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Plasma cholinesterase activity as an environmental impact biomarker in juvenile green turtles (*Chelonia mydas*)[1]

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ABSTRACT.- Fonseca L.A., Orozco A.M.O., Souto P.C., Dornelas L.R.S., Filho W.P.C., Girardi F.M., Ermita P.A.N. & Fagundes V. 2020. **Plasma cholinesterase activity as an environmental impact biomarker in juvenile green turtles (*Chelonia mydas*).** *Pesquisa Veterinária Brasileira 40(1):72-76.* Departamento de Veterinária, Universidade Federal de Viçosa, Av. Peter Henry Rolfs s/n, Campus Universitário, Viçosa, MG 36570-900, Brazil. E-mail: leandroabreu@ufv.br

The objective of this study was to evaluate the enzymatic activity of plasma cholinesterase in *Chelonia mydas* marine turtles belonging to two populations, according to their capture sites, under the absence and probable influence of anthropic effects. A total of 74 animals were used and later divided into two groups, based on the capture site. Blood samples were collected from all captured animals, which were then released into the sea at the site of capture. A descriptive statistical analysis of the plasma cholinesterase activity values and an analysis comparing these values based on the capture site were performed. Samples of heparinized plasma from animals captured at the two different sites were analyzed. Plasma cholinesterase activity ranged from 121 to 248U/L, with a mean and standard deviation of 186.1±30.68U/L. When comparing plasma cholinesterase activity values in individuals based on the capture site, a significant difference was observed. Establishing reference values for different sea turtle populations is necessary to interpret future sampling results and to allow sea turtles to be used as sentinels of ecosystem health. Future studies are needed to evaluate other populations and the activity of plasma cholinesterase in juvenile marine turtles, in relation to environmental contamination.

INDEX TERMS: Plasma cholinesterase, environmental impact, biomarker, green turtles, *Chelonia mydas*, cholinesterase, marine turtle, biochemical markers, turtles, wildlife animals.

RESUMO.- [**Atividade da colinesterase plasmática como biomarcador de impacto ambiental em tartarugas verdes juvenis (*Chelonia mydas*).**] O objetivo desse estudo foi avaliar a atividade enzimática da colinesterase plasmática em tartarugas marinhas da espécie *Chelonia mydas* em duas populações de acordo com o local de captura, sob ausência e provável influência de efeito antrópico. Foi utilizado um total de 74 animais e posteriormente divididos em dois grupos de acordo com o local de captura. Foram coletadas amostras de sangue de todos os animais capturados e em seguida liberados ao mar no mesmo local. Foi realizada uma análise estatística descritiva dos valores da atividade plasmática de colinesterase do total de animais e análise comparando os valores de acordo com o local de captura. Foram analisadas amostras de plasma heparinizado de animais capturados em dois locais distintos. Os valores da atividade plasmática de colinesterase variaram de 121 a 248U/L, com média e desvio padrão de 186.1±30.7U/L. Quando comparados os valores de atividade plasmática da colinesterase nos indivíduos de acordo com o local de captura, foi observada diferença significativa. O estabelecimento de valores de referência para diferentes populações de tartarugas marinhas são necessários para

interpretar os futuros resultados amostrais e permitir que as tartarugas marinhas sejam usadas como sentinelas da saúde do ecossistema. Estudos futuros são necessários para avaliar outras populações e a atividade da colinesterase plasmática de tartarugas marinhas juvenis em relação à contaminação ambiental.

TERMOS DE INDEXAÇÃO: Colinesterase plasmática, biomarcador, impacto ambiental, tartarugas verdes, *Chelonia mydas*, tartaruga marinha, marcadores bioquímicos, animais selvagens.

INTRODUCTION

With modernization, industrial and urban development has been expanding, which brings important environmental impacts. In light of this, it is necessary to control the emission of pollutants, since they can compromise water resources, which are of great importance to ecosystems (Freitas 2009). The production of potential synthetic compounds from industrial, agricultural and domestic activities is responsible for much of the contamination of the waters that reach rivers and oceans. Accordingly, a multitude of chemical products in various concentrations (Freire et al. 2008) accumulate in animals living in marine environments (Coito et al. 2007).

Monitoring the aquatic environment is necessary. For this, biomarkers have been used to identify the presence of contaminating agents and their interaction with the organisms (Sarkar et al. 2006) that use these environments, in a predictive manner, thus avoiding the occurrence of irreversible environmental damage (Cajaraville et al. 2000). Some environmental monitoring programs have used the inhibition of acetylcholinesterase enzymatic activity in fish and other animals as a biomarker indicative of environmental contamination by organophosphates and carbamates (Oliveira et al. 2007).

Cholinesterases are enzymes that are responsible for the hydrolysis of choline esters. There are two types of cholinesterases: I) acetylcholinesterase (AChE) or erythrocyte cholinesterase, which is most concentrated in the central nervous system, striated muscle and erythrocyte membrane; and II) butyrylcholinesterase (BChE) or plasma cholinesterase, which is most abundant in plasma (Kramer & Hoffmann 1997). According to other studies, acetylcholinesterase may also be inhibited by heavy metals and surfactants (Coito et al. 2007).

According to Fonseca et al. (2015),marine turtles of the *Chelonia mydas* species can be used as sentinels of exposure to pollutants that inhibit the enzymatic activity of plasma cholinesterase, since these animals have detectable activities of this enzyme. However, these authors suggest evaluating the enzymatic activity in this species under different conditions and exposures, both *in situ* via comparison between sites and *ex situ* through bioassays.

The objective of this study was to evaluate the enzymatic activity of plasma cholinesterase in marine turtles belonging to the *C. mydas* species; these were in two populations, according to their capture sites, under the absence and probable influence of anthropic effects.

MATERIALS AND METHODS

Experimental design. A total of 74 animals were used in our study and later divided into two groups based on their capture sites: G1 and G2. All captured animals were banded; biometric data and blood were collected immediately after capture. The animals were then released into the sea at the same site. The animals were manually captured at low tide by fishing, using a hand net, or diving, in strategic locations and monitored by GPS. Licenses for the collection, transport and handling of biological material were granted by the Biodiversity Information and Authorization System (SISBIO)/ICMBio - IBAMA, under registration number 26080.

A descriptive statistical analysis of the plasma cholinesterase activity values of the animals and an analysis comparing these values based on the capture sites were performed. The G1 comprised 35 individuals captured in the Bay of Sueste in the Archipelago of Fernando de Noronha, state of Pernambuco (3°51′26″ S and 32°25′33″ W), Brazil. The region is characterized as an important feeding and spawning area of *Chelonia mydas* (Bellini et al. 1996, Baptistotte 2007). Because the animals were in the open sea, the manual capture involved continuous, prolonged chases and required diving and pursuit durations of over 60 minutes until the individuals could be captured and transported to the beach. During this pursuit, the capture was realized when the individuals, worn out by the fugue, rose to the surface to breathe and could not escape the capture. Following the capture, the animals were transported to the beach for blood collection, banding, biometry measurements, and subsequent release.

The G2 comprised 39 individuals captured from the final effluent of a steelworks company in the city of Vitória, state of Espírito Santo (20°15′49″ S and 40°13′43″ W), Brazil. In this region, seawater is collected and used for cooling in industrial operations, thus causing an increase in the local average temperature of the water, in addition to receiving domestic and industrial sewage after treatment. The high water temperature and the richness of organic matter favor the growth of algae. According to Torezani et al. (2010), these conditions lead to noticeable growth, mainly of green algae *Enteromorpha flexuosa*, but also of *Pterocladiella* sp., *Jania* sp., *Arthrocardia* sp. and *Chaetomorpha* sp. Four capture points were previously defined on both sides of the effluent channel. This effluent channel is 500m long and approximately 33m wide, with an average depth of 2m.

Table 1. Characteristics of study groups (G1 and G2) of the present study

Criteria	G1	G2
Capture and manipulation time	Average over 60 minutes	Average less than 60 minutes
Average water temperature	28°C[a] (82.4°F)	22°C (71.6°F), increased by 8.75°C[b] (47.8°F)
Capture method	Diving with continuous pursuit	Fishing hand net
Number of animals	35	39
Site	Fernando de Noronha/PE	Vitória/ES

[a] Accoding to Mendes (2006), [b] According to Torezani (2010).

As the animals were aggregated in the effluent channel, and by the use of fishing hand nets, the process involved surprising the animals without persecution (Table 1).

The areas used in our study were chosen and characterized using the information described by Dos Santos et al. (2010), who evaluated the environmental quality of both localities using the ecological evaluation index (EEI) (Orfanidis et al. 2001, 2003) and concluded that the area used in our study to capture individuals from G1 was classified as of good quality, in contrast to the area used to capture individuals from G2 that was characterized as of bad quality.

Collection of blood and biometric data. From all the captured individuals, carapace size data (in centimeters); curvilinear carapace length (CCL) and weight (in grams) were obtained to characterize the age of the animal, and blood was collected. Recaptured animals were evaluated, measured, weighed, and released without further blood collection. The sex of the animals was not determined due to the absence of external sexual dimorphism at this stage of the life cycle. Peripheral blood collection was performed by venipuncture of the cervical sinus or jugular vein, preceded by adequate cleaning and antisepsis with iodinated alcohol. Blood was immediately transferred to a 2ml heparin-containing tube (Vacutainer®) and was kept cool in an ice container until arrival at the laboratory.

Sample processing. In the laboratory, heparin tube-conditioned blood was centrifuged for 10 minutes at 4000rpm, and the obtained plasma was transferred to 1.7ml microtubes for cholinesterase dosing. Heparinized plasma was frozen at -20°C (68°F) until the samples were processed. To measure the cholinesterase enzyme activity, we followed the method of Ellman et al. (1961), modified by Sturm et al. (1999), which allows for the quantification of enzyme activity in plasma samples (Fonseca et al. 2015). The determination of cholinesterase plasma activity was performed using the kinetic enzymatic method. The reaction occurs due to the catalytic action of cholinesterase on the hydrolysis of butyrylthiocholine to thiocoline and butyrate. Thus, thiocholine reduces hexacyanoferrate III (which is yellow in color) to hexacyanoferrate II (colorless). The decrease in absorbance was measured at 405nm and represents the intensity of the enzymatic activity. For this dosage, the test had a minimum detection limit of 50U/L and the reaction was linear up to 25000U/L.

Statistical analysis. The results were submitted to descriptive analysis to obtain means and standard deviations. The symmetry of the data distribution was evaluated using a Shapiro-Wilk test, and the homogeneity of the variances was evaluated using a Levene's test. For the determination of the reference range, the outliers were detected and discarded, according to the Tukey procedure, where a result is defined as extreme when its value is less than the first quartile minus 1.5 times the interquartile range, or greater than the third highest quartile plus 1.5 times the interquartile range, similar to that used by Friedberg et al. (2007). After the outliers were removed, the 95% confidence interval was obtained. The procedure was performed using SPSS 20 software (IBM SPSS Statistics). The influence of the capture site on plasma cholinesterase levels was assessed using a Student's t-test for independent samples. The statistical package Minitab 17 (Minitab Inc.) was used and significance was considered when $p < 0.05$.

RESULTS

All captured animals were juveniles, according to the criteria proposed by Hirth (1971), i.e., curvilinear carapace length (CCL) was less than 73.5cm. The animals presented CCLs ranging from 28.4 to 56.3cm, with a mean and standard deviation of 51.8±11.3cm.

Heparinized plasma samples from the 74 animals captured from two different sites were analyzed. All analyzed samples showed some enzymatic activity of plasma cholinesterase. Plasma cholinesterase activity ranged from 121 to 258U/L, with a mean and standard deviation of 186.1±30.7U/L. The results of the descriptive statistical analysis of plasma cholinesterase activity are presented in Table 2 and a graphical representation is presented in Figure 1. When comparing plasma cholinesterase activity values between individuals according to the capture site, a significant difference was observed.
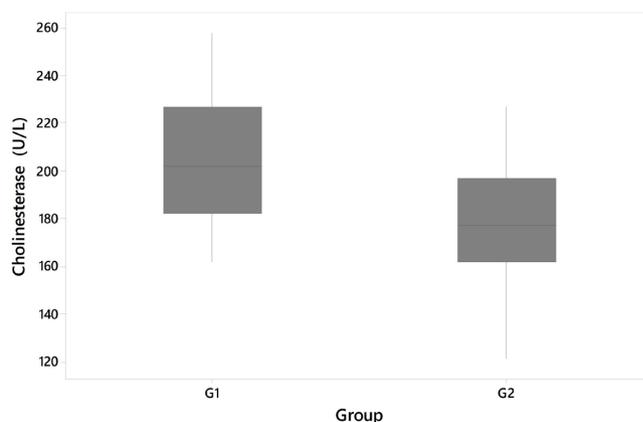

Fig.1. Graphical representation for cholinesterase activity (U/L) in green turtle (*Chelonia mydas*) from Brazil.

Table 2. Descriptive statistics for cholinesterase activity (U/L) in green turtle (*Chelonia mydas*) from Brazil

Statistic	Plasma cholinesterase activity (U/L)		
	Total	Group 1	Group 2
Number of samples	74	35	39
Mean + SD	186.1 + 30.7	178.2 ± 26.9[a]	194.1 ± 32.5[b]
95% Lower confidence interval	178.8	200.1	170.3
95% Upper confidence interval	193.4	232.7	186.7
Minimum	121	162	121
Maximum	258	258	227

SD = Standard deviation; [a,b] Different letters on the same line demonstrate significant statistical difference.

DISCUSSION

In veterinary medicine, the determination of reference biochemical values is indispensable for the monitoring of the health status of individuals of different species. Some of these parameters can be used as biomarkers, and according to Atkinson Junior et al. (2001), their measurement serves as an indicator of a normal biological process, pathogenic, or in response to a therapeutic intervention. In this study, the method used to measure cholinesterase activity was the same as that used by Fonseca et al. (2015) and all animals in the study had some type of activity for this enzyme.

As the animals in the G1 were captured in an environmental conservation unit and did not present any evidence that could directly or indirectly interfere with plasma cholinesterase activity, the data obtained in our study can be proposed as a reference for this site for future studies of *Chelonia mydas* juveniles, as there is no data in the current literature for the studied species and age group.

Animals are often exposed to toxins of various origins. The evaluation of biomarkers permits the evaluation of an individual's exposure to these substances and their health effects on the body (Myers et al. 2017). The difference in the values found between the groups in our study demonstrates that the activity of this enzyme can vary according to the environmental conditions in which the animals live.

Cholinesterase activity has been used as an indicator of environmental contamination in several species and situations. In dogs, the inhibition of cholinesterase activity is an indicator of exposure to organophosphorus toxins (Ferré et al. 2015) and carbamates (Saldeña et al. 2017). In birds, studies have evaluated the activity of this enzyme. In northeast Mexico, cholinesterase activity decreased by 29-49% because of possible exposure to pestivcides (Ruvalcaba-Ortega et al. 2017). In Spain, Oropesa et al. (2017) observed a decrease in plasma cholinesterase activity under *in vitro* conditions in samples of griffon vultures (*Gyps fulvus*) exposed to carbamates, suggesting that it is a suitable biomarker for monitoring the exposure of this toxin.

Similarly, in aquatic environments, the enzymatic activity of cholinesterase has been studied as an indication of environmental contamination (Oliveira et al. 2007). Accordingly, some aquatic species can be considered sentinels. According to the results obtained by Coito et al. (2007), marine sponges belonging to the species *Spongia officinalis* and *Spongia agaricina* show cholinesterase activity and can be used as biomarkers of pesticide exposure. Omar-Ali et al. (2017) found a significant decrease in plasma acetylcholinesterase activity in *Atractosteus spatula* (alligator gar) under chronic exposure to organophosphate pesticide (diazinon). However, in our study, no evidence of environmental contamination by pesticides was measured.

However, plasma cholinesterase activity may vary as a function of exposure to other toxic compounds, depending on the exposure period. When studying the effect of exposure to iron on acetylcholinesterase (AChE) enzyme activity in fish brains and livers, Sant'Anna et al. (2011) observed an increase of this enzyme 24 hours after exposure. Carvalho et al. (2017) obtained similar results when they evaluated the activity of this enzyme in the brain of tadpoles after 48 hours of exposure to different types of heavy metals. However, after 16 days, a significant decrease of the enzymatic activity was demonstrated, which suggests that AChE can be used as a predictive biomarker in heavy metal-contaminated environments.

Environmental contamination by iron ore is an old, widely-discussed problem in the locality in which the G2 resides (Pinheiro 2013). In addition, as already mentioned, the locality of G2 also has an elevated water temperature and the presence of abundant organic matter of algae. In our study, animals from the G2 showed an increase in enzymatic activity, when compared to those of the G1. This difference may suggest an anthropogenic influence on the habitat in which the G2 inhabited. Thus, among some possibilities, it can be assumed that environmental contamination by iron ore, a problem experienced for years by the local population (Nassar et al. 2003), is a possible cause of the elevation of enzymatic activity. However, although analyses of the composition of the local water are carried out periodically, the results were not available in full. Further studies on the composition of the heavy metals present in this water and the time the animals are exposed to the toxics present in the effluent need to be performed.

Das (2007) and Silva et al. (2012) report that acetylcholinesterase and butyrylcholinesterase can be considered biomarkers of low-grade inflammation in various clinical conditions. This is because acetylcholinesterase and butyrylcholinesterase reduce the levels of acetylcholine, a molecule that plays an anti-inflammatory role locally and systemically by inhibiting the production of tumor necrosis factor, interleukin-1, inhibitory macrophage migration factor, and other proinflammatory cytokines. Thus, with increasing levels of acetylcholinesterase and butyrylcholinesterase, there is an increase in inflammatory events in the body.

Finally, according to Fonseca et al. (2015), because of the longevity of *C. mydas* sea turtles and their herbivorous feeding behavior, together with the fact that they have detectable plasma cholinesterase activity, this species can be proposed as a bioindicator of exposure to pollutants that influence the activity of this enzyme, especially in environments influenced by industrial activities. For this, it is necessary to evaluate enzymatic activity under different conditions and exposures, both *in situ* by comparison between sites and *ex situ* through bioassays. The possibility of using blood samples permits the development of studies that use minimally invasive techniques and minimize the ethical considerations of animal use in research.

CONCLUSIONS

In the present study, significant differences in plasma cholinesterase activity were observed among the evaluated populations.

Group 2 turtles showed higher activity of AChE in relation to Group 1; this could be attributed to an altered state caused by the anthropic effect on the habitat of these animals.

Establishing reference values for different sea turtle populations is necessary to interpret future sampling results and allows sea turtles to be used as sentinels of ecosystem health.

Future studies are necessary to evaluate other populations and the effect of different toxins on plasma cholinesterase activity in juvenile sea turtles.

Conflict of interest statement.- The authors have no competing interests.

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