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Stress Biomarkers in the Giant Manta *Mobula birostris* Associated to Tourism in the Revillagigedo National Park, Mexico

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Abstract

A constant increase in dive tourism over the past years in the Revillagigedo National Park, Mexico, could result in a stressful scenario for giant mantas (*Mobula birostris*). The purpose of this study was to determine the degree of oxidative stress in terms of changes in catalase units (CAT) and muscle glycogen concentration in this species during two periods of different tourism intensity in this protected area. A total of 21 muscle biopsies were collected in March (peak tourism) and November (lower tourism), 2019. Stress biomarkers were analysed by commercial kits from the company Cayman Chemical. Oxidative stress (catalase activity) was significantly higher during the period with lower tourism ($p = 0.002$), compared to the period with more tourism, suggesting the presence of the general adaptation syndrome. In males, there was a significant difference ($p = 0.0005$) in oxidative stress between periods of different tourism intensity, suggesting that the reproductive season may be a stressor. Morphotypes showed different oxidative stress ($p = 0.031$); however, the reason is unknown. No statistical differences were detected in glycogen concentrations between the tourism periods ($p = 0.123$), probably because this polysaccharide is not a proper indicator of chronic stress in giant mantas. Based on these findings, giant mantas may have an adequate response in terms of oxidative stress due to an increase in tourism; however the observed increase in catalase suggests that it is within the tolerance range of these organisms.

Keywords

Catalase, Conservation, Elasmobranchs, Glycogen, Oxidative Stress

1. Introduction

A state of stress begins in all vertebrates when a threatening physical and/or psychological event occurs, altering many physiological processes necessary to maintain homeostasis [1]. Stress is defined as the negative effect on an individual that exceeds its control systems and increases its vulnerability [2]. The direct effects of a stressor on an organism are metabolic and can alter cellular components, such as enzymes and membranes, thus affecting functions like breathing, circulation, immune response, osmoregulation and hormonal regulation [3] [4]. Previous studies that have assessed the response to stress in elasmobranchs have reported the use of different bioindicators, for example, electrolytes, cell markers, leukocyte response, and hormones, among others [4] [5].

Oxidative stress occurs when there is an imbalance between the increased production of reactive oxygen species (ROS) and the inability of the antioxidant system to maintain equilibrium [1]. Catalase is a ubiquitous antioxidant that is present in most aerobic cells [6]. Vélez-Alavez *et al.* [7] determined the antioxidant system in the muscle of three species of elasmobranchs and three species of teleosts, showing that the activity of catalase was higher in elasmobranchs compared to teleosts due to differences related to their phylogeny and low molecular weight. Also, when analyzing the total antioxidant capacity and the total oxidative state, among other bioindicators, of southern rays (*Dasyatis americana*) in places with and without tourism, Semeniuk *et al.* [1] reported that the exposure to tourism significantly increases total ROS while reducing the antioxidant capacity.

On the other hand, muscle glycogen functions as a glucose reserve and is used as energy for muscle contraction [8] [9]. Bashamohideen and Parvatheswararao [8] showed that extreme osmotic changes, as a stressful environment, resulted in decreased glycogen in liver and muscle in Mozambique tilapia (*Tilapia mossambica*). However, there is no evidence of glycogen changes in elasmobranchs exposed to any kind of stressors.

The considerable increase in diving tourism in the Revillagigedo National Park went from 1594 tourists in 2012 to 4900 in 2019 (CONANP, unpublished data), which could result in a stressful condition for the giant manta (*Mobula birostris*), a vulnerable and emblematic elasmobranch of the park.

2. Material and Methods

Fieldwork

Sampling of giant mantas was undertaken in the Revillagigedo National Park (RNP), Colima, Mexico, in 2019. The National Commission of Protected Natural

Areas (CONANP) restricts the passage of tourist boats from July to October due to the hurricane season [10], therefore, the first sampling of giant mantas was carried out in March with the highest number of tourists (146 divers, $\bar{x}$ 12 days), and the second sampling in November with the lowest number of tourists (55 divers, $\bar{x}$ 12 days).

The mantas were individually identified by photos and videos using underwater cameras. The pigmentation patterns of the ventral zone of giant mantas were recorded for the two morphotypes (black and chevron) [11]. The size of mantas was estimated using two submersible laser pointers and a GoPro® camera by directing the laser points to the ventral area of the animal and taking a photo of the total diameter of the ventral surface. Using point-to-point laser, a 20 cm reference was registered for each manta and through the AxioVision/Zeiss image software the total sizes were obtained by extrapolation. Mantas were sexed by the presence or absence of claspers. Skeletal muscle samples were taken using a Reeb & Best biopsy system [12] with a pole spear by SCUBA diving and within 5 min the diver went up to give the sample to a person in a zodiac that stored it in 4 ml cryovials inside a liquid nitrogen container (−172˚C) to avoid degradation of the metabolites. A total of 21 samples were obtained in the two seasons from 21 different mantas: 14 in March (high tourism) and 7 in November (low tourism).

Laboratory

An average of 130 mg of the muscle biopsies were used, and 2 mL of lysis buffer (TRIS 1 M pH 8, NaCl 5 M, EDTA 0.5 M pH 8, SDS 10% and distilled water) was added. The samples were sonicated in a CVX 130 PB, Vibracell sonicator using a wave amplitude of 40% directly on the tissue inside the cryovials which were immersed in crushed ice. The process of sonication consisted of 10 sec of sonication by 10 sec of rest for a total of 5 min. The samples were then cryocentrifuged at 14,000 rpm for 10 min at 4˚C, and the supernatant was separated and frozen at −4˚C.

Oxidative stress was estimated in relation to catalase units under activity, representing acute stress due to his accelerated metabolism. This was determined using the Catalase assay kit (Cayman Chemical®, USA). The chronic stress was estimated in relation to the glycogen concentration using the Glycogen assay kit (Cayman Chemical®, USA) with some modifications. Briefly, 50 μL of the hydrolysis enzyme (amyloglucosidase) with 10 μL of sample were added, then was incubated for 120 min at 37˚C in a Humboldt oven. Later, 50 μL of ultrapure water were added to give the sample more volume. Glycogen concentration was obtained using an automated analyzer (BioSystems A15).

Statistical Analysis

Differences in biomarkers between tourism levels were determined using the Mann-Whitney U test including sex and morphotypes in the model. Regression and correlation multiple linear analyses were used for the total length through the JMP Statistical Discovery software.

3. Results

The results were contrary to what was expected. Giant mantas showed significantly higher CAT during the low tourism period (p = 0.002) compared to the high tourism period, which means an increase of oxidative stress in muscle, related to an acute stress response at the beginning of the tourism season in these islands (Figure 1). In the period of less tourism, male mantas presented higher activity of catalase (p = 0.0005), compared to the period of greater tourism; females didn't present any significant difference. Both the chevron morphotype and black morphotype mantas showed significantly higher CAT units (p = 0.006 and 0.014, respectively) in the period with less tourism intensity. Also, the comparison of CAT activity between morphotypes was carried out, finding a statistical difference between the chevron morphotype and the black one (p = 0.031). No significant differences were found in the CAT between size and tourism period (higher tourism R^2 = 0.050, p = 0.442/less tourism R^2 = 0.069, p = 0.569). No significant differences were found in glycogen concentration between tourism periods (p = 0.123), sexes (F = 0.260/M = 0.878), sizes (higher tourism R^2 = 0.037, p = 0.511/lower tourism R^2 = 0.007, p = 0.862), or morphotypes (C = 0.796/B = 0.152).

4. Discussion

The lack of significant difference in catalase between the experimental and control groups in the multiple studies on oxidative stress in muscle [7] [13] [14], and the significant difference with this project, may be because of the time elapsed from the capture of the organism until the muscle sample is collected in

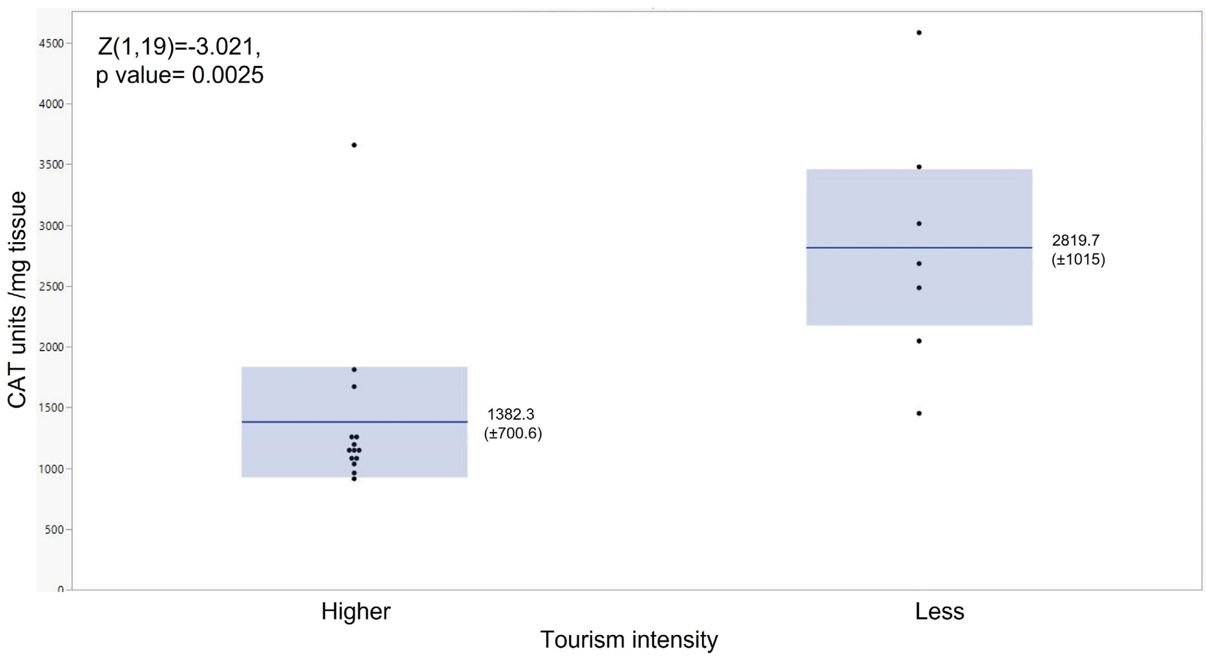

Figure 1. Units of catalase per milligram of tissue between periods with different intensity of tourism. Dots represent the result of catalase units of every giant manta.

the fishing fields is not mentioned, or the time it took to take the samples is not taken into account [1], which, can influence the metabolism of reactive oxygen species (ROS). Also, the capture of organisms influences the metabolism of ROS, since the animal enters a state of stress due to physical exertion [15] and initiates a process of general physiological catabolism, altering all metabolic compounds to study [16]. Differences in catalase units between periods with varying tourism intensity, may be due to an effective response of muscle cells to oxidative stress [7]. It has been suggested that the statistical difference between the CAT of mantas is within the tolerance ranges of the antioxidant in this species [17]. There is evidence to suggest that giant mantas are an intelligent species, with the largest brain of any fish and with specific adaptations, such as countercurrent brain warming mechanisms [18]. This may be related to their great social capacity, and they may adapt easily to tourism despite its continuous increase [19].

Filho *et al.* [20] suggested that the level of antioxidants in elasmobranchs are lower than teleosts and reflects the level of oxygen consumption. This pattern was confirmed by López-Cruz *et al.* [13], who worked with three shark species: shortfin mako (*Isurus oxyrinchus*), silky shark (*Carcharhinus falciformis*) and smooth hammerhead (*Sphyrna zygaena*) where the level of antioxidants against oxidative stress was directly related to the level of swimming activity. The most active sharks (shortfin mako shark and silky shark) presented a significantly higher antioxidant defense than the less active smooth hammerhead. Biomarkers of defense against oxidative stress after harmful dangerous stress can provide information, not only about the ability of fish to resist future oxidative stress, but also about the degree to which these defenses have been depleted [21].

Contrary to the results obtained in this work, Semeniuk *et al.* [1] demonstrated that in the most popular tourist sites in the Cayman Islands, southern skates (*Dasyatis americana*) exhibit decreased total antioxidant capacity (TAC) and increased total oxidative status (TOS), compared to non-tourist sites that presented a lower number of parasites and had the highest TAC, demonstrating a better response to ROS. This difference between the presentation of greater antioxidant capacity between Semeniuk *et al.* [1] with the results of CAT present in giant mantas, may be due because tourism in the Cayman Islands is continuous, and tourists offer food to rays. The response of the organisms to stress varies with the nutritional changes during the annual cycle, which may affect antioxidant defenses [22]. In the same way, in the southern rays, the fact of not having rest periods from tourism may be a factor that makes a difference in the response to oxidative stress compared to the giant mantas of the Revillagigedo National Park, which have four months of absence of tourists due to the presence of hurricanes.

Findings suggest that giant mantas in the RNP show a General Adaptation Syndrome (GAS), described by Selye [23], and still in use, which is a physiological response based on the sum of all non-specific systemic reactions in the body that result from exposure to a stressor and helps the body to adapt to fight or flight [24].

The GAS is divided into three stages [23] [25]:

1) The alarm reaction is the sum of all nonspecific systemic phenomena caused by a sudden exposure to stimuli to which the mantas are not quantitatively or qualitatively adapted; it is a general state of catabolism. This reaction might occur in November, when the arrival of divers starts (period with less tourism), and higher amounts of CAT are recorded (**Figure 2**).

2) The resistance stage represents the sum of all nonspecific systemic reactions caused by prolonged exposure to stimuli to which the body has adapted because of continuous exposure. It is characterized by greater resistance to the agent to which the body is exposed to and less resistance to other types of stress. It may occur when there is an increase in divers. Therefore, an effective response to the stressor is inferred by giant mantas, decreasing CAT levels through adaptation to the presence of the divers (**Figure 2**).

3) The exhaustion stage represents the sum of all nonspecific systemic reactions (eg. shock, inflammation, anoxia), which eventually develop because of prolonged exposure to stressors that have been overcompensated for and those compensating mechanisms can no longer be maintained; consequently, the organism perishes. There are no records of this stage in giant mantas of the RNP.

The occurrence of hurricanes at the RNP limits the access of boats and divers, hence a zero-tourism season exists at these islands when mantas have no contact with tourists. Therefore, in the presence of the stressor, at the beginning of the tourism season, it is presumed that mantas initiate a process of general catabolism starting the alarm reaction, registering the highest levels of catalase. With the persistence and increase of tourism, giant mantas overcompensate the homeostatic levels, presenting the resistance stage. Over time, levels become stable again, and this is reflected by mantas getting used to divers, decreasing the production of reactive oxygen species and catalase consequently (**Figure 3**).

The CAT response in different sexes could be attributed to the reproductive events of the giant mantas in RNP. Courtship behavior was observed in October (M. Hoyos, personal observation), prior to the sampling in November. This

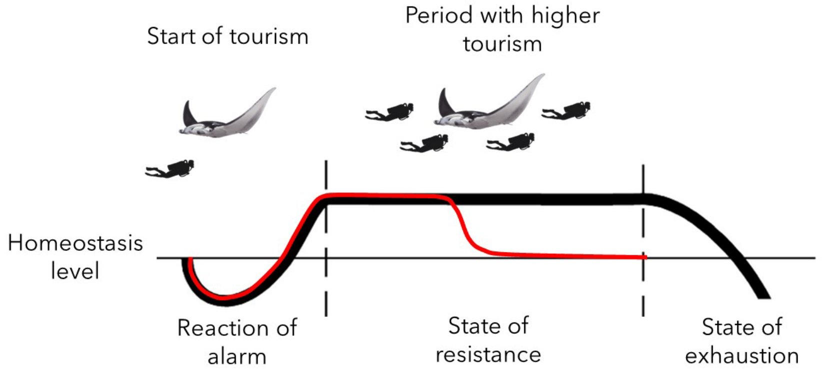

Figure 2. Phases of the General Adaptation Syndrome (GAS). The thin middle line represents the level of homeostasis; the thick black line represents the physiological changes during a complete GAS event. The red line suggests the physiological response of the recorded CAT units in giant mantas.

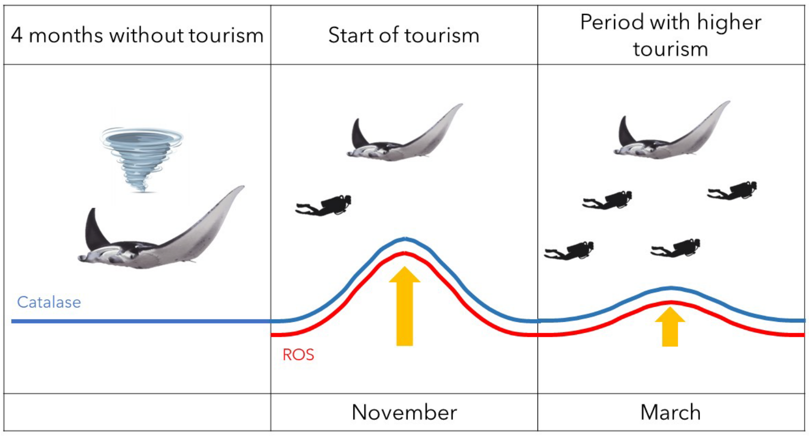

Figure 3. The box figure showing catalase units and reactive oxygen species (ROS) in giant mantas over time and their response to tourism. It is inferred that there is no significant CAT response regarding ROS in the four months without tourism, since the stressor considered in this project is not present.

behavior is more stressful for males, because they have to compete with other males to be selected by the female [26], and this could be raising the levels of ROS and therefore of CAT [27] [28]. As well as Manire *et al.* [27], where they found an increase in serum corticosteroids in male shovelhead hammerheads (*Sphyrna tiburo*) due to reproductive events in the months of August and October. The same response obtained in this paper could be attributed to the small size of the males sampled in the period with less tourism, which is very low (n = 2) and these values create a bias. It is recommended to obtain a larger number of samples.

The difference among morphotypes may be the result of a bias due to the small number of black morphotype samples (n = 2), therefore it is recommended to increase the number of morphotype samples. To date, the only difference established between morphotypes is the color pattern [11]. This study reports the first physiological difference between *Mobula birostris* morphotypes, unknowing the cause.

There are individual differences in the response to stress, based on the individual's experience in early and adult life [29]. Positive or negative experiences throughout life can bias an individual toward a positive or negative response in a new situation [29]. This experience over time and with the brain development of this species [18], led us to expect differences between sizes, assuming that adults present less stress (acute and chronic) than juveniles, but even though we had samples of both sizes, no statistical differences were obtained in this project. However, as in this work, Semeniuk *et al.* [1] found no statistical differences in antioxidants and oxidative status in the blood of southern skates (*Dasyatis americana*) between the different grown stages in the presence of tourism, reflecting that the antioxidant response is not related to the age of the organisms.

In the muscle, the glycogen hydrolysis releases glucose aim to cover energy requirements. This type of stress is mainly known as "stress of exercise", and is commonly observed in fish capture [8] [30] [31]. Different studies reported a

29.05% decrease in muscle glycogen due to stress in carps [32] and up to 53% in mammals [33]. It should be mentioned that mammals have a higher concentration of glycogen than fish [34]. Positive values were obtained in the muscle samples of hydrolyzed glycogen in the giant mantas; however, these did not show a significant difference between tourism levels. Muscle glycogen may not be a representative metabolite for assessing chronic stress due to tourism in giant mantas. There were no statistical differences in this polysaccharide between adults and juveniles. It is important to mention that, in almost all organisms, the body adapts over time to a stressor, and even if it is still present, glycogen can return to a maintenance level [30].

It is necessary to increase the sample size and the periodicity of the sampling, as Black and Malcolm [30] suggest that the general swimming activity varies from month to month in relation to changes in currents and this can be a key point in periodic glycogen changes. It is recommended to evaluate other variables or supplement with other metabolites that respond to stress to obtain a better understanding of what is happening physiologically in giant mantas and how they are responding to external agents.

5. Conclusion

The low presence of tourists in November seems to be a trigger sufficiently stressful to produce oxidative stress in giant mantas' muscle tissue, unlike the period of greater tourism. This evidence suggests an adaptation by these animals to diver presence that visit the Park, despite the annual increase of tourism. There is a higher CAT activity in males that suggests a cross-reaction of tourism stress with the breeding season. The first physiological difference between chevron and black morphotypes regarding oxidative stress was recorded, where the chevron morphotype is more stressed than black morphotypes. Glycogen was not found to be an efficient metabolite for inferring the chronic stress status of mantas in presence of a stressor such as tourism.

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Author Contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by Edgar M. Hoyos-Padilla, Fernando R. Elorriaga-Verplanken, and Felipe Galván-Magaña; analysis was performed by Guillermo Valdivia-Anda, Renato Peña and James T. Ketchum. The first draft of the manuscript was written by Carolina Hernández-Navarro and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available due to data misappropriation but are available by request to the corresponding author.

Ethical Approval

Protocol number 16022, UC Davis Institutional Animal Care and Use Committee.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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