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RESEARCH PAPER

Protective Effects of Ectoine on Physiological Parameters of *Daphnia magna* Subjected to Clove Oil-Induced Anaesthesia

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Abstract

Clove oil (CO) used as an anaesthetic in aquaculture causes toxic effects in fish and invertebrates since it has a narrow margin between effective and lethal concentrations. Ectoine (ECT) is an osmoprotectant produced by halophilic bacteria in response to stressful factors. It was hypothesized that ECT may attenuate physiological alterations induced by CO in aquatic animals. The aim of the present study was to determine if ECT induces protective effects in Daphnia magna subjected to CO-induced anaesthesia. Daphnids were treated with 50 or 150 µL/L CO alone or in the combination with 10, 25 or 50 mg/L ECT. The results showed that both concentrations of CO alone induced anaesthesia, heart arrhythmia, decreased heart rate, increased diastole/systole ratio and duration of diastole. On the other hand, ECT attenuated alterations evoked by CO with the most significant effects in the combination of 50 µL/L CO+25 mg/L ECT. The animals showed a prolonged TtA and attenuated depression of thoracic limb activity, heart rate, arrhythmia, decreased diastole/systole ratio and duration of diastole. The results indicate that ECT may be a cardioprotective agent in anaesthesized crustaceans.

Keywords: Ectoine, clove oil,*Daphnia magna*, anaesthesia, heart activity.

Introduction

Clove essential oil (CO) is a disstillate of flowers, stems and leaves of *Syzygiumaromaticum* inhabiting the Eeastern Hemisphere or *Eugenia aromaticum* and *Eugenia caryophyllata* the Western Hemisphere. Composition of CO is variable, however eugenol (4-allyl-2-methoxyphenol) ($C_{10}H_{12}O_2$) and isoeugenol (4-propenyl-2-methoxyphenol) are its basic ingredients, making up 70-98% of CO total weight (Isaacs, 1983). The other components are eugenol acetate (>17%), and kariofilen 5 (~12%) (Isaacs, 1983; Sladky *et al*., 2001)The oil possesses anaesthetic, antioxidant, antifungal, antiinflammatory, cytotoxic and antimicrobial properties towards pathogenic fungi, bacteria, and viruses (Norton *et al*., 1996; Arina and Iqbal, 2002; Prashar *et al*., 2006; Chaieb *et al*., 2007; Fu *et al*., 2007; Akbari *et al*., 2010).

CO has been used for anaesthesia in fish and invertebrate animals such as shrimps for stress reduction during transportation (Norton *et al*., 1996; Akbari *et al*., 2010; Jaganathan *et al*., 2011; Javahery *et al*., 2012). As an anaesthetic, it has a very narrow margin of safety between effective and toxic concentrations. Some studies indicated that CO may be lethal to fish if used at excessive concentrations or if regulations about time of aneshesia specific for a single fish species are not followed (Sladky *et al*., 2001).This compound also causes various toxic effects in human colon cancer cell lines in the *in vitro* conditions and in insects, therefore it has been used as an insecticide (US EPA, 2004; Boyer *et al*., 2009).The potential harmful effects of CO on non-target aquatic animals such as invertebrates was also reported. For example, a reduction growth and bleaching of corals was observed after repeated field application of the oil (Pastor *et al*., 2013).

Halophilic microorganisms respond to osmotic stress by changing the composition of membrane lipids and by selective intracellular accumulation of low molecular weight solutes. As a result of the increased intracellular level of solutes the cells maintain osmotic balance under hyperosmotic stress and avoid water loss and subsequent irreversible dehydration (da Costa, 1998; Roessler and Müller, 200). There exist various mechanisms in bacteria to maintain the intracelllularosmolarity, such as accumulation of inorganic ions (for example KCl) or very soluble compounds of low molecular weight, known as "compatible solutes" which do not interact with cellular processes. Compatible solutes can be

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divided into several structural groups: sugars (trehalose, surcose), polyols (glicerol, sorbitol, mannitol, α-glucosyl-glycerol, mannosyl-glycerol, mannosyl-glyceramide), N-acetylated diamino acids (like N-acetylglutaminylglutamine amide), betaines (such as glycine betaine and derivatives), amino acids (proline, glutamate, glutamine, alanine, ectoines like ectoine and hydroxyectoine) and derivatives (Galinski *et al*., 1985; Pastor *et al*., 2010).

Ectoine (ECT) (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is produced by aerobic, chemoheterotrophic, and halophilic bacteria to survive under extreme conditions (Lippert and Galinski, 1992; Nagata and Wang, 2001). Microorganisms such as *Marinococcus* sp. ECT1 synthesize and accumulate intracellular ECT in response to osmotic stress during unfavourable environmental conditions, such as high temperature, salinity (Wei *et al*., 2011; Bownik *et al*., 2014). This amino acid protects bacterial cell membranes, enzymes and nucleic acids against dehydration (Galinski *et al*., 1985). There are also results on effects induced by ECT in aquatic animals. Our previous studies revealed its low toxicity with the modulatory influence on heart activity, thoracic limb movement in *Daphnia magna* and protective effects in heat-stressed daphnids (Bownik *et al*., 2014; Bownik *et al*., 2015).

It is generally accepted that anaesthetics affect cardiovascular performance in lower vertebrates (McKenzie *et al*., 1989; Hill and Forster, 2004).Side-effects were reported in CO-anaesthesized fish such as ventilator failure and medullary collapse (Sladky *et al*., 2001). *Daphnia magna* was suggested as a model animal for studying anaesthetic potency of certain drugs (ASTM, 1986; McKenzie *et al*., 1992). On the basis of the previous study by Bownik (2015) showing anaesthesia, depression of thoracic limb movement, decrease of heart rate and arrhythmia in *Daphnia magna* exposed to CO it was hypothesized that ECT with its osmoprotective properties may attenuate alterations of physiological parameters caused by the oil. Therefore, the aim of the present study was to determine effects of CO alone and the combinations of CO and ECT on time to anaesthesia (TtA) thoracic limb activity and some endpoints of heart functioning of *Daphnia magna*: heart rate, diastole/systole heart area ratio and diastole duration.

Materials and Methods

Animals

Daphnia magna were cultured for several generations in five 6 l tanks with 5 l of aerated culture medium on the window ledge in a laboratory under light: dark period of 16 h: 8h. *Daphni*a culture medium was prepared following the ASTM standards (ASTM, 1986). The medium was synthetic freshwater (48 mg of $NaHCO_3$, 30 mg of $CaSO_4 \cdot 2H_2O$, 30 mg of $MgSO_4$ and 2 mg of KCl per litre of deionized water adjusted to a pH of 7.4), with a temperature of 23±2ºC. The number of cultured daphnids was about 30 animals per litre.The animals were fed once daily with a few drops of powdered *Spirulina* (2mg/L water) per tank and supplemented with a few drops of baker’s yeast per tank (from 10 mg/L stock suspension). Feeding was stopped 24 hours before the experiment.

Chemicals

Clovebud (*Eugenia caryophyllus*) essential oil (CO) was purchased as a commercial preparation with from PharmaTech. The quality of the oil was ascertained to be equal to the standards of European Pharmakopeia (75-88% eugenol, 5-14% β-caryophyllene, 4-15% acethyleugenol). Since CO has poor solubility in water therefore it was diluted in pure dimethyl sulfoxide (DMSO) (>99% analytical grade, PolskieOdczynnikiChemiczne) in 1:9 ratio to form a stock solution (Barbosa *et al*., 2003). Although CO has good solubility in ethanol our preliminary tests showed that even low levels of this solvent alone may affect thoracic limb movement and heart activity in *Daphnia magna*, therefore DMSO was used in the study as an alternative solvent with its documented low toxicity to daphnids and the ability to solubilize CO (Damiani *et al*., 2003; Dobre *et al*., 2011; Rana *et al*., 2011). Since CO is light-sensitive, the stock solution was stored in a light-protected vial in a dark place before the experiment.

Pure ECT (≥99 purity) produced by *Halomonaselongata* was purchased from Sigma-Aldrich. The amino acid is water-soluble therefore and aqueous stock solution was prepared for further mixing with CO.

Daphnids were treated with 50 or 150 µL/L CO alone and in the combination with various concentration of ECT (10, 25 and 50 mg/L). Both concentrations of CO for *Daphnia magna* were chosen on the basis of the preliminary experiment to obtain two different times to anaesthesia. ECT concentrations were selected on the basis of the previous research with respect to toxicity of the amino acid (Bownik *et al*., 2015). Appropriate amount of CO stock solution was diluted with 100 mL of *Daphnia* culture medium with ECT to obtain the following combinations: 50 µL/L CO+10 mg/L ECT, 50 µL/L CO+25 mg/L ECT, 50 µL/L CO+50 mg/L ECT and 150 µL/L CO+10 mg/L ECT, 150 µL/L CO+25 mg/L ECT, 150 µL/L CO+50 mg/L ECT. Control daphnids were also exposed to the same amount of DMSO that was added to the medium in the experimental groups.

Determination of TtA

30 *Daphnia magna* neonates were used to determine TtA for each experimental group. 3 mL of

CO alone or the combination of CO+ECT at the appopriate concentration were transferred from the the vial to a well (35 mm in diameter and 20 mm of hight) of a flat, clear-bottom polystyrene 6-well microplate (Nunc). After a single daphnnid was placed in the well the timer was started. When the animals were immobilized the timer was stopped. The animal was treated as anaesthesized when it was immobilized and did not show motility within 15 seconds and did not respond to external stimulation (touching of the animal with a pipette tip).

Determination of Heart Functioning and Thoracic Limb Activity

10 daphnids (in triplicate) was used for one experimental group in the study. Heart functioning and thoracic limb activity were determined at 0, 10, 20, 22, 25 and 30 min of the immersion in 50 µL/L CO or in the combination with various concentrations of ECT. Since 150 µL/L CO alone induced more rapid changes, particularly at the initial stages of the immersion the parameters were determined at shorter time intervals: 0, 2, 4, 6, 8, 9, 10, 15, 20 and 30 min of the exposure. One single daphnid was placed in one well of a 24-well flat-bottom microplate (Nunc) in 2 mL of medium containing appropriate concentration of CO alone or the mixture CO+ECT and a timer was started. At the appropriate time the daphnid was transferred from the microplate well in a 50 µL droplet of to a microscope slide for a microscopic examination of the physiological parameters. Animal movements in the droplet were limited by cotton wool fibers placed on the microscope slide. The microscopic view of a daphnid was recorded (with the speed of 30 frames per second) with a digital camera Nikon D3100 mounted on a microscope. The microscopic magnification (30-100x) and camera resolution allowed to perform the analysis with a good visibility of the heart. One video clip of the microscopic view of immersed *Daphnia magna* was recorded for a mininum of 1 minute. After the examination the daphnid returned to the appropriate well of the microplate for continuation of the exposure. Heart rate was assessed with Tracker® by a frame-by-frame video analysis and counting separate heart contractions per 1 minute. Using this method the irregular heart contractions could be easily recognized. Thoracic limb movement was determined by a frame-by-frame video analysis and counting the separate movements (beats) of the limbs per 1 minute.

Digital analysis of the video clip with beating heart of treated daphnids was used for a graphic representation of an exemplary of heart arrhythmia. Each frame of the video clip (with the speed of 30 frames per second) showing an image of heart cycle was represented by a value on y axis. Diastole was represented by 0 value and each step of heart contraction was plotted on the graph against the number of video frames on x axis. Systole (contracted heart muscle reaching its smallest area) at each heart cycle was represented as a peak in the cardiogram. After systole, each step of heart relaxation was also plotted on the graph and represented by decreasing values on y axis against the number of video frames on x axis. The heart was treated as arrhythmic when diastole and systole occured at non-regular time intervals.

Determination of Diastole/Systole Heart Area Ratio

The most significant differences of the effects on heart rate between CO alone and the combinations of CO+ECT were noted at 30 minute of the exposure. Digital analysis of the video clip with beating heart of daphnids was used for the determination of diastole/systole heart area ratio. The video analysis was performed with Tracker® software to select the frames with heart in diastole or systole. Afterwards, separate frames were saved as image files and the heart area was calculated with the use of Image Tool® software after drawing an ellipse-like shape along the heart contour in the digital image. The heart was considered to be during systole when it covered the smallest area. The heart was treated to be during diastole when the organ covered the largest area. Differences of diastole/systole ratio were compared between the experimental and control groups.

Duration of Diastole

Time of heart remaining in diastole (relaxation) was measured in all experimental groups by video analysis using Tracker® software. The video clip with the beating heart of *Daphnia magna* at 30 minute of the exposure was analysed with a frame-by-frame method and time of heart remaining in relaxation was calculated by multiplying the duration of separate video frame (33 ms) by the number of video frames with heart recorded in diastole.

Statistical Analysis

The results are presented as means±standard deviation (SD). Data were tested for homogeneity of variance for ANOVA assumptions. Experimental data were analyzed using ANOVA followed by Tukey's test to detect differences among means. All statistical analyses were completed using Develve® software. Values were statistically significant when $P<0.05$.

Results

Time to Anaesthesia (TtA)

The results showed that daphnids exposed to the combinations of 50 µL/L CO and various conccentrations of ECT showed a prolonged TtA when compared to those immersed in CO alone

(Figure 1a). The longest TtA (25.3 ± 2.1min) was observed in the animals exposed to the combination of 50 µL/L CO+25 mg/L ECT when compared to 50 µL/L of CO alone (TtA=16.2±0.5 min).

Daphnids exposed to the higher concentration of CO alone (150 µL/L) showed the most significantly abbreviated TtA (2.34±0.22 min) among all experimental groups in the experiment (Figure 1b), however, the animals treated to the same concentration in the combination with ECT needed substantially more time for induction of anaesthesia. The longest TtA was noted in daphnids immersed at 150 µL/L+25 mg/L ECT (10.1±1.5 min).

Thoracic Limb Activity

Daphnids exposed to 50 µL/L CO alone showed a rapid decrease of limb activity after 10 minutes of the treatment (78±15 bpm) when compared to non-treated control daphnids (227±21 bpm) (Figure 2a). After 30 minutes of the exposure the limb activity was reduced to 24±25 bpm. On the other hand, less decreased activity was observed in the groups of daphnids treated with combinations of CO+ ECT. The highest attenuation of the decrease was noted at 50 µL/L+25 mg/L ECT and 50 µL/L+50 mg/L of ECT.

Microcrustaceans treated with 150 µL/L of CO alone for 10 minutes presented a significant decrease of thoracic limb activity to 50±22 bpm (Figure 2b). After 22 minutes of the exposure the limb activity was reduced to 10±10 bpm and remained unchanged until 30 minute. The limb movement was less decreased in the group of daphnids exposed to the mixtures of CO and ECT. The most substantial attenuation of the decrease was seen at 150 µL/L of CO+50 mg/L of ECT (192±21 bpm, 72±24 bpm after 10 and 30 minutes, respectively).

Heart Rate

Daphnids exposed for 30 minutes to 50 µL/L of CO alone showed a decrease of heart rate to 204±21 bpm after 10 minutes and 90±bpm after 30 minutes (Figure 3a). However, the inhibition was significantly attenuated when the animals were treated with the combinations of CO+ECT. The lowest reduction of heart rate was observed at 50 µL/L CO+ 25 mg/L ECT (348±13 and 294±12 bpm after 10 and 30 minutes, respectively). A significant attenuation of heart rate decrease after 30 minutes was also noted at 50 µL/L CO+10 mg/L ECT and 50 µL/LCO+50 mg/L ECT (132±21 bpm and 210±23 bpm).

Exposure of daphnids to 150 µL/L of CO alone resulted in a rapid decrease of heart rate to 42±15 bpm and 36±17 bpm after 30 minutes (Figure 3b). On the other hand attenuation of heart rate inhibition was noted after 30-minute exposure to the combinations of 150 µL/L CO+10 mg/L ECT (60±21 bpm) and 150 µL/L+25 mg/L ECT (90±14 bpm).

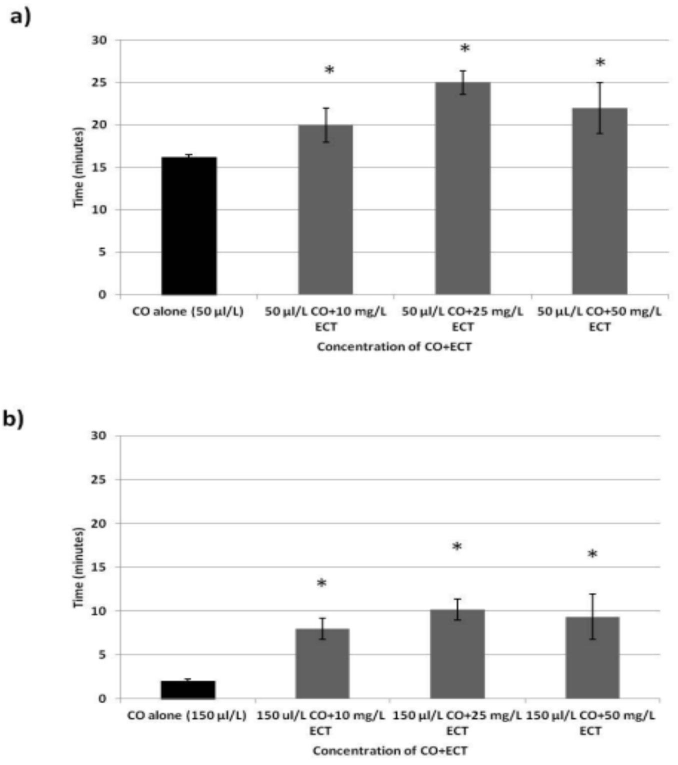

Figure 1. Time to anaesthesia (TtA) of *Daphnia magna* treated with clove oil (CO) at concentrations of 50 µL/L (panel "a") and 150 µL/L (panel "b") alone or in the combination with different concentrations of ectoine (ECT). Results are presented as means±SD, n=30, statistical significance, P<0.05.

Heart Arrhythmia

The results of our study revealed that daphnids exposed to 50 µL/L of CO alone showed irregular heart beat which occurred in 12% of daphnids after 10 minutes of immersion. 30% of the crustaceans showed arrhythmia after 20 minutes and 93% after 30 minutes of the exposure (Figure 3c). On the other hand, lower percentage of daphnids treated with CO+ECT showed heart arrhythmia. Reduced number of daphnids with arrhytmic hearts was seen at 50 µL/L CO+25 mg/L ECT and at 50 µL/L CO+50 µL/L ECT, however no significant differences between these two experimental groups were noted. 15% of arrhythmia-positive daphnids were noted at 20 minute, however the it was 10% at 30 minute of the exposure.

Daphnids treated with 150 µL/L of CO alone also showed a significant heart arrhythmia (Figure 3d). Heart beartirreglarity was noted in 93% daphnids after 20 minutes and all daphnids were arrhythmia-positive at 30 minute of the exposure. On the other hand, there was a significantly lower number of animals with arrythmia in the groups that were treated with the combinations of 150 µL/L CO with different concentrations of ECT. An exemplary of *Daphnia magna* heart contraction at 30 minute of immersion in different concentrations of CO alone or CO+ECT is presented in Figure 4.

Diastole/Systole Heart Area Ratio

The results of the present study showed that treatment of daphnids with 50 µL/L of CO alone resulted in the increased diastole/systole ratio after 30 minutes (1.53±0.2) when compared to the control (1.21±0.14) (Figure 5a). However, the animals exposed to the combinations of CO+ECT showed alleviated increase of the ratio with the most significant level in the combination of 50 µL</L CO+50 mg/L ECT (1.28±0.16).

The highest diastole/systole ratio after 30 minutes of exposure showed d<aphnids treated with 150 µL/L CO alone (1.91±0.17) when compared to the control (Figure 5b). On the other hand, a significant decrease of the ratio was observed in the crustaceans exposed to the combinations of 150 µL/L CO and different concentrations of ECT. However the difference between the groups with ECT was not statistically significant.

Duration of Diastole

The present study revealed that the duration of diastole was significantly increased in daphnids exposed for 30 minutes to 50 µL/L alone CO alone (640±66 ms) when compared to the unexposed control group (66±33 ms) (Figure 6a). The mixture of CO and the highest concentration of ECT (50 µL/L CO+50 mg/L ECT) induced the most significant reduction of diastole duration (320±33 ms). The longest time of diastole was observed in daphnids treated for 30 minutes with 150 µL/L of CO alone (700±33 ms) (Figure 6b). On the other hand, combinations of the oil with different concentrations of ECT induced a significantly shortened time of

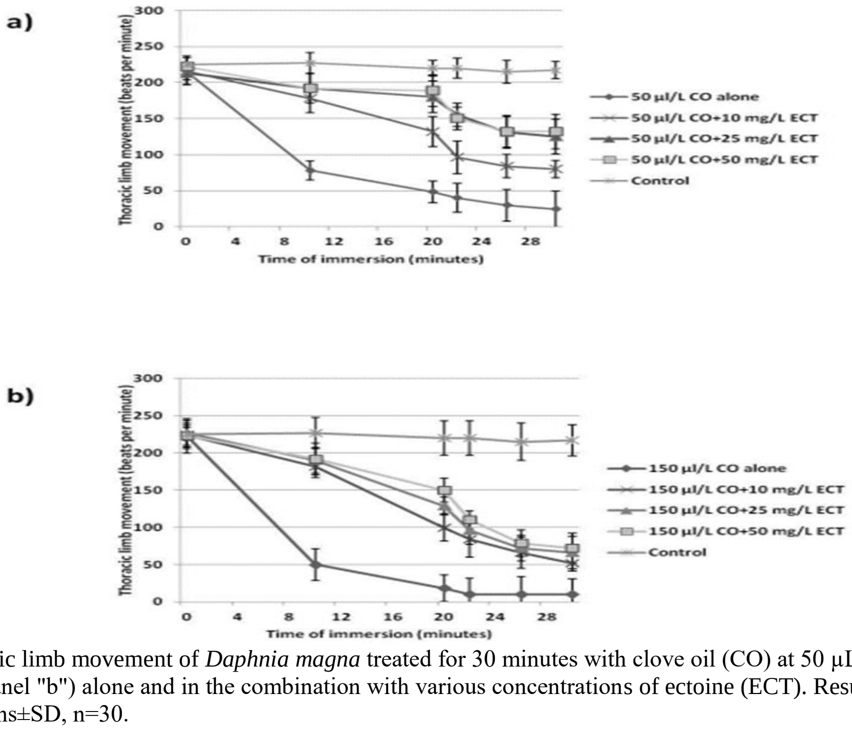

Figure 2. Thoracic limb movement of *Daphnia magna* treated for 30 minutes with clove oil (CO) at 50 µL/L (panel "a") and 150 µL/L (panel "b") alone and in the combination with various concentrations of ectoine (ECT). Results are presented as means±SD, n=30.

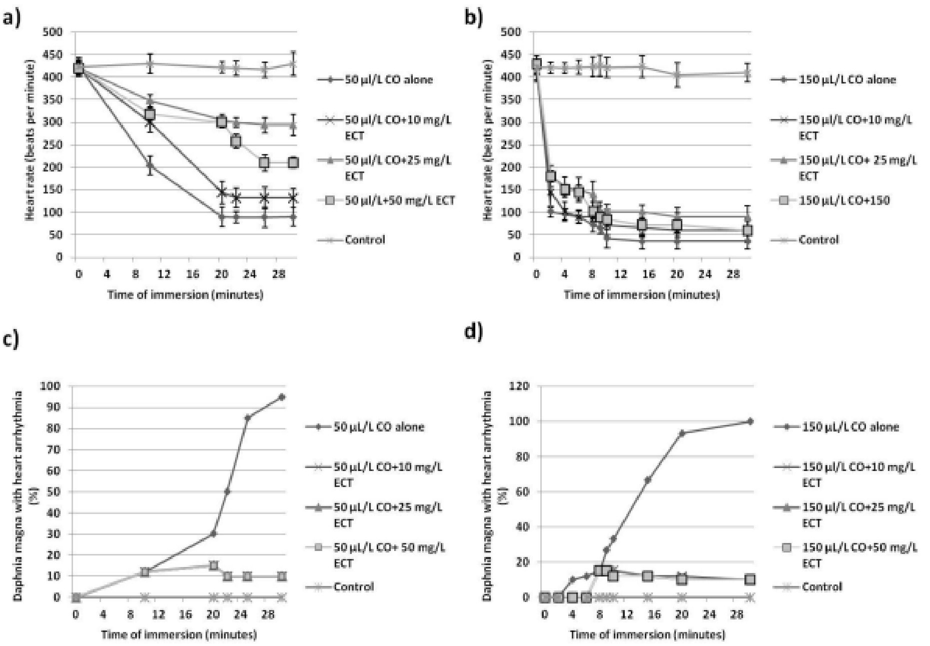

Figure 3. Heart rate and percentage of *D. magna* with heart arrhythmia during 30-minute exposure to clove oil (CO) alone and in the combination with various concentrations of ectoine (ECT). Panel "a" presents heart rate of daphnids exposed to 50 µL/L CO+ different concentrations of ECT. Panel "b" shows heart rate of daphnids exposed to a mixture of 150 µL/L of CO and different concentrations of ECT. Panels "c" and "d" present percentage of daphnids with heart arrhythmia treated with CO at 50 and 150 µL/L, respectively in the combination with different concentrations of ECT, n=30.

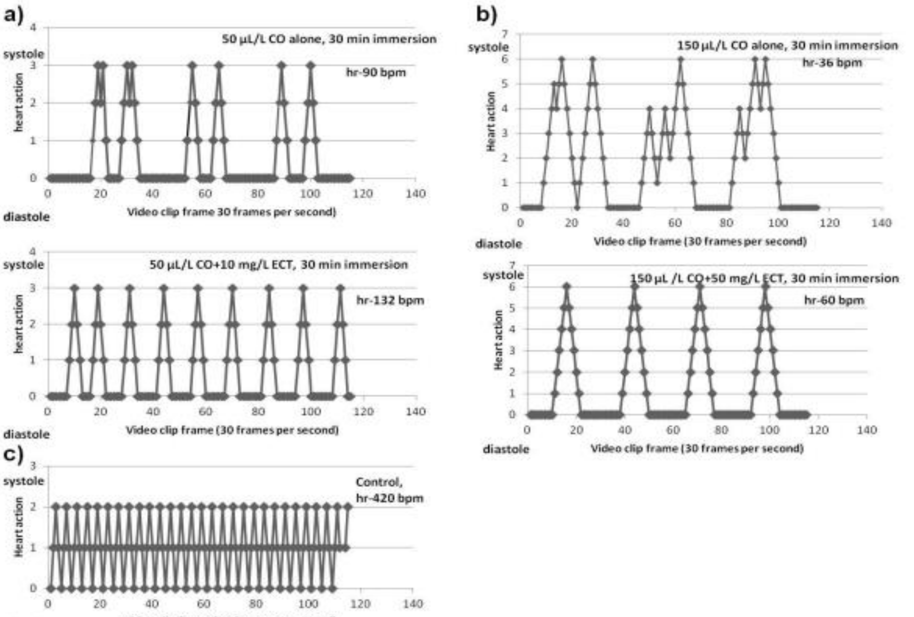

Figure 4. Graphic representation of exemplary heart muscle contraction of *D. magna* exposed for 30 minutes to CO alone and the combination of CO+ECT. Panel "a" shows the heart action of daphnids exposed to 50 µl/L CO alone (upper graph of the panel "a") and the combination of 50 mg/L of CO+10 mg/L ECT (lower graph). Panel "b" presents graphs with the cardiac cycle of daphnids exposed to 150 µl/L of CO alone (upper graph) and the combination of 150 µl/L of CO+ 50 mg/L ECT (lower graph). Panel "c" shows heart action in the non-exposed control group. The results were obtained after a frame-by-frame video analysis of the recorded heart muscle contraction. The video resolution of 30 frames per second allowed to detect several stages of heart muscle contraction. Diastole (heart relaxation) is represented as 0 value on y axis. Systole is represented by the "peak" of each cardiac cycle. Daphnids exposed to both concentrations of CO alone showed heart rhythm irregularity and prolonged time of relaxation in comparison to those exposed to the combination of CO+ various concentrations of ECT which manifested regular heart rhythm and a shorter time of diastole. "hr" -heart rate.

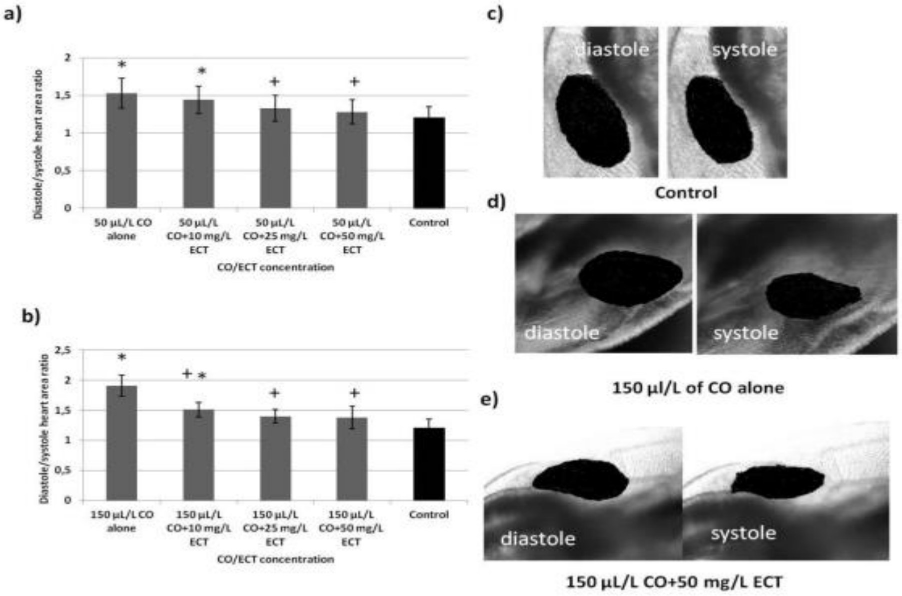

Figure 5. Diastole/systole heart area ratio in *Daphnia magna* at 30 minute of exposure to 50 µl/L CO alone or the combination of 50 µL/L CO and various concentrations of ECT (panel "a") and 150 µl/L CO and the combinatiorial exposure of 150 µL/L CO and various concentrations of ECT (panel "b"). The right side of the figure presents microscopic images of exemplary hearts in diastole and systole of daphnids in the control group (panel "c"), treated with 150 µL/L CO alone (panel "d") and the combination of 150 µL/L CO+50 mg/L ECT (panel "e"). Heart area (presented as dark area of ellipsoidal shape) was estimated by the analysis of heart digital images from the video clip of the trated animals with the use of Image Tool® software. The heart area ratio in diastole/systole was calculated by the division the value of the heart area in diastole by the value of heart area in systole. Results are presented as means±SD, n=30. "*"-statistical significance between the control and experimental groups. "+"-statistical significance between CO alone and CO+ECT (P<0.05).

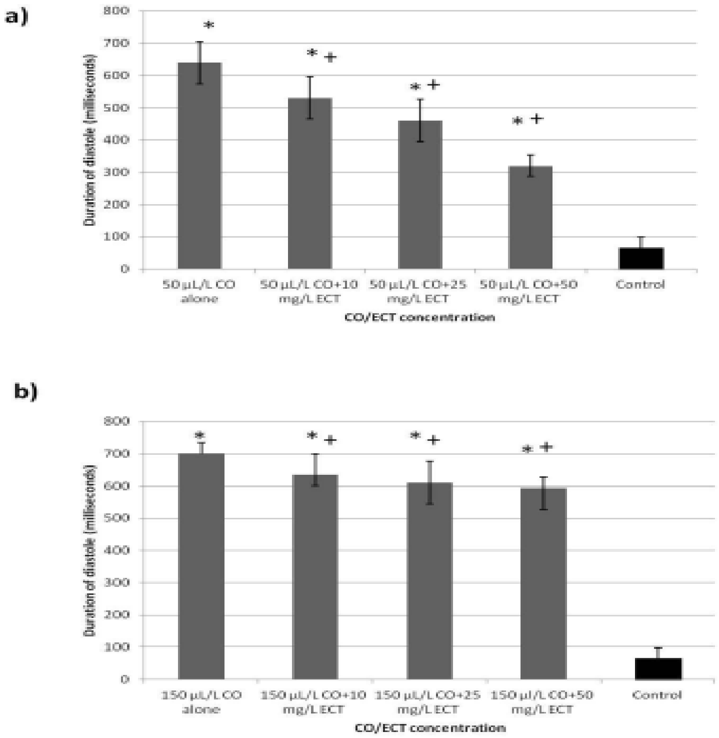

Figure 6. Duration of *Daphnia magna* heart relaxation during 30-minute combinatotial treatment with 50 µL/L (panel "a") and 150 µL/L clove oil (CO) (panel "b") and various concentrations of ectoine (ECT). The results are presented as means±SD, n=30. *-statistical significance between the experimental and control groups."+" - statistical significance between CO alone and the combination of CO+ECT (P<0.05).

diastole when compared to that in 150 µL/L alone. However the differences between the groups with ECT were not significant.

Discussion

Time to Anaesthesia

Most studies on the effects induced by CO or eugenol were performed on mammals, fish and some invertebrate species (Hamackowa *et al*., 2006; Venarsky and Wilhelm, 2006; Lovern *et al*., 2007; Parodi *et al*., 2012) but very little is known on the effects of this essential oil on *Daphnia magna*. Daphnids seem to be more sensitive to eugenol than other crustaceans since its effective concentrations to induce anaesthesia in post-larvae or sub-adult shrimp, *Litopannaeus* in the study by Parodi *et al*. (2012) were higher than those which were used in the present study. The increased susceptibility of *Daphnia magna* to eugenol may be explained by their lower body mass in comparison to other crustaceans. The results showed that daphnids exposed to the combination of CO+ECT were more resistant to anaesthesia than those treated with CO alone. However, the decreased sensitivity of daphnids to CO was not concentration-dependent since TtA of daphnids exposed to CO combined with the highest concentration of ECT (50 µL/L ECT) had slightly shorter TtA than those exposed to 25 mg/L ECT. It may be a result of harmful effects of higher concentrations of ECT on crustacean nervous system (Berthoud and Reilly, 1992) and they may be also augmented by the presence of neurotropic agents, such as CO.

Thoracic Limb Activity

Thoracic limb movement by which daphnids maintain their ventilation and filter out food particles from surrounding water is considered by some authors as a reliable endpoint of microcrustacean activity (Pirow *et al*., 1999; Cho *et al*., 2008). The present study showed that both concentrations of CO alone significantly decreased the limb acivity which may be a consequence of anaesthetic effects of CO depressing the nervous and muscular systems. On the other hand, exposure to the combinations of CO and ECT attenuated the inhibition of limb activity which may be explained by a reduction of eugenol-induced blockade of ion channels in the membrane of nerve cells (Bekker and Krijgsman, 1951; Chung *et al*., 2008).The protective effect of ECT was more pronounced in daphnids which were subjected to the combinations with the lower concentration of CO. Although a study by Bownik *et al*. (2015) showed that ECT alone at higher concentrations may slightly decrease the limb activity the present results indicate that the amino acid did not elicit additive inhibitory effects with CO but oppositely, it attenuated the CO-induced inhibition of thoracic limb activity. It suggests that effects of ECT on physiological parameters of *Daphnia magna* may be modulated by some certain factors.

Heart Activity

Daphnia magna, unlike most arthropods, has a myogenic heart responding to a variety of agonists and antagonists, in a similar way to that in vertebrates, therefore it has been used by some authors as a model organ for determination of effects induced by bioactive agents (Stein *et al*., 1966; Postmes *et al*., 1989; Villegas-Navarro *et al*., 2003; Campbell *et al*., 2004; Okushima *et al*., 2014). The present study showed a negative chronotropic effect in *Daphnia magna* induced by CO alone at both concentrations, however more increased bradycardia was noted at the higher concentration of CO. The effects are similar to those observed by other authors who found that CO and other anaesthetics induce hypotension and bradycardia in fish and mammals (Lahlou *et al*., 2004; Mitchell *et al*., 2009; Huang *et al*., 2012). It is known that inhibition of heart rate caused by CO is associated with biological properties of eugenol which affects cardiac muscles acting as a Na^+ and Ca^{2+} channel blocker, however its mechanisms of action on ionic channels both in vertebrates and invertebrates are not completely understood (Bekker and Krijgsman, 1951; Dyson *et al*., 1998; Chung *et al*., 2008). Nevertheless, since heart of *Daphnia magna* is myogenic, CO-induced alterations of its activity may be linked to the blockade of ion channels.

The results of the present study showed that the negative chronotropic effect induced by CO in daphnids was attenuated in daphnids exposed to the combinations of CO with ECT. Although the mechanisms of this effect are not known, it can be hypothesized that since ECT is an osmoprotectant, it may regulate the level of intracellular ions and may stabilize cell membranes and ion channels against eugenol-induced blockade. It may be speculated that ECT modulates heart rate and the effects seem to depend on whether the amino acid acts alone or in the combination with other agents. The previous study by Bownik *et al*. (2015) showed ECT alone at higher concentrations inhibits heart rate in daphnids. On the other hand, the present study showed that the same concentration of the amino acid used in the combination with CO attenuated heart rate decrease. Interestingly, the most distinct attenuation was observed during the exposure to CO+25 mg/L ECT with slightly lower depressive effect of ECT at 50 mg/L used in our study, which may be a result of its detrimental effects caused at the highest concentration of the amino acid.

Cardiac arrhythmias induced by anaesthetics are a complication observed in humans and various animal species (Kuner *et al*., 1967; Boudoulas *et al*., 1979; Kalra, S. and Hayaran, 2011). The present study revealed heart beat irregularity in daphnids exposed to both concentrations of CO alone. The percentage of daphnids with arrhythmia was concentration-dependent and it was more pronounced with increasing time of the exposure. It was also noted that ECT at all concentrations used in the study reduced the number of daphnids with CO-induced heart arrhythmia. The attenuating effects were obtained even in the combinations with the lowest concentration of ECT, however higher amounts of this amino acid did not enhance the protective effect. Interestingly, in some daphnids a transient arrhythmia was seen at 10 minute of immersion, but it was reduced during further exposure.

Studies in mammals showed that some agents, such as cardioactive chemicals may alter the duration of diastole (Katz and Lorell, 2000). The present study showed that CO alone increased duration of heart muscle relaxation in *Daphnia magna* in a concentration-dependent manner. It was presumably caused by eugenol-induced reduction of ions influx initiating the heart contraction (McCowley, 1955; Kuner *et al*., 1967). This effect was more significant at its higher concentration and longer duration of diastole correlated with more decreased heart rate. Studies on mammals showed that eugenol-induced relaxation of heart muscle results from the blockade of calcium inward currents (Satoh, 1994; Hamackova *et al*., 2006). On the other hand, ECT seems to attenuate CO-induced positive lusitropic effect since the period of heart relaxation was decreased in daphnids exposed the combination of CO+ECT and the difference was more pronounced in the groups treated with the combinations containing the lower concentration of CO. Daphnids with more attenuated heart rate treated with the combination of CO+ECT also showed a shorter time of diastole when compared to those exposed to CO alone, except for the animals treated with the mixture of CO and the highest concentration of ECT.

Optical analysis of heart area in two cadiac phases: systole and diastole in *Daphnia magna* exposed to different chemicals was previously performed by Villegas-Navarro *et al*. (2003). Since systole heart area was not altered in all experimental groups, the differences in distole/systole ratio were dependent on diastole area. Our studies showed that daphnids treated with CO alone or in the combination with ECT showed no significant changes of their heart area in systole, but had increased area of the organ during diastole in a concentration-dependent manner. Daphnids exposed to the combination of CO+ECT showed a decreased diastole/systole area ratio with more pronounced differences between the experimental groups exposed to the higher concentration of CO. Crustaceans treated to the combination of CO with higher concentrations of ECT showed very similar ratio to that in the control group which suggests that the amino acid decreases CO-induced heart relaxation in *Daphnia magna*. No significant differences of diastole/systole heart area ratio were found between the groups with ECT indicating that further increase of its concentration did not enhance the attenuating effect.

Currently, little data exist on the influence of ECT on heart functioning. Some cardioprotective effects in mammals were found to be induced by another amino acid and a compatible solute, taurine (Birdsall, 1998: Xu *et al*., 2008). High concentrations of this compound are found in the mammalian heart where it regulates the intracellular level of ions (Eby and Halcomb, 2006). Since ECT, like taurine is an osmoprotectant, the mechanisms of its cardioprotective action may be associated with regulation of intracellular ion influx to the cardiomyocytes.

Conclusion

The present study revealed that ECT reduced susceptibility to anaesthesia and attenuated depressive effects induced by CO on thoracic limb activity of *Daphnia magna* and attenuation of bradycardia and cardiac arrhythmia during anesthesia, however mechanisms of its protective action are still an open question and require additional studies. The results indicate that ECT may be considered as an agent regulating heart activity in animals subjected to CO-induced anaesthesia.

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