Fish Physiol Biochem
DOI 10.1007/s10695-016-0276-5

Talking to the dead: using *Post-mortem* data in the assessment of stress in tiger sharks (*Galeocerdo cuvier*) (Péron and Lesueur, 1822)

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Received: 9 April 2016 / Accepted: 8 August 2016

Abstract Sharks are very sensitive to stress and prone to a high mortality rate after capture. Since approximately 50 million of sharks are caught as bycatch every year, and current recommendations to reduce the impact of commercial fishing strongly support immediate release, it is imperative to better understand post-release mortality caused by the stress of capture and handling. Blood samples allow the assessment of stress levels which are valuable tools to reduce mortality in commercial, recreational and scientific fishing, being essential for the improvement in those conservation measures. Biochemical analyses are widely used for sharks as stress indicators, with secondary plasma parameters (lactate, glucose and ions) being the most often employed assays. However, it is virtually impossible to determine baseline plasma parameters in free-ranging sharks, since blood withdrawal involves animal capture and restrain, which are stressful procedures. This study aims at analyzing secondary parameters of five healthy tiger sharks captured with circular hooks and handlines in Fernando de Noronha (Northeastern Brazil) and comparing them with secondary parameters of three dead tiger sharks caught off Recife (also Northeastern Brazil). The results showed that the analysis of some plasma constituents in dead animals may be an efficient tool to assess stress and lethality. However, traditional parameters such as glucose and calcium, need to be used with caution. The results also demonstrated the extreme importance of urea and phosphorus for assessing stress response and mortality in tiger sharks, both parameters frequently neglected and of utmost importance for shark's homeostasis.

Keywords Elasmobranch · Shark · Stress physiology · Post-release recovery

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Introduction

Sharks are extremely sensitive to human activities, especially interactions with fishing (commercial and recreational), which expose them to a variety of

Published online: 22 August 2016

stressors, causing physiological and behavioral changes that can seriously compromise their survival (Skomal 2007). Overfishing is the primary threat to elasmobranchs, and approximately 50 % of the sharks caught annually are part of bycatch (Dulvy et al. 2008), being traditionally defined as incidental capture of nontarget species or unwanted size ranges of target species through a non-selective fishing process (Crowder and Murawski 1998; Davies et al. 2009). For species that are brought on board alive, air exposure combined with the stress of capture can reduce fitness and survival, decreasing the efficiency of compensatory practices, such as immediate release, currently proposed as a way to reduce bycatch impacts (FAO 2011).

Primary blood parameters such as catecholamines and corticosteroids are widely used as stress markers for vertebrates, with the role of cortisol well established for mammals, birds and fishes (e.g., Wingfield and Romero 2001). However, for elasmobranchs, the use of primary stress parameters has proved to be an additional challenge since, unlike teleosts, elasmobranchs do not produce cortisol (Skomal and Bernal 2010; Anderson 2012). In sharks and rays, the functional corticosteroid expressed is 1α-hydroxycorticosterone. This glucocorticoid was first described by Idler and Truscott (1966). However, the unique chemical nature of 1α-hydroxycorticosterone brings distinct disadvantages to its detection, mostly because it is difficult to synthesize (Anderson 2012) (besides being currently unavailable since it is not being produced in any laboratory), thus limiting its use as a stress marker. Some studies employed 1α-hydroxycorticosterone to access its secretory dynamics (Hazon and Henderson 1984) and the effects of dietary protein restriction on this corticoid (Armour et al. 1993). Other studies used corticosterone (CS) analysis to access the reproductive status (Rasmussen and Gruber 1993; Manire et al. 2007), maturity (Rasmussen and Crow 1993) and stress response in sharks (Manire et al. 2007; Hoffmayer et al. 2012) as an alternative to 1α-hydroxycorticosterone. However, since CS corresponds to only 10 % of the glucocorticoid levels in elasmobranchs, and none of the published papers was able to truly prove the role of CS in the stress response, its use is still controversial.

Traditionally, secondary parameters such as glucose and lactate have been commonly used as tools for stress analysis in sharks (Cliff and Thurman 1984; Wells et al. 1986; Hoffmayer and Parsons 2001; Manire et al. 2001; Mandelman and Farrington 2009; Heberer et al. 2010; Brooks et al. 2012; Mandelman and Skomal 2009; Marshall et al. 2012; Gallagher et al. 2014). Plasma ion analysis has also been used and suggested as reliable stress markers for free-ranging and captive sharks. Potassium for instance has its increased levels related to stress response (Wells et al. 1986; Manire et al. 2001; Moyes et al. 2006; Mandelman and Farrington 2007; Brooks et al. 2012).

Regardless of which physiological parameters are used for assessing the stress of capture and handling, baseline parameters of non-stressed animals have always proved to be elusive, due to the impossibility of obtaining them from free-ranging animals without any level of stress. Some alternatives have been proposed, such as the use of captive animals (Skomal and Mandelman 2012), reduced time of capture (Marshall et al. 2012), behavioral analysis (swimming capacity) (Manire et al. 2001; Skomal et al. 2007; Hyatt et al. 2016; Whitney et al. 2016) and mathematical modeling (Skomal 2006). However, none of them was proved to be fully effective. The issue of a lack of baseline parameters is that stress studies exhibit a significant amount of data but without control groups. For that reason, most of the discussion about stress in sharks ended up describing numerical results, such as increase or decrease in ion concentrations in plasma, without clear conclusions about whether the animals were in fact stressed.

In this context, the aim of this study was to analyze and propose the use of *Post-mortem* data as putative "control/reference" groups. While baseline data of healthy unstressed individuals are challenging to obtain, the use of negative data (lethal) may be a novel and useful control tool. Blood analyses of dead animals can help to establish ranges or limits of values that can indicate when animals are reaching potentially alarming conditions, improving that way compensatory release protocols. This alternative "relationship to reference values" would mean that, instead of "the closer to basal control levels, the less stressed," we would have "the closer to dead-reference levels, the more stressed and vulnerable." To validate this new approach, data from tiger sharks that died in longlines were compared to data from healthy tiger sharks caught, tagged and released alive and in good condition.

Materials and methods

Live sharks capture and containment

Five tiger sharks were caught in Fernando de Noronha, Pernambuco, Brazil (3°51′ 13.71″S, 32°25′25.63″W) in July 2014. Capture was performed individually from a small vessel using baited handlines equipped with circle hooks about the 20-m isobath. After the capture, sharks were conducted to the M/V OCEARCH and placed on a hydraulic lift in order to remove them out of the water for sampling and tagging. Eye coverage and pump insertion with constant water flow in the animal's mouth were used in order to reduce stress (Smith et al. 2004). Sex, life stage and size (total length—cm) are presented in Table 1.

Live sharks blood sampling

The first blood sampling (capture group) was taken immediately after the animal was securely restrained (Mandelman and Skomal 2009), occurring approximately 10 min after capture and 2 min after lifting. Parasites were removed from the gills, mouth and cloaca using tweezers, and a fin clip sample was taken using scissor as part of ongoing studies non-related to the analysis presented here. SPOT tags and internal acoustic tags were deployed in all sharks, and signals started 2 days after the procedures. The second blood sample (handling group) was obtained after tagging in order to allow the appraisal of the stress caused by sampling and time out of the water. All procedures were performed within approximately 14 min, avoiding prolonged air exposure. The capture and sampling of live animals were approved by the Ethics Committee on Animal Use (CEUA) 23082.025519/2014—UFRPE.

Table 1 Sex, life stage and size (total length—cm) of live and dead tiger sharks analyzed in the present study

Tiger sharks	Sex	Stage	TL (cm)
Live 1	Female	Juvenile	259
Live 2	Male	Juvenile	182
Live 3	Male	Juvenile	204
Live 4	Male	Juvenile	244
Live 5	Female	Juvenile	243
Dead 1	Female	Juvenile	105
Dead 2	Female	Juvenile	144
Dead 3	Male	Juvenile	284

Dead sharks blood sampling

Tiger sharks were caught by the R/V *Sinuelo*, a boat operating a shark control program in Recife, Pernambuco, Brazil (see Hazin and Afonso 2013, for details). Blood from three dead tiger sharks caught with longlines equipped with baited 17/0 circle hooks was used as *Post-mortem* data. Average soak time of the longline gear was about 12 h, but the exact time the animal was caught could not be precisely determined. Sex, life stage and size (total length—cm) are presented in Table 1. Blood samples were collected immediately after hauling the animal on board. Blood analysis and dissection were carried out in order to access the cause of death. All animals were healthy before capture. The reduced sample size reflects the new practices employed here, aimed at reducing lethal sampling of apex predators for scientific purposes (animals accidentally died). The capture and sampling of dead animals were approved by the Brazilian Ministry of Environment (permit number 15083-8) and by the Ethics Committee on Animal Use (CEUA) 23082.003679/2009—UFRPE.

Plasma assays

After blood withdrawal, the sample (~5 mL) was immediately centrifuged for 7 min at room temperature (20 °C). Plasma was separated and kept frozen at −20 °C until analysis at the Laboratory of Comparative Physiology of Osmoregulation in Curitiba, Paraná. Osmolality, urea, total proteins, glucose, lactate, pH and ionic concentrations (Na^+, Cl^-, K^+, Mg^{2+}, Ca^{2+}, P^{3+}) were assayed in plasma samples. Osmolality was determined using a vapor pressure osmometer (VAPRO 5520, Wescor, USA) in undiluted samples. Urea (Labtest, Brazil, catalog n. 27; wavelength 600 nm), total proteins (catalog n. 99; wavelength 545 nm), glucose (catalog n. 133-1/500; wavelength 505 nm), lactate (catalog n. 138-1/50; wavelength 550 nm), chloride (catalog n. 49; wavelength 470 nm), calcium (catalog n. 90; wavelength 570 nm), phosphorus (catalog n. 42; wavelength 650 nm) and magnesium (catalog n. 50; wavelength

505 nm) levels were quantified colorimetrically (Ultrospec 2100 PRO, Amersham Pharmacia biotech, Sweden). Plasma samples were appropriately diluted in ultrapure water: 1:50 for urea, 1:2 for chloride and 1:15 for magnesium. Sodium and potassium were quantified using flame photometry (CELM FC-180, Brazil) in samples diluted (1:200) in ultrapure water. For pH measurements, the pH meter (inoLab, Level 1, WIW, Germany) was calibrated using buffers (Neon, Brazil) of pH 4.0, 6.86 and 9.18. After calibration, the samples were read in room temperature (20 °C).

Statistical analyses

A Student's *t*-Test (paired two-sample) was used to assess differences between plasma parameters in live sharks, comparing values for each same shark after capture and after handling in the boat. A Student's *t*-Test (unpaired two-sample comparison) was applied to verify differences between independent live and dead sharks. All tests were performed with a limit of significance of 0.05, using R software for statistical computing and graphics (R Development Core Team 2016).

Results

Levels of pH, glucose, lactate and potassium

Live sharks

The mean pH for live sharks after capture was 6.85 ± 0.21 (S.E) and 6.82 ± 0.24 (S.E) after handling (Fig. 1). Glucose, lactate and potassium within live sharks (after capture vs. after handling) showed no significant statistical differences (Fig. 1).

Dead sharks

For dead animals, pH showed a sharp decrease to 5.71 ± 0.57 (S.E) when compared to live animals (Fig. 1). Glucose, lactate and potassium showed statistical differences between dead and live sharks. Glucose showed wide variation among animals, without a clear pattern. Plasma lactate and potassium showed a sharp increase in dead animals (Fig. 1).

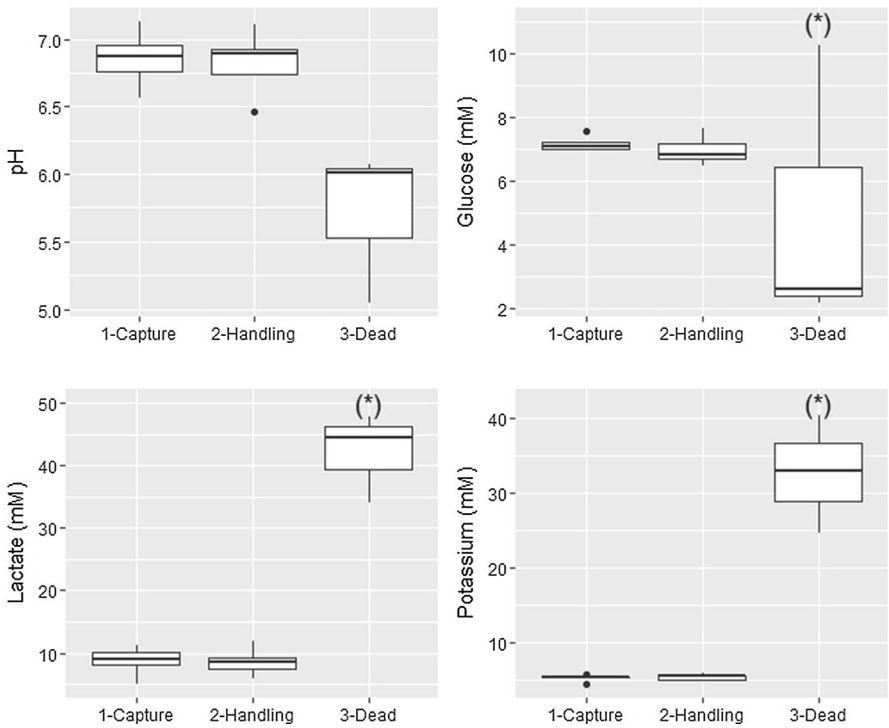

Fig. 1 Levels of pH, glucose, lactate and potassium in plasma for live (capture and handling) and dead tiger sharks. The *black points* represent outliers. The *symbol* (*) represents statistical differences (Student's *t*-Test, $p = 0.05$) between live ($n = 5$) and dead sharks ($n = 3$)

Osmolality, osmolytes (Na^+, Cl^-, Mg^{2+}, Ca^{2+}, P^{3+}, urea) and total proteins

Live sharks

There were no differences for any of the osmolytes or osmolality analyzed between captured and handled samples, except for chloride that showed an increase after handling. Total proteins in plasma were also similar within live sharks (Figs. 2, 3).

Dead sharks

Urea plasma concentration was lower in dead animals when compared to live sharks. Sodium, chloride, phosphorus and osmolality were higher in dead sharks, when compared to live animals. Magnesium, calcium and total proteins values of dead sharks did not show differences when compared to values in live sharks (Figs. 2, 3).

Discussion

The comparison between live and dead sharks demonstrated that plasma pH, lactate, potassium, phosphorus, osmolality, urea, sodium and chloride showed significant alterations. Thus, it is possible to infer that the parameters cited above are indeed relevant tools for comparative stress studies in tiger sharks. In contrast, total plasma proteins, magnesium and calcium showed no differences between live and dead sharks and therefore seem not to be adequate to infer severe stress in this species. For glucose, the difference between live and dead sharks was at an individual level, without a clear pattern of modulation during stress response. As observed in behavioral studies, stressful situations lead to responses at the individual level (Cockrem 2013; Vitousek et al. 2014; Brajon et al. 2016). Abrupt stressful situations have proved to trigger off even more particular responses (Edwards 1988). Coping strategies are also described as a way to deal with stressful situations, such as salinity changes (Marshall 2003) and captivity (Braithwaite and Ebbesson 2014). So, it is possible that the variable pattern observed in plasma glucose may be related not only to the amount of stress experienced by the individual (at a "personality" level), but also on how the organism in question *copes* with stress (Edwards 1988).

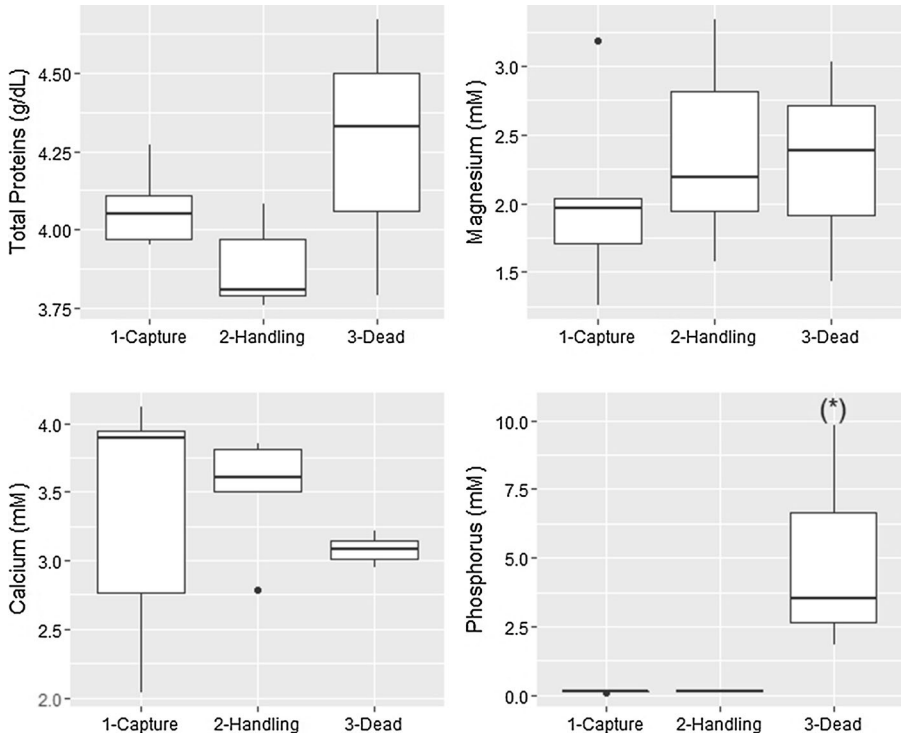

Fig. 2 Total proteins, magnesium, calcium and phosphorus concentrations in plasma for live (capture and handling) and dead tiger sharks. The *black points* represent outliers. The *symbol* (*) represents statistical differences (Student's *t*-Test, $p = 0.05$) between live ($n = 5$) and dead sharks ($n = 3$)

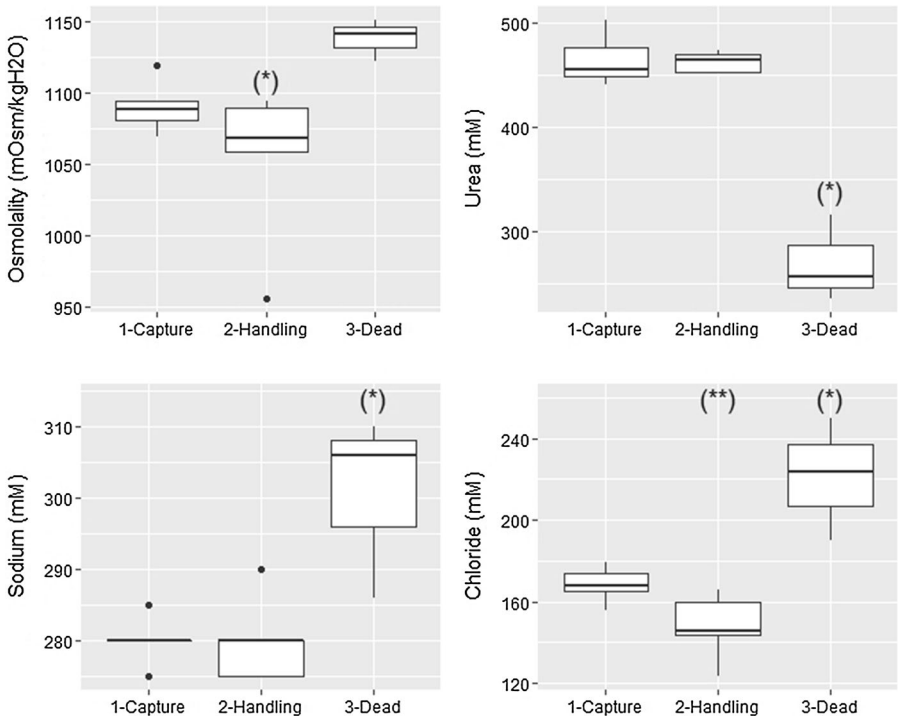

Fig. 3 Osmolality, urea, sodium and chloride concentrations in plasma for live (capture and handling) and dead tiger sharks. The *black points* represent outliers. The *symbol* (*) represents statistical differences (Student's *t*-Test, $p = 0.05$) between live ($n = 5$) and dead sharks ($n = 3$). The *symbol* (**) denotes statistical differences between capture ($n = 5$) and handling ($n = 5$) in live sharks

Problematic of control group in stress studies and *Post-mortem* as a promising field in research

The practice of blood withdrawal causes discomfort to the animal, potentially generating physiological alterations with respect to ideally unstressed animals (Pickering et al. 1982; Barton and Iwama 1991; Foo and Lam 1993). Thus, the need for manipulation makes it impossible to obtain baseline data as controls. The use of anesthetics in sharks during field trips is not recommended, since the time spent out of the water needs to be minimized in order to avoid the deleterious effects of prolonged air exposure and due to the fact that the animals need to be released back to nature in a responsive state. However, since data from anesthetized animals can bring new information and improve the understanding of elasmobranch stress response, whenever it is possible, blood withdrawal using anesthesia (e.g., in small-sized shark's species that can be maintained in captivity for scientific purposes) should be addressed.

Since it is difficult to establish a baseline reference, the use of negative control through *Post-mortem* analysis might become a viable option since it is possible to verify the extent to which the animal can withstand the physiological stress of capture without succumbing to death. The closer the parameters from live animals are from those levels associated with dead animals (i.e., lethal levels of internal parameters), the less healthy the animal are, and death will be then more likely to ensue, caused, at least partly, by the stress of capture.

Currently, *Post-mortem* data are rarely used and poorly understood. Living organisms are complex systems composed of highly refined functions (Pozhitkov et al. 2016). The complexity and proper functioning of an organism are attributes closely related to a series of genetic and physiological regulatory paths, responsible for the maintenance of the homeostasis and link between molecules, cells, organs and systems. Obviously, the traditional approach in the research field is to focus on genetic expression and physiological responses of living organisms. However, there has been a growing interest on the use and assays of biological material from recently deceased animals. It is relevant to evaluate how and for how long physiological systems at organs, tissues and cellular levels keep working after death.

Genetic and physiological responses in dead animals are closely related to the time that it takes to dissipate stored energy and also deceleration of feedback loops (Pozhitkov et al. 2016). A recent study

showed that expression and upregulation of genes related to the maintenance of homeostasis, such as HSP 70, solute carrier family 26 anion exchanger member 4 (*Slc26a4*), potassium channel voltage-gated subfamily H (*Kcnh2*) and urea transporter 2 (*Slc14a2*), occur for hours to days after death (Pozhitkov et al. 2016). This leads us to believe that a better and more complete understanding of *Post-mortem* parameters may bring new and as yet undiscovered genetic and physiological patterns that can help to unveil gaps in studies made with live animals. *Post-mortem* levels may increasingly be considered as reference endpoints for extreme stress in live animals.

Homeostatic profile of tiger sharks

By definition, homeostasis is the state of internal stability (physical–chemical), within certain limits, even in the face of alterations imposed by the environment (Cannon 1929). Vertebrates are essentially osmoregulators in that energy is spent in assuring "constant" or at least stable extracellular osmotic concentrations. Stressful situations can cause an imbalance of osmotic/ionic homeostasis. Thus, knowledge of the main osmotically active components in plasma is essential to understand the recovery process or death caused by a stressor. Currently, the majority of stress studies in sharks does not measure the main molecules involved in osmoregulation process so it is virtually impossible to determine the homeostatic profile and the role of each osmolyte for the species in question (Table 2). The analysis conducted here shows that urea has a major role in assuring homeostasis in the species, followed by plasma sodium. Plasma osmolality in elasmobranchs is essentially the sum of NaCl and urea (and TMAO, not assayed here) (Marshall and Grosell 2006; Hammerschlag 2006; Ballantyne and Robinson 2010; Wright and Wood 2015), and these were very different in the dead animals, when compared to both groups of living animals. The result was expected and entirely consistent with cessation of salt secretion by the rectal gland, causing plasma NaCl levels to increase, and breakdown of urea reabsorption by the kidneys, causing urea titers to decrease in plasma. Other studies of stress of capture performed with *G. cuvier* reported lower values for lactate (around 4 mM—Marshall et al. 2012) (3 mM—Gallagher et al. 2014), sodium (267 mM), calcium (2.4 mM) (Marshall et al. 2012) and magnesium (1 mM) (Marshall et al. 2012), similar values for glucose (6.4 mM) and potassium (5.3 mM) (Marshall et al. 2012) and higher values for chloride (263 mM) (Marshall et al. 2012) than those observed in the live animals of the present study. Despite the possible comparisons mentioned above, it is unclear whether the differences in the concentrations observed in other studies actually mean deviation from a homeostatic state, since only few ions and/or osmotic molecules were analyzed.

Lactate values were exceptionally high in dead sharks when compared to living individuals. Increases in plasma lactate are expected since an increase in the energy demand is observed during capture as a physiological response to fight (partly supported by anaerobic metabolism in white muscle) (Bone 1988; Smith 1992; Pikering and Pottinger 1995; Hoffmayer and Parsons 2001). The synthesis and release of lactic acid lead to a decrease in blood pH causing a variety of deleterious effects at the cellular level (Albers1970; Smith 1992). A negative correlation was observed between lactate and pH (Pearson's $R^2 = -0.90, p = 0.03$). In live animals, the lower plasma lactate concentration was consistent with higher pH values. Dead animals also showed increased lactate, followed by plasma acidification. Stress and exhaustive exercise from fighting in animals hooked leads to markedly increased lactate and consequent muscle and plasma acidosis, coupled to the inability to ventilate efficiently, resulting in a total loss of acid–basic balance (Wood et al. 1983; Bonga 1997; Thomas et al. 1999). Plasma lactate and pH are thus closely related parameters that should be evaluated in elasmobranchs stress studies, as also demonstrated by Awruch et al. (2011) and Gallagher et al. (2014).

Comparing the present data with others studies performed using other species of sharks in good condition versus moribund/dead animals (Manire et al. 2001; Moyes et al. 2006), it was possible to observe that, even within a similar time frame after death (i.e., ~hours), the pattern of loss in homeostasis is not identical (Table 3). The most typical "death markers" are lactate and potassium, which always show a sharp increase. Differences among the species were noted in plasma calcium and magnesium; in tiger sharks, these parameters were not different between dead and live animals. The opposite was observed for other species of sharks. The complexity of the regulation of these two cations coupled with the variability *Post-mortem* suggests that further studies are needed in order to

Table 2 Plasma concentrations of urea, sodium, chloride, magnesium, potassium, calcium, phosphorus, osmolality, glucose, lactate and total proteins in several sharks exposed to stress of capture

Shark species	Urea (mM)	Na^+ (mM)	Cl^- (mM)	Mg^{2+} (mM)	K^+ (mM)	Ca^{2+} (mM)	P^{3-} (mM)	Osmolality	Glucose (mM)	Lactate (mM)	Total proteins
Carcharhinus plumbeus[a]	−	+	+	+	+	+	−	−	+	+	−
Rhizoprionodon terraenovae[a]	−	+	−	−	+	+	−	−	+	+	−
Carcharhinus limbatus[a]	−	+	−	−	+	+	−	−	+	+	−
Carcharhinus longimanus[a]	−	+	−	−	+	+	−	−	+	+	−
Carcharhinus obscurus[a]	−	+	+	+	+	+	−	−	+	+	−
Isurus oxyrinchus[a]	−	+	−	−	+	+	−	−	+	+	−
Prionace glauca[a]	−	+	−	−	+	+	−	−	+	+	−
Carcharhinus falciformis[a]	−	+	−	−	+	+	−	−	+	+	−
Carcharhinus perezii[b]	+	+	+	+	+	+	−	−	+	+	−
Prionace glauca[c]	+	+	+	+	+	+	−	+	+	+	−
Alopias vulpinus[d]	−	+	+	+	+	+	−	−	+	−	−
Rhizoprionodon terraenovae[e]	−	−	−	−	−	−	−	+	+	+	−
Carcharhinus limbatus[f]	−	−	−	−	−	−	−	−	−	+	−
Carcharhinus leucas[f]	−	−	−	−	−	−	−	−	−	+	−
Sphyrna mokarran[f]	−	−	−	−	−	−	−	−	−	+	−
Negaprion brevirostris[g]	−	−	−	−	−	−	−	−	−	+	−
Squalus acanthias[h]	+	+	+	+	+	+	−	−	+	+	−
Heterodontus portusjacksoni[i]	+	−	−	−	+	−	−	−	+	+	−
Mustelus antarcticus[i]	+	−	−	−	+	−	−	−	+	+	−

Data used to build the table came from: [a] Marshall et al. (2012), [b] Brooks et al. (2012), [c] Wells et al. (1986), [d] Heberer et al. (2010), [e] Hoffmayer and Parsons (2001), [f] Gallagher et al. (2014), [g] Brooks et al. (2011), [h] Mandelman and Farrington (2007), [i] Frick et al. (2010)

provide a clearer picture of their loss of homeostasis. Otherwise, NaCl showed differences for tiger sharks but not for other species when comparing dead and live animals. As discussed above, the increase in their plasma levels suggests failure of the rectal gland secretory function. The lack of difference in NaCl levels in dead individuals of other species (see Table 3) with respect to their live counterparts may be ascribed to a higher sensitivity of these other species to capture and handling when compared to tiger sharks. Indeed, tiger sharks are known to be very robust and reportedly show high survival rates facing either accidental capture in commercial fisheries (catch and release, Beerkircher et al. 2002; Morgan and Burgess 2007) or specific capture for scientific purposes (Gallagher et al. 2014).

Table 3 References used: *S. tiburo*, *C. limbatus* and *C. leucas* (Manire et al. 2001); *P. glauca* (Moyes et al. 2006); *G. cuvier*, current study (same data depicted in Figs. 1, 2, 3)

Plasma osmolyte	Shark species										
	*S. tiburo**		*C. limbatus**		*C. leucas**		*P. glauca#*		*G. cuvier#*		
	Live (4)	Dead (12)	Live (9)	Dead (8)	Live (11)	Dead (5)	Live	Dead	Capture (5)	Handling (5)	Dead (3)
Osmolality	–	–	–	–	–	–	–	–	1090 ± 8.3	1053 ± 25.1	1138 ± 8.5
Urea	–	–	–	–	–	–	357 ± 4 (11)	352 ± 11 (8)	464 ± 11	462 ± 4.4	269 ± 23.8
Sodium	312 (306–317)	301 (286–306)	321 (313–329)	328 (321–333)	288 (285–294)	283 (278–291)	263 ± 1 (11)	264 ± 4 (7)	280 ± 1.58	280 ± 2.73	300 ± 7.4
Chloride	207 (206–209)	210 (205–213)	208 (207–212)	212 (210–214)	202 (201–204)	199 (198–200)	240 ± 4 (11)	236 ± 5 (7)	168 ± 4.02	147 ± 18.7	221 ± 17.4
Potassium	6.4 (6.0–7.1)	16.1 (11.4–17.5)	4 (3.7–4.3)	7.3 (7.0–9.7)	6.3 (5.7–6.7)	10.1 (9.4–12.9)	5.12 ± 0.44 (11)	7.01 ± 0.66 (9)	5.26 ± 0.2	5.32 ± 0.18	33.53 ± 4.5
Calcium	16.5 (15.9–17.2)	16.8 (15.8–17.5)	17.1 (16.3–17.7)	19.9 (18.4–21.7)	17.3 (16.0–17.6)	18.6 (17.2–19.0)	3.13 ± 0.11 (11)	3.70 ± 0.14 (9)	3.3 ± 0.4	3.48 ± 0.1	3.06 ± 0.07
Magnesium	–	–	–	–	–	–	0.98 ± 0.05 (11)	1.57 ± 0.08 (9)	2 ± 2.95	2.35 ± 2.94	2.26 ± 0.4
Lactate	3.9 (2.2–5.3)	12 (12.0–12.0)	4.7 (4.1–6.4)	12 (11.2–12.0)	6.3 (4.5–11.8)	12 (12.0–12.0)	5.80 ± 2.96 (11)	27.7 ± 24.07 (9)	8.72 ± 1.04	8.62 ± 1.01	42.13 ± 4.1
Glucose	183 (175–192)	125 (115–141)	62 (48.5–67.3)	44 (31.5–60.0)	54.5 (41.0–64.0)	22 (11.5–32.3)	4.75 ± 0.40 (11)	4.16 ± 0.69 (9)	7.16 ± 1.06	6.98 ± 2.05	5.01 ± 2.6
T. Proteins	3.2 (3.0–3.3)	3.9 (3.7–4.3)	2.2 (2.2–2.6)	2.4 (2.1–2.7)	2.9 (2.8–2.9)	2.6 (2.3–2.8)	–	–	4.08 ± 0.05	3.9 ± 0.06	4.26 ± 0.25
Phosphorus	–	–	–	–	–	–	–	–	0.14 ± 0.01	0.15 ± 0.01	5.05 ± 2.4

* Values are median (25th–75th quartiles) and # mean ± SEM (*n*)

Glucose: the need for caution when employed as stress marker

In vertebrates, as a primary response to stress, catecholamine and corticosteroids are released into the bloodstream (de Roos and de Roos 1978). As a result, the glycogen produced and stocked in the liver is mobilized in order to face the energy demands of the animal. However, in sharks, the major source of oxidative fuel are ketone bodies and amino acids (Speers-Roesch and Treberg 2010; Ballantyne 2015), and not glucose as observed in mammals and birds (Boonstra 2004; Romero and Butler 2007; Wright et al. 2011). Nevertheless, hyperglycemia is commonly used in studies as an indicator of stress in sharks (Table 2). Characterization of urea transporters in sharks demonstrates that during hyperglycemia, glucose receptors located in the rectal gland are activated (Walsh et al. 2006; Deck et al. 2016). Thus, high plasma glucose concentration in sharks is probably responsible to maintain the secretory mechanism (NaCl), using glucose as a metabolic fuel in an attempt to maintain the homeostatic balance and not for aerobic metabolism. Also, a recent study hypothesized the use of plasma glucose by the shark's brain as crucial for the metabolic coupling between glia and neurons (Balmaceda-Aguilera et al. 2012). There was no correlation between lactate and glucose, showing that glucose decrease (normally correlated to aerobic metabolism as a stress response) has no connection with lactate increase (closely related to anaerobic metabolism facing exhaustive exercise).

Unlike commonly reported, the present study showed *Post-mortem* hypoglycemia in two of three analyzed dead sharks (possibly related to the exhaustion from capture). Studies performed with bonnethead (*S. tiburo*), blacktip (*C. limbatus*) and bull (*C. leucas*) sharks showed hypoglycemic response in moribund/death sharks (Manire et al. 2001), corroborating at least partially to the findings of the present study. Post-release survival analysis performed in blue sharks (*P. glauca*) showed no difference between plasma glucose concentrations in moribund and live sharks (Moyes et al. 2006), proposing that hyperglycemia is not directly related to lethality. Atlantic sharpnose sharks (*Rhizoprionodon terraenovae*) exposed to different stressors also did not exhibit hyperglycemia as response (Hoffmayer et al. 2015). The high glucose concentration often observed in stress studies may be related to a series of factors not related to capture or handling, such as genetic predisposition, circadian rhythms (Gutierrez et al. 1984), feeding responses or as an immediate (short-term) reaction to a non-resting state. It is hypothesized that sharks are at an earlier stage in the glucose regulation evolutionary path (Ballantyne 2015). The "poor" regulation of glucose is probably related not only to the inability of kidneys to properly regulate the molecule, but also for its reduced role in elasmobranch's energy intake (Ballantyne 2015). Such is the lack of pattern for glucose in tiger sharks that the same concentrations observed in the present study (<30 min hooked) were observed in tiger sharks captured by longline hooked for 2–12 h (Marshall et al. 2012). Also, stressors such as severe hypoxia (Routley et al. 2002; Speers-Roesch and Treberg 2010; Speers-Roesch et al. 2012) and long-term fasting (de Roos et al. 1985) did not cause changes in glucose levels, suggesting the weak association between this compound and the main stress response mechanisms. Finally, Mommsen et al. (1999) discussed how the role of glucose is often overrated in its importance in fish. Thus, the use of glucose to assess stress should be treated with caution, since there are many variables that may affect its concentration.

Urea and phosphorus as stress parameters

Despite the proven importance of urea as the most significant molecule responsible for extracellular osmotic stability in the group (Ballantyne and Robinson 2010; Ballantyne 2016; Treberg and Speers-Roesch 2016), few studies have analyzed plasma alterations of urea upon the stress of capture and handling in sharks (Moyes et al. 2006; Mandelman and Farrington 2007; Frick et al. 2010; Brooks et al. 2012) (Table 2). Such is the importance of this compound for sharks that about 14 % of the oxygen consumption is reserved for urea synthesis (Kirschner 1993) and the ability to decrease urea in plasma seems to be determinant for the evolutionary occupation of freshwater environments (Ballantyne and Robinson 2010). A reduction of 57 % in urea concentration was observed between live and dead sharks, clearly demonstrating an inability to maintain the urea balance and as a consequence, concentrations compatible to life. The decrease observed was probably caused by membrane rupture, loss of retention

capacity and reduced renal reabsorption of urea via the countercurrent system, as suggested above, with the cessation of blood pumping by the heart. These results suggest that the analysis of plasma urea might be useful to assess homeostatic changes caused by stress (death) in sharks and should not, therefore, be neglected.

Plasma phosphorus, which has been even more overlooked (Table 2), shows values five times higher in dead animals when compared to concentrations found in live animals. It is a compound involved in cell growth/differentiation, cell membrane formation and phospholipids production. Phosphorus imbalance can also lead to changes in calcium regulation, since both are closely related (Andrigueto et al. 1990). Hyperphosphatemia is detrimental, since it leads to phosphate crystals formation which block arteries, causing heart failure (Neves et al. 2004). The higher concentrations of phosphorus in plasma observed in the present study may be related to the disintegration of cellular membranes, renal failure and gill collapse. Although rarely used, plasma phosphorus also seems to be an effective and unexpensive tool to assess stress level in this species.

Conclusions

By comparing surviving and dead tiger sharks, it was possible to conclude that: (1) *Post-mortem* data may be used as endpoint references in studies of stress in *G. cuvier*; (2) the sharks caught, sampled and released alive in the present study did not show loss of plasma osmotic/metabolic homeostatic balance when compared to *Post-mortem* data, indicating that the procedures did not generate a significant stress response; (3) the use of glucose as a tool for stress analysis should be treated with caution since: (a) hyperglycemia observed after capture may be related to other factors and (b) elasmobranchs do not use glucose as metabolic fuel. Finally (4) urea and phosphorus concentrations, often neglected, are in fact very relevant for osmotic and ionic balance and, for that reason, should be analyzed in stress studies.

Acknowledgments We would like to thank Chris Fisher and OCEARCH crew for sponsorship, access to the vessel, tools, sharks and professional fishing experience, CAPES for the fellowship grant to NW (99999.006477/2015-01) and ASA (BJT-A049/2013), FAPESP for the fellowship grant to HB (2013/25930-0), Fernando Ferradosa for reviewing the English and both anonymous reviewers for their significant and valuable contributions to the final version of this manuscript.

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