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Short Communication

Oxidative stress and RNA/DNA ratio following longline capture in the silky shark *Carcharhinus falciformis* (Müller & Henle, 1839)

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ABSTRACT. This study used transcriptomics tracers and measurements of oxidative stress to examine the effect of capture by longline on the silky shark *Carcharhinus falciformis*. From February 2010 to May 2012, *C. falciformis* individuals captured in the eastern Pacific Ocean were initially examined for sex, degree of maturity, and morphometric data. Samples were then taken from muscle tissue to determine RNA, DNA, and protein concentrations as well as RNA/DNA and protein/DNA ratios. The levels of three oxidative stress indicators [superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA)] were determined in muscle. The analysis of the 159 specimens (93 juveniles and 66 adults) showed low RNA/DNA ratios at the different stages of maturity. This basal level of transcriptomics capacity may have been caused by the physiological stress resulting from capture. In juveniles, the low RNA/DNA and protein/DNA ratios were associated with low levels of cellular damage related to oxidative stress, whereas in adults the level of cell damage due to oxidative stress was high, especially in older females. This pattern indicated decreased antioxidant response capacity with increasing age in elasmobranchs.

Keywords: *Carcharhinus falciformis*, reactive oxygen species, RNA/DNA ratio, protein/DNA ratio, superoxide dismutase, malondialdehyde.

Biochemical/molecular tools can be used to determine the physiological condition of fish and thereby explore possible relationships associated with somatic growth, nutritional status, and exposure to pollutants (Balza *et al.*, 2007). DNA and RNA as well as the relationship between them have long been used as reliable molecular markers of increased cell numbers and protein synthesis. The RNA/DNA ratio reflects the cellular transcriptomics capacity and is directly related to tissue formation. It is thus a key indicator of cell metabolic intensity and a practical tool to infer growth levels and responses (Sulkin *et al.*, 1975; Buckley *et al*., 1999; Chícharo & Chícharo, 2008).

The highly dynamic behavior of elasmobranchs implies the early induction of oxidative stress responses, including the production of reactive oxygen species (ROS), the levels of which are proportional with age. ROS participate in the functional impairments associated with aging, by causing oxidative damage to lipids, proteins, and nucleic acids (Martínez-Álvarez *et al*., 2005). Prooxidant activity is exacerbated by extreme conditions such as capture, which induces a wide range of physiological alterations related to physical overexertion, including hypoxia and hemorrhage (Gelsleichter, 2004). Mandelman & Skomal (2009) reported differences in the blood acid-base status of five species of carcharhinid sharks caught by longline fishing. The physiological response to capture differed significantly between species.

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Chronic stress is reflected in an organism's RNA/DNA and protein/DNA ratios, as demonstrated in various marine species (Buckley *et at.*, 1999; Gil *et al*., 2003; Reef *et al*., 2010). The aim of this study was to identify possible relationships between these transcriptome tracers and oxidative stress in the silky shark *Carcharhinus falciformis*. Individuals of different ages caught by longline fishing in the Mexican central Pacific were evaluated. The results shed light on the relationships between: 1) the levels of nucleic acids and oxidative tracers and critical processes indicative of physiological exhaustion due to the physical effort exerted during capture, and 2) specific physiological stress conditions and the maturity, age, and sex of the silky shark.

During February 2010 to May 2012, six field surveys and samplings were conducted on-board vessels of a medium-scale Mexican longline fleet fishing for sharks in the eastern Pacific Ocean. The boats departed from and returned to the port of Manzanillo, Colima, Mexico. The fishing area is located between the 15º76'-17º31'N and 103º29'-105º05'W.

The total length (TL, in cm) and sex of each shark was recorded, together with the degree of maturity, based on the scale described by Castro (1993). Muscle tissue samples (0.5 g) were taken from the antero-dorsal portion of live sharks, preserved in the tissue preservative All protect tissue reagent (Qiagen) at a concentration of 100 mL per 10 mg of muscle, and stored in the laboratory at -50°C until analyzed. Blood samples (2 mL) were preserved in EDTA (1 mM) and kept at 4°C until the analysis.

Nucleic acids and proteins were extracted and purified for total DNA/RNA/protein analyses using the Nucleo Spin® TriPrep commercial kit, which ensures efficient, high-quality separation of the three cellular components. RNA and DNA concentrations were determined by measuring the absorbance at 260 nm (A260) using a spectrophotometer (Lambda 35 Perkin, New York, USA). Oxidative stress in muscle was evaluated by measuring the levels of the metalloenzyme SOD, which catalyzes the dismutation of superoxide anion to molecular oxygen and hydrogen peroxide, a crucial component of the cellular antioxidant defense mechanism (Malstrom *et al*., 1975). SOD was measured using a SOD assay kit (Cayman Chemical, USA; cat. no. 706002). The antioxidant glutathione (GSH) was measured using a glutathione assay kit (Cayman Chemical Company; cat. no.703002). Lipid peroxidation was assessed using a thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical Company; cat. no. 10009055) based on the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA) at high temperatures (90-100°C) under acidic conditions.

The size structure of shark sampling was described using sex-specific length-frequency histograms that groups individuals by 10 cm TL classes. The ages of the examined specimens were estimated based on growth parameters previously reported for *C. falciformis* in the study area (Zea de la Cruz, 2007) and using the equation of von Bertalanffy (Sparre & Venema, 1997):

$$t = t_0 - (1/K) * ln(1 - L_t/L_\infty)$$

where L_t is the observed length, L_∞ is the asymptotic length, t_0 is the theoretical age at zero length, and K is the growth rate. The sex-specific age structure of the specimens was also described using length-frequency histograms. The growth rate at each age was calculated using the following equation (Sparre & Venema, 1997):

$$\frac{\Delta L}{\Delta t} = (L_\infty - L_i)\, K$$

where L_i is the length at age t in years.

Biochemical data (SOD, GSH, and MDA) as well as RNA/DNA and protein/DNA ratios were tested for normality and homogeneity of variance. Data that failed the assumption of normality were log-transformed. A two-way analysis of variance (ANOVA II, $\alpha = 0.05$) was used to statistically compare the mean values of the biochemical indexes with respect to sex and maturity stage. In addition, relationships between age and growth rate and between age and RNA/DNA were examined for evidence of the trends in the respective slopes (positive or negative) by linear regression analysis and by testing the significance of the slopes (t-test, $\alpha = 0.05$). The sex-specific relationships between TL (in cm) and the levels of the three oxidative indices (SOD, GSH, and MDA) were similarly evaluated for their slope trends by linear regression analysis. The statistical analyses are described in detail in Zar (1999).

In fish, the stress response involves multiple physiological reactions whose products can be measured in blood and muscle tissue. According to the criteria of Mendoza-Alfaro *et al.* (2002), in their study of the RNA/DNA ratio in fish larvae, a value of 1.0 indicates a physiological condition in which the RNA content of the organism has decreased to the limit necessary to survive. In the particular case of elasmobranchs, Tavares *et al.* (2006), studied the transcriptomic relations in the dog shark *Mustelus canis* and reported a range of 1.5-2.7 for adult males and 1.1-3.6 for females. In our study, the mean values ranged from 0.41 to 0.48 in both sexes (Table 1).

Table 1. Mean ± SD, RNA/DNA and protein/DNA ratios in *Carcharhinus falciformis*, analyzed by sex and maturity stage.

Sex/Maturity stage	RNA/DNA	n	Protein/DNA	n
Males				
Young	0.48 ± 0.30	53	62.39 ± 48.07	50
Adult	0.47 ± 0.25	36	52.72 ± 43.22	38
Females				
Young	0.41 ± 0.19	40	49.56 ± 36.21	35
Adult	0.43 ± 0.21	30	74.61 ± 54.12	29

The lower values in our samples may reflect a poorer physiological condition of the sharks evaluated in the present study, due to stress factors such as the physiological attrition derived from being fished and captured, nutritional status, and the time elapsed after digesting the last prey (Mendoza-Alfaro *et al.*, 2002; Buckley *et al.*, 2004). Kieffer (2000) reported that an inevitable consequence of shark fishing is the extreme muscular fatigue of the fish. An additional, high-level source of physiological stress is the out-of-water exposure accompanied by the physical trauma derived from hook damage.

Prolonged food deprivation of the silky shark could also lead to a decline in the assayed molecular indicators, in turn compromising the antioxidant defense system. However, the level of SOD activity in the adult females sampled was within the range reported in adult muscle of this species by López-Cruz *et al.* (2010). Those authors reported an activity for SOD of 0.05 U mg^{-1} protein, which is very similar to the activity measured in the present investigation (Table 2). The antioxidant response was also highest in adult females, as evidenced by the highest concentrations of GHS. Moreover, transcriptomic activity, determined by calculating the RNA/DNA and protein/DNA ratios, were higher in this group than in males and young females (Table 1). This result is explained by the presence of three pregnant females among the 30 adult females. The physiological processes active during pregnancy likely include the capacity to increase energy reserves to levels that are higher than during other life-history stages (Ebert, 2002). In a *t*-test, the slopes for antioxidants were significant only in females (Table 3). By contrast, in males, SOD concentrations were relatively constant (Table 3). However, MDA concentrations were higher than the levels considered to be high (1.5-4.5 μmol g^{-1}) as reported for elasmobranchs by Wilhelm & Boveris (1993). The increased levels of GSH in adult females (Fig. 1) suggest that in this stage, as in many other organisms, non-enzymatic antioxidants of exogenous origin provide an increased functional reserve. An increase in GSH levels with increasing age was reported for the shark *Squalus acanthias* by Rudneva-Titova (1997). This author showed that a lack of CAT and SOD activity in erythrocytes was accompanied by an increase in the levels of urea, GSH and vitamin K, presumably to compensate for the limited capacity of the enzymatically based antioxidant system.

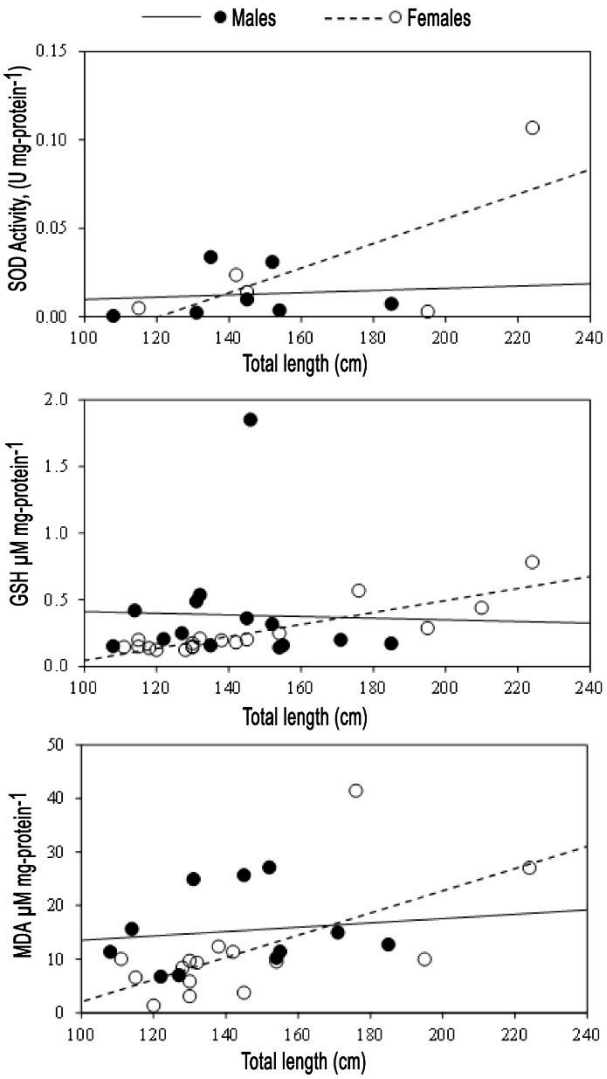

Figure 1. Relation between the total length and three indicators of oxidative stress (SOD, GSH and MDA) for males and females of *Carcharhinus falciformis*. Regression lines fitted to the data are also shown.

Paon & Kenchington (1995) and Pérez-Camacho *et al.* (2003) suggested that the high costs of reproduction in females required increased levels of protein synthesis. Barrera-García *et al.* (2013) measured higher levels of glutathione peroxidase and CAT activity in immature than mature females of the blue shark (*P. glauca)*, in contrast to the higher SOD levels in adult than young females. The levels of MDA in these speci-

Table 2. Mean ± SD, RNA/DNA and protein/DNA ratios in *Carcharhinus falciformis* analyzed by sex and maturity stage.

Sex/maturity stage	SOD U (mg protein^{-1})	n	GSH (µM mg protein^{-1})	n	MDA (µM mg protein^{-1})	n
Males						
Young	0.013 ± 0.014	6	0.42 ± 0.47	12	15.54 ± 8.20	9
Adult	0.007	1	0.18 ± 0.02	2	13.84 ± 1.57	2
Females						
Young	0.014 ± 0.009	3	0.17 ± 0.04	14	7.58 ± 3.48	12
Adult	0.054 ± 0.073	2	0.52 ± 0.21	4	25.15 ± 15.75	3

Table 3. Trends in the slopes obtained by linear regressions of length (TL) *vs*. oxidative indices (SOD, GSH and MDA) in *Carcharhinus falciformis* as determined in a *t*-test. Significant *P*-values are indicated in bold.

Relation/sex	Slope (b)	*t*-test	*P*
Length *vs* SOD			
Males	0.000	0.242	0.819
Females	0.001	1.936	**0.043**
Length *vs* GSH			
Males	-0.001	-0.106	0.918
Females	0.005	6.921	**0.000**
Length *vs* MAD			
Males	0.040	0.397	0.701
Females	0.207	2.963	**0.011**

mens evidenced the extensive cellular damage due to oxidative stress (Fig. 1).

In the present investigation, the sharks collected by longline fishing remained alive for an average of 3 h. The resulting physiological stress was manifested not only as low RNA/DNA and protein/DNA ratios in muscle tissue but also as increases in the stress markers discussed above. In general, the defense system that protects fish against oxidative stress weakens with age (Martínez-Álvarez *et al.*, 2005), resulting in a gradual imbalance between increased ROS production and decreases in enzymatic and non-enzymatic antioxidant defenses. In turn, there is damage to lipids, proteins, and nucleic acids and thus functional deteriorations associated with aging. In our *C. falciformis* specimens, this sequence of events was suggested by comparing adults and juveniles. In the latter, SOD activity was much lower than the value reported by López-Cruz *et al.* (2010). The MDA concentrations in juveniles were also low. Moreover, elasmobranchs also have a non-enzymatic line of defense to compensate for oxidative imbalances associated with the dynamics of oxygen consumption and periods of starvation (Solé *et al.*, 2009).

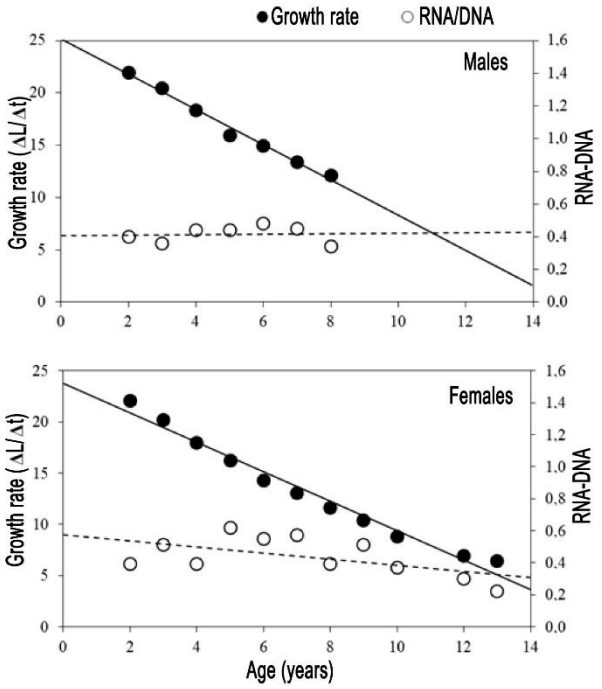

Figure 2. Relation between age and the growth rate and between age and the mean RNA/DNA ratio for males and females of *Carcharhinus falciformis*. Regression lines fitted to the data are also shown.

Our results suggest that this system becomes more functional as the organisms reach greater stages of maturity.

The analysis of age *vs* the RNA/DNA ratio showed that both the growth rate and the RNA/DNA ratio decreased with increasing age, in males as well as females (Fig. 2). However, the relationship was significant only in females, perhaps because of the larger age structure of the females the males evaluated in this study (Table 4). In a study by Tavares *et al.* (2006) that included *M. canis* juveniles, the RNA/DNA ratio decreased as the size of the shark increased. The discrepancy in these results can perhaps be attributed to

Table 4. Trends in the slopes obtained by linear regressions of age (years) *vs* the growth rate or the RNA/DNA ratio of *Carcharhinus falciformis*, as determined in a *t*-test. Significant *P*-values are indicated in bold.

Relation/sex	Slope (b)	*t*-test	*P*
Age *vs* growth rate			
Males	-1.685	-19.701	**0.000**
Females	-1.438	-19.871	**0.000**
Age *vs* RNA/DNA			
Males	0.001	0,136	0.897
Females	-0.019	-2.033	0.073

the time at which the specimens were caught, their biological habits, environmental conditions, the number of organisms analyzed, and their degree of maturity. Our study included both juveniles and adults, such that the size and age structures were large. The low RNA/DNA ratio at the different stages of maturation may have reflected a basal state in transcriptomics capacity imposed by the physiological stress related to efforts to escape being fished.

Age-related changes in the biochemical indicators were determined in this study. Thus, in juveniles, the low RNA/DNA and protein/DNA ratios occurred together with low levels of cellular damage due to oxidative stress. By contrast adults were characterized by both high-level transcriptomic activity and cell damage due to oxidative stress, especially in older females. These results are consistent with a pattern of a decreasing antioxidant response capacity with increasing age in elasmobranchs.

The different stress factors associated with the response to being fished, such as time on the line, hypoxia, and hemorrhage, are manifested by changes in the levels of various biochemical stressors. Future research will benefit from the development of a sampling scheme in which the organisms are not stressed during their capture.

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