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Stress reaction in crayfish: chlorides help to withstand stress in high nitrite concentration conditions – preliminary study

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ABSTRACT

A non-invasive method of recording cardiac activity (heart rate – HR) and stress reaction (stress index – SI) was used to understand the immediate and ongoing stress reaction of crayfish to the chemical stimuli. This method detects changes in the shape and amplitude parameters of the response to the stress factors, which characterized the crayfish functional state. Experimental animals (*Astacus leptodactylus*) were divided to the two groups with (400 mg·L^{-1} Cl$^-$) and without added chlorides and then exposed to a stepwise increased level of nitrite to the final (sublethal-lethal) concentration of 60 mg·L^{-1} N-NO$_2^-$ within 24 hours. The course of crayfish reaction was evident and provided information about their reaction to the sublethal-lethal concentration over time. As expected, a less prominent stress reaction was detected in the group with chlorides. The non-invasive method successfully evaluated the sensing of chemical stimuli in water through HR and SI changes.

RÉSUMÉ

Réaction au stress chez l'écrevisse : les chlorures aident à résister au stress dans des conditions de fortes concentrations en nitrite – étude préliminaire

Une méthode non-invasive d'enregistrement de l'activité cardiaque (rythme cardiaque – HR) et de réaction au stress (indice de stress – SI) a été utilisée pour comprendre la réaction au stress immédiate et ultérieure de l'écrevisse à des stimuli chimiques. Cette méthode détecte les changements dans la forme et l'amplitude des paramètres de la réponse aux facteurs de stress, qui caractérisent l'état fonctionnel de l'écrevisse. Les animaux de l'expérience (*Astacus leptodactylus*) ont été divisés en deux groupes avec (400 mg Cl$^-$·L^{-1}) et sans chlorures ajoutés puis exposés à des niveaux croissants par paliers de nitrite jusqu'à la concentration finale (sub-létale–létale) de 60 mg·L^{-1} N-NO$_2^-$ pendant 24 heures. La réaction de l'écrevisse est évidente et fournit des informations sur les réactions au cours du temps à une concentration sub-létale. Comme escompté, une réaction au stress moins marquée est détectée dans le groupe avec des chlorures. La méthode non-invasive a déterminé correctement l'impact du stimulus chimique de l'eau par les changements de HR et SI.

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INTRODUCTION

Toxicity tests usually describe a direct impact of several chemicals on model organisms. The results then express the concentration of a given chemical which caused mortality of tested organisms in a limited time period (Buikema *et al.*, 1982). Nevertheless, much lower (sublethal) concentrations of a chemical could influence the physiological and functional state or behaviour of treated organisms (Kleerekoper, 1976; Schober and Lampert, 1977; Vernberg *et al.*, 1977). Sublethal poisoning may be detectable after a longer time period or even be unnoticeable. Moreover, it may be difficult to detect stress reactions in such cases. Several invasive and non-invasive techniques could be used to evaluate the physiological state of fish (*e.g.* Kane *et al*., 2004, 2005) and crayfish (*e.g.* Bierbower and Cooper, 2009). Both of techniques give accordingly good results. Invasive monitoring is based on method of electrocardiography and uses crayfish as measuring device including animal directly to the measuring circuit (Bierbower and Cooper, 2009). But transduction of physiological data involved invasive procedures such as electrode implantation, which increase physiological disturbance such as unneeded rising of heart rate (Aagaard *et al.*, 1991). One of the non-invasive methods (used in our study) based on the evaluation of the physiological state by measurements of heart rate and cardiac activity patterns was developed for such purposes by Kholodkevich *et al*. (2007). Heart rate and cardiac activity patterns are the key indicators of the condition of tested organisms and can be expressed as stress dependent factors. These measurements are included in all general clinical assessments in medical science (Swash and Mason, 1984) and are therefore used also for the stress index calculation (Kholodkevich *et al*., 2008). At present, data regarding fish or crayfish stress reactions to sublethal-lethal levels of poisoning are often lacking. The non-invasive method of detection of heart rate and stress reaction could open a new window of knowledge in toxicology and biomonitoring. It is supposed that crayfish are capable to react quickly to any chemical changes by a stress reaction rather than toxicity thresholds resulting in death of specimens. Numerous authors have found heart rate to be a useful indicator of changes in physiological state, even in crustacean, molluscs and fish (*e.g.* Depledge and Andersen, 1990; Kholodkevich *et al*., 2008).

Nitrites (NO_2^-) are usually found together with nitrates and ammonia in water. Ammonia (NH_3) is considered one of the most important contaminants in aquatic environment because of its toxic nature and ubiquity in surface water system. It is discharged into the environment in large quantities from industrial, municipal and agricultural sources (Bloxham *et al.*, 1999). In aquatic ecosystems, nitrite concentrations are elevated by pollution with nitrogenous wastes and imbalances in bacterial nitrification and denitrification processes (Eddy and Williams, 1987). The average concentration of $N-NO_2^-$ in ground water ranges from 0.004 to 0.179 mg·L^{-1}. Higher concentrations (more than 1 mg·L^{-1}) occur in wastewater (Pitter, 1999) and in intensive aquaculture recirculating systems usually in newly activated biofilters (Masser *et al*., 1999). In fish, nitrites are absorbed by the gills and oxidize the iron in the haemoglobin molecules to methemoglobin. The result of nitrite poisoning is methaemoglobinemia caused by a reduction in the blood oxygen carrying ability (Svobodová *et al*., 2005). It is likely that a similar type of reaction to that occurring with the iron of haemoglobin also occurs with the copper in crustacean hemocyanin (Colt and Armstrong, 1981). Nitrites have an affinity for the active chloride uptake mechanism by chloride cells in the gills. Chloride cells excrete ammonia or H^+ ions for Na^+ and bicarbonate (HCO_3^-) for Cl^- (Love, 1980). Nitrites thus have an affinity to Cl^-/HCO_3^- exchange. Part of chloride demand is replaced by NO_2^- when available in water. The higher concentration of chlorides therefore protects fish against the toxic impact of nitrite *via* the competition between chloride and nitrite ions transporting across the gill membrane (Jensen, 2003). The positive effect of chloride on tolerance to nitrites has been demonstrated in other studies in fish (Hilmy *et al*., 1987; Atwood *et al*., 2001; Huertas *et al*., 2002; Fuller *et al*., 2003; Taveres and Boyd, 2003) and crayfish (Beitinger and Huey, 1981; Jeberg and Jensen, 1994; Kozák *et al*., 2005).

This study was concerned with 1) a preliminary testing of the reactivity of crayfish to the common chemical, nitrite, to gain information about sensitiveness to an increasing level of chemical stimuli up to the sublethal-lethal concentration and 2) to evaluate if chlorides could

help crayfish to withstand stress in high nitrite concentration conditions. The sensitivity of a non-invasive method for heart rate and stress index monitoring was also evaluated. This study can offer some suggestions and recommendations for future research of heart rate and stress monitoring in crayfish or fish.

MATERIAL AND METHODS

The experiment was conducted in the Laboratory of Experimental Ecology of Aquatic Systems, Saint-Petersburg Scientific Research Center for Ecological Safety, Russia.

> EQUIPMENT

A fibre-optic method originally developed for recording cardiac activity of Crustacea (Decapoda) and Mollusca was used (Fedotov *et al.*, 2000; Kholodkevich *et al.*, 2008). The optical signal modulated by the heart of the tested animal contains information on cardiac activity which is processed by a personal computer (for more details see Kholodkevich *et al.*, 2008) (Figure 1). The results create a photoplethysmogram, which can be further analyzed by various mathematical and statistical methods. The variation pulsometry method was used to study the distribution of cardiac intervals, and analyze relationships between its shape and the functioning of the cardiac system (Kholodkevich *et al.*, 2008) (Figure 2). For this method the following characteristics were chosen as biomarkers: heart rate (HR), and stress-index (SI), which is defined by the formula:

$$SI = 1/(2 {}^*CI_m {}^*SD^2),$$

where CI_m = mean cardiac interval, which is related to HR, with HR = $60/CI_m$; SD = standard deviation of heart rate (Kholodkevich *et al.*, 2007, 2008).

> ANIMALS AND EXPERIMENTAL CONDITIONS

Narrow-clawed crayfish originating from Sevan Lake (Armenia) were used in the experiment. The animals were acclimatized to the controlled conditions for three weeks before the experiment. They were kept in a trough ($2.5 \times 0.5 \times 0.3$ m) with shelters in a recirculating system. During the experiment, each crayfish was maintained in a separate 5-liter aquarium with a shelter, which allowed the crayfish to stay inside (Figure 3). The water level was set at 15 cm. Crayfish were not fed during the experiment. The non-treated water quality parameters were as follows: temperature 22 °C, oxygen saturation 80–100%, pH 7.5, HCO_3^- 32.5 mg·L^{-1}, SO_4^{2-} 19.3 mg·L^{-1}, Cl^- 7.8 mg·L^{-1}, Ca^{2+} 11.3 mg·L^{-1}, Mg^{2+} 2.9 mg·L^{-1}, Na^{2+} 3.3 mg·L^{-1}, K^+ 1.5 mg·L^{-1}. The experiment was performed at the September photoperiod (D:N = 14:10).

> EXPERIMENTAL DESIGN

The study was conducted in two groups, with added chlorides (Cl^-; 400 mg·L^{-1}) and without chlorides. The chloride level of 400 mg·L^{-1} was chosen as relatively safe for crayfish (Kozák *et al.*, 2009) and helpful against a negative impact of nitrites (Kozák *et al.*, 2005). Each group consisted of three adult males, with total length (TL) = 131.3 ± 3.5 mm, carapace length (CL) = 70.7 ± 2.2 mm and weight (w) = 63.7 ± 4.1 g in the chlorides group, and TL = 130.7 ± 3.9 mm; CL = 71.0 ± 2.6 mm and w = 70.3 ± 5.5 g in the group without chlorides, respectively. One monitoring system at full capacity monitored all six treated specimens during the 24 hours period of treatment.

Animals were stocked in aquaria for acclimation in the evening before the day of the experiment. The concentration of 60 mg N-NO_2^-·L^{-1} was chosen as a possible sublethal-lethal
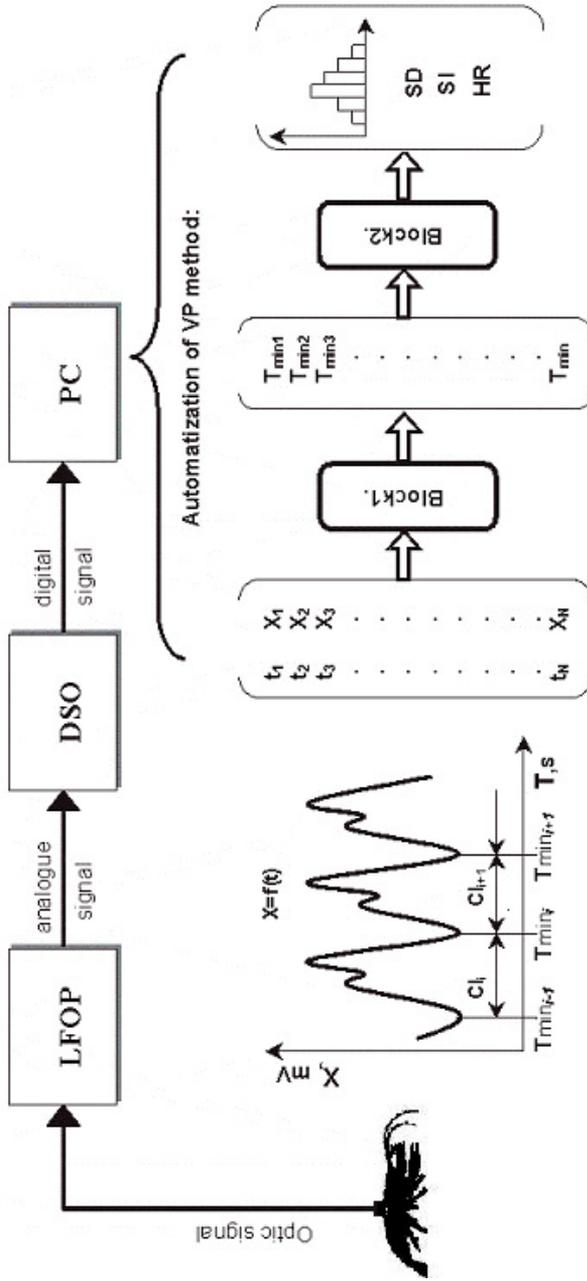
Figure 1
Block-scheme of cardiac activity registration, signal transformation and automatic data processing in real time. LFOP: laser fibre-optic photoplethysmograph, DSO: digital storing oscilloscope, PC: personal computer, VP: variation pulsometry, SD: standard deviation, HR: heart rate, SI: stress-index (Kholodkevich et al., 2008).

Figure 1
Bloc-diagramme de l'enregistrement de l'activité cardiaque, transformation du signal et traitement en temps réel des données. LFOP : photopléthysmographe à fibre optique laser, DSO : oscilloscope à mémoire, PC : ordinateur personnel, VP : variation de pulsations, SD : déviation standard, HR : rythme cardiaque, SI : indice de stress (Kholodkevich et al., 2008).
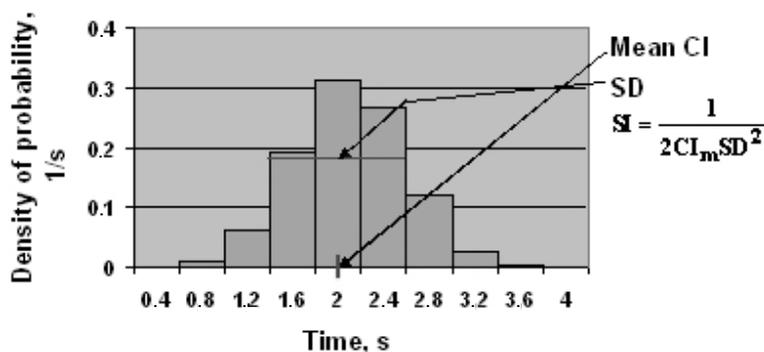
Cardiac intervals distribution

Figure 2

Variation pulsometry method to study relations between cardiac system functioning and the cardiac intervals distribution law (Kholodkevich et al.*, 2008).*

Figure 2
Méthode de variation de pulsations pour étudier les relations entre le fonctionnement cardiaque et la loi de distribution des phases d'activité cardiaque (Kholodkevich *et al.*, 2008).
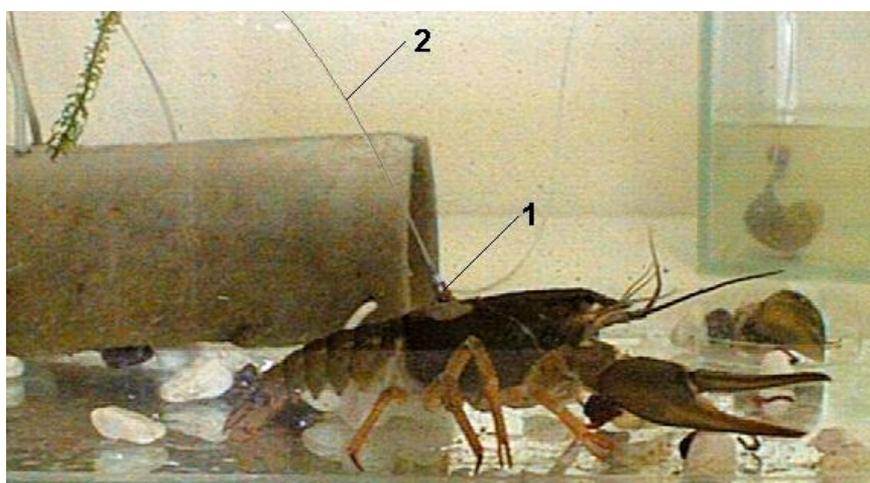

Figure 3

*Narrow-clawed crayfish (*Astacus leptodactylus*) in 5-L aquarium with a shelter, which allowed the crayfish with sensor (1) and fibre (2) attached to stay inside (Kholodkevich* et al.*, 2008).*

Figure 3
Écrevisse à pattes grêles (*Astacus leptodactylus*) dans un aquarium de 5 L avec un abri qui accueille l'écrevisse équipée de senseur (1) et fibre (2) de (Kholodkevich *et al.*, 2008).

concentration for crayfish in the short time exposure according to the literature and previous experiments with *Orconectes limosus* (Kozák *et al*., 2005). The relevant amounts of Cl^-, dissolved in 100 mL of water and the first concentration of nitrite (3.75 mg $N\text{-}NO_2^-\cdot L^{-1}$), were applied together to aquaria. Nitrite concentrations were increased every two hours from the initial level of 3.75 mg $N\text{-}NO_2^-\cdot L^{-1}$ through concentrations of 7.5, 15, 30 mg $N\text{-}NO_2^-\cdot L^{-1}$, to the final concentration of 60 mg $N\text{-}NO_2^-\cdot L^{-1}$. Crayfish exposure in each nitrite concentration therefore lasted for two hours. Crayfish were then exposed to the highest concentration for 16 hours. Crayfish were then placed in fresh water without any additional chlorides or nitrites for one day. Samples of water for chemical analysis were taken regularly from each concentration.

Figure 4
The hanging of crayfish – crayfish is hanged on wire above the floor, provided to evaluate their physiological stage.

Figure 4
Écrevisse suspendue au-dessus du fond pour évaluer son état physiologique.

> *DATA ANALYSIS*

Data were edited and examined using ANOVA for repeated measurements with Tukey *post-hoc* test in program Statistica 9.0 (StatSoft., Inc.). Data are presented as means ± SE.

RESULTS AND DISCUSSION

We demonstrated the effectiveness of the non-invasive method for monitoring cardiac activity of crayfish exposed to the sublethal-lethal nitrite concentration. Crayfish reaction to excess nitrite was detectable (Figure 5). It can be presumed that a similar type of reaction as known in fish, *i.e.* accumulation of nitrites resulting in subsequent methaemoglobinemia (Kroupová *et al*., 2005; Svobodová *et al*., 2005), occurs in copper of crustacean hemocyanin (Colt and Armstrong, 1981; Rainbow, 2002; Kouba *et al*., 2010). We found a difference in both heart rate and stress index and the "chloride" group could be distinguished on the basis of obtained data. The stress index (SI) was already found to be elevated from the 2nd tested $N\text{-}NO_2^-$ level (7.5 mg·L^{-1}) in the group without chlorides. Reactions in crayfish to the treatment with increased chlorides were observed from the $N\text{-}NO_2^-$ level of 30 mg·L^{-1}. The course of reactions to increasing nitrite levels, in both SI and HR, was significantly influenced by the presence of chlorides. Heart rates of animals without added salt presented fluctuating values independently of nitrite exposure as a probable impact of their toxicity. Regarding heart rate, the loss of its circadian rhythmicity has been presented as a potential early warning indicator for mortality in freshwater crab *Potamon potamios* and noble crayfish (*Astacus astacus*) after toxication (Styrishave and Depledge, 1996).
Generally, crayfish in the group without chlorides showed more distinct reactions. Such status is presumably related to maintaining homeostasis in both crayfish and fish (Maetz, 1971; Wheatly and Gannon, 1995), and the related competition of chloride and nitrite ions in the gill chloride cells. Crayfish in both groups were insensible and "near to death" at the end of observations, but with different stress reactions (Figures 4, 6−8). Such harmful effects are not surprising when we consider that values of 96hLC50 for $N\text{-}NO_2^-$ usually range from 0.66
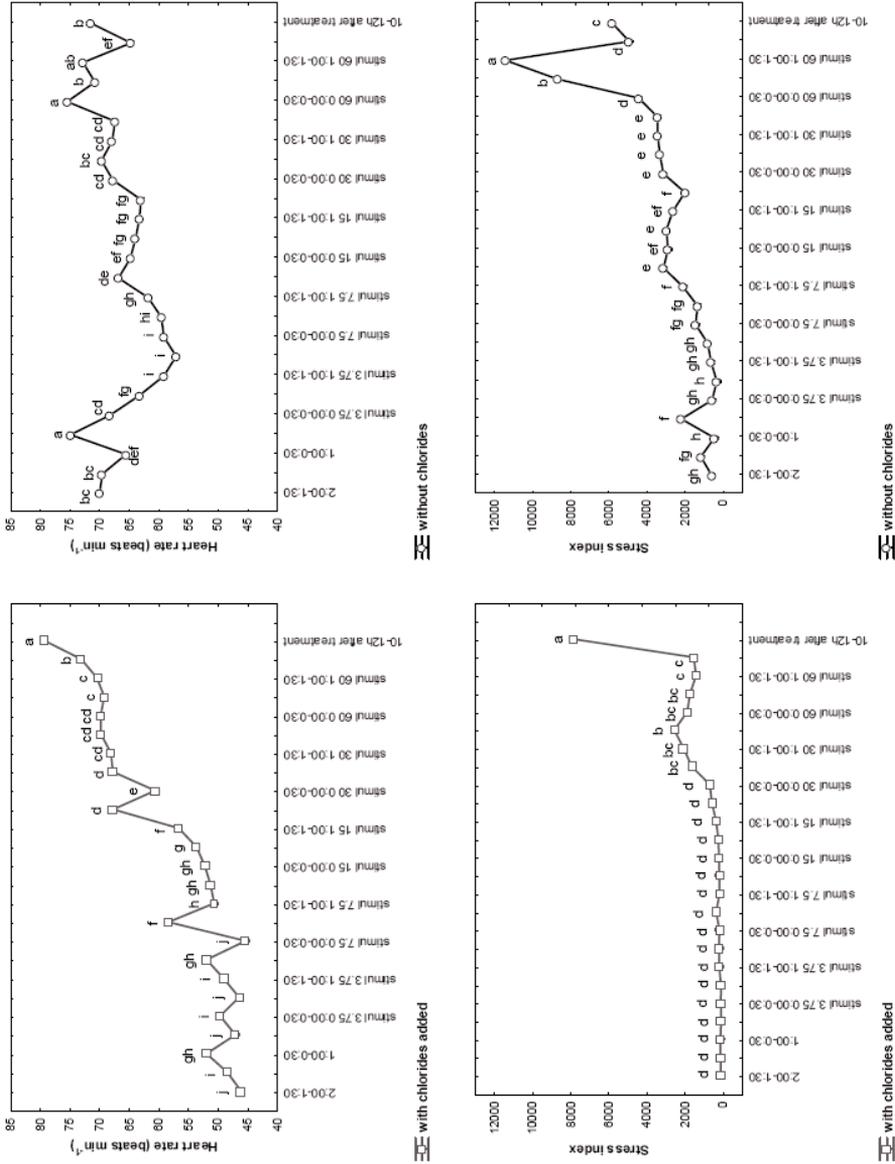
Figure 5

The course of crayfish reaction (heart rate, stress index) at different nitrite levels during experiments. Right column represents group with (400 mg Cl⁻·L⁻¹) and left column shows data for the group without an addition of chlorides. Data are presented as mean ± SE. Different superscripts differ at α = 0.05 (ANOVA, F = 400.1, P < 10⁻⁶).

Figure 5

Déroulement de la réaction de l'écrevisse (rythme cardiaque, indice de stress) à différentes concentrations de nitrite pendant les expériences. La colonne de droite représente le groupe dans l'eau avec chlorures (400 mg Cl⁻·L⁻¹) et celle de gauche le groupe sans addition de chlorures. Les données sont des moyennes avec écart-type. Les différentes lettres diffèrent au niveau α = 0,05 (ANOVA, F = 400,1 ; P < 10⁻⁶).
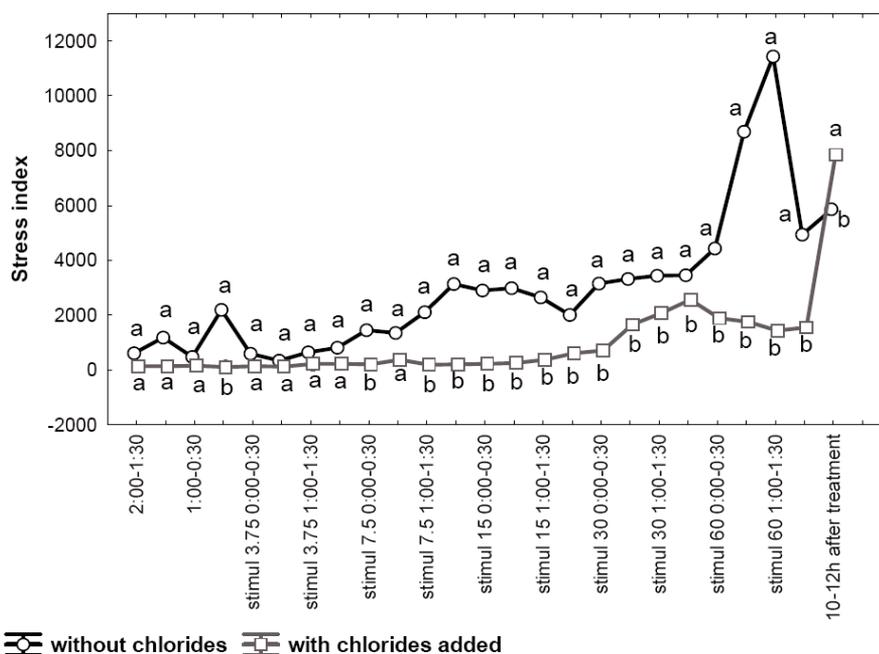
Figure 6

The comparison of crayfish stress index at different nitrite levels between group with (400 mg Cl⁻·L⁻¹)
and without an addition of chlorides. Data are presented as mean ± SE. Different superscripts differ at
$\alpha = 0.05$ *(ANOVA, F = 238.7, P < 10⁻⁶).*

Figure 6

Comparaison des indices de stress de l'écrevisse soumise à différentes concentrations de nitrite entre le
groupe dans l'eau avec chlorures (400 mg Cl⁻·L⁻¹) et celui sans addition de chlorures. Les données sont
des moyennes avec écart-type. Les différentes lettres diffèrent au niveau $\alpha = 0,05$ (ANOVA, $F = 238,7$;
$P < 10^{-6}$).

to 200 mg·L⁻¹ in freshwater fish, or commonly between 8.5 and 15.4 mg·L⁻¹ for crustaceans
(Boyd, 1990 in Rouse *et al*., 1995). In general, the N-NO₂⁻ concentration of 60 mg·L⁻¹ was
critical for both groups. However, crayfish were placed into fresh water for 24 hours following
nitrite exposure. While all crayfish from the group without chlorides died within this time, the
other group was capable of fast recovery under fresh water conditions and survived. Chloride
presence therefore enabled crayfish to withstand and survive very high nitrite concentration.

On the other hand, toxic chemicals such as nitrite have visible negative effects when ex-
ceeded – the danger of direct death of animals. However, sublethal effects of nitrites or other
chemicals should not be underrated. It is known that elevated concentrations of nitrites results
in reduced fitness of animals because of ionic imbalance of extra cellular fluids or increased
energy costs in the process of maintaining additional transport sites (Harris and Coley, 1991).
Rouse *et al*. (1995) presented that 24-hour exposure to nitrite concentrations of 0.4 mg·L⁻¹
and 0.6 mg·L⁻¹ decreased subsequent growth of redclaw crayfish (*Cherax quadricarinatus*)
by 17% and 67% and increased mortality by 5% and 48%, respectively. It can be expected
that it is a result of the stress reaction caused by nitrite treatment.

Finally, we can safely claim that the presented method of stress monitoring can and should
be used to fill knowledge gaps about sublethal effect of toxicants in aquatic environment and
give real levels of chemicals which do not cause any disruptions or deteriorations in health,
behaviour or chemical communication of cultured or wild (free living) organisms.
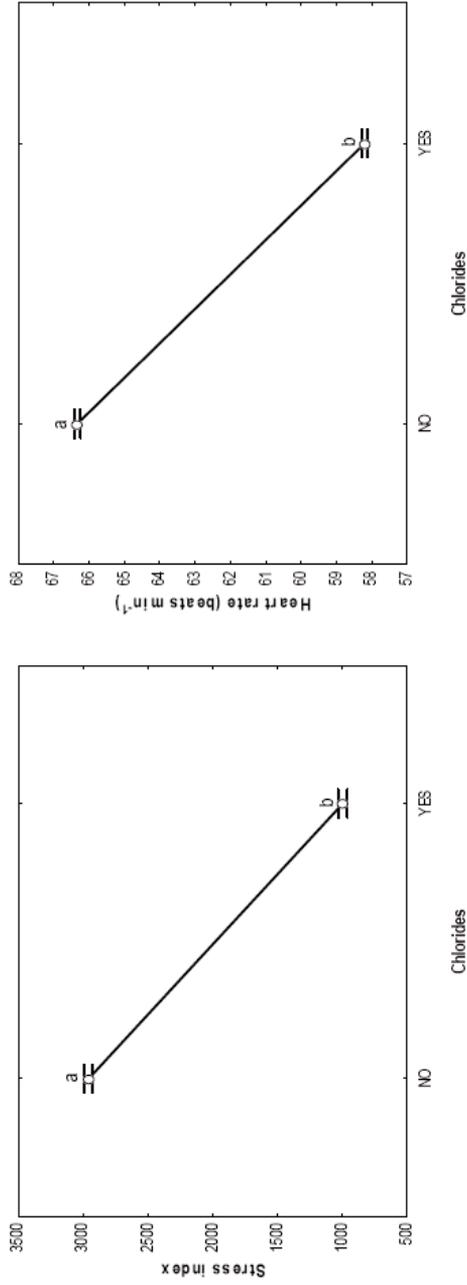
Figure 7
The comparison of mean values (heart rate, stress index) reached in group with (400 mg Cl⁻·L⁻¹) and without an addition of chlorides. Data are presented as mean ± SE. Different superscripts differ at α = 0.05 (ANOVA, F = 2345.5, P < 10⁻⁶).

Figure 7
Comparaison des valeurs moyennes (rythme cardiaque, indice de stress) des écrevisses soumises à différentes concentrations de nitrite entre le groupe dans l'eau avec chlorures (400 mg Cl⁻·L⁻¹) et celui sans addition de chlorures. Les différentes lettres diffèrent au niveau α = 0,05. Les données sont des moyennes avec écart-type. (ANOVA, F = 2345,5 ; P < 10⁻⁶).
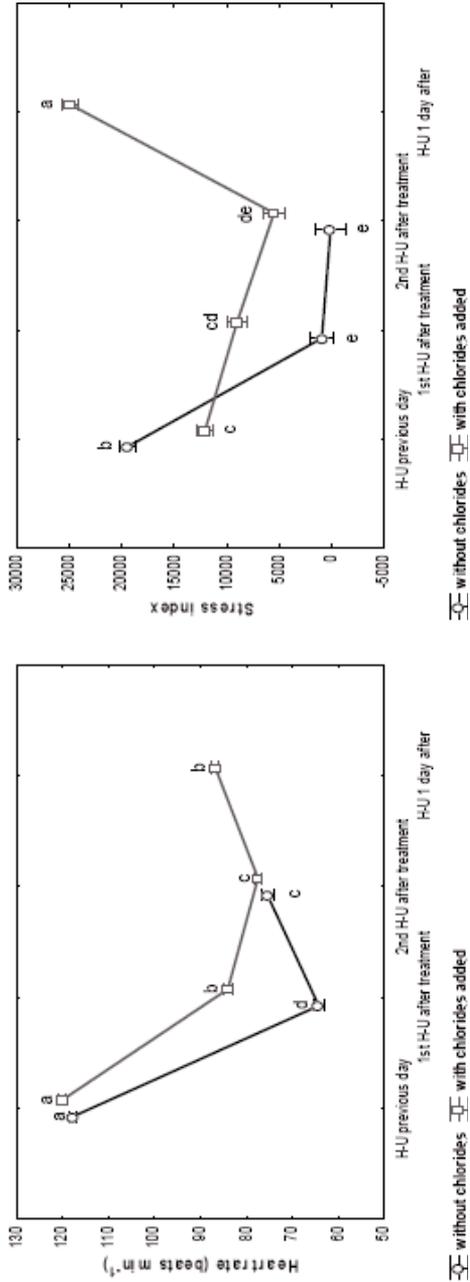
Figure 8
The comparison of values (heart rate, stress index) reached in group with (400 mg Cl⁻·L⁻¹) and without an addition of chlorides during particular hang-ups (H-U). Data are presented as mean ± SE. Different superscripts differ at α = 0.05 (ANOVA, F = 42.3, P < 10⁻⁶).

Figure 8
Comparaison des valeurs (rythme cardiaque, indice de stress) atteintes par des écrevisses soumises à différentes concentrations de nitrite entre le groupe dans l'eau avec chlorures (400 mg Cl⁻·L⁻¹) et celui sans addition de chlorures pendant les moments de suspension de l'écrevisse. Les données sont des moyennes avec écart-type. Les différentes lettres diffèrent au niveau α = 0,05 (ANOVA, F = 42,3 ; P < 10⁻⁶).

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