www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 15: 157-166 (2015) DOI: 10.4194/1303-2712-v15_1_17

PROOF

Clove essential oil from *Eugenia caryophyllus* Induces Anesthesia, Alters Swimming Performance, Heart functioning and Decreases Survival Rate During Recovery of *Daphnia magna*

Adam Bownik[1,*]

[1] The John Paul II Catholic University of Lublin, Faculty of Biotechnology and Environmental Sciences, Department of Animal Physiology and Toxicology, Kontstantynów 1 „I" 20-708 Lublin, Poland.

* Corresponding Author: Tel.: +48.814 545458; Fax: ;
E-mail: adambownik@wp.pl

Received 25 April 2014
Accepted 31 March 2015

Abstract

Clove oil is a common anesthetic used in aquaculture. However little is known on its effects on non-target crustaceans living in fish ponds and other natural reservoirs. The purpose of the investigation was to determine survival, time to anesthesia (TtA) and recovery (TtR), swimming activity, heart functioning during 5 and 45-min exposure of *Daphnia magna* to clove oil at concentrations of 150, 100, 50, 25, 10 and 1 µl/L. Swimming behavior and heart activity of *Daphnia* were evaluated by video image analysis with the use of Tracker® v.4.82 and Image Tool® software.

The results showed that the oil induced anesthesia at three highest concentrations: 150, 100 and 50 µl/L. TtA and TtR were dependent on the size of the animal and concentration of the oil. The shortest TtA and the longest TtR were noted for the smallest daphnids anaesthetized for 45 minutes at 150, 100 and 50 ul/L. Lethality of small and large daphnids previously exposed to anesthetic concentrations for both times was observed at 24th hour of recovery period suggesting latent toxicity of clove oil. The oil inhibited heart rate, heart muscle contraction and swimming velocity at anesthetic (150, 100, 50 µl/L) and at non-anesthetic concentrations of 25, 10 and 1µl/L. Recovery period after previous 45-min anesthesia showed temporal increase however and subsequent decrease of *Daphnia* swimming velocity and heart activity resulting probably from latent toxicity.

The results of the present study indicate that clove oil induces rapid immobilization and latent toxicity in *Daphnia magna*, at lower concentrations that those recommended for fish anesthesia and suggest that excessive use of the oil in natural ponds or other water reservoirs may be detrimental to coexisting cladocerans leading to disturbance of ecological balance.

Keywords: Daphnia, clove oil, anesthesia, swimming behavior, heart activity

Introduction

Clove essential oil is a disstillate of flowers, stems and leaves of *Syzygium aromaticum* found in Eeastern Hemisphere or *Eugenia aromaticum* and *Eugenia caryophyllata* in Western Hemisphere. Composition of clove oil is variable, however eugenol (4-allyl-2-methoxyphenol) and isoeugenol (4-propenyl-2-methoxyphenol) are its active ingredients, making up 70-98% of total weight (Issacs *et al*., 1983), other components are eugenol acetate (>17%), and kariofilen 5 (~12%) (Issacs *et al*., 1983; Soto and Burhanuddin, 1995). The oil possesses anesthetic, antioxidant, antifungal, antiinflammatory, cytotoxic and antimicrobial properties towards pathogenic fungi, bacteria, and viruses (Arina and Iqbal, 2002; Chaieb *et al*., 2007; Fu *et al*., 2007; Prashar *et al*., 2006).

Clove oil is approved by for US Food and Drug Administration (FDA) for safe for use in humans as mild topical anesthetic or analgesic to reduce toothache, headache joint pains and also as a food additive (Alqareer *et al*., 2006; FDA, 2007). Although the oil is not approved by FDA to use in fish (FDA, 2007), it has advantages over other anesthetics for its high efficacy, low cost and low toxicity to fish and some invertebrate species (Akbulut *et al*., 2010). Therefore, it has been used in aquaculture as an anesthetic for surgery and as an agent reducing stress during blood sampling, measurement of length and weight (Svobodova and Kolarova, 1999), transport (Munday and Wilson, 1997; Shima *et al*., 2006) and also in field conditions for fish collection (Prince and Powell, 2000). Studies performed in fish indicated that immersion time shorter than 5 min to concentrations of 40 and 120 mg/L does not affect their swimming performance of either life stage (Munday and Wilson, 1997).

Clove oil has also been used for anesthesia in invertebrate animals for stress reduction during transport, but similarly to fish there are species-

dependent differences in their sensitivity (Akbari *et al*., 2010; Norton *et al*., 1996). Promising results of using clove oil with short times to anesthesia and recovery were obtained in an amphipod *Gammarus minnus* (Venarsky *et al*., 2006) and in stress handling of freshwater prawns *Macrobrachium rosenbergii* (Coyle *et al*., 2004; Saydmohammed and Pal, 2009).

Eugenol, apart from its anesthetic properties, it is known to induce various toxic effects, therefore it has been used as an insecticide (US EPA, 2004). It also kills human colon cancer cell lines in the *in vitro* conditions (Jaganathan *et al*., 2011). Side-effects of the clove oil in fish such as ventilator failure and medullary collapse which is associated with toxic action of eugenol (Sladky *et al*., 2001). Some studies indicated that it may be lethal for fish if used at high concentrations or if regulations about time of anaesthesia specific for a single fish species are not followed. The potential harmful effects of clove oil on non-target aquatic animals such as invertebrates should also not be excluded and its uncontrolled field application may be detrimental to sensitive non-target organisms. For example, a reduction of coral growth and bleaching was observed after repeated field application of the oil (Boyer *et al*., 2009).

There is a lack of knowledge on susceptibility to anesthesia and possible toxic effects induced by clove oil in *Daphnia magna*, a custacean that is widely distributed in aquaculture reservoirs and plays important ecological roles in aquatic ecosystems. Assessment of behavior and physiological parameters in *Daphnia* after the exposure to clove oil could indicate if the oil used for fish anesthesia in field conditions could induce harmful effects in this non-target cladoceran. The aim of the present study was to determine time to anesthesia and recovery of daphnids exposed to different concentrations to the oil and evaluate its influence at anesthetic and non-anesthetic concentrations on the swimming performance and heart activity of *Daphnia magna*.

Materials and Methods

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 16h: 8h. *Daphnia* culture medium was prepared following the ASTM standards (American Society of Testing and Materials, 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 liter 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 liter. The animals were fed once daily with a few drops of powdered *Spirulina* (2mg/L water) per tank and supplemented with a few drops per tank of 10 mg/L stock suspension of baker's yeast. Feeding was stopped 24 hours before the experiments.

Clove bud (*Eugenia caryophyllus*) essential oil was purchased as commercial preparation with 100% purity from PharmaTech (Poland). The quality of the essential was equal to the standards of European Pharmakopeia. The stock solution of the oil was done by its dilution with ethanol with the ratio 1:9 (clove oil, ethanol) (Ögretmen and Gökcek, 2013). Appropriate amounts of the oil from the stock solution were diluted with 100 ml of water in 150 ml beakers to reveal the following concentrations: 150, 100, 50, 25, 10 and 1 µl/L. 15. 3 ml of the oil at appropriate concentration were transferred from the the vial to a well (35 mm diameter) of the flat, clear-bottom polystyrene 6-well microplate (Nunc, Denmark). A single daphnid was placed in the well with appropriate concentration of the oil and the timer was started. When the animals were immobilized the timer was stopped and time to anesthesia (TtA) was recorded. The animal was treated as immobilized when it did not show any movement within 15 sec and did not respond to external stimulation (touching of the animal with a pipette tip).

During 1, 5, 10, 20 and 30 min of anesthesia a single daphnid was transferred in a 50 µl drop of the tested solution of the oil to a microscope slide for determination of heart activity and length. A light microscope with a 10 µm ruler on microscopic stage and magnification of 75 x was used with a mounted digital camera Nikon D 5100 which allowed to record a minimum 1 min long video (with the speed of 30 frames per second) of the microscopic view of *Daphnia*. The microscopic magnification and camera resolution allowed to perform the analysis with a good visibility of the heart. Using this method of analysis irregular heart contractions (arrhythmia) could be easily recognized. The heart area and time of systole and diastole was measured with the use of Tracker® and Image Tool®. First, Tracker® was used for video file analysis of the heart at systolic or diastolic phase. Then, separate frames with visible heart at systole or diastole were saved as image files and analysed in Image Tool®. The heart areas at the two cardiac phases were calculated with the use of software after drawing an ellipse-like form along the heart shape in the digital image. The heart was considered to be at systole at its lowest volume covering the smallest area in the cycle and when the organ covered the largest area was treated to be in diastole. The heart area in systole and diastole was compared among daphnids exposed to different concentrations of the oil during anesthesia and recovery period and to that of the animals from the control group.

Length of the anesthetized *Daphnia* was determined by the analysis of its digital image with the use of Image Tool® by computing the difference between the line of known distance (10 µm ruler) and the line drawn from the head to the base of the apical spine.

After the measurements the daphnid returned to the well for continuation of anesthesia. After the

anesthesia, the animals were transferred for recovery to a well containing 10 ml of clean medium. Once the animal was placed in clean water the timer was started to count TtR and when *Daphnia* began to swim, the timer was stopped and the time was recorded. The animals that showed no movement was taken for microscopic analysis were treated as dead when their heart beat and thoracic limb movement was stopped.

The swimming velocity was analyzed according to the experimental setup described by Shimizu *et al*. (2002) with some modifications (Bownik *et al*., 2014). *Daphnia magna* neonates <24 h old of 2nd–5th generations are more sensitive to various stressors than adults, therefore further studies regarding analysis of swimming behavior were restricted to small bodied individuals. 10 daphnids were transferred from the culture tanks to the observation well containing 3 ml of the appropriate concentration of clove oil. Control wells contained clean water only. Swimming velocity of the control and exposed animals in all observation wells was recorded for minimum 1 minute every 5 minutes during immersion in the anesthetic and every 5 minutes from the beginning of recovery period at 60, 120 min and 6, 12 and 24 hour. The video clips of swimming daphnia at each replicate were recorded at 30 frames/sec with a digital camera Nikon D5100 mounted on a stand over the microplate illuminated underneath with an illuminator of cold light of 3500 lux intensity and processed with motion analysis software. Swimming velocity of the individuals was tracked by the computer program. Vertical movement of *Daphnia* was negligible because of very small depth of the solution present in the microplate observation well. The video file with the recorded trajectories of swimming *Daphnia* was analyzed frame-by-frame with Tracker®. By clicking with the cursor on *Daphnia* image in separate frames, the program plotted the whole trail left by a single *Daphnia* (treated by the program as a mass point) measuring its maximal, minimal and mean velocity (v) expressed in millimeters per second (mm/s). Since the animals moved virtually only in two dimensions swimming behavior analysis was based on the trajectory represented by x and y coordinates. The velocities of ten daphnids were plotted in the separate graphs which were then superimposed for comparison between the experimental groups. Since swimming speed was not equal for all individuals in each experimental group and the control, the mean velocity (v) of 10 daphnids from each experimental group was meaned and treated as one result. The same procedure of microscopic examination was repeated at 2, 5 minute for 5-min anesthesia and 10, 20, 30, 45 min for the 45-min anesthesia and also at 30, 120 min, 6 and 12 and 24 hours of recovery period.

Statistical Analysis

All results are presented as means±standard deviation (SD). All data were assessed for homogeneity of variance for ANOVA assumptions. For statistical analysis of velocity magnitude, heart rate, heart area and times of diastolic and systolic phases using least squares regression were examined first. Experimental data were analyzed using ANOVA followed by Tukey's test to detect differences among means. All analyses were completed using Develve® statistical software. Values were statistically significant when $P<0.05$.

Results

Time to Anesthesia (TtA)

Immobilization (anesthesia) of daphnids was observed after application of three concentrations of clove essential oil: 150, 100 and 50 µl/L. TtA correlated with body length (Figure 1a). Smaller daphnids had shorter times than the large-bodied ones. The largest *Daphnia* of ≥3 mm exposed to the oil at 150 µl/L had TtA of 3-5 min. TtA of medium-sized >2 to <3 mm and the smallest daphnids of ≤2 mm long were 1.5-4.2 min and 1.05-2.35 min, respectively. The longest TtA exhibited animals exposed to clove oil at 50 µl/L. Daphnids ≥3, >2 to <3 and ≤2 mm showed TtA of 50-57, 32-56 and 14.3-45.3 min, respectively. Anesthesia was not observed at three lower concentrations 25, 10 and 1 µl /L of the oil.

Time to Recovery (TtR)

TtR was dependent on *Daphnia* size and duration of anesthesia (Figure. 1b, Figure 1c). The longest TtR showed the smallest daphnids previously immobilized for 45 min. Daphnids of ≥3, >2 to<3 and ≤2 mm length previously anesthesized at 150 µl/L showed TtR of 8.53-9.5, 14.4-15.6 and 14.2- 15.6 min. 5-min anesthesia resulted in shorter TtR of 3.17-8.3, 8.1-9.31, 9.16-12.4 for *Daphnia* length of ≥3, >2 to<3 and ≤2 mm, respectively. Shorter TrR showed daphnids anesthesized at a concentration of 50 µl/L of the clove oil. Lethality after 24 hours of RP in the group of daphnids anesthesized for 5 and 45 min was similar (Figure. 2)

Swimming Velocity

Swimming velocity of *Daphnia* was markedly reduced after 2 minutes of exposure to clove oil to 0±0.07 mm/s at 150 µl/L, 0.5±0.05 mm/s at 100 µl/L and 2.3±0.4 mm/s at 50 µl/L in comparison to the control group (4.36 ±0.3 mm/s) (Figure. 3a). Lower concentrations of the oil had lower inhibitory potential (Figure. 4 a). The swimming velocity of the animals increased after their transfer to clean medium. Daphnids that were previously anesthetized for 45 minutes at 150 and 100 µl/L (represented as ToR150 and ToR100, respectively) showed the velocities

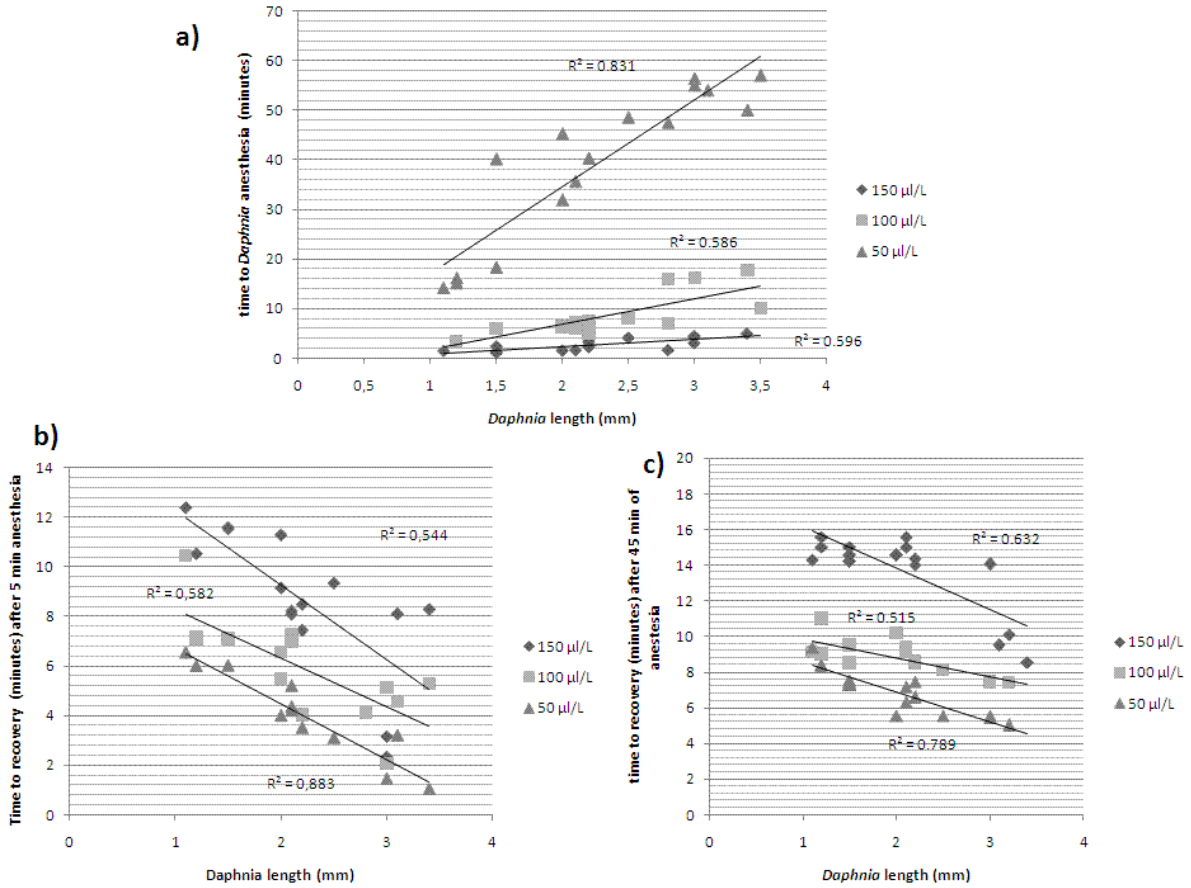

Figure 1. Correlation between body length of *Daphnia magna* versus time to anesthesia (TtA) a) and time to recovery (TtR) after 5 b) or 45 minutes c) at various concentrations of clove essential oil, n=15. The strongest correlation of TtA and body length (R^2=0.831) is seen at 50 µl/L (triangles). Moderate coefficients of correlation are for 100 (squares) (R^2= 0.586) and 150 µl/L (rhombs) (R^2= 0.596). Strong correlation is also seen between TtR and body length of *Daphnia* previously anesthetized for both 5 and 45 minutes at 50 µl/L (R^2= 0.883 and 0.789, respectively). Moderate correlation for TtR and body length is for both periods of anesthesia at 100 (R^2= 0.582, 0,515 for 5 and 45-min, respectively) and 150 µl/L (R^2= 0.544, 0,632 for 5 and 45-min, respectively).

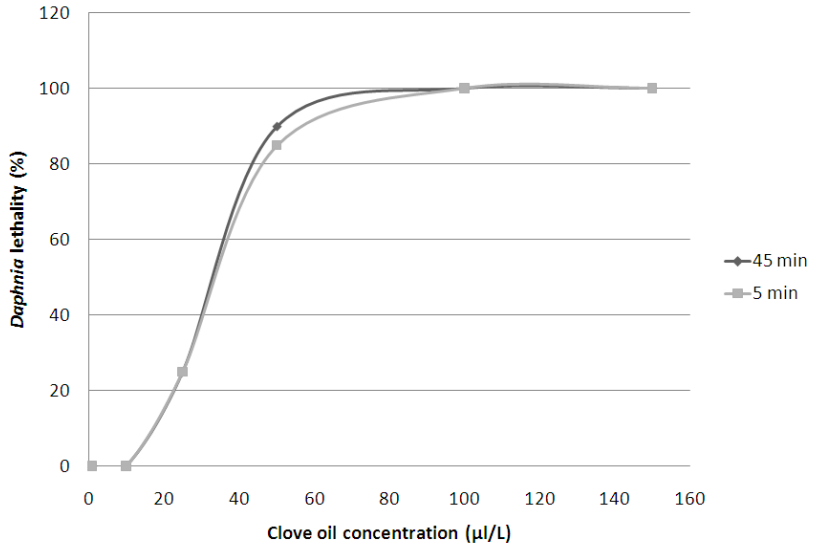

Figure 2. Percentage of letality observed at 24th hour of recovery period of *Daphnia* previously exposed to 150, 100 and 50 µl/L of clove oil. Anesthetic concentrations of clove oil induced latent toxicity observed at 24th hour of recovery period. Results are presented as means±SD, n=15.

1±0.2 and 1.1±0.3 mm/s at 15 min and 9 min of RP respectively. However after 12 h it was reduced to 0.2±0.02, 0.23±0.01 mm/s at concentrations of 150 and 100 µl/L, respectively.

The animals previously anesthetized for 5 min in clove oil at those concentrations (represented as ToR150 and ToR100, respectively) needed less time for recovery (Figure 3b). Daphnids previously immobilized at 50 µl/L showed their swimming velocity to be 0.9 ±0,03 mm/s after 5 min of RP. Velocity values of the individuals at RP after the exposure to three highest concentrations were elevated until 60 min (2.2±0.1, 2.3±0.3 and 3.2±0.1 mm/s, respectively) however they dropped at 12th h to 0.04±0.005, 0.1±0.8 mm/s at concentrations of 150 and 100 µl/L, respectively.

Heart Rate

Clove oil at concentrations of 150, 100, 50 and 25 µl/L induced a negative chronotropic effect in the heart of *Daphnia magna* after 2 min of the exposure. Less pronounced decrease of heart rate was seen at concentrations of 50 and 25 µl/L (Figure. 5). *Daphnia* transferred to clean water after 45-min anesthesia showed a rapid elevation of their heat rate at 15 min of RP in the groups of daphnids previously exposed to 150, 100, 50 and 25 µl/L (304±5, 330±8, 366±4 bpm) when compared to the control group (432±12 bpm), however at 12th hour of RP it dropped to 60±3, 65±2 and 175±5 bpm, respectively with distinct symptoms of heart arrhythmia at the two highest concentrations (Figure 5a). 5-min anesthesia in the oil did not shortened much the time needed for recovery of heart rate (Figure 5b). A significant inhibition of heart was observed in the group of *Daphnia* previously exposed to 150 and 100 µl/L after 120 min of RP reaching 64±4 and 70±7 bpm at 150 and 100 µl/L after 12 h.

Heart Area

A significant increase of heart area during systole after both 5 and 45-minute anesthesia to clove oil was observed at concentrations of 150, 100, 50 and 25 µl/L (Figure. 6a, Figure 6b). At 45th min of exposure to 150 µl/L the systole area increased to 23±1 µm2, however 5-min exposure to the same concentration induced lower increase (16±1 μm^2) as compared to the control (11±2μm^2). Slight increase of heart area in systole also showed *Daphnia* treated for 45 min with the oil at 50 and 25µl/L (15±0.2 and 14,5±0.2 μm^2, respectively). The heart area at systole

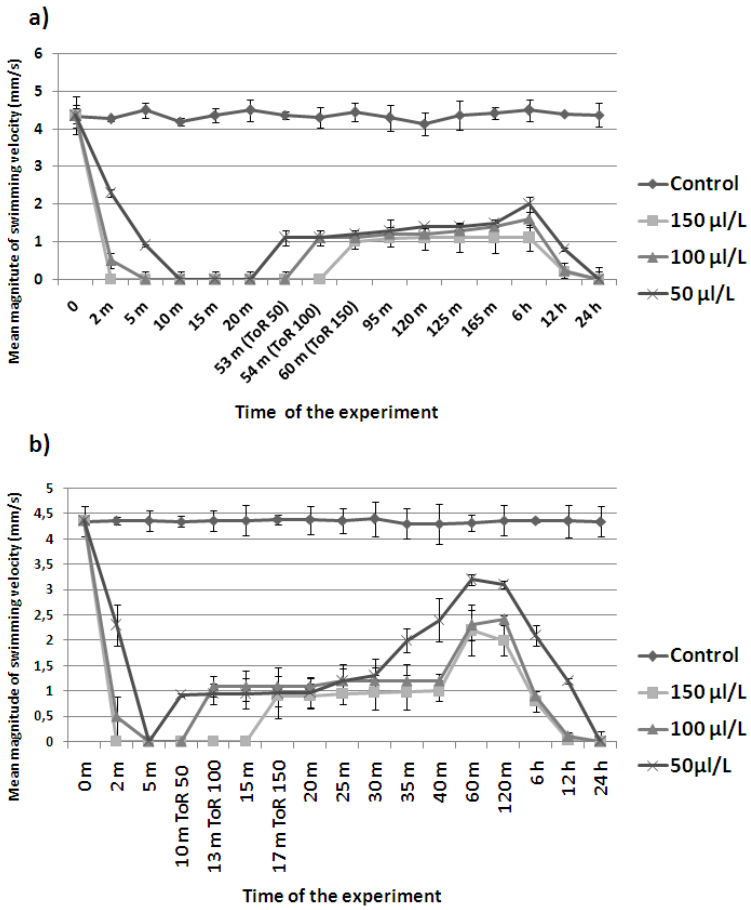

Figure 3. Swimming velocity of Daphnia magna anesthetized for 45 (a) and 5 minutes (b) in clove essential oil. ToR50, ToR100, ToR150 are times of recovery for daphnids previously exposed to concentrations of 50, 100 and 150 µl/L, respectively. Mean velocities of 10 daphnids from each experimental group was treated as one result. Results are presented as means±SD, n=10.

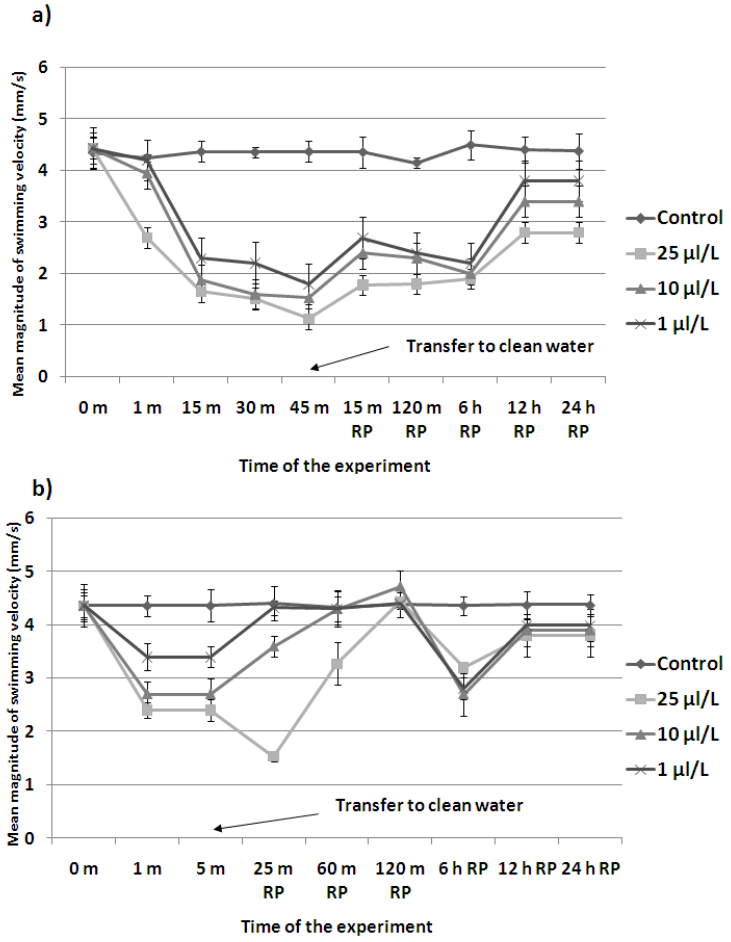

Figure 4. *Daphnia magna* swimming velocity after 45 (a) and 5-min (b) exposure to non-anesthetic concentrations of clove essential oil and subsequent transfer to clean water. RP (recovery period) was started after transfer of daphnids to clean water. Recovery period was initiated after transfer of daphnids to clean water after 45 (a) and 5 (b) min of anesthesia. Mean velocities of 10 daphnids from each experimental group was treated as one result. Results are presented as means±SD, n=10.

was reduced to the control values at all experimental groups after 15 min of RP in clean water in the groups previously exposed for both 5 and 45 min.

Discussion

The present study has demonstrated high susceptibility of *Daphnia magna* to clove oil. Short TtA of *Daphnia* could be explained by quick absorption of highly lipophilic eugenol targeting the fat tissue and nervous system. The results indicating decrease of TtA with increasing concentrations of clove oil are consistent with those obtained in vertebrates and other invertebrates (Velisek *et al*., 2005; Venarsky *et al*., 2006). The anesthetic effects of clove oil was observed in freshwater amphipod *Gammarus minnus* (Venarsky *et al*., 2006). These invertebrates showed similar times to anesthesia (1.7-7 min) to those of *Daphnia*. Eugenol was also found to induce anesthesia in a whiteleg shrimp *Litopenaeus vannamei* (Parodi *et al*., 2012). Sub-adults had similar TtA when they were treated with 50 µl/L of the oil to those for the smallest daphnids, however shrimps seem to need less time to recover than *Daphnia*. On the basis of current results and those obtained by Venarsky *et al*. (2006) it can be concluded that much lower concentrations of clove oil are required for anesthesia in comparison to those for other crustacean, amphipod, *Gammarus pulex* with commonly used anesthetic, MS-222 (300-2000 mg/L and TtA of 15-26 minutes) (Ahmad, 1969).

The present study revealed that there is a correlation between TtA or TtR with *Daphnia* size. Smaller daphnids (neonates) were more sensitive to clove oil with shorter TtA and longer TtR than large-bodied (adult) daphnids. It seems that smaller daphnids are more susceptible to anesthesia than the larger ones because of their faster absorption of the anesthetic in relation to lower body mass. The recovery rate of daphnids was also size-dependent. Smaller daphnids needed more time to recover than the larger ones. This is in opposite to the results obtained in *Gammarus minus* by Venarsky *et al*. (2006), who observed higher recovery rates of smaller amphipods. According to the literature, time to anesthesia and recovery depend on many factors, such as: animal species, body weight or length, as it was shown in the studies in fish and mammals (Houston

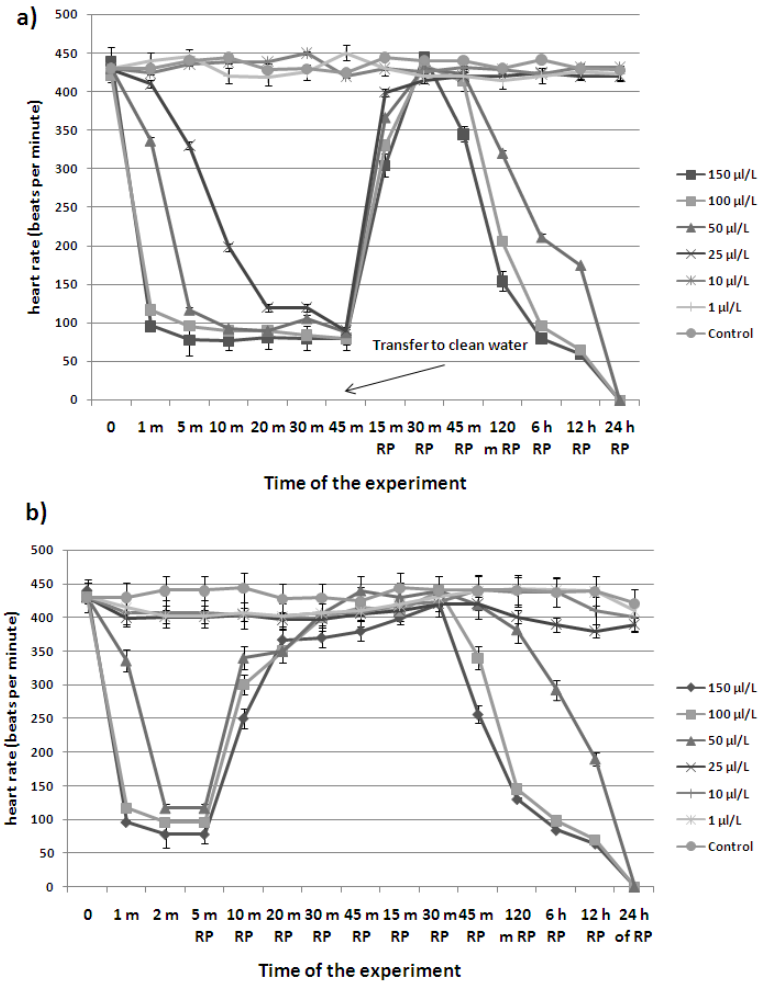

Figure 5. Heart rate of *Daphnia magna* exposed for 45 (a) and 5 minutes (b) to different concentrations of clove essential oil and subsequent transfer to clean water for recovery. Results are presented as means±SD, n=10.

and Corlett, 1976, Coyle *et al*., 2004). The relation between body size and time to anesthesia/recovery seems to be different in various species. For example, large white fish, *Coregonus lavaretus* showed shorter times to anesthesia than smaller fish, however, *Oncorhynchus mykiss* manifested the opposite type of sensitivity (Hoskonen and Pirhonen, 2004).

High lethality of daphnids previously exposed to anesthetic concentrations of the oil was observed after 24 h of the recovery period. Interestingly, the lethal effect was not dependent on time of anesthesia. There is no data in the literature on possible latent toxicity of clove oil during recovery. A lack of oil toxicity to amphipods treated with similar concentrations of the oil (Venarsky *et al*., 2006) may be explained by their larger size (4-14 mm) compared to *Daphnia* of 1-3.5 mm.

Observation of behavioral changes proved to be a useful tool in evaluation of toxic effects of various compounds in different vertebrate and invertebrate animals. Determination of swimming parameters such as velocity may give sufficient data on the neurotoxic effects of various substances (Dodson and Frey, 2001; Tierney, 2011). The inhibition of swimming velocity during RP seems to be a result of latent neurotoxic changes. The inhibition of swimming performance may be explained by depressive action of eugenol on neuromuscular synapses as it was shown in studies in crayfish which showed that eugenol inhibits the neuromuscular transmission (Ozeki, 1975). Clove oil-induced decrease of swimming velocity was also observed in fish (Anderson *et al*., 1997) and this phenomenon was associated with depression of nervous system.

Changes in swimming behavior may result from neurotoxic action of the oil inhibiting neuromotor activity of *Daphnia*, however further investigation is needed to explain mechanisms of its toxicity in *Daphnia*. When the oil is used in natural water reservoirs for fish anesthesia, the alterations in swimming behavior of daphnids may have serious ecotoxicological implications since the exposed cladocerans with motility deficits may be unable to mate or be more visible and thus more susceptible to be eaten by predators.

Daphnia are known to have myogenic heart responding to a variety of agonists and antagonists, similarly to vertebrate animals and optical

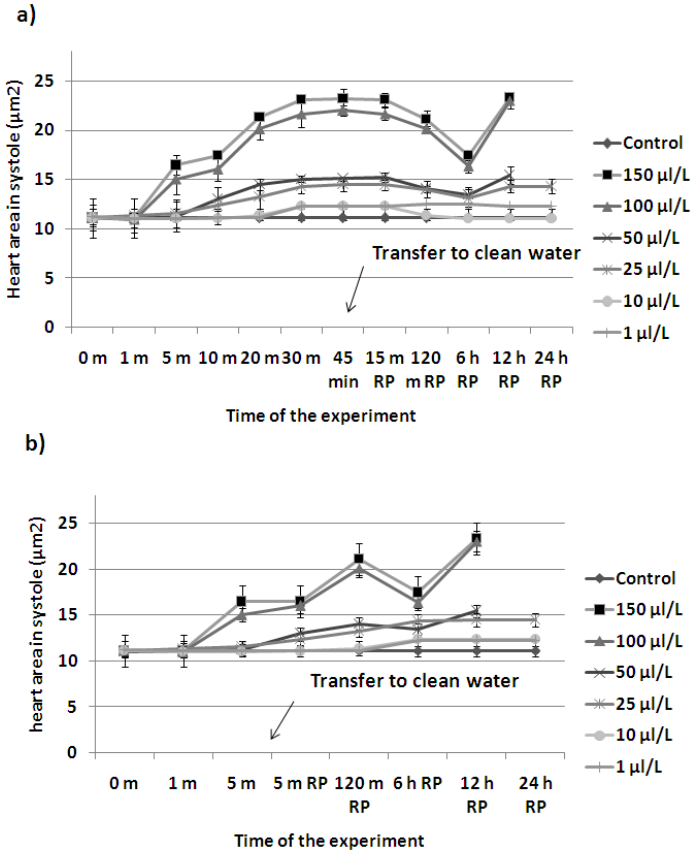

Figure 6. Heart area in systole of *Daphnia magna* anesthetized for 45 (a) and 5 (b) minutes at different concentrations of clove essential oil and in recovery period. Results are presented as means±SD, n=10.

examination of this organ after treatment with bioactive agents has been used by some authors (Bekker, Krijgsman, 1951; Villegas-Navarro *et al*., 2003; Campbell *et al*., 2004; Bownik *et al*., 2015).This organ is clearly visible through transparent exoskeleton in a light microscope and it is possible to measure its activity. Video analysis of heart functioning in slow motion proved to be a reliable method for a precise determination of heart rate and measure times of cardiac phases without the necessity of slowing down the heart rate by lowering the ambient temperature (Campbell *et al*., 2004). Alteration of heart rate in *Daphnia* was previously documented as a response to environmental conditions or bioactive substances (Navarro *et al*., 2003; Campbell *et al*., 2004; Bownik *et al*., 2014; Bownik and Stępniewska, 2015) however, there is a lack of data on such effects induced by clove oil. Results from the present study indicate similarity of heart response to clove oil of *Daphnia* to that in aquatic vertebrates (Cooke *et al*., 2004). Some authors showed that active ingredient of the oil eugenol, a calcium and potassium channel blocker affects the cardiac contractile proteins and in a consequence causes heart rate inhibition and heart relaxation (Sensch *et al*., 2000; Lahlou *et al*., 2004; Damiani *et al*., 2004).

Measurement of cardiac area changes was previously used as an optical method to study the effects of various drugs on heart functioning in *Daphnia* (Villegas-Navarro *et al*., 2003). The present study showed that clove oil at the highest concentration increased the heart area during systole when compared to the control values suggesting lower magnitude of heart muscle contraction as a result of heart relaxation. As eugenol was reported to induce smooth muscle relaxation in mammals by a blockade of calcium and potassium channels, this mechanism may also occur in daphnids. Longer duration of diastole and increased heart area in systole may be the effect of alterations in functioning of contractile proteins resulting from modification of ion channel activity. (Sensch *et al*., 2000; Villegas-Navarro *et al*., 2003).

The main advantage of using clove oil in comparison to other anesthetics is its relatively low price and a wide margin of safety between lethal and effective concentrations and a lack of distress in anesthetized fish. The recommended concentration of clove oil for safe and effective anesthesia of adult rainbow trout is 30 mg/L (Prince and Powell 2000; Svoboda and Kolarova, 1999) which is much higher than those which induced toxicity in *Daphnia*. Use of such or even lower amounts of the oil in natural reservoirs for fish anesthesia may induce massive mortality of coexisting cladocerans leading to reduction of food to smaller fish and to alterations in trophic relations in the aquatic ecosystem.

References

Ahmad, MF. 1969. Anaesthetic effects of tricane methane sulphonate (MS 222 Sandoz) on *Gammarus pulex* (L.) (Amphipoda). Crustaceana, 16:197–201.

Akbari S, Khoshnod, M.J., Rajaian, H. and Afsharnasab, M. 2010. The use of eugenol as an anesthetic in transportation of with indian shrimp (*Fenneropenaeus indicus*) post larvae. Turk. J. Fish. Aquat. Sci., 10:423-429.

Akbulut, B., Cakmak, E., Aksungur, N., and Cavdar, Y. 2010. Effect of exposure duration on time to recovery from anaesthesia of clove oil in juvenile for Russian sturgeon. Turk. J. Fish. Aquat. Sci., 11:463–467.

Alqareer, A., Alyahya, A. and Andersson, L. 2006. The effect of clove and benzocaine versus placebo as topical anesthetics. J. Dent., 34:747-750.

American Society of Testing and Materials. 1986. Standard practice for conducting static acute toxicity tests on wastewaters with Daphnia: Annual book of ASTM standards. Vol. 11.04. Philadelphia, 4229-4284.

Anderson, W.G., McKinley, S.R. and Covalecchia, M. 1997. The use of clove oil as an anesthetic for rainbow trout and its effects on swimming performance. N. Am. J. Fish. Manage, 17: 301-307.

Arina, B. and Iqbal, A. 2002. *In vitro* fungitoxicity of the essential oil of *Syzygium aromaticum*. World J. Microbiol. Biotech., 18:317-319.

Bekker JM, Krijgsman BJ. 1951. Physiological investigations into the heart function of *Daphnia*. *J. Physiol*. 115: 249-257.

Boyer, S.E., White, J.S., Stier, A.C. and Osenberg, C.W. 2009. Effects of the fish anesthetic, clove oil (eugenol), on coral health and growth. J. Exp. Mar. Biol. Ecol., 369:53–57.

Bownik, A., Stępniewska, Z. and Skowroński, T. 2014. Protective effects of ectoine on heat-stressed *Daphnia magna*. J. Comp. Physiol B., 184: 961-976.

Bownik, A. and Stępniewska, Z. 2015. Protective effects of ectoine on behavioral, physiological and biochemical parameters of *Daphnia magna* subjected to hydrogen peroxide. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 170: 38-49 doi:10.1016/j.cbpc.2015.02.002

Bownik, A., Campbell, A.K., Wann, K.T.and Matthews, S.B. 2004. Lactose causes heart arrhythmia in the water flea *Daphnia pulex*. Comp. Biochem. Physiol. Part B, 139: 225-34.

Chaieb K, Zmantar T, Ksouri R, Hajlaoui H, Mahdouani K, Abdelly C, Bakhrouf A. 2007. Antioxidant properties of essential oil of *Eugenia caryophyllata* and its antifungal activity against a large number of clinical *Candida* species. Mycosis, 50: 403-406.

Cooke, S.J., Suski, C.D., Ostrand, K.G., Tufts, B.L. and Wahl, D.H. 2004. Behavioral and physiological assessment of low concentrations of clove oil anaesthetic for handling and transporting largemouth bass (*Micropterus salmoides*). Aquaculture, 239: 509-529.

Coyle, S.D., Durborow, R.M. and Tidwell, J.H. 2004. Anesthetics in aquaculture. SRAC Publication, No. 3900.

Coyle, S.D., Dasgupta, S., Tidwell, J.H., Beavers, T., Bright, L.A. and Yasharan, D.K. 2005. Comparative efficacy of anesthetics for the freshwater prawn *Macrobrachium rosenbergii*. J. World Aquacult. Soc., 36: 282–290.

Damiani, C.E., Moreira, C.M., Zhang, H.T., Creazzo, T.L. and Vassallo, D.V. 2004. Effects of eugenol, an essential oil, on the mechanical and electrical activities of cardiac muscle. J. Cardiovasc. Pharmacol., 44:688-695.

Dodson, S.I. and Frey, DG. 2001. Cladocera and other branchiopoda. In: Thorp JH, Covich AP, (Eds.), Ecology and classification of North American Freshwater Invertebrates. Elsevier, 778 pp.

FDA, 2007. Guidance for industry concerns related to the use of clove oil as an anesthetic for fish. Food and Drug Administration Center for Veterinary Medicine, Rockville, MD. Available on the internet at www.fda.gov/downloads/animalveterinary/guidancecomplianceenforcement/guidanceforindustry/ucm052520.pdf

Fu, Y., Zu, Y., Chen, L., Shi, X., Wang, Z, Sun, S. and Efferth, T. 2007. Antimicrobial activity of clove and rosemary essential oils alone and in combination. Phytother. Res., 21: 989-994.

Hoskonen, P. and Pirhonen, J. 2004. Temperature effects on anaesthesia with clove oil in six temperate-zone fishes. J. Fish Biol., 64: 1136–1142.

Houston, A.H. and Corlett, J.T. 1976. Specimen weight and MS-222. J. Fish Res. Board Can., 33: 1403–1407.

Isaacs, G. 1983. Permanent local anaesthesia and anhydrosis after clove oil spillage. Lancet, 1:882–883.

Jaganathan, S.K., Mazumdar, A., Mondhe, D. and Mandal, M. 2011. Apoptotic effect of eugenol in human colon cancer cell lines. Cell Biol. Int., 35:607–615.

Lahlou, S., Interaminense, L.F., Magalhães, P.J., Leal-Cardoso, J.H. and Duarte, G.P. 2004. Cardiovascular effects of eugenol, a phenolic compound present in many plant essential oils, in normotensive rats. J Cardiovasc Pharmacol, 43: 250– 257.

Munday, P.L. and Wilson, S.K., 1997. Comparative efficacy of clove oil and other chemicals in anesthetization of *Pomacentrus amoboinensis*, a coral reef fish. J. Fish Biol., 51: 931–938.

Norton, J.H., Dashorst, M., Lansky, T.M. and Mayer, R.J. 1996. An evaluation of some relaxants for use with pearl oysters. Aquaculture, 144:39–52. www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_florals.htm

Ögretmen, F. and Gökcek, K. 2013. Comparative Efficacy of three anesthetic agents on juvenile African Catfish, *Clarias gariepinus* (Burchell, 1822). Turk J Fish Aquat Sci., 13: 51-56.

Ozeki, M. 1975. The effects of eugenol on the nerve and muscle in crayfish. Comp. Biochem. Physiol. Part C, 50: 183-191.

Parodi TV, Cunha MA, Heldwein CG, de Souza DM, Martins ÁC, Garcia LO., Wasielesky WJr, Monserrat JM, Schmidt D, Caron BO, Heinzmann B, Baldisserotto B. 2012. The anesthetic efficacy of eugenol and the essential oils of *Lippia alba* and *Aloysia triphylla* in post-larvae and sub-adults of *Litopenaeus vannamei* (Crustacea, Penaeidae). *Comp. Biochem. Physiol*. Part C, 155:462–468.

Prashar, A., Locke, I.C. and Evans, C.S. 2006. Cytotoxicity of clove (*Syzygium aromaticum*) oil and its major components to human skin cells. Cell Prolif., 39: 241-248.

Prince, A. and Powell, C. 2000. Clove oil as an anaesthetic for invasive field procedures on adult rainbow trout.

N. Am. J. of Fish. Manag., 20: 1029–1032.

Saydmohammed, M. and Pal, A.K. 2009. Anesthetic effect of eugenol and menthol on handling stress in *Macrobrachium rosenbergii*. Aquaculture, 298: 162-167.

Sensch, O., Vierling, W., Brandt, W. and Reiter, M. 2000. Effects of inhibition of calcium and potassium currents in guinea-pig cardiac contraction: comparison of b-caryophyllene oxide, eugenol, and nifedipine. Brit. J. Pharm., 131: 1089–1096.

Shima, J.S., Osenberg, C.W., Mary, C.M. and Rogers, L. 2006. Implications of changing coral communities: do larval traits or habitat features drive variation in density-dependent mortality and recruitment of juvenile reef fish? Proc. of the 10th Int. Coral Reef Symp., 226–231 pp.

Shimizu, N., Ogino, C., Kawanishi, T. and Hayashi, Y. 2002. Fractal analysis of *Daphnia* motion for acute toxicity bioassay. Inc. Environ. Toxicol., 17: 441-448.

Sladky, K.K., Swanson, C.R., Stoskopf, M.K., Loomis, M.R. and Lewbart, G.A. 2001. Comparative efficacy of tricaine methanesulfonate and clove oil for use as anesthetics in red pacu (*Piaractus brachypomis*). Amer. J. Vet. Res., 62: 337–342

Soto, C.G. 1995. Clove oil as a fish anaesthetic for measuring length and weight of rabbitfish (*Siganus lineatus*). Aquaculture, 136: 149–152.

Svoboda, M. and Kolarova, J. 1999. A survey of anaesthetics used in the fish farming. Health Protection of Fish – Proceeding of Papers. Research Institute of Fish Culture and Hydrobiology VodAany, 49-72. (In Czech):

Tierney, K.B. 2011. Behavioral assessments of neurotoxic effects and neurodegeneration in zebrafish. Biochim. Biophys. Acta (BBA) – Mol. Basis Dis., 1812: 381-389.

US EPA. 2004. Floral Attractants, Repellents, and Insecticides. US Environmental Protection Agency.

Velisek, J., Svobodova, Z. and Piackova, V. 2005. Effects of clove oil anaesthesia on rainbow trout (*Oncorhynchus mykiss*). Acta Vet. Brno, 74: 139-146.

Venarsky, M.P. and Wilhelm, F.M. 2006. Use of clove oil to anaesthetize freshwater amphipods. Hydrobiologia, 568:425-432.

Villegas-Navarro, A., Rosas, L.E. and Reyes, J.L. 2003. The heart of *Daphnia magna*: effects of four cardioactive drugs. Comp. Biochem. Physiol. Part C, 136: 127–134.