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The Toxic Effects of Chloroform Stress Exposure on the Mediterranean Green Crab (*Carcinus aestuarii)*

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Abstract

The presence of different chemical substances in the aquatic environment such as chloroform, present high concern for potential adverse effects on non-target species. Chloroform, is known more formally as trichloromethane ($CHCl_3$). It is a volatile organic compounds (VOCs) which can be founded most frequently in both surface and ground water. In Albania, studies which estimate the effect of chloroform in aquatic living organisms are not present. The current study evaluates the physiological response of Mediterranean green crab C*arcinus aestuarii* against to chloroform exposure by measuring the haemolymph glucose level enzimatically. 20 animals were used for this experiment. Ten green crabs were assigned as the eyestalk-ablated group and the remaining ten animals as an intact group. Animals were exposed to the diluted chloroform solution on the ratio 1:1000 for 15 min (concentration: 0.005g/L). During experiments, the time exposure was recorded. Before and after exposure, the haemolymph glucose level was measured and the results showed that haemolymph glucose level, which was measured into intact animals, was significantly increased ($F= 8.93$, df=1, 10, $p=0.014$ so $p<0.05$) while into eye-stalk animals the haemolymph glucose level is slightly decreased, ($F=4.571$, df=1, 10, $p=0.058$ so $p>0.05$). The results of chloroform stress exposure indicate an aquatic environmental risk of this chemical substance for living organisms even in low concentrations. Obtained data on the biological effect of chloroform on Mediterranean green crab, *Carcinus aestuarii***,** showed that these species can be used as bio indicator for bio-monitoring pollution of the aquatic environment.

Keywords: chloroform, eyestalk, *Carcinus aestuarii*, hyperglycemic hormone, glucose.

1. Introduction

Different studies are done about toxic effects of chemical substances on terrestrial and aquatic wildlife worldwide [11]. Some studies report about the toxicity of chloroform in different species [30, 22].Chloroform is found to be the most frequently detected volatile organic compound (VOC) of both surface- and ground-water sources of drinking water and is used for different purposes such as: fumigant, insecticide, a precursor for dyes and pesti cides, and for the manufacture and processing of pharmaceuti cals. [12, 19] Many low cost methods are used in order to evaluate the toxic effects of different contaminants on the metabolism of aquatic animals. Several researchers consider the use of biomarkers as an 'early warning' tool for the detection of pollution degree on the environment where they live and the biological effects on the"non-target" animals [7, 9, 20, 29, 2].The toxicity induced by chloroform has been reported in different living organisms such as: aquatic bacteria, algae, invertebrates, fish, and amphibians. As a result of *Microcystis aeruginosa* for 6 day exposure to chloroform in the amount of 185 mg/L, an initial reduction in the level of cell multiplication is performed [8]. While into green algae *Scenedesmus subspicatus*, a 48-h EC10 for biomass of 225 mg/L (EC50 560 mg/L was evaluated [17] .Many studies related to the effects of chloroform on fish are done. A study done in rainbow trout *Oncorhynchus mykiss*, 4-day post-hatching LC50s resulted in a range of 1.24 mg/L (200 mg calcium carbonate/L) to 2.03 mg/L (50 mg calcium carbonate/L) [6]. The toxic effects of chloroform are evaluated for human health too. The U.S. Environmental Protection Agency (USEPA**)** proposed these aquatic criteria for the protection of human health against the probable carcinogenic effects of chloroform: 5.7 µg/L in water for people who consume organisms and water and for these who consume the organisms only 470 µg/L. These values

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include the use of the Bio-concentration Factor (BCF) in order to evaluate the concentrations of the compound that can be accumulate in the aquatic organisms tissues taking into the consideration the trophic levels that they occupy in food chains [28]. In Albania there are few studies relating the concentrations of volatile organic compounds in water bodies and their effects on organism's physiology. The toxic effects of chloroform in crabs are not well known and there are only some few studies done about the use of crustaceans as bio-indicators of water pollution [3, 21, 4].

2. Materials and methods

2.1 *Collection site*

Narta Lagoon is a lagoon located in the west part of Albania. It is the second largest lagoon in Albania referring to size and is located about 3 kilometers north of the city of Vlora. The lagoon is part of the Narta-Vjosa Protected Landscape and is connected by two short canals to the Adriatic Sea. Narta Lagoon has an area of 4180 hectares and a water surfacecovering 42 km². It has a maximum depth of 1.5 m and an average depth of 0.7 m. 20 adult male and female of specimens of crab *Carcinus aestuarii* were collected by a fisherman during April 2015 in Narta Lagoon, Vlora, Albania. They were immediately placed into buckets with sea water of the collection site and they were transferred quickly in the laboratory. The animals were allowed to acclimate to the laboratory conditions for 3 days before the chloroform exposure.

2.2 *The experiment exposure*

Animals were kept in 30 L aquaria filled with continuously aerated seawater (SNSW, Nutri-SeaWater®Aquarium Saltwater, pH: 8 ± 0.1; salinity: 36±1 ppt; temperature: 17 ± 1°C) for acclimatization for 4 days before starting the experimental procedure. Water was changed every two days in order to reduce the effect of O_2 level decreasing on the glucose level of animals. Animals were fed once a day with algal slurry (liquifry marine, Interpet, Dorking, England). After acclimatization, crabs were divided in two groups: an intact group (n=10) kept in artificial sea water and the eye-stalk ablated group (n=10) exposed to chloroform which was added to the container. The chloroform was diluted in the ratio 1:1000 ml (0,005 g/L). For both groups, the haemolymph glucose level was measured before and after the time exposure.

2.3 *Collection of haemolymph and measurement of the glucose level*

The haemolymph was collected at the base of the fourth moving leg of the crab, and it was withdrawn through the athrodial membrane into a 2.5 mL hypodermic syringe fitted with a 25 gauge needle and containing 0.5 mL of physiological Ringer solution for crustaceans: [20 mM (4.77g) Hepes, 436 mM (25.48g) NaCl, 53 mM (13.06g) MgSO4, 10 mM (0.75g) KCl, 10 mM (1.47g) CaCl2), pH 7.4]. The obtained solution was discarded into a 2.0 mL siliconised (Sigmacote) Eppendorph tube held in ice water.Animals were manipulated very carefully in order to avoid excessive stress. Measurement of the haemolymph glucose levels in the crabs was done enzimatically using the glucometer (One Touch-Ultra).

2.4 *Statistical Analysis*

One way ANOVA was used to analyze the results obtained before and after treatment with chloroform. Explanation of the results is done according to the value of p. The result is considered significant when $p<0.05$ and non-significant when $p>0.05$. Results are shown on the table below by using mean ± SEM.

3. Results and Discussions

The haemolymph glucose levels alteration after exposure to chloroform are shown in Table 1. There is an significant increased of haemolymph glucose levels in intact animals after exposure to the proposed dose of chloroform. While in eyestalk ablated crabs, the haemolymph glucose level change is nonsignificant.

Table 1. Results of haemolymph glucose level after chloroform stress exposure

Animals	No. animals	Before exposure	After exposure
Intact	N= 10	34.5±1.23	80.53±1.53*
Eyestalk ablated	N=10	20.16±0.98	18.83 ± 0.47^{NS}

In the intact group, as a result of the presence of the hormone was noticed that glucose level is drastically increased after the chloroform exposure of animals for 10 min from 34.5±1.23 mg/dl haemolymph to 80.3±15.3 mg/dl haemolymph (F= 8.93, df=1, 10, p=0.014 so p<0.05). While in eyestalk-ablated animals, the glucose level is not changed significantly as a result of the hormone absence (from 20.16±0.98 mg/dl haemolymph to 18.83±0.47 mg/dl haemolymph (F=4.571, df=1, 10, p=0.058 so p>0.05).

The following graph shows the results obtained.

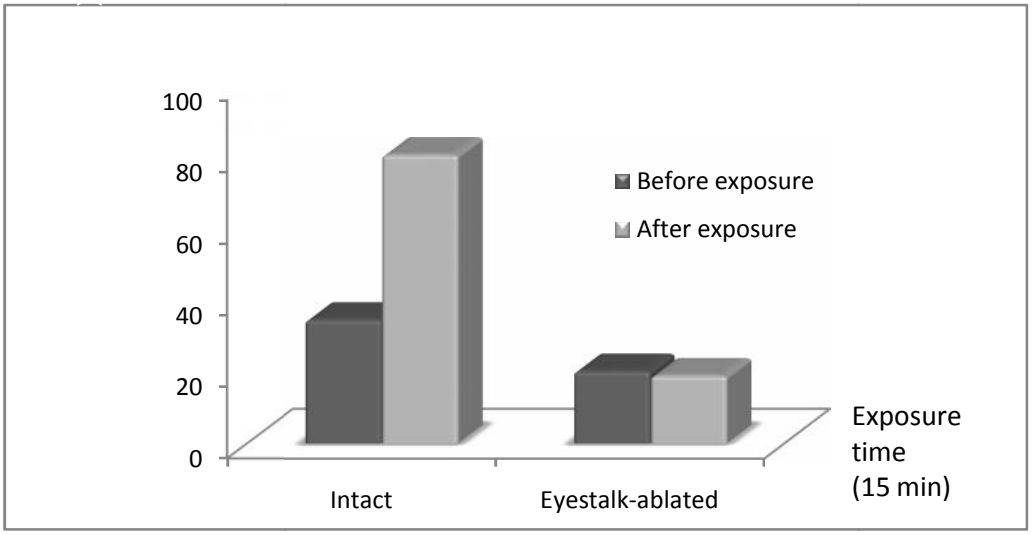

Figure 1. Glucose levels in haemolymph observed for intact and eyestalk-ablated group of studied crabs, in control condition and in presence of 0.005g/l chloroform. The difference between control and treatment is significant (p< 0.05)

The necessary time to the chloroform exposure is 2-3 minutes for a concentration of 1:500 or maybe even longer for a solution which is more diluted [1] like in our experiment in which the chloroform concentration was 0.005g/L.The present study aimed to investigate the effects of chloroform on the haemolymph glucose level in the Mediterranean green crab *Carcinus aestuarii*. Many changes of the environmental factors may induce stress in aquatic animals which lead on behavioral and physiological alterations in aquatic animals as a result of homeostatic regulation. The glucose level in the haemolymph of crustaceans changes significantly due to the changes of physiological and environmental conditions [15].The exposure of intact animals to the chloroform stress for 15 min, induces a significant increase of the haemolymph glucose level, even in low concentration (the glucose level is increased 2.5 times compared with the glucose level before the exposure to chloroform). While into eyestalk-ablated animals, the haemolymph glucose level was slightly decreased as a result of removed eyestalks [18]. Webster in 1996 has done similar studies in the species *Cancer pagurus* and he noticed that chloroform causes a significant increase of the haemolymph glucose level on intact animals. When the animal is under the stress exposure, the metabolic processess goes faster with results in an increase of glucose level produced from hepatopancreas. Sinus glands play an important role on this mechanism because they control the glucose level by producing the crustacean hyperglycemic hormone, cHH. Many experiments done in crustaceans, have showed the role of crustacean hyperglycemic hormone, cHH, has on the regulation of the glucose level [24]. The way how crabs are able to increase the glucose level in their heamolymph as a result of different factors, indicates us that maybe this response is similar to the hyperglycemic response of mammals [31]. Many of researchers have reported that surgical anesthesia provokes in mammals an immediate hyperglycemic response. Also results of this experiment shows that chloroform provokes a similar response into crab *Carcinus aestuarii*. Exposure of animals for a long time to the chloroform stress may lead to a cardiac arrest which can cause a lack of oxygen and maybe, the nervous centers which control the effect of chloroform over sinus glands, may control the effect of hypoxia into the sinus glands too [14]. The process which involves the cyclic nucleotides in the physiological response of crabs is not well known. Many stressors induce the cHH release, which make

possible the glucose level alteration. The prime target tissues of cHH which is a pleiotropic hormone, are hepatopancreas and muscle tissues [10]. Many studies shows a correlation between the hyperglycemic activity of cHH and glycogenolysis in the mentioned tissues; processes of synthesis and degradation of glycogen are controlled by cHH [25]. Researchers proposed that cHH performs it's hyperglycemic activity as a result of using up the source of glycogen found in many tissues, especially in muscles by activating an enzyme called phosphorylase and/or inhibition of glycogen synthase [13]. It seems that cHH has similar activity in invertebrates as glucagon and catecholamines in vertebrates which use cAMP as a second messenger [23]. A study which was done to observe the activation of phosphorylase by cAMP, reports that cyclic nucleotides maybe are involved in the metabolic activity of cHH and the induced hyperglycemia in *Orconectes* as a result of cAMP and cGMP activity [5, 27]. Comparing vertebrate tissues with arthropod tissues, the levels of cGMP dependent protein kinases result higher in arthropod tissues which make us to think that cGMP maybe more important in invertebrate than vertebrates [16, 26].The hyperglycemic response by animals studied on this research, make us to think that maybe even in *Carcinus aestuarii,* cGMP plays an important role on the mechanism of haemolymph glucose regulation. In order to provide more data about the amount of cGMP produced and the mechanism used in the species *Carcinus aestuarii* during the physiological response induced by toxic effects of chloroform and other pollutants factors, more studies in molecular level should be done.

4. Conclusions

Chloroform stress exposure as many other water pollutants provoke a physiological response into Mediterranean crab by increasing drastically their haemolymph glucose level. This makes *Carcinus aestuarii* a good bio indicator organism of the environment stress and helps us to better understand the molecular mechanism of carbohydrate metabolism under the different stressful factors.

Also, many of other aquatic animals which share the same aquatic environment with *Carcinus aestuarii***,** may be effected from chloroform.

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