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Life after death? Exploring biochemical and molecular changes following organismal death in green turtles, *Chelonia mydas* (Linnaeus, 1758)

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HIGHLIGHTS

- Levels of biomarkers were analyzed in turtles sampled up to 24 h after euthanasia.
- Antioxidant activity and MDA levels were stable for up to 24 h *post mortem.*
- Carbohydrate's metabolism did not change within a 24 h *post mortem* period.
- Green turtles found stranded up to 24 h after death can be used for biomonitoring.

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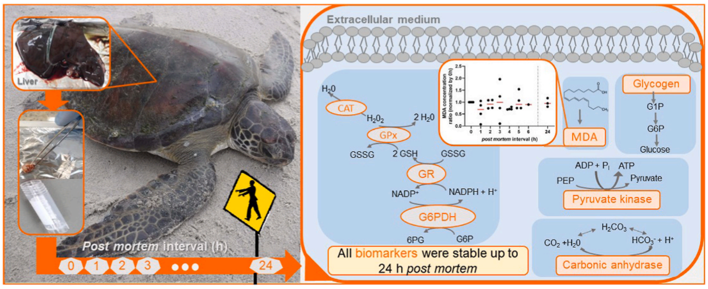

ABSTRACT

Green turtles, *Chelonia mydas*, have been included in biomonitoring efforts given its status as an endangered species. Many studies, however, rely on samples from stranded animals, raising the question of how death affects important biochemical and molecular biomarkers. The goal of this study was to investigate *post mortem* fluctuations in the antioxidant response and metabolism of carbohydrates in the liver of *C. mydas*. Liver samples were obtained from six green turtles which were submitted to rehabilitation and euthanized due to the impossibility of recovery. Samples were collected immediately after death (t = 0) and at various time intervals (1, 2, 3, 4, 5, 6, 12, 18 and 24 h *post mortem*), frozen in liquid nitrogen and stored at −80 °C. The activities of catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH) were analyzed, as were the levels of lipid peroxidation, glycogen concentration, RNA integrity (RNA IQ) and transcript levels of carbonic anhydrase and pyruvate carboxylase genes. Comparison between *post mortem* intervals showed a temporal stability for all the biomarkers evaluated, suggesting that changes in biochemical and molecular parameters following green turtle death are not immediate, and metabolism may remain somewhat unaltered up to 24 h after death. Such stability may be associated with the overall lower metabolism of turtles, especially under an oxygen deprivation scenario such as organismal death. Overall, this study supports the use of biomarkers in sea turtles sampled within a period of 24 h *post mortem* for biomonitoring purposes, though it is

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recommended that *post mortem* fluctuations of particular biomarkers be evaluated prior to their application, given that proteins may show varying degrees of susceptibility to proteolysis.

1. Introduction

Green turtles, *Chelonia mydas* (Linnaeus, 1758), are found around the globe in tropical waters and, to a lesser extent, in subtropical areas (Broderick and Patricio, 2019). The species is highly migratory, with nesting areas in a great number of countries. In Brazil, green turtles are known to nest in oceanic islands and/or archipelagos such as Trindade, Rocas Atoll and Fernando de Noronha (Almeida et al., 2011). Nonetheless, the species is considered the most coastal among the sea turtles occurring in Brazil, frequently occurring in estuaries and river mouths (Marcovaldi et al., 2011). Indeed, more than 26 thousand green turtles have been found stranded along southeastern coast of Brazil in the last five years (data from https://simba.petrobras.com.br/simba/web). The majority of stranded animals are juveniles that use the coastal areas to forage, while adults and youngsters are more commonly found in oceanic waters or close to their original reproductive ground (Bolten, 2003; Musick and Limpus, 1993).

Due to its proximity to the coast, *C. mydas* is exposed to a variety of human impacts, with bycatch from fisheries representing a major cause of death among juveniles (Marcovaldi et al., 2011). Habitat degradation, ingestion of debris (as plastics), climate change and exposure to pollutants also poses a significant threat to the conservation of this species. Previous studies have shown the bioaccumulation of chemical contaminants in tissues of green turtles found in Brazil, with a prevalence of PCBs and organochlorine pesticides (OCPs), such as lindane, heptachlor and DDTs, as well as metals, such as Cu, Fe and Pb (da Silva et al., 2016; Filippos et al., 2021; Jarcovis et al., 2021; Sánchez-Sarmiento et al., 2017).

The impact of anthropic activities on survival and health of *C. mydas*, associated with their long-life cycle and late sexual maturation has prompted their inclusion on the IUCN Red List of Threatened Species as an endangered species in some regions of the globe. Likewise, green turtles are included on the Official Brazilian List of Endangered Fauna Species (Ministério do Meio Ambiente, 2014). The potential risks associated with the exposure to pollutants has also encouraged their inclusion as a target species in the ongoing Projeto de Monitoramento de Praias na Bacia de Santos (PMP-BS, in English "Santos Basin Beach Monitoring Project"), carried out by PETROBRAS to satisfy environmental constraints of the federal environmental licensing of PETROBRAS' activities of production and outflow of oil and natural gas in Santos Basin, conducted by IBAMA in Brazil.

Given legal, logistic and mainly the ethical constraints of collecting individuals of this endangered species for the purpose of environmental biomonitoring, PMP-BS and other monitoring efforts rely greatly on samples from stranded green turtles. However, the use of dead animals for biomarker analysis may be biased due to the biochemical and molecular changes following the organismal death.

Many studies have described *post mortem* changes in biochemical and molecular parameters in vertebrates. For instance, in a review of studies with porcines, Scheffler and Gerrard (2007) reported that the ATP synthesis – following mammal death – was maintained in skeletal muscles through the catabolism of glycogen, generating a buildup of lactate. In bovines, the *post mortem* interval (0–10 days of display) was associated with an increase in the levels of lipid peroxidation and variations in antioxidant activity (Pastsart et al., 2013). In human brain samples, levels of lipid peroxidation also increased during the *post mortem* period (up to 26 h), though antioxidant activity remained stable in this tissue (Harish et al., 2011).

Regarding molecular parameters, Pozhitkov et al. (2017) reported, in a comparative study between zebrafish and rat, that while the total abundance of gene transcripts decreased along the *post mortem* period, transcripts of more than a thousand genes became more abundant after the organismal death. The most abundant transcripts were associated to stress, immune response, inflammation and apoptosis-related genes, suggesting that *post mortem* alterations might depend on the biological role of the gene and/or protein. Interestingly, the *post mortem* changes in gene transcription were different between the two analyzed species, suggesting that organismal death may influence the biochemical and molecular parameters in a species-specific manner.

Like other reptiles, turtles are able to tolerate anoxic conditions much longer, showing less metabolic changes than mammals, which can be related to several metabolic traits. As ectotherms, turtles display a significantly lower metabolism than endotherms, with similar body temperature and size, and are capable to reduce it even further when facing anoxic conditions (Jackson, 2000). For instance, in hepatocytes from the freshwater turtle *Chrysemys picta bellii*, ATP production under anoxia is reduced by 90% of those observed under aerobic conditions (Buck et al., 1993). The reduction in ATP synthesis is accompanied by a reduction in ATP hydrolysis, balancing the system (Hochachka et al., 1996). Turtles are also capable of neutralizing the acidosis generated by the increased levels of lactate produced under anaerobic metabolism, using extracellular (pericardial and peritoneal fluids) and intracellular fluids as buffers and, under extreme conditions, through the release of carbonate from the shell and bones (Jackson, 2000).

Given the turtle's metabolic features, the present study aimed to investigate the *post mortem* changes of biochemical and molecular parameters involved in metabolic pathways expected to be influenced by the lack of oxygen which follows *C. mydas* death, such as: carbohydrate metabolism (through transcript levels of carbonic anhydrase (*CA*), pyruvate carboxylase (*PYR*), and glycogen levels), oxidative stress (through the activity of the antioxidant enzymes catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glucose 6-phosphate dehydrogenase (G6PDH)) and lipid peroxidation (through malondialdehyde (MDA) levels).

CAs are responsible for the hydration of CO_2, participating in pH regulation, and other processes such as the synthesis of urea and glucose, especially under an increased lactate production (Chegwidden et al., 2000; Dodgson, 1991). PYR catalyzes the carboxylation of pyruvate to oxaloacetate, a crucial step for gluconeogenesis. Under fasting and/or increased lactate production, both gluconeogenesis and glycogen breakdown are expected to act as the main sources of glucose (Rotman et al., 1991).

Among biomarkers of oxidative stress, CAT is responsible for the decomposition of hydrogen peroxide (H_2O_2) into water and molecular oxygen. GPx also acts to degrade H_2O_2, catalyzing its reduction as well as the reduction of other reactive organic peroxides through the concomitant oxidation of GSH to GSSG. GSSG is reconverted to GSH through GR activity, which uses NADPH as an electron donor. NADPH is, in turn, produced by G6PDH via the pentose phosphate cycle (Regoli et al., 2011). Lastly, the lipid peroxidation biomarker included in this study, MDA is a secondary lipid peroxidation product, extensively used as an indicator of injury associated with oxidative stress (Valavanidis et al., 2006).

Taken together, the results of the present study are expected to enhance our knowledge about the *post mortem* metabolic responses in turtles and help to establish the best *post mortem* period to quantify biomarkers to be assessed in pollution biomonitoring that can express *in vivo* levels.

2. Material and methods

2.1. Sample collection

Samples of *C. mydas* ($n = 6$) were obtained from animals euthanized at rehabilitation facilities maintained by either the TAMAR Project (Brazilian Center for the Protection and Research of Sea Turtles Foundation) or by the PMP-BS. The physical characteristics of each specimen included in this study are shown in Table 1.

The decision to euthanize the animals was based solely on their clinical condition and recovery perspectives, determined by veterinarians. Five animals were maintained under rehabilitation for at least one week before being euthanized, while one green turtle (ID number 49550; Table 1) was euthanized on the same day of arrival at the rehabilitation facility due to its severe clinical condition (Table S1, Supplementary Material). The details regarding the drugs administered during rehabilitation and for euthanasia of the green turtles are presented in Table S2 (Supplementary Material).

Immediately after euthanasia, a liver sample was obtained and fractioned into subsamples, individually stored in cryovials and either immediately frozen in liquid nitrogen ($T = 0$) or maintained at room temperature under laboratory conditions and placed in liquid nitrogen after pre-determined *post mortem* intervals (1, 2, 3, 4, 5, 6 and 24 h after euthanasia). Samples were preferably taken near to the hepatic portal vein, in accordance with the procedure normally conducted by the veterinarians of PMP-BS for chemical and biomarker analyzes. Two turtles (127048 and 138975) were sampled at differing intervals (*post mortem* intervals specified in Table 1) and thus, were not included in statistical analysis. All samples were maintained at −80 °C until analysis.

2.2. Enzyme assays

For catalytic assays, liver samples were homogenized in Tris buffer (Tris-HCl 50 mM, pH 7.4, 150 mM KCl, 1 mM DTT and 0.5 mM PMSF) and centrifuged at 9000×*g* for 30 min at 4 °C. The supernatant, corresponding to the S9 fraction, was maintained at −80 °C and used for assays to determine the activities of the following enzymes involved in the antioxidant system: catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glucose-6-phosphate dehydrogenase (G6PDH).

CAT activity was determined following to the method described by Aebi (1984). For this assay, samples were combined with an assay mixture (1 M Tris-HCl, 5 mM EDTA, pH 8.0) containing hydrogen peroxide (H_2O_2) at a final concentration of 10 mM. The decomposition of H_2O_2 was accompanied colorimetrically at 240 nm.

GR and GPx activities were determined based on the consumption of NADPH, measured at 340 nm (Carlberg and Mannervik, 1985; Flohé and Günzler, 1984). For GR activity assay, samples were first incubated at 30 °C for 10 min with an assay mixture (100 mM Potassium phosphate buffer, 5 mM EDTA, 2 mM GSSG and 1.5 mM GSH, pH 7.0). 20 μL of a NADPH solution (final concentration of 0,12 mM) were then added to each sample and NADPH consumption was accompanied at 340 nm. For GPx assay, samples were also incubated at 30 °C for 10 min with an assay mixture (100 mM Potassium phosphate buffer, 5 mM EDTA, 1 mM GSH and 0.2 mM NADPH, pH 7.0). A cumene peroxide (CuOOH) solution (final concentration of 1 mM) was then added to each sample and NADPH consumption was accompanied at 340 nm.

G6PDH activity was also determined at 340 nm, following the protocol established by Glock and McLEAN (1953). Briefly, an assay mixture (100 mM Tris-HCl, 200 mM $MgCl_2$, 2 mM G6P and 0.13 mM $NADP^+$, pH 7.4) was added to each sample, and $NADP^+$ reduction was accompanied at 340 nm.

All catalytic assays were performed in duplicates at 30 °C in 96-well plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). Enzyme activity was normalized by protein concentration (mg/mL), determined according to Bradford (1976) and calculated as mU/mg prot.

2.3. Lipid peroxidation

The levels of lipid peroxidation were evaluated following the protocol based on the formation of malondialdehyde (MDA) and thiobarbituric acid (TBA) adducts (De Almeida et al., 2004). Briefly, liver samples were homogenized in buffer containing butylated hydroxytoluene (BHT) (0.1 M Tris, 0.05% BHT). A thiobarbituric acid solution (TBA, 0.4% in 0.2 M HCl) was added to each sample, and the mixture was incubated at 90 °C for 40 min. Subsequently, n-butanol was added to each sample, which were then centrifuged at 5000×*g* for 3 min (4 °C). The resulting supernatant was applied in a High Performance Liquid Chromatography (HPLC) with Diodes Array Detection (DAD), Ultimate 3000 model, coupled with Acclaim 120C18 column (250 mm × 2.1 mm × 5 μm) (Thermo Fisher Scientific), using 50 mM potassium phosphate buffer (pH 7.0) with 40% methanol as the mobile phase. The results were evaluated using the software Chromeleon 7 and MDA estimation was carried out using a MDA standard calibration curve obtained by the hydrolysis of tetramethoxypropane (TMP). MDA levels were calculated as nmol/g tissue.

2.4. Glycogen concentration

Glycogen concentration in liver samples was measured colorimetrically at 460 nm using an iodine binding assay (Dreiling et al., 1987). Briefly, liver samples were homogenized in perchloric acid 10%, centrifuged at 15,000×*g* for 30 min (4 °C) and the supernatant used for glycogen determination.

Table 1
Characteristics of green turtles (*Chelonia mydas*) included in this study.

ID	Sex	Post mortem intervals (h)**	Weight (kg)	CCL (cm)	Body condition	Histopathological findings***	Age	Stranding location (LAT/LONG)
49550	F	0, 1, 2, 3, 4, 5, 6, 24	4.4	32.1	Good	Cerebral and Renal hemorrhage due to trauma	J	−25.5310763/-48.3099532
57374	F	0, 1, 2, 3, 4, 5, 6, 24	2.0	31.9	Cachetic	Myositis from bacterial origin	J	−24.7758038/-47.590484
58546	UN	0, 1, 2, 3, 4, 5, 6, 24	2.8	30.4	Thin	Parasitical pancreatitis; Bacterial pneumonia	J	−25.0002899/-47.8640382
59784	F	0, 1, 2, 3, 4, 5, 6, 24	2.8	32.5	Thin	Pulmonary atelectasis leading to respiratory failure	J	−24.8826078/-47.735092
127048*	F	0, 1, 2, 3, 6, 12, 18, 24	2.5	34	Cachetic	Pulmonary atelectasis leading to respiratory failure	J	−26.326261/-48.55383
138975*	F	1, 2, 3, 6, 12, 18, 24	9.0	43.1	Good	Cranial trauma with secondary lesions to the liver and lymph nodes	J	−28.4955959/-48.7615138

Physical characteristics and the location where the specimens were found during PMP-BS monitoring are shown for each green turtle identified and included in this study. The sex of 58546 was undefined due sexual immaturity verified by gonadal analysis. Abbreviations: F: Female, UN: undefined, CCL: curved carapace length, J: Juvenile, LAT: latitude, LONG: longitude. * Samples were included in graphs, but not included in statistical analyzes due to the differing *post mortem* intervals. ** *Post mortem* interval was counted from the moment of euthanasia. ***Main finding of histopathological analysis. Full report available at https://simba.petrobras.com.br/simba/web/sistema/pmp/1/exam/with the ID provided in Table 1.

To determine glycogen concentration, 260 μL of an iodine reagent (1,3 mL of: 0.26 g of iodine and 2.6 g of potassium iodide in 10 mL distilled water + 100 mL of saturated $CaCl_2$) was added to 40 μL of each sample and glycogen standards (Sigma, US). Mixtures were incubated for 10 min before absorbance measurements. Glycogen concentration in samples was calculated based on the linear equation obtained from glycogen standards ($r = 0.999$). The assay was performed in 96-well plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA).

2.5. Molecular biomarkers

2.5.1. RNA total extraction, RNA integrity and cDNA synthesis

For total RNA extraction, 100 mg of liver samples were homogenized in 1 mL of Qiazol Lysis reagent (Qiagen, US) and incubated at 20 °C for 30 min 200 μL of chloroform was added to each sample. The homogenate was vigorously mixed, incubated at room temperature for 3 min, and centrifuged at 14,000×*g* for 30 min (4 °C). The resulting aqueous supernatant was then transferred to a new microtube, mixed with 500 μL of 2-propanol, incubated for 10 min at room temperature and centrifuged at 14,000×*g* for 30 min (4 °C). The resulting pellet, containing the RNA, was cleaned with ethanol 75%, dried and eluted in molecular grade water.

The concentration and purity of the total RNA extracted from each sample was assessed in a NanoDrop ND1000 (Thermo Fisher Scientific, US) using the absorbance values obtained at 260 nm and the ratios between the values obtained at 260/280 and 260/230, respectively.

The integrity of the extracted RNA was also assessed, using a QubiT 4 fluorometer (Thermo Fisher Scientific, US) and the RNA IQ Assay kit (Thermo Fisher Scientific, US), to evaluate the influence of *post mortem* intervals over the degradation of RNA (data presented in Supplementary Table S3). Total RNA was stored at −80 °C until complementary DNA (cDNA) synthesis.

cDNA was synthesized from 1 μg of total RNA using the QuantiTect® Reverse Transcription kit (Qiagen, US) and stored at −20 °C until quantitative reverse transcription PCR analysis (qRT-PCR).

2.5.2. Primers design and real time quantitative reverse transcription PCR analysis (qRT-PCR)

Two genes were included in this study as biomarkers of the metabolism of carbohydrates: pyruvate carboxylase (*PYR*) and carbonic anhydrase (*CA*). Specific primers for each of these target genes were designed with the PrimerQuest Tool® (IDT, US), using sequences deposited in NCBI (https://www.ncbi.nlm.nih.gov/genbank/) and prioritizing primer pairs that were designed in distinct exons or in exon/exon junctions. Primers were evaluated using the OligoAnalyzer™ Tool (IDT, US) and FastPCR 6.5 software (PrimerDigital, Finland). Final primer pairs are presented in Supplementary Table S4.

qRT-PCR assays were performed in a Rotor-Gene Q Thermocycler (Qiagen, US) using QuantiFast SYBR Green PCR Kit (Qiagen, US) with amplification protocol consisting of one cycle for enzyme activation at 95 °C (5 min) and 35 cycles of denaturation at 95 °C for 10 s combined with primer annealing/elongation at 60 °C for 30 s. All samples were analyzed in duplicate. Reference blanks and a standard curve for the target gene, used to assess primer efficiency (E) and amplification linearity, were included in each assay. To evaluate the identity and specificity of PCR products, a melting curve analysis with temperature range from 60 to 95 °C was included at the end of each assay.

Quantification cycle (Cq) values obtained for each sample were normalized by cDNA load in each reaction, measured fluorimetrically at 80 °C using the Quant-iT™ OliGreen™ ssDNA assay kit (Thermo Fisher Scientific, US) as described by Rhinn et al. (2008).

2.6. Statistical analysis

Due to the low number of samples in each group (each *post mortem* interval, $n = 6$), data normality tests are not reliable. Hence, to evaluate if *post mortem* interval influenced the biomarkers responses, the non-parametric Friedman's test for paired data was applied. Differences were considered significant when $p < 0.05$. Due to the great inter-individual variation in biomarker data, for graphic representations, biomarker data from all intervals were normalized by the value obtained at $T = 0$ h for each turtle. All statistical analyzes were performed in GraphPad Prism 9.3.1.

3. Results and discussion

The main finding of this study is the apparent stability of biochemical biomarkers up to 24 h after the death of green turtles, *C. mydas*. The activities of antioxidant enzymes GPx (F-statistic = 9.833, $p = 0.1982$) (Fig. 1C), GR (F-statistic = 9.333, $p = 0.2296$) (Fig. 1B) and CAT (F-statistic = 6.583, $p = 0.4735$) (Fig. 1D) were stable, showing very little fluctuation between *post mortem* intervals for 24 h. G6PDH activity, whilst visually showing a tendency for higher values in samples collected 3 h after organismal death, did not vary significantly between post *mortem* intervals (F-statistic = 11.50, $p = 0.1182$) (Fig. 1A). MDA levels were also stable in samples collected up to 24 h after organismal death (F-statistic = 5.357, $p = 0.4989$) (Fig. 1F).

These results were unexpected since after organismal death, the oxygen stores are quickly depleted, forcing the cells to turn to anaerobic metabolism for ATP production. As a result, intracellular membranes become less selective and the cellular pH tends to decrease, activating hydrolytic enzymes and, consequently, leading to the denaturation of molecules and membranes, and to the loss of enzyme activities (Butzbach, 2010).

The collapse of enzymatic activity is known to influence many biochemical pathways. For instance, previous studies have attributed the establishment of oxidative stress in *post mortem* chicken thigh muscle to the loss of activity of antioxidant enzymes (Carvalho et al., 2019). Indeed, in rat liver the activity of SOD, GPx, GST and CAT, as well as GSH content, decreased from 0 to 6 h after death, while the MDA levels increased after 2 h *post mortem* (Sener et al., 2012). Similar results were obtained in a study with duck muscle, which reported a decrease in SOD and GPx activity and an increase in MDA content in a time dependent manner after death (Zheng et al., 2020).

In contrast to previous literature reports, our results do not suggest neither a decrease in antioxidant activity nor an increase in MDA levels following death in liver tissue of green turtles. Our results appear to be similar to those obtained from anoxia studies with turtles. Anoxia can be considered similar to the *post mortem* changes in the sense that both conditions ensue oxygen deprivation to tissues. In a study carried out with the freshwater turtle *Trachemys scripta elegans*, Willmore and Storey (1997) observed that, during exposure to anoxia and following recovery to aerobic metabolism, antioxidant activity in the liver remained practically unaltered, with CAT and GPx activities similar to those of the control group (not exposed to anoxia). Levels of lipid peroxides in the liver also remained stable under anoxia and after aerobic recovery. Only SOD showed a 30% decrease in activity in anoxic conditions (Willmore and Storey, 1997). Collectively, these results suggest that oxygen deprivation and aerobic recovery do not represent a challenge to oxidative balance in the liver of freshwater turtles. A possible explanation might be the overall low metabolism in the absence of oxygen and a high constitutive antioxidant capacity in these animals. These attributes can also be associated with the *post mortem* stability of both antioxidant activity and MDA levels in the liver of green turtles observed in the present study, suggesting that the *post mortem* challenges to homeostasis enforced by the lack of oxygen do not affect the cellular oxidative balance in *C. mydas* up to 24 h after death.

Interestingly, the *post mortem* anaerobic scenario also does not seem to influence significantly the metabolism of carbohydrates in green turtles. Glycogen concentrations (F-statistic = 6.333, $p = 0.5014$) (Fig. 1E) remained stable up to 24 h *post mortem*, as did the transcript levels of *PYR* gene (F-statistic = 10.33, $p = 0.1705$) (Fig. 2A).

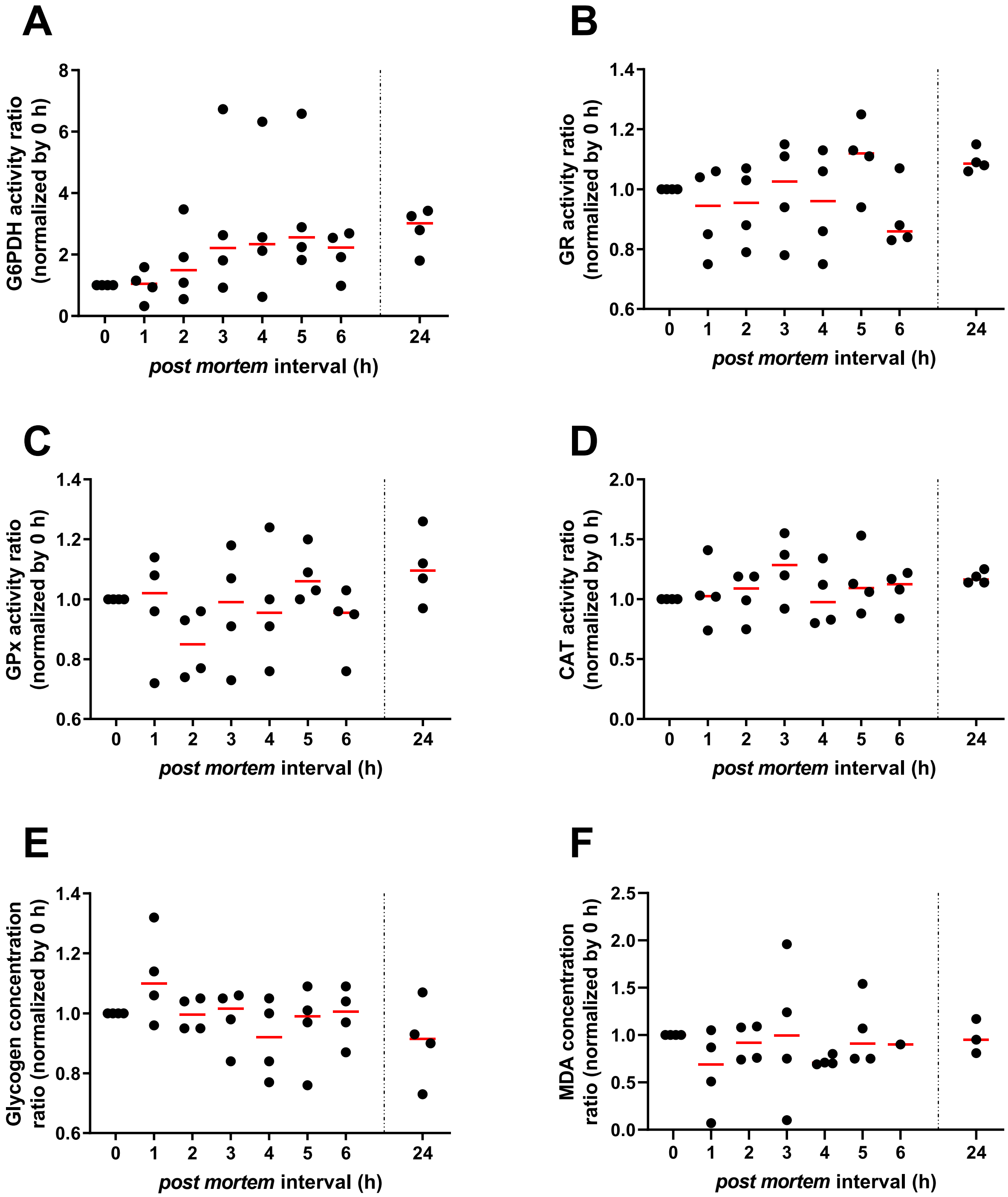

Fig. 1. Ratios of biochemical biomarkers: (A) G6PDH activity, (B) GR activity, (C) GPx activity, (D) CAT activity, (E) glycogen concentration, and (F) MDA levels in liver samples of green turtles, *Chelonia mydas*, at *post mortem* intervals (0, 1, 2, 3, 4, 5, 6 and 24 h). Values were normalized by levels at T = 0 h for each turtle individually. Dots represent the samples and red lines represent medians for each *post mortem* interval. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

After the death of an organism – in the absence of blood flow and oxygen supply, the cellular ATP production is maintained primarily through the anaerobic catabolism of hepatic glycogen stores in a process known as glycogenolysis (Ferguson and Gerrard, 2014). Thus, glycogen concentrations in *C. mydas* liver were expected to decrease after death in a time dependent manner. Indeed, the glycogen content in pork muscle decreased *post mortem* reaching 45% of starting levels after 24 h (Guo et al., 2021). However, Gümus et al. (2016) reported that, in mice, hepatic, renal and muscle glycogen reserves did not change up to 60 h *post mortem,* possibly due to autolysis processes that can culminate in the degradation of key enzymes in glycogenolysis and glycolysis pathways. On the other hand, glucose levels did decrease in a time dependent manner in both liver and muscle of mice, which indicates a reduction of glucose synthesis after death in these organs (Gümus et al., 2016). However, our results concerning glycogen content and *PYR* transcript levels suggest that both hepatic glycogenolysis and gluconeogenesis metabolism remained unaltered after *C. mydas* death.

Autolysis could also explain the observed *post mortem* stability of hepatic glycogen concentrations of *C. mydas.* However, considering the singular metabolic characteristics of turtles, this apparent stability can

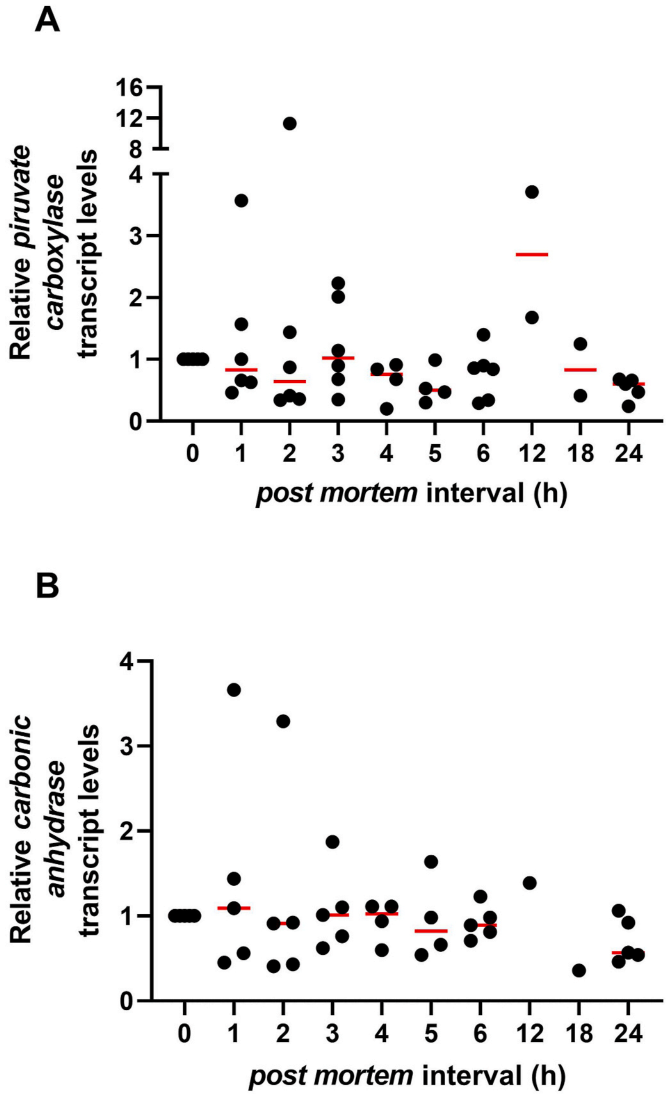

Fig. 2. Ratios of transcript levels of: (A) pyruvate carboxylase and (B) carbonic anhydrase genes in the liver of green turtles, *Chelonia mydas*, at various *post mortem* intervals. Values were normalized by levels at T = 0 h for each turtle individually. Dots represent samples, red lines represent medians for each *post mortem* interval. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

also be associated with the low metabolism of turtles, especially under oxygen deprivation (Williard, 2013), decreasing the requirement for ATP and postponing the cellular need to degrade glycogen.

Interestingly, transcript levels of carbonic anhydrase (*CA*) also remained stable up to 24 h *post mortem* (F-statistic = 9.250, p = 0.2352) (Fig. 2B). CAs are zinc-metalloenzymes that catalyze the hydration of CO_2, playing an important role in pH regulation and other pathways (Chegwidden et al., 2000). For instance, in the liver of guinea pigs and rats, mitochondrial CAs are responsible for providing HCO_3- to both carbamoyl phosphate synthase and pyruvate carboxylase, participating in the synthesis of urea and glucose, especially under an increased lactate production (Dodgson, 1991). While the metabolic function of CA is still not established in reptiles, the absence of *post mortem* fluctuations in the *CA* and *PYR* transcript levels in the liver of *C. mydas* supports that the cutoff of blood flow and oxygen supply after death is not followed by an increase in glycogen breakdown and anaerobic glycolysis at least up to 24 h after death.

It is worth noting that, the absence of *post mortem* influence over the biomarkers evaluated here may also be at least partly associated with the *ante mortem* conditions of the turtles included in this study. Only two out of four *C. mydas* were in good body condition upon arrival at the rehabilitation facilities, while the other animals were either thin or cachectic (Table 1). Fasting and, consequently, cachexia, can affect both physiological and biochemical parameters, especially regarding energy metabolism. In fact, decreased glucose in blood in *C. mydas* and *Lepidochelys kempii* was observed during two weeks of food deprivation (Moon et al., 1999), while glycogen reserves tended to decrease in *Chrysemys dorbigni* after 30 days of fasting (Partata and Marques, 1994).

In that sense, the observed *post mortem* stability of glycogen content in *C. mydas* can be due to an increased *ante mortem* consumption of glycogen stores.

Also, while mean CAT (16.80 ± 7.81 U.mg prot^{-1}) and GR (0.023 ± 0.003 U.mg prot^{-1}) values obtained at T = 0 were similar to those reported for healthy *Lepidochelys olivacea* (CAT – 18.19 ± 14.26 U.mg prot^{-1}; GR – 0.027 ± 0.023 U.mg prot^{-1}) (Cortés-Gómez et al., 2018), they were lower than those reported for *C. mydas agassizii* of a pristine spot (CAT – 78.1 ± 12.2 U.mg prot^{-1}) (Valdivia et al., 2007). This may suggest that the values obtained at T = 0 for antioxidant enzymes may be somewhat affected by the conditions of the turtles *ante mortem.*

In addition to their body condition, five out of six turtles were medicated with a myriad of antibiotics, analgesics and/or anti-inflammatory drugs for at least one week prior to being euthanized. Several drug classes are known to alter biochemical parameters. For instance, catalase activity was shown to increase in fish (*Danio rerio*) exposed to a mix of pollutants (Araújo et al., 2022), which included antibiotics such as amoxicilin, used in some of the treated turtles. Also, Li and collaborators (2018) reported that pigs treated with enrofloxacin, an antibiotic used in several of the turtles included in this study, displayed higher levels of MDA in comparison to control animals. In contrast, in *Danio rerio* exposed to enrofloxacin, GPx and GR activity remained unaltered (Sehonova et al., 2019).

Another drug, Tramadol, used in many of the specimens, has been shown to increase lipid peroxidation and reduce the activity of antioxidant enzymes such as CAT, GPx and GR on the liver of mice and dogs treated with the compound (Nazifi et al., 2019; Sheweita et al., 2018). And flunarizine, used in one particular turtle, was associated with lower TBARS levels in humans, given its antioxidant properties (Ciancarelli et al., 2004).

Therefore, the putative influence of such parameters on the biomarkers assessed constitutes a limitation of the present study, though it is enforced by the ethical constraints of sampling live and healthy sea turtles. However, despite this limitation, we believe that the results presented here do contribute to a better understanding of cellular changes after organismal death in green turtles, *Chelonia mydas*. Furthermore, our results suggest that *C. mydas* found stranded, with an estimated time of death within 24 h, can be successfully used in biomonitoring studies in coastal and oceanic environments.

4. Conclusion

The present study showed that the antioxidant activity, levels of lipid peroxidation, glycogen content and the transcript levels of carbonic anhydrase and pyruvate carboxylase genes in liver of green turtles were unaltered for up to 24 h after death. Such results suggest that the metabolic changes following organismal death may happen slowly in *C. mydas*, supporting the use of biomarkers within a period of 24 h *post mortem* for biomonitoring purposes. Nonetheless, we recommend that *post mortem* fluctuations of particular biomarkers be evaluated prior to their application, given that proteins may show varying degrees of susceptibility to autolysis.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136569.

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