Resazurin/DCF-based method to quantify ETS activity and ROS production in animals, using brine shrimp (*Artemia salina*) as an animal model

Juan Rafael Buitrago Ramírez[1,2]; Robson Matheus Marreiro Gomes[1,2]; Alan Carvalho de Sousa Araujo[1,2]; Sonia Astrid Muñoz Buitrago[1,2]; José María Monserrat[1,2,3]
1 Universidade Federal de Rio Grande, FURG
2 Laboratorio Bioquimica Funcional de Organismos Aquaticos, BIFOA;FURG
3 Instituto de Ciencias Biologicas ICB; FURG

Introduction

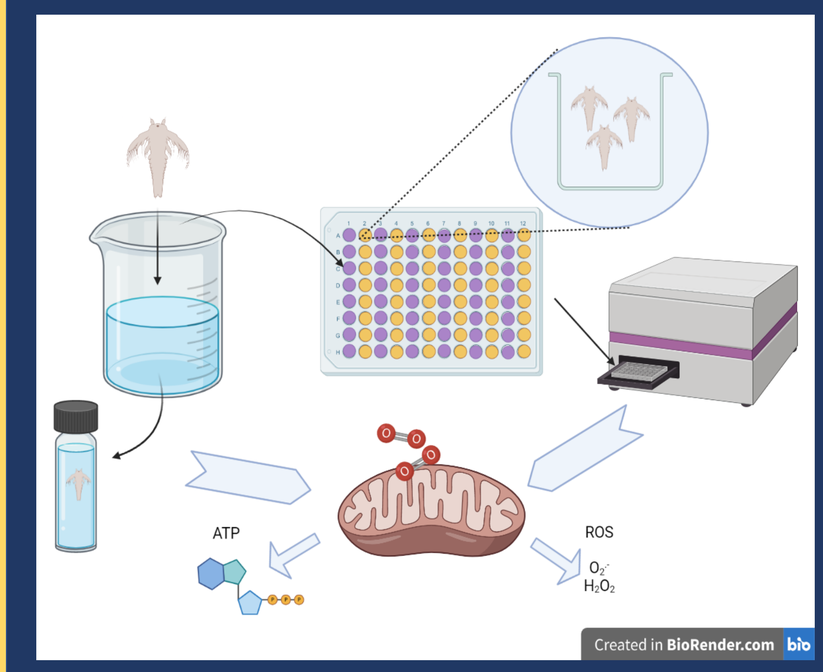
In bioenergetics and toxicology the seek for rapid and accurate methods to screen physiologic and metabolic responses of animals to xenobiotics compounds is an important issue in fields as nutrition, bioactive compounds research or anthropogenic environmental impacts. All related intrinsically with aquaculture activity.

The proposed method here is based in Resazurin and DCF fluorescence reactions. Resazurin it's a common reagent used as a viability pigment, in cellular and bacterial assays. This which after been reduced by ETS in resorufin, emits a fluorescence that can be measure as a aerobic metabolism index. DCF as well is commonly used in oxidative stress measure protocols mostly as a ROS production marker in homogenized biological samples, cells cultures, and animals like Daphnia, and Rotifers (Rodrigues et al., 2021; Ulm et al., 2015; Zhang et al., 2004).

Aim

This work proposes an *in-vivo* method to screen the electron transport system (ETS) activity using resazurin and reactive oxygen species (ROS) production using DCF in 12h post-eclosion brine shrimps nauplii as model under different environmental conditions.

Graphical Abstract



Methods

Experiment 1: It was developed a method to assay the ETS activity in brine shrimp. first, a factorial design was made to establish the optimum levels of resazurin concentration and animal density in 96 well-plates, using the resazurin finals concentrations of 5, 7.5, and 10 µg mL^{-1} and animal densities of 60, 120, and 240 mL^{-1}. Was calculated the kinetic velocity for comparisons and polynomic regression with velocities means.

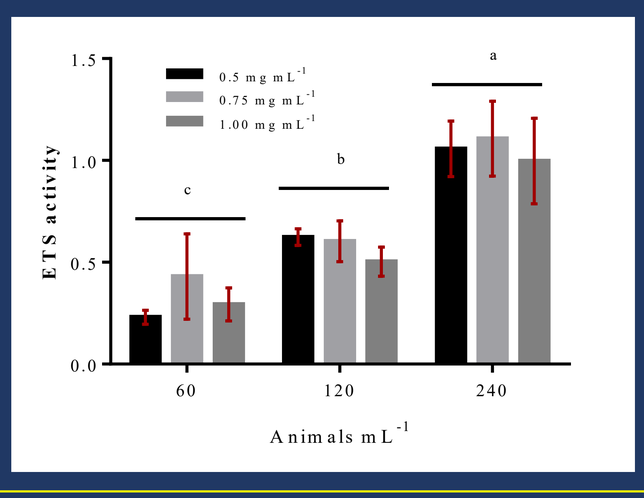
Experiment 2: Then it was done an exposure of artemia to potassium cyanide (ETS complex IV inhibitor) at concentrations of 50, 100, 150, and 200 µg L^{-1} to observe a dose-response effect in the fluorescence kinetics of both ETS and ROS, the kinetic velocities were calculated for 2h exposure and used to determine the effect of the concentration of KCN in the metabolic status of the animals.

Experiment 3: At last, a lipoic acid, a well known antioxidant exposure assay in a 24h in the concentrations, 0, 2, 4, 6, 8, and 10 µM, two samples of 100 µL was taken of each beaker with a frequency of 6h for resazurin, ROS, and total ammonia nitrogen (TAN) concentration and O_2 consumption just at the end of the experiment.

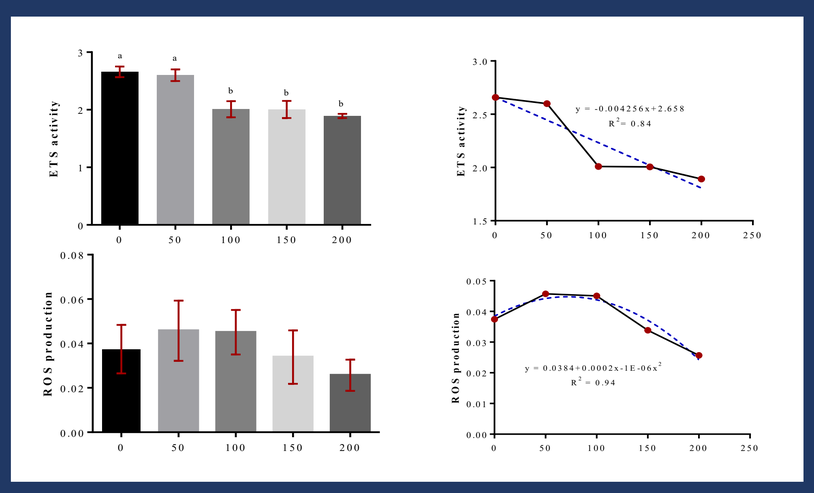
Statistical analysis: Parametric assumptions (normality; Shapiro-Wilk and homocedasticity; Levene) were tested. Experiment 1 and 3 data were analyzed, with factorial ANOVA, multiple comparisons were done with Tukey test. To the experiment 2 data were analyzed with a one-way ANOVA, Tukey (ETS activity) and Kruskal-Wallis and Dunn´s test (ROS).

Results

In the first experiment, resazurin concentration didn't affect the kinetic velocities, and kinetic responses were proportional to the density of animals, as expected.

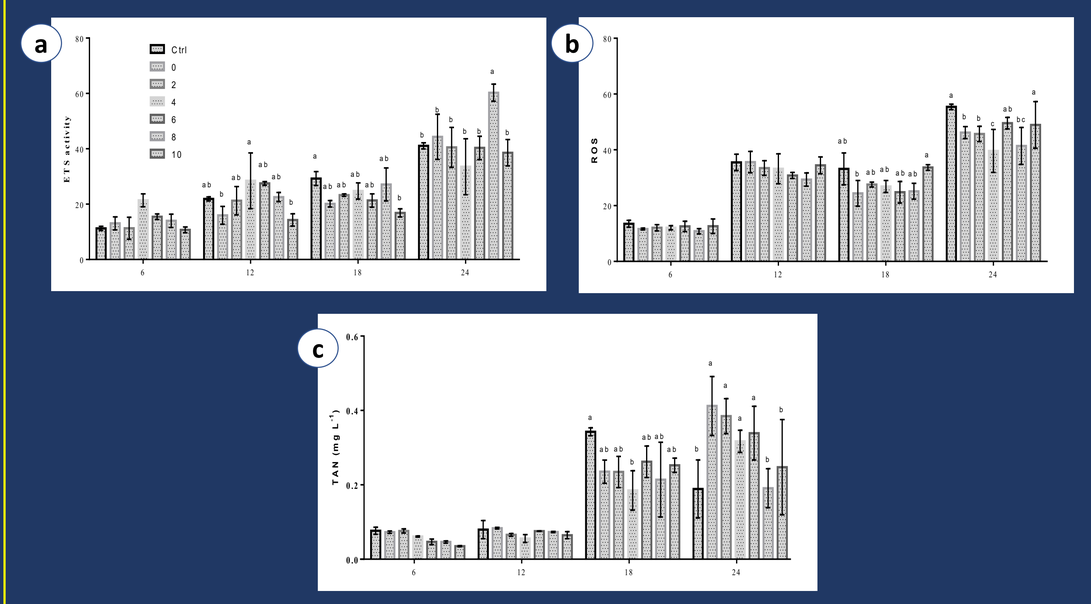


In the KCN experiment, significant differences were detected in ETS activity but not in ROS production may be because high SDs presented. About polynomic regressions both resazurin, and ROS diminished their velocities in a dose-response manner (R2 =85%, 94% respectively), but in the ROS case, the KCN concentrations produce a quadratic behavior of regression velocities means, due a slight, but not significative, increase in ROS production in 50 and 100 µg L-1 KCN concentrations.

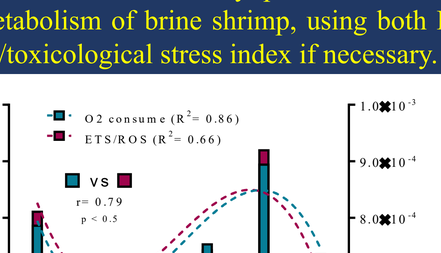


ETS and ROS production of brine shrimp nauplii exposed to different KCN concentrations. (a, and b) each graph column represents kinetic velocity means o fluorescence increase in different KCN concentrations in µg L-1 (X-axis) letters indicate statistic differences. (c) linear regression of ETS activity vs KCN concentrations, with the linear function and determinant coefficient (R2).

ETS activity, ROS production, and TAN medium concentration presented significant differences between treatments. LA dose, experimental time, and interaction between them were significant sources of variability ($p<0.05$). Besides, at 12 h, ETS activity results, are pointing to LA hormetic effect, but this should be tested in furthers analysis.



At 24h, *Artemias* oxygen consumption presented significant differences between treatments, being 8 µM LA dose which presents the higher O_2 consumption ratio.



No significant correlations were found between the response variables, except between oxygen consumption and ETS / ROS, which were well correlated ($r = 0.79$; $p <0.05$). This supports the idea of using ETS / ROS activity production method as an alternative to screening for the aerobic metabolism of brine shrimp, using both ROS production and ETS activity-relativized oxidative/toxicological stress index if necessary.

Conclusion

The Resazurin/DCF-based method can be used for ETS activity and ROS production *in-vivo* quantification in artemia. This method, like respirometry, must be applied with complementary methods to meet more conclusive information.

References

Rodrigues, R.V., Maltez, L.C., Ferreira, C.C. *et al.* ROS in vivo determination and antioxidant responses in rotifers *Brachionus plicatilis* fed with commercial yeast *Saccharomyces cerevisiae* or microalgae *Nannochloropsis oculata*. *Aquacult Int* **29,** 1657–1667 (2021). https://doi.org/10.1007/s10499-021-00707-4

Ulm, L., Krivohlavek, A., Jurašin, D. *et al.* Response of biochemical biomarkers in the aquatic crustacean *Daphnia magna* exposed to silver nanoparticles. *Environ Sci Pollut Res* **22,** 19990–19999 (2015). https://doi.org/10.1007/s11356-015-5201-4

Zhang HX, Du GH, Zhang JT. Assay of mitochondrial functions by resazurin in vitro. Acta Pharmacol Sin. 2004 Mar;25(3):385-9. PMID: 15000895.