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Brief Report

Lipid Peroxidation in Hepatopancreas, Gill, and Hemolymph of Male and Female Crabs *Platyxanthus Orbignyi* after Air Exposure

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Abstract: Levels of lipid peroxidation in hepatopancreas (HP), gill (G), and hemolymph (HYM) of stone violaceous crab *Platyxanthus orbignyi* (Milne Edwards and Lucas (1843)) were performed to examine the effect of short exposure to air. After four hours animals were collected, 14 from exposure to air and 10 from seawater were dissected and their lipid peroxidation (LPO) levels were evaluated using the ferrous oxidation-xylenol orange (FOX) method, in gill, hepatopancreas, and hemolymph. The total mortality of those crabs was evaluated after seven hours at 22 ± 1 °C on exposure to air conditions. Levels of LPO in hepatopancreas (female/male = 4.68 ± 1.60/5.12 ± 1.59 Eq-H_2O_2/g wet tissue) and hemolymph (female/male = 1.48 ± 1.42/1.28 ± 1.06 Eq-H_2O_2/g wet tissue) displayed no significant differences, in contrast, gills displayed significant differences (male/female = 5.63 ± 0.83/4.63 ± 0.44 Eq-H_2O_2/g wet tissue, $p < 0.05$). The results showed that air exposure in the short term in this study induces a different response in oxidative stress levels and damage could be accompanied by accumulation of peroxide lipids (LOOH). These results suggest that different organs can show different responses to oxidative stress between male and female crabs to this species.

Keywords: *Platyxanthus orbignyi;* lipid peroxidation; air exposure; hepatopancreas; gill; hemolymph

1. Introduction

The stone violaceous crab *Platyxanthus orbignyi* [1] is an important resource for artisanal fisherman in the southeastern Pacific (Ecuador, Peru, and Chile) [2]. The violaceous crab inhabits sandy and stony substrates in the supratidal zone, where only the highest tides reach [3]. After being captured, crabs are exposed to air for several hours in plastic containers until their arrival to the market, therefore, water flow through the gills is interrupted. At this time an increase of reactive oxygen species (ROS) begins, producing oxidative stress, including lipid peroxidation (LPO) [4,5]. From a commercial point of view, crabs exposed to air for several hours can produce oxidation in their tissues, and this phenomenon can influence the final taste of the meat [4].

Physiological, morphological and behavioral adaptations are found in animals residing in stressful environments, such as exposure to air [6,7]. In crustaceans, the most common adaptations under these conditions are the activation of anaerobic metabolism, metabolic rate depression, reduction of heart rate, and even of locomotion [7]. These physiological imbalances caused by stressful environments can lead to increased ROS formation, and even attempting to recover balance through reoxygenation could increase ROS levels [8].

ROS are potentially dangerous when interacting with proteins, nucleic acids, carbohydrates, and lipids [8]. These species, when interacting with lipids in tissues, can trigger LPO which consists of

lipid degradation [9]. Since cell membranes are predominantly composed of lipids, this degradation could compromise cell membrane function [10]. Oxidative stress is an unfavorable condition and can occur when crabs living in aquatic environments are exposed to air for longer periods than air exposure behavior (normogenic conditions of 1 to 2 min) [11]. Studies in the porcelain crab *Petrolisthes cinctipes* and *P. eriomerus* show that the animals that inhabit intertidal zones were able to modify their physiology and morphology in order to withstand overexposure to air, even with high levels of LPO in gills and muscles [12].

In crabs *Paralomis granulosa*, exposure to air for 6 h showed high levels of LPO in HP [4], even with less exposure to air (3 h) similar levels of stress were found in HP, G, and muscle (M) of *Callinectes danae* and *C. ornatus* [5]. However, different sexes of crabs could be able to present differences in their responses to stress, as shown in the species *Callinectes amnicola* and *Geothelphusa dehaani*, where males present greater oxidative damage and, as a consequence, experience a reduction in their population [13].

In this brief work, the levels of lipoperoxidation in hepatopancreas, gills, and hemolymph were evaluated in males and females of adult crabs *Platyxanthus orbignyi*, when they were exposed to air for 4 h, considering the transportation time from capture to market. The aim was to verify the physiological differences caused by increased levels of stress between both sexes that could modify the quality of the final product, mainly in gills and hepatopancreas, which are commonly consumed by the southern Pacific coastal population.

2. Material and Methods

Ammonium iron (II) sulfate hexahydrate (Sigma, >99%), hydrogen peroxide solution (Sigma, 25%–35%), xylenol orange tetrasodium salt (Sigma, >99%), methanol (Sigma, > 99.9%), sulfuric acid (Sigma, 98%) and cuvette 1.5 mL UV grade of polymethylmethacrylate (Thomas Scientific) were purchased for this assay.

Animal collection. Male and female *Platyxanthus orbignyi* (Milne Edwards and Lucas, 1843) [2] specimens were collected 8 km offshore from the Oquendo beach, Callao, Lima, Perú coast, (11°59'17.6"S 77°11'57.3"W) in December 2018 (reproductive season, summer), using fishing nets and transported into plastic container 30 × 30 × 100 cm for 3 h at 22 °C ± 1, in the dark. All crabs were in intermolt and of legal size, i.e., >7.2 cm of carapace length [14]. One group (n = 14) of animals were transported without seawater (air exposure), and another group (n = 10) was transported in seawater 35 ‰, pH 8.1 at 22 °C ± 1. Immediately, the living animals were brought to the Universidad Nacional de Ingeniería (Lima campus), being transported for 1 h at 22 °C ± 1. The individuals were divided into four groups: i) Female crabs for control (9.6 ± 1.0 cm, 176 ± 28 g, n = 5) in seawater, ii) male crabs for control (9.5 ± 0.4 cm, 163 ± 13 g, n = 5) in seawater, iii) male crabs on-air exposure (7.3 ± 0.7 cm, 104 ± 20 g, n = 7) and iv) female on-air exposure (7.2 ± 0.3 cm, 113 ± 14 g, n = 7) [15] .

Sample collection. The animals were dissected using stainless steel scissors. HP and G samples were collected in plastic Eppendorf (1.5 mL) and immediately, weighed (0.03c0.05 g) and frozen at –20 °C to avoid LPO decomposition. HYM (100–150 µL) was collected in plastic Eppendorf (1.5 mL), using sterile insulin syringes (500 µL) to penetrate the intersegmental membrane between the posterior of the carapace and the coxa of the leg, previously disinfected with alcohol 70% (Aldrich, USA) [15] , and was immediately added to methanol 100% (1:9 ratio, sample:methanol), previously cooled at 5 °C, after that, the methanolic suspensions were homogenized using vortex, and stored at –20 °C.

Biochemical analysis. The LPO levels in HP, G, and HYM were assessed by a modified Fox method as hydrogen peroxide equivalents (Eq-H_2O_2) [9,15–17]. Hydrogen peroxide was used considering its greater reactivity in the FOX method (100%) compared to t-Butyl hydroperoxide (96%), cumene hydroperoxide (98%), di-cumyl peroxide (12%), benzoyl peroxide (9 %), and lauroyl peroxide (21%). In addition, its sensitivity and stability were considered in relation to linoleic hydroperoxide as a control, which remained stable throughout the experiment (1 h) [16].

Frozen HP and G tissue samples were rapidly weighed in a plastic Eppendorf (1.5 mL) and homogenized in cold 100% methanol (5 °C) (1:9 w:v). Simultaneously, HYM samples were rapidly

thawed and kept at 5 °C. Then, all samples (HP, G, and HYM) were rapidly centrifuged (10,000 rpm) at 5 °C for 10 min. For the standard assay, the following reagents were added sequentially in a plastic cuvette: 270 µL, 1.0 mmol L^{-1} iron (II) salt (ferrous ammonium sulfate), 105 µL, 0.25 mol L^{-1} sulfuric acid, 105 µL, 1.0 mmol L^{-1} xylenol orange, and 525 µL of water. A sample of tissue methanolic extract (45 µL) was then added, and the final volume was 1.05 mL. Blanks were prepared to replace tissue extracts by methanol (45 µL). Samples were incubated at 22 ± 1 °C for 1 h, and absorbances were obtained at 580 nm (A_{sample}) in bringing a Perkin Elmer UV Vis Spectrophotometer Lambda 25 (USA). The cuvettes were covered to avoid losing liquid by evaporation. After that, 30 µL of H_2O_2 (175 mmol L^{-1}) was added to the sample and absorbance was read again after 15 min ($A_{sample + H_2O_2}$). LPO levels were calculated according to the following equation (1):

$$\frac{\text{Eq} - H_2O_2}{\text{wet tissue (g)}} = \left(\frac{A_{\text{sample,580 nm}}}{A_{\text{sample+ } H_2O_2 \text{,580 nm}}}\right) \times \frac{5 \text{ nmol } (H_2O_2) \times 1.05}{10 \text{ V}} \quad (1)$$

where, V = sample volume and 10 is a correction factor of dilution made during the preparation of methanol extract (1:9, weight:volume).

Statistical analysis. Data are presented as means ± standard error. Kruskal–Wallis one-way analysis of variance on ranks was performed to determinate LPO levels on each condition to air exposure. Data were checked for normality and homogeneity of variance by Holm–Sidak test performed with Stat 3.2 software (Systat Software, San Jose, CA, USA) [15]. Significant differences ($p < 0.05$) were compared using the Tukey post hoc test [18].

3. Result

The LPO levels were analyzed in hepatopancreas (HP), hemolymph (HYM), and gills (G) of crab *Platyxanthus orbignyi* in both males and females. The results displayed higher LPO levels in HP, however, there was no significant difference between experimental and controls (Figure 1 and Table 1).

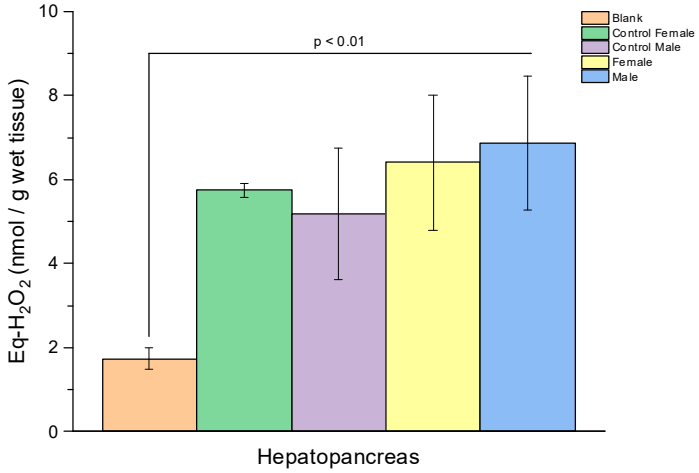

Figure 1. Level of lipid peroxidation (measured as H_2O_2 equivalent per g wet tissue) in the hepatopancreas of female and male crab *Platyxanthus orbignyi* at 22 ± 1 °C. Blank is represented by the absence of tissue extracts. Average ± SD, n = 7 (ANOVA, $p < 0.05$).

Table 1. Level of lipid peroxidation in hepatopancreas, gill, and hemolymph of female and male crab *Platyxanthus orbignyi* at 22 ± 1 °C. Average ± SD, n = 7 for sample and n = 5 for control. (n.d. = not detected). Blank was subtracted from each Lipid peroxidation value.

		Lipid peroxidation (Eq-H_2O_2 (nmol/g wet tissue))		
Sex	**Description**	**Hepatopancreas**	**Gill**	**Hemolymph**
Female	Control	4.62 ± 0.13	0.72 ± 0.56	0.46 ± 0.39
	Sample	4.68 ± 1.60	2.90 ± 0.45	1.48 ± 1.42
Male	Control	3.44 ± 1.27	1.66 ± 0.84	0.19 ± 0.10
	Sample	5.12 ± 1.59	3.75 ± 0.83	1.28 ± 1.06

G also showed LPO. However, their levels were lower in relation to HP, and higher in comparison to HYM (Figure 2 and Table 1). In G, our results also show that levels of LPO were significantly higher in males exposed to air than in females ($p < 0.05$, Figure 2).

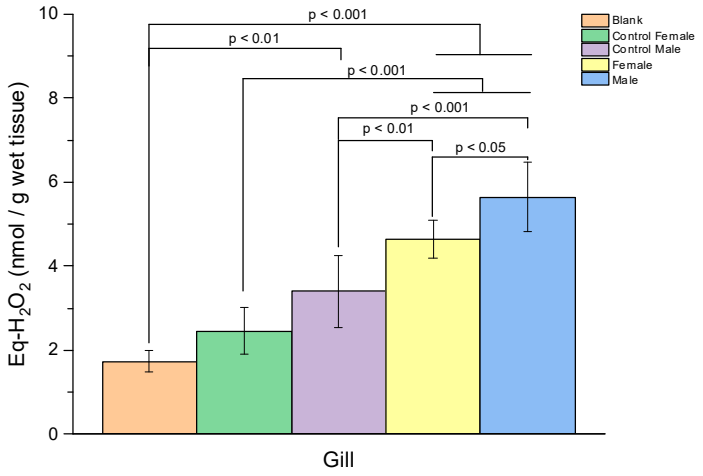

Figure 2. Level of lipid peroxidation (measured as H_2O_2 equivalent per g wet tissue) in the gill of female and male crab *Platyxanthus orbignyi* at 22 ± 1 °C. Blank is represented by the absence of tissue extracts. Average ± SD, n = 7 (ANOVA, $p < 0.05$).

The lowest LPO levels were found in HYM (Figure 3 and Table 1), with no significant differences between controls and experimental.

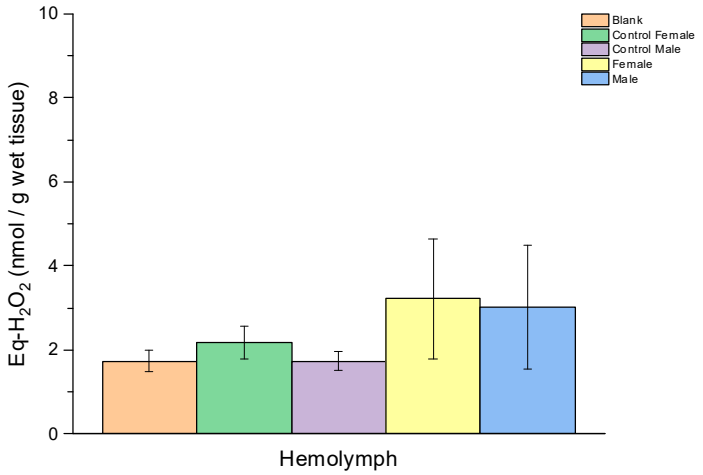

Figure 3. Level of lipid peroxidation (measured as H_2O_2 equivalent per g wet tissues) in the hemolymph of female and male crab *Platyxanthus orbignyi* at 22 ± 1 °C. Blank is represented by the absence of tissue extracts. Average ± SD, n = 7 (ANOVA, $p < 0.05$).

After evaluating the lipid peroxidation levels in HP, G, and HYM of crab *Platyxanthus orbignyi* in both males and females after 4 h exposure to air, our results suggest a decreasing order of oxidative damage on these tissues as shown below:

$$\mathrm{LPO}_{\mathrm{male}}^{\mathrm{HP}} \cong \mathrm{LPO}_{\mathrm{female}}^{\mathrm{HP}} > \mathrm{LPO}_{\mathrm{male}}^{\mathrm{G}} > \mathrm{LPO}_{\mathrm{female}}^{\mathrm{G}} > \mathrm{LPO}_{\mathrm{male}}^{\mathrm{HYM}} \cong \mathrm{LPO}_{\mathrm{female}}^{\mathrm{HYM}} \quad (2)$$

Therefore, the sex-specific affinity in relation to redox activity still needs to be studied in greater detail.

4. Discussion

Swimming crabs of the genus *Platyxanthus* inhabit interdital and subdital rock habitats [19], and they can tolerate exposure to air only for short periods [20]. Short periods of exposure of this species can lead to highly stressful situations, and consequently to their death, as we intend to demonstrate in this work. So far, there is no work related to over-exposure to air in this species. Similar work on another species has been previously developed with the porcelain crab *Petrolisthes cinctipes e P. eriomerus* and showed that animals inhabiting the intertidal region are resistant to abiotic factors, they were able to develop morphological and physiological mechanisms that allowed survival in stressful environments [12]. It means, not all species that live in similar habitats could have the same resistance to over-exposure to air. Exposure to air is a situation of great stress in crabs, and it can cause significant physiological disturbances [12], which lead to the formation of reactive oxygen species (ROS), and can cause oxidative damage through LPO [4].

Studies performed on crab *Paralomis granulosa*, a species found in Tierra del Fuego (Chile), displayed that HP was the organ most affected by LPO process when the animal was exposed to air for 6 h [4]. Similarly, studies in *Callinectes danae* and *C. ornatus* also displayed high levels of LPO in HP, in relation to G, M, and HYM when the animals were exposed to the air for 3 h [5]. The results displayed in this brief work corroborate with those of previous studies, which could suggest a tendency of greater LPO levels in hepatopancreas in relation to the other organs. That trend could be explained by the high levels of unsaturated lipids found in HP, mainly due to arachidonic acid [21], which could undergo LPO in the presence of free radicals [22]. This difference in levels of LPO could be a response to a greater accumulation of metals in hepatopancreas that catalyzes the formation of reactive oxygen species [23].

The imbalance between antioxidants and reactive oxygen species can be generated by stressful conditions as a result of exposure to air, which is confined to the G compartments during aerobic metabolism [24–26]. This phenomenon was observed in crab *Sinopotamon henanense*, where the generation of LPO in gills was catalyzed by the presence of cadmium [24,25]. Studies on mangrove crabs *Ucides cordatus* from regions contaminated with cadmium also showed significant levels of LPO in G, under conditions of oxygenation in saltwater [26]. Thus, our results shown in this work can verify this phenomenon observed in gills which are described in previous works under similar conditions.

Our results displayed high LPO levels in male G in relation to female G. Similar results were shown in *Callinectes amnicola* where a sex-specific affinity was observed in relation to the redox activity [13]. This affinity could lead to reproductive difficulties, with the quantitative diminution of males in a redox environment [13]. Studies performed in crab *Geothelphusa dehaani* also found sexual and reproductive abnormalities in males in relation to females, both in environments under stress [13].

In HYM, lower LPO levels could be a result of the low levels of unsaturated fatty acids present in this tissue, or because of the presence of LPO inhibitory proteins [27,28]. Studies on insects have shown that the presence of apo-proteins in HYM can inhibit the LPO of linoleic acid and lipoforine (major lipoproteins found in HYM) [27,28]. Thus, it is suggested that crustaceans could present similar proteins, which would possibly inhibit LPO in HYM.

In fact, *Platyxanthus orbignyi* are affected at 4 h of air exposure although they appear to be in good condition. The levels of LPO were higher in HP, followed by G ($LPO_{male} > LPO_{female}$) and HYM. This should be considered in order to mitigate the stressing conditions generated by the commercial capture and transport process. Particularly, these data could be useful to those needing to know the environmental conditions (time) in which these animals can be exposed to the air without suffering any irreversible damage. Furthermore, future studies on the quantification of antioxidants levels should be done, for a better understanding of different responses of lipid peroxidation levels in this species.

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