the river littoral 15-20 cm deep with the almost imperceptible flow, (3) deeper littoral zone (up to 1-1.2 m) with a well-developed phytal, mostly soft macrophytes (*Myriophyllum* sp., *Nymphaea* sp., *Lemna* sp., etc.), and with a flow velocity of 0.1-1 m/sec, and (4) benthic zone, without vegetating macrophytes, covered with a layer of plant detritus.

Mollusks were acclimated for 14 days (Khlebovich, 1981) in 10 L the aquarium, with a planting density of 5 specimens L^{-1}, water temperature 21-23°C, pH 7.2-7.8, DO 7.8-8.6 mg O_2 L^{-1}. The water was replaced every day. The mollusks were fed by soft vegetation from their sampling places (*Cladophora* sp. and *Myriophyllum spicatum*).

The toxicological experiment was done according to Alekseev (1981). The volume of the aquarium was 100 L, water temperature 20-23°C, pH 7.2-8.2, DO 7.6-8.9 mg O_2 L^{-1}. Toxic substances were $CuCl_2{\cdot}2H_2O$, $ZnCl_2{\cdot}2H_2O$, $NiCl_2{\cdot}6H_2O$ and $MnCl_2{\cdot}4H_2O$ (puriss. p. a.) in concentrations of 0.001–1000 mg L^{-1} (calculated by the cation). Exposition time was 48 hours, with daily water replacement. The results were evaluated in 10 and 30 minutes, 1, 2, 4, 6, 24 and 48 hours after the beginning of the experiment. Toxic effects on experimental animals were indicated by visual observation of their behavior and general

Table 2. Mortality (%) of allospecies under the exposure of heavy metal ions.

Concentration, mg L^{-1}	"Western" allospecies				"Eastern" allospecies			
	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}
0.0001	0	0	0	0	0	0	0	0
0.001	0	0	0	0	10	0	0	0
0.01	40	0	0	0	100	10	0	0
0.1	100	20	0	0	100	40	20	0
1.0	100	100	20	0	100	100	40	0
10	100	100	70	0	100	100	100	0
100	100	100	100	30	100	100	100	40
1000	100	100	100	100	100	100	100	100

Table 3. Latent period (h) of *Planorbarius corneus s. lato* allospecies under the exposure of heavy metal ions.

Concentration, mg L^{-1}	"Western" allospecies				"Eastern" allospecies			
	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}
0.001	14	24.1	25.5	-	12	21.0	23.1	-
0.01	4.2	10.3	24.2	25	1.3	8.3	21.0	24
0.1	1.4	2.1	20.5	4.1	0.5	1.5	16.5	4.0
1.0	0.5	1.5	2.1	2	0.3	1.1	1.4	1.5
10	0.3	1.0	1.1	1.1	0.15	0.4	0.5	1.0
100	0.1	0.1	0.1	0.35	0.1	0.1	0.1	0.3
1000	-	-	-	0.3	-	-	-	0.25

condition. As indicators, we evaluated the degree of the body covering epithelium damage, the intensity of mucus secretion by the outer epithelium cells, motion behavior, and nutrition intensity. Persistence coefficient (PC) was calculated according to Vyskushenko (2002) by the formula of $PC=E_k/E_n$, where E_k is a time to the death of the last test animal; E_n is the time to the death of the first test animal. Adaptation coefficient was calculated according to Malacea (1968) by the formula of $AC=E_e/E_c$, where E_e is an average time to the death of all the test animals, E_c is an average time to the death of all the control group animals.

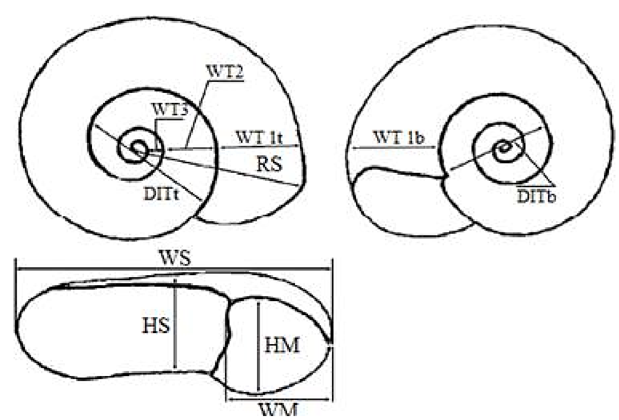

Figure 3. Measurements of the shells (scheme): HS – shell height; WS – shell width; HM – mouth hight; WM – mouth width; WT 1t – width of the last whorl (top view); WT 1b – width of the last whorl (bottom view); WT2 – width of the penultimate whorl; WT3 – width of the third whorl; DITt – diameter of the internal whorl (top view); DITb – diameter of the internal whorl (bottom view); RS – shell radius (Garbar, 2009).

Results

Western allospecies (Sapohivka River, Pripyat basin) were collected on 11.08.2020. The average shell size (mm) were 25.99±0.32, the aperture height 13.28±0.11. Eastern allospecies (Psel River, Dnieper basin) were collected 05.09.2020, the shell diameter (mm) was 24.96±0.15, and aperture height 12.29±0.08.

The threshold concentrations of all toxicants for eastern allospecies were an order of magnitude lower than for western allospecies (Table 1). The same order was for the main toxicological indicators, LC_0, LC_{50} and LC_{100}. We also found the difference in mortality between the allospecies under heavy metal ion poisoning (Table 2). Thus, in the water with 0.001 mg L^{-1} Cu^{2+}, western allospecies had 100% survival, while 10% of eastern allospecies specimens died at the end of the experiment. The duration of the latent

Table 4. Adaptation coefficient (h) of *Planorbarius corneus s. lato* allospecies under the exposure of heavy metal ions.

Concentration mg L^{-1}	"Western" allospecies	"Eastern" allospecies
Cu^{2+}	4	3.1
Zn^{2+}	1.30	1.20
Ni^{2+}	1.15	1.10
Mn^{2+}	1	1

Table 5. Persistence coefficient (h) of *Planorbarius corneus s. lato* allospecies under the exposure of heavy metal ions.

Concentration, mg L^{-1}	"Western" allospecies				"Eastern" allospecies			
	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}
0.1	1.14	1.25		-	1.20	1.24	-	-
1	1.32	1.34	2.05	-	1.40	1.42	2.25	-
10	2.47	1.56	3.12	-	3.15	2.30	3.34	-
100	3.12	2.87	2.21	-	3.41	3.05	5.53	-
1000	-	-	-	3.57	-	-	-	3.73

Table 6. Rating of heavy metal ion concentrations (mg L^{-1}) according to the effect on *Planorbarius corneus s. lato* allospecies

Ion	Subthreshold	Sublethal	Chronic lethal	Acutely lethal
		"Western" allospecies		
Cu^{2+}	10^{-5} and lower	0.001-0.0001	0.05-0.01	1-0.1
Zn^{2+}	0.01 and lower	1-0.01	2-1	5-3
Ni^{2+}	0.01 and lower	2-0.1	6-13	40-10
Mn^{2+}	0.3-0.003	10-1	40-20	80-50
		"Eastern" allospecies		
Cu^{2+}	10^{-6} and lower	10^{-4}–10^{-5}	0.01-0.001	1-0.05
Zn^{2+}	10^{-5} and lower	0.001-0.0001	0.1-0.01	3-1
Ni^{2+}	10^{-4} and lower	0.01-0.001	0.4-0.1	10-1
Mn^{2+}	0.01 and lower	5-0.1	30-5	70-35

Table 7. Time-to-death (h) of *Planorbarius corneus s. lato* allospecies under the exposure of heavy metal ions.

Concentration, mg L^{-1}	"Western" allospecies				"Eastern" allospecies			
	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}
0.001	37	-	-	-	35	-	-	-
0.01	30	41.0	43.3	-	27	39.2	39.3	-
0.1	12	23.2	29.1	-	10	21.3	25.3	-
1.0	0.5	18.4	19.3	-	0.3	17.1	17.3	1
10	0.3	6.3	8.1	29.1	0.15	6.0	7.2	27
100	0.1	0.4	0.5	16	0.1	0.1	0.1	15
1000	-	-	-	1.4	-	-	-	1.1

poisoning periods also differs (Table 3). Under the toxicant concentrations of 0.001-10 mg L^{-1}, the latent periods for eastern allospecies were lower than for western one. Adaptation coefficients in the environment polluted by Cu^{2+}, Zn^{2+}, and Ni^{2+} (Table 4) were also lower for eastern allospecies. As for Mn^{2+}, there was no difference between the allospecies. The absolute values of the persistence coefficient (Table 5) were higher for eastern allospecies. For both allospecies, the values of the persistence coefficient increased with the increase of toxicant concentration in the water.

The data presented in Table 6 show the toxic resistance of each allospecies under the exposure of heavy metals in concentrations from subthreshold to acutely lethal. For all the studied toxicants, in eastern allospecies the symptoms of poisoning appeared earlier and were caused by lower concentrations. Time-to-death decreased when the toxicant concentration in the environment increased (Table 7).

Table 8. Mean time-to-death (h) of *Planorbarius corneus s. lato* allospecies under the exposure of heavy metal ions.

Concentration,	"Western" allospecies				"Eastern" allospecies			
mg L^{-1}	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}
0.01	43	-	-	-	40	-	-	-
0.1	23.1	40	42	-	21	37	40	-
1.0	9.2	20	29	-	8.2	18	28	-
10	3.2	11	15	-	2.5	10	13	-
100	1.1	3	5	46	1	2.5	4	43
1000	-	-	-	-	-	-	-	3

At the same concentrations, the time-to-death of eastern allospecies was higher. For the average time-to-death, a similar pattern was observed (Table 8).

Discussions

Heavy metals enter the body of aquatic organisms mostly percutaneously in an osmotic way (Golovko et al., 2018) and much less with their food objects (Romanenko, 2001). They are able to accumulate in mollusks with subsequent redistribution between organs and tissues with haemolymph. The primary accumulation of heavy metals occurs in hepatopancreas. The heavy metals used in our experiments (Cu, Zn, Ni, and Mn) are biophilic, i.e. extremely necessary for viability processes of these aquatic organisms. However, they are extremely dangerous and often lethal at high concentrations, because their accumulation in the tissues and organs disrupts a number of important biochemical processes and physiological functions (Frias-Espericueta et al., 2003; Wong and Pak, 2004; Kyrychuk, 2010, Grubinko, 2011).

For each studied allospecies, we clarified the action limits of the above-mentioned toxicants depending on concentrations (from subthreshold to acutely lethal), which allowed to determine the zones of their toxic effects on test animals according to Dudnik and Yevtushenko (2011). The symptoms for each phase of the poisoning pathological process on aquatic mollusks are well-known (Vyskushenko, 2002; Pinkina, 2010; Stadnychenko et al., 2021). However, the expressiveness and overtime changes of these symptoms for *P. corneus s. lato* has not been studied yet. We found that Cu^{2+}, Zn^{2+}, Ni^{2+}, and Mn^{2+} below the threshold concentrations did not affect the ethological or physiological parameters of the studied mollusks. Such a reaction to the polluted environment is usually regarded as a latent phase that is an initial stage of the poisoning process (Veselov, 1968, 1984). This is the longest phase of this process, which is typical for all endogenous toxicants, including heavy metals. The first ethological and physiological changes were observed when experimental specimens were kept in an environment with sublethal toxicant concentrations. The response to the toxic effect includes reversible pathological changes both in the mollusks’ behavior and their physiological systems' functioning. The first ones were manifested by a significant increase of motion activity in attempts to leave the environment, and the second ones in the suppression of their food and sexual behavior. These indicators are the ground for the determination of toxicants’ threshold limit values for aquatic organisms. In Ukraine, for fishery reservoirs, they are equal to 0.005 mg L^{-1} for Cu^{2+}, 0.01 mg L^{-1} for Zn^{2+} and Ni^{2+}, 1 mg L^{-1} for Mn^{2+} (Resolution of the Cabinet of Ministers of Ukraine, 2013). All the above-mentioned pathological changes in mollusks were registered in the next phase of the poisoning, stimulation. In eastern allospecies, such changes occur 1-1.5 hours earlier and at much lower toxicant concentrations than in western allospecies. However, they developed cumulative toxicosis after prolonged chronic exposure to the same sublethal concentrations. During this phase, both allospecies demonstrated the stimulation of feeding and sexual behavior. The protective physiological response included increased secretory function of outer epithelial glandular cells that decreased the rate and the volume of toxicants' percutaneous penetration into the body. Over time, the layer of skin mucus did not thicken, but thinned due to its partial coagulation, exfoliation, and split-off of different shape and size coagulants, laying bare affected areas of the mollusk body surface. Under Zn^{2+} exposure, only 55% of western allospecies specimens and 63% of eastern allospecies specimens remained covered by intact mucus at the end of the experiment.

Under the exposure of chronic lethal concentrations, the mortality at the end of the experiment was 36% for western allospecies, and 45% for eastern allospecies. The surviving mollusks were clearly depressed having a mild to moderate swelling of the body, which indicated the disruption of their water balance. This is one of the animals' rapid protective physiological reactions (Pinkina, 2010; Pinkina and Pinkin, 2018), aimed to decrease the toxicant damaging effects by diluting the toxic substances.

In experiments with acute lethal concentrations, mollusks tried to avoid the toxic environment crawling on the aquarium walls, and, being above the surface of the water, immobilized, clinging to the walls. Some individuals, exhausted by unsuccessful attempts to get out of the poisoned environment, sank to the bottom and lay there motionless. In this case, 45% of western allospecies specimens and 55% of eastern allospecies specimens had a dropout reaction. Their body volume increased (1.5-2 times above normal) due to its watering, the columellar muscle tone decreased, and they could not retract the body into the shell, so their head and leg hung out through the aperture. This reaction is usually associated with impaired renal function due to decreased osmotic concentration of their excreta (Kolupaev, 1989). In such cases, the continuity of the tissue structures, especially cell membranes, is usually injured, which in turn leads to the destruction and death of cells and tissues. Such damages indicated the depressive and sublethal phases of the poisoning process. For the eastern allospecies, these symptoms were much clearer and started earlier than for the western allospecies. At the end of this experiment, during the lethal phase, the mortality reached 100%. Thus, we established the time-to-death values for each allospecies. Its duration decreased proportionally for both allospecies with increasing heavy metals concentrations. Most early the death had come under the Cu^{2+} poisoning and latest in Mn^{2+} treatment. Eastern allospecies were more sensitive to the effects of all the toxicants used in the experiments: with increasing concentrations, its time-to-death was much less (1-2 hours) compared to western allospecies.

Under the same concentrations of all heavy metal ions, eastern allospecies mortality was always higher than the western one. We assume that one of the reasons for this is the different ecological conditions in their usual natural habitats. After all, they are usually much more difficult for eastern allospecies due to the greater aridity of Left Bank Ukraine climate, especially in the extreme south of the steppe zone. Therefore, the toxic action limits from threshold to maximum lethality of *P. corneus* s. lato allospecies differ significantly for each heavy metal ion. Eastern allospecies are affected by lower concentrations and earlier than western allospecies indicating their lower toxic resistance. Eastern allospecies are more sensitive and less resistant to all heavy metal ions used in the experiment. This fact is confirmed by such ecotoxicological indicators as the latent period duration, time-to-death, and mean time-to-death, as well as adaptation and persistence coefficients. According to the latter, the first symptoms of poisoning and the symptoms of irreversible poisoning appear much earlier in eastern allospecies, and western allospecies are more viable than the eastern one. With increasing toxicant concentrations, the latent period begins earlier for both allospecies, and the time-to-death decreases. The toxicological reactions' comparison between western and eastern genetic allospecies of *P. corneus s. lato* was the first study to estimate the physiological differences between them. Therefore, this study encourages further investigations of the ecology and especially ecotoxicology of these mollusks.

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