MULTIDISCIPLINARY SCIENCE JOURNAL

RESEARCH ARTICLE
Published Online: October 5, 2022
https://doi.org/10.31893/multiscience.2022020

Iron deposition in the liver and guts of giant South American freshwater turtle *Podocnemis expansa* (Schweigger 1812) from a commercial breeding farm in Acre, Brazil

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Abstract Wildlife turtles are endangered species because of the traditional capture of eggs and adult animals in Northern Brazil, such as the South American freshwater turtles (*Podocnemis expansa*). Successful conservation efforts in order to keep them in captivity and fed commercial fish chow may lead to physiological changes and systemic pathologies. Thus, in the present study, were evaluated the liver and digestive tract of 14 giant South American freshwater turtles microscopically and described the histopathological changes related from commercial breeding and captivity. There was the presence of ferric ions in the stomach epithelium and macrophages with ferric beads in crypts and intestinal villi. In the liver, it was observed disruption of hepatic architecture and liver hemosiderosis. There was the presence of large melano-macrophage centers (MC) filled with ferric ions throughout the liver parenchyma. The size of MC ranged from 25.3 x 31.3 µm up to 196.7 x 168.7 µm and occupied between 9.0 ± 2.9% and 37.5 ± 9.8% of the liver parenchyma area. Those findings can be influenced by age, malnourishment, or ammonia intoxication with posterior anemia, but all those hypotheses must be tested in the future.

Keywords: melano-macrophage centers, bioindicators, hemosiderosis, giant South American turtle, *Podocnemis expansa*

1. Introduction

The turtles represent the most threatened group of vertebrates on the planet; approximately 60% of all currently recognized modern turtle and tortoise species are either already extinct or threatened with extinction (Turtle Taxonomy Working Group 2014, 2017). Giant South American turtle *Podocnemis expansa* (Schweigger 1812) is an omnivorous species that historically faced many threats associated with intense predation of adults and eggs, causing a reduction in natural populations in the Amazon, Orinoco, and Essequibo basins, leading almost to extinction (Terán et al 1995; Cantarelli et al 2014). In the mid-1970s, Brazilian government conservation efforts supported its reproduction in captivity and also *in situ* conservation programs. Afterward, commercial breeding farms were legalized, accounting for more than 80 farms in Northern and Midwest Brazil (Vogt 2008; Almeida and Abe 2009; Cantarelli et al 2014).

Despite its roots as environmental science, wildlife captivity still needs further investigation as well as the effects caused by the maintenance of *P. expansa* specimens in artificial conditions. Enlightening this issue, a wide range of studies described the possible consequences of food on anatomical and physiological changes for freshwater turtles and tortoises kept in captivity and fed commercial fish chows (Sá et al 2004; Tavares-Dias et al 2008; Moura et al 2012; Kanghae et al 2014; de Oliveira et al 2020).

Iron is a trace element absorbed in the small intestine proximal region and stored as ferritin in enterocytes (Waldvogel-Abramowski et al 2014) and gut macrophages, with final deposition in the liver as hemosiderin (Iancu 1992). The melano-macrophages are cells of the reticuloendothelial system in the liver and spleen of reptiles. They attach and turn into melano-machrophage centers (MC), which may accumulate melanin, lipofuscin, and hemosiderin (Gopalakrishnakone 1986; Agius and

 Received: July 26, 2022 | Accepted: September 22, 2022

Roberts 2003). Henninger and Beresford (1990) and Johnson et al (1999) considered the presence of iron in melano-macrophages as a defensive response against reactive oxygen species, but recently, the hypothesis is that MCs represent a primitive site of adaptive immune system activation (Steinel and Bolnick 2017). Thus, this study aimed to evaluate the liver and the digestive tract of *P. expansa* kept in captivity and to describe the histopathological changes, discussing its possible underlying causes.

2. Materials and Methods

Fourteen giant South American turtles (*Podocnemis expansa*) of both sexes, ranging from 3 to 8 years old and weighing between 5 and 22 kg (mean of 7.5 kg), were investigated. These samples were obtained from a commercial breeding farm in the Rio Branco municipality, Acre State, Northern Brazil. The turtles were euthanized following ethics procedures (Dornelles and Quintanilha 2007), under the authorization of the Acre Environmental State Institute (15/2011) and the Brazilian Federal Institute of Environment and Renewable Natural Resources (IBAMA).

Liver, stomach (cardiac region), and small intestine (duodenum) samples of *P. expansa* were collected. Tissues were fixed in 10% formaldehyde, dehydrated in alcohol, and embedded in resin (HistoResin, Leica, Germany). Sections with 3 µm thickness were stained with hematoxylin and eosin (HE) and the Prussian Blue technique. The histopathological evaluation of the sections was carried out under Olympus BX60 light microscope (Olympus, Japan) and AxioCam HRc camera (Zeiss, Germany).

For each collected liver, ten fields of the stained slides using the Prussian Blue technique were photomicrographed at 40x magnification (surface area = 14,9774.16 μm^2), and the percentage of the area occupied by melano-macrophages centers (MC) was calculated by the AxioVision 4.8 software (Zeiss, Germany). Data were evaluated by descriptive (mean ± standard deviation) and did not show normal distribution (Shapiro-Wilk test, turtle #10, $p>0.05$). Therefore, the non-parametric Kruskal-Wallis test with 95% confidence interval was used by the software *GraphPad Prism 6* (GraphPad Software Inc., California, USA), which showed differences among turtles' iron storage areas ($p<0.0001$).

3. Results

In all analyzed livers, hydropic and fatty degeneration of the hepatocytes, congestion, loss of tissue architecture with the presence of liver fibrosis, and hepatocytes with pyknotic nuclei were observed (Figure 1A). In the HE staining, large MC with eosinophilic were detected throughout the parenchyma and showed globular, pyriform, and reniform shapes. The Prussian blue allowed the detection of the intracytoplasmic hemosiderin deposition (hemosiderosis) in hepatocytes and melano-macrophages across the hepatic parenchyma, with an intense concentration of iron in hepatocytes closer to the portal triads (Figure 1B).

The histopathological evaluation of the stomach and small intestine stained with HE and Prussian blue staining revealed intracytoplasmic ferric ions present in the stomach epithelium (cardiac region), the intestinal villi, and crypts across the board (Figure 1C).

The quantification of the occupied area by MC varied between 9.0 ± 2.9% and 37.5 ± 9.8% ($p<0.0001$; Shapiro-Wilk normality test with Kruskal-Wallis non-parametric post hoc test) (Table 1) in the analyzed areas of the regions examined, with the presence of MC with areas ranging between 25.3 x 31.3 µm and 196.7 x 167.8 µm.

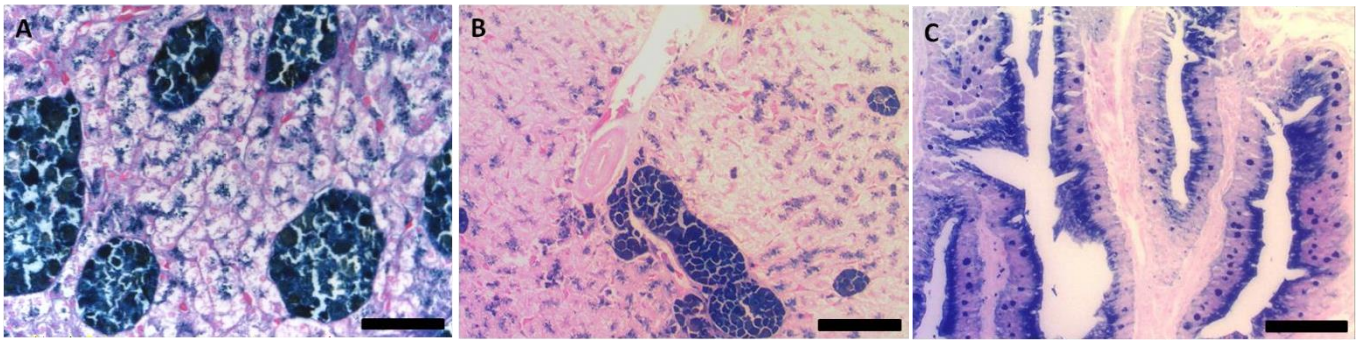

Figure 1 A: the liver of *P. expansa* turtles showed hepatocytes with hydropic and fatty degeneration and fibrotic septa with large melano-macrophage centers throughout the liver parenchyma. B: the melano-macrophage centers had a close relationship with the portal spaces. C: in the intestine, enterocytes and macrophages are involved in the storage and recycling of ferric ions. Prussian blue staining. Bars: A - 50 µm, B and C - 100 µm.

4. Discussion

Liver diseases and their histological modifications in turtles and tortoises still need more studies to be elucidated. Hepatocytes surrounding portal triads receive blood from central veins with lower oxygen levels, being those cells more susceptible to undesirable liver microenvironmental conditions (Brunt et al 2014), triggering inflammatory responses in turtles

(Johnson et al 1999; Johnson et al 2005). Parallel with mammals, liver dystrophy and fatty liver deposition are the early stages of liver diseases as seen here in *P. expansa* turtles and diagnosed in marine turtles (Sinn 2004). Non-alcoholic fatty liver disease (NAFLD) is closely linked to nutrition, as seen in humans (Angulo 2002). The increase of the generation of reactive oxygen species in the hepatocytes turns into non-alcoholic steatohepatitis (NASH) with inflammatory infiltrate in the liver parenchyma over time (Angulo 2002). Sartori et al (2022) demonstrated that the aggravation of NAFLD into NASH in *Chelonoidis carbonaria* tortoises is associated with a compromised mitochondrial function such as lower mitochondrial oxygen consumption and lower Ca^{2+} retention capacity.

Table 1 Occupied area (%) by the hepatic melano-macrophage centers.

Turtle	Occupied area (%)
1	28.4 ± 5.8[a]
2	22.0 ± 8.3[a]
3	13.5 ± 2.3[b]
4	18.4 ± 6.1[b]
5	37.5 ± 9.8[a]*
6	12.5 ± 2.8[b]
7	15.3 ± 6.2[b]
8	10.3 ± 4.3[b]
9	9.8 ± 2.5[b]
10	18.7 ± 8.9[b]
11	13.5 ± 5.4[b]
12	9.0 ± 2.9[b]**
13	12.2 ± 6.8[b]
14	15.5 ± 4.7[b]

In lines, different letters indicate statistical significance among occupied areas by melano-machrophages in the turtle livers (Kruskal-Wallis test, $p<0.0001$). * Oldest turtle is around eight years old; ** younger turtle is around three years old.

As we observed, melano-macrophages are found in the liver of *P. expansa* kept in captivity (Moura et al 2012) and turned into higher clusters (MCs) especially seen in the older turtle, as a defense against reactive oxygen species (Henninger and Beresford 1990; Johnson et al 1999), mainly for the presence of melanin in melano-machrophages (Gopalakrishnakone 1986, Agius and Roberts 2003). The final stage of those conditions is chronic hepatitis and liver cirrhosis with iron deposition, as reported by Rosskopf and Shindo (2003) in desert tortoises *Gopherus agassizii*. But the relationship between MCs and turtle age was reported by Christiansen et al (1996), and iron content increases with age in tortoises and freshwater turtles reported by Kienzle et al (2006), Sartori et al (2022), and in this report. Rosskopf and Shindo (2003) hypothesized two etiologies to explain that pathology: blood breakdown in disease and enzyme deficiency – partially unveiled by Sartori et al (2022), which should be tested in the future for *P. expansa* turtles.

The other possibility for iron deposition is the nutritional imbalance in rations. Even though there are few reports detailing iron levels in diets for reptiles (Kik et al 2003) and tests for the determination of iron requirements for turtles (Chu et al 2007), it was observed that the iron requirements for turtles are higher than for fish (Watanabe et al 1997). The possibility in our case is that *P. expansa* turtles were fed fish chow, and at the moment of the euthanasia, as a consequence, malnourished turtles may present normocytic-hypochromic anemia and liver hemosiderin deposition caused by iron deficiency (Tavares-Dias et al 2009). As recently reviewed by Rawski et al (2018), standardized nutritional requirements for most freshwater turtles are not available, and until now, no micromineral requirements are available for *P. expansa* turtles. Indeed, data from biochemical and enzymes of the energy metabolism show that *P. expansa* turtles have a herbivorous profile, therefore feeding commercial omnivorous chow for fishes should be avoided (Duncan and Marcon 2009; Fonseca et al 2016).

Another factor that can interfere with iron deposition in the liver is the environmental ammonia levels in chelonian ponds. The intensification of cheloniculture systems is closely linked to the enrichment of ammonia, nitrite, and nitrate in water leading to environmental stress (Agius and Roberts 2003). In these systems, the major source of ammonia is the high protein content chows utilized in animal feeding. The excess feed usually sinks and is degraded by nitrogen cycle bacteria (Avnimelech 1999). Indeed, it should be considered that *P. expansa* contributes to water ammonia as freshwater turtles can excrete more than 40% of the total nitrogen products as ammonia (Shoemaker and Nagy 1977). Nitrogen waste products bind stably to erythrocyte hemoglobin and form methemoglobin, which prevents gas exchange (Eddy and Williams 1987). However, detoxification of methemoglobin is accomplished by the enzyme methemoglobin reductase present in erythrocytes of fish, reptiles, birds, and mammals (Board et al 1977; Jensen 2009).

Other xenobiotic chemicals affecting *P.expansa* turtles are reported in the literature. Mercury was found stored in *P.expansa* livers and muscles, which can be ingested by riverine people (Schneider et al 2010). Tests in eggs artificially incubated and exposed to pesticides such as fipronil and glyphosate resulted in lowered body mass at hatch, impaired development and bone malformation (Mendonça et al 2022) and methyl parathion led to reduced levels of total fat in the shells and reduced egg

hatchability (Valdes et al 2015). Chronic exposition to those pesticides may undergo to metabolic bone disease in mature *P. expansa* turtles (Araújo et al 2019).

Unfortunately, it was not possible to determine if the iron deposition in the liver was physiological or pathological (Ganz 2013; Waldvogel-Abramowski et al 2014), in the same way as reported by Gyimesi and Howerth (2004) for the crocodile lizard *Shinisaurus crocodilurus*. Some ways for future works are the determination of nutritional requirements (Rawski et al 2018), populational studies in liver alterations and diseases prevalence for *P. expansa* reared in captivity (Rosskopf and Shindo 2003; Kienzle et al 2006, Sartori et al 2022).

5. Conclusions

In conclusion, this was the first study that detected the presence of iron ions in the stomach and small and large intestines, in addition to quantifying the deposition of iron in the liver of giant South American turtles, *P. expansa*, from commercial breeding farms.

In the present report, the outcome of this process was detected as hepatic hemosiderosis. As a non-pathognomonic sign in the liver, it is not discarded as an underlying cause for iron deposition, a chronic intoxication by ammonia or xenobiotics, nutritional iron imbalance, and NAFLD-like in turtles associated with liver enzyme deficiencies. Moreover, this species of wildlife suffer from mercury deposition in the liver and muscles that may lead to chronic mercury intoxication in riverine people that consume turtles' meat. This fact must be explored in future research with the inhabitants of Rio Branco municipality.

Conflict of Interest

The authors declare no conflict of interest.

Funding

This research did not receive any financial support.

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