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Evaluation of the use of alternative biomarkers as indicators of post-release mortality in Blacktip Sharks (Carcharhinus limbatus)

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EVALUATION OF THE USE OF ALTERNATIVE BIOMARKERS AS INDICATORS OF POST-RELEASE MORTALITY IN BLACKTIP SHARKS (*CARCHARHINUS LIMBATUS)*

By

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CERTIFICATE OF APPROVAL

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ABSTRACT

The release of sharks caught in recreational fisheries or as by-catch in non-target commercial fisheries is generally regarded as a sustainable fishing approach. However, post-release mortality can occur in these fish due to physiological damage sustained during capture. It is important to determine the amount of mortality specifically attributed to capture so losses can be accounted for in population management practices. Previous studies have used electronic tagging and/or measurement of secondary stress indicators in plasma (e.g., pH, levels of lactate, glucose, pCO_2) to estimate rates of post-release mortality. These methods may not always be the best approaches, as electronic tagging can be costly, and some secondary stress markers may not consistently correlate with survivorship. Consequently, there has been a call for alternative indicators of post-release mortality in elasmobranchs. To address this, the present study evaluated several possible indicators of post-release mortality in plasma from blacktip sharks (*Carcharhinus limbatus*) captured via commercial and recreational methods, using individuals for which survivorship outcomes were confirmed via electronic tagging. These indicators included biomarkers of oxidative stress-induced macromolecule damage such as lipid peroxidation (malondialdehyde), protein carbonylation (PCs) and DNA oxidation (8-hydroxy-2'-deoxyguanosine); alternative measures of stress-induced energy mobilization (ketone bodies); indicators of rhabdomyolysis (myoglobin and creatine kinase); and general cell damage (cell-free DNA). Data suggested that 8-OHdG may increase with mortality in certain circumstances, but levels of plasma cell-free DNA and MDA did not differ with survivorship. Assays for PCs and indicators of rhabdomyolysis could not be validated. Concentrations of ketone bodies appeared to correlate with secondary stress indicators, but not with survivorship. This study demonstrates

that 8-OHdG may be a useful biomarker when assessing mortality in prolonged stressful situations but not in shorter capture encounters. There is still a need for the exploration of additional biomarkers to determine a reliable predictive indicator of post-release mortality.

INTRODUCTION

Elasmobranchs, the subclass in which sharks and rays belong, are frequently caught unintentionally as by-catch in both recreational and commercial fisheries. Most elasmobranchs are released after capture if they are not the intended target of fishing effort. Determining the impact that catch-and-release fishing has on shark populations requires an understanding of the stress response in elasmobranchs. The accepted definition of stress is a physiological state produced by an environmental factor that exceeds the normal adaptive responses of the animal or disturbs normal functioning to such an extent that the chances for survival are significantly reduced (Brett, 1958). It is important to note that the source of the stress can change the severity of the response. Under natural circumstances, fluctuations in environmental conditions including temperature may cause small bouts of stress that elicit very little response, whereas sudden changes like capture in fishing gear can cause acute stress, or an increased response, in elasmobranchs (Skomal and Mandelman, 2012). Capture stress can cause many physiological and metabolic changes in elasmobranchs that can result in a state of vulnerability in a released individual that may persist for up to twenty-four hours (Skomal, 2007). This may make the animal susceptible to opportunistic predators, as well as increases the chance the animal may experience mortality if it is unable to reverse the physiological damage resulting from capture. Individuals may experience muscle fatigue from exhaustive struggling, prolonged exposure to air, and external and/or internal physical trauma (Skomal, 2007; Skomal and Mandelman, 2012). Captured animals may also undergo cycles of oxygen deficiency (hypoxia) and re-oxygenation

during the struggle, which can increase the amount of stress and metabolic change the animal will experience and ultimately cause damage to tissue and organs (Skomal and Mandelman, 2012).

Elasmobranchs are captured by both the commercial and recreational fisheries. Commercial fishing is defined as the activity of catching fish for commercial profit and in many cases, this is done to provide catch for consumption. Recreational fishing, sometimes also referred to as sport fishing, is the act of fishing for competition or enjoyment. Some elasmobranch populations have experience and/or are experiencing population declines related to commercial fishing, as well as increased mortality rates related to recreational fishing (Whitney *et al*., 2017). In this case, sharks are exposed to anaerobic stress during the fight against capture, increased handling stress in an anoxic environment, and the potential for damage to the gill lamellae from exposure to air which may reduce the animal's ability to recover from respiratory acidosis (Casselman, 2005; Mandelman and Skomal, 2009).

During capture, sharks and other elasmobranchs undergo waves of sequential physiological responses. The first is the primary stress response, which occurs during and immediately after capture. This response is defined as an increase in the amount of circulating stress hormones, catecholamines and corticosteroids, in the bloodstream (Skomal and Mandelman, 2012). The secondary response is generally categorized by changes in biochemical factors in the blood and muscle, such as accumulation of lactate in muscle, decrease in intracellular and extracellular blood pH due to respiratory acidosis, changes to osmotic homeostasis and other physiological alterations (Skomal and Mandelman, 2012). The tertiary stress response has been generally viewed as the long-term effect of acute and chronic stress,

which can impact behavior, fitness and survivorship and is usually considered to be a response of the whole organism (Skomal and Mandelman, 2012). These effects can also be observed in whole populations in cases where a large number of individuals are affected, an example being the response to pollutant exposure (Skomal and Mandelman, 2012). Behavior has been shown to be atypical for days after an event of acute stress in other vertebrates, and elasmobranchs have been shown to exhibit listlessness and erratic behavior following capture (Skomal and Mandelman, 2012; Whitney *et al*., 2016). Hematological and immunological changes have also been noted in sharks after capture and have been attributed to the tertiary stress response (Van Rijn and Reina, 2010; Skomal and Mandelman, 2012).

Currently, little is known about the primary stress response in elasmobranchs. As in other vertebrates, catecholamines such as epinephrine and norepinephrine are expected to trigger the fight-or-flight response, mobilizing energy sources (deRoos and deRoos, 1978; Skomal and Mandelman, 2012; Pires *et al.,* 2011). However, very few studies have attempted to measure catecholamine levels as an indicator of stress in elasmobranchs because these hormones dissipate rapidly from the blood stream and prove unreliable when compared to other biomarkers of stress (Skomal and Mandelman, 2012; Pires *et al.,* 2011). Corticosteroids are other important hormones that are generally increased in vertebrates during the stress response. Elasmobranchs produce a unique corticosteroid, 1-α-hydroxycorticosterone (1α-OHB), which is thought to play a similar role to cortisol; this is frequently used in teleosts to quantify stress response (Skomal and Mandelman, 2012; Hazon and Balment, 1998; Romero, 2002). However, no reliable assay to quantify 1α-OHB currently exists (Skomal and Mandelman, 2012). Renshaw *et al.* (2012) also argues that attempting to measure 1α-OHB during the stress response may be difficult as it appears to be important in other metabolic processes and therefore not only reflect stress levels.

Manire *et al*. (2007) has also reported that 1α-OHB may plays roles in the reproductive biology of elasmobranchs, further confounding how much of the hormone may be present due to stress or other physiological functions. Due to the challenges in investigating the primary response, most stress related research within elasmobranchs has focused on the secondary stress response (Skomal and Mandelman, 2012).

Biomarkers of the secondary stress response (also called traditional indicators of stress in the current study) include but are not limited to the accumulation of lactate in the blood and muscle, increased partial pressure of carbon dioxide and subsequently respiratory acidosis, and intra- and extracellular pH changes due to acidosis (Skomal and Mandelman, 2012). In a study done by Mandelman and Skomal (2009), it was found that the severity of the physiological changes attributed to the capture event depended on the species of shark in question. Blood acid-base chemistry including pH, pCO_2 and lactate, were found to have the farthest deviation from baseline for the dusky shark *Carcharhinus obscurus*, the blacktip shark *Carcharhinus limbatus*, and the Atlantic sharpnose shark *Rhizoprionodon terraenovae*. These three species were found to be the most physiologically altered and had the highest at-vessel mortality rates. The study did not extend after release, so the post-release survival was unknown. It was also noted that there were changes to the blood-gas chemistry of the tiger shark *Galeocerdo cuiver* and the sandbar shark *Carcharhinus plumbeus*, although the changes were much less dramatic than other species and fewer cases of at-vessel mortality were observed. A study done by Mandelman and Farrington (2007) tested the blood chemistry of the spiny dogfish *Squalus acanthais* captured via otter-trawl and held in captivity at various intervals for 30 days to observe the impact of capture, transport, and captivity on the stress response. They found that the blood chemistry composition for the trawl-captured sharks mirrored the composition observed when the same species were

caught on hook-and-line, excluding glucose. It was noted that after the initial stressor of capture, a large increase in pCO_2 and an inversely related drop in blood pH was observed. Lactate levels measured immediately after capture were only moderately elevated, but increased significantly during transportation, suggesting that the initial moderate level of lactate was the onset of lactic and respiratory acidosis, and that the peak level took longer to manifest (Mandelman and Farrington, 2007). Glucose levels were lower than expected and did not reflect the levels found in animals caught on hook-and-line. Mandelman and Farrington (2007) attribute this hypoglycemia as a response to capture by trawl which depleted glucose levels more than hook-and-line fishing and was then further exacerbated by transport.

While some traditional markers have been shown to correlate with survivorship under some circumstances, they have been shown to be inconsistent. This may be due to a large amount of variation in the hypoxia tolerance between species of elasmobranchs (Renshaw *et al*., 2012). For example, during periods of hypoxia, lactate is known to be elevated. Using lactate as a proxy of stress may not reveal the complete level of stress due to the variation in hypoxia sensitivity across species of elasmobranchs (Skomal and Mandelman, 2012; Renshaw *et al*., 2012). Marshall *et al.* (2012) collected eleven species of sharks on longline gear to observe stress related physiological changes. It was noted that glucose did not always correlate with mortality, with dead sharks having some of the highest and lowest levels of glucose recorded during the study. The researchers comment that there were interspecific trends associated with glucose for longline captured sharks but that more research needed to be done. Lactate levels varied between species as well in this study, and a clear threshold for mortality could not be elucidated. Marshall *et al.* (2012) argues that this may be due to the differences in activity levels of each species, and how each species is able to deal with the accumulation of lactate during bouts of high energy

exercise. Due to the inconsistency in secondary stress markers, others have proposed use of alternative markers of stress (Renshaw *et al*., 2012). The current study attempted to address this call by exploring the use of alternative markers including plasma-borne indicators of oxidative stress, indicators of damaged cells and muscle, and alternative energy sources, ketone bodies. This was accomplished by evaluating differences in these markers in blacktip sharks for which survivorship outcomes were determined using electronic tagging, following capture and release via commercial- and recreational- based fishing. In order to provide sufficient background on the nature of this approach, a review of the use of tagging for determining elasmobranch survivorship outcomes and summaries of the potential value of using the proposed indicators in provided below.

Use of tagging in assessing post-release mortality in fishery-caught sharks

In using blood-borne biomarkers to determine occurrences of post-release mortality, many studies use external tagging to corroborate survival or mortality outcomes (Skomal, 2007; Whitney *et al*., 2016). Efforts to observe the behavior and movement of elasmobranchs post-release use a variety of tags to track location and survival. These tagging approaches include, but are not limited to: conventional tagging, acoustic tracking, the use of archival and pop-off satellite tags (PSATs), and use of accelerometer data loggers (ADLs) (Skomal, 2007; Whitney *et al*., 2016). Conventional tagging usually refers to a low-tech, low cost tag that typically requires a small hole for attachment and utilizes an identification number for the individual as well as directions to report the recovery of the individual after recapture. Conventional tagging relies on the recapture of an animal to determine its movements and survivorship, resulting in data deficiencies if animals are not recaptured (Skomal, 2007). Tags may be shed, individuals may die of unrelated causes, or individuals may emigrate, and contributes to the low recapture rate

associated with conventional tagging which makes it hard to derive any capture related mortality from this strategy (Skomal, 2007). Acoustic tags are typically more expensive than conventional tagging, and do not require recapture of the individual to collect data but must have the shark swim within range of acoustic receivers to collect movement data. Acoustic tags are also more invasive as they require a surgical incision to implant the tag into the body cavity of the individual (Skomal, 2007). Archival and PSATs are useful for long term tracking of movement but are limited by their deployment costs as well as size related complications for the animal (Skomal, 2007). These tags require larger areas of attachment to a shark and are more expensive but can transmit GPS coordinates along with other data whenever the tag surfaces (Skomal, 2007). According to Skomal (2007), greater than 100 tags would be needed to generate usable data for a post-release survivorship study which would make it quite a costly endeavor. ADLs are midline in both the cost and tech categories, and measure acceleration as well as body orientation, swimming dynamics and behaviors associated with swimming (Whitney *et al*., 2016). ADLs attach to the shark via a plastic cable with a timed galvanic release through a small hole in the dorsal fin. The tag is equipped with a float package to allow the tag to disengage from the shark after galvanic release (between 12 hours and 3 days) and float to the surface for recovery (Whitney *et al*., 2016). ADLs can be used to determine the survival of an animal post-release and give information about its condition through its orientation and swimming behavior (Skomal, 2007; Whitney *et al*., 2016).

An overview of oxidative stress and its potential value for measuring post-release mortality in sharks

One of the responses to physiological stress is oxidative stress, which occurs when organisms experience an increase in the production of reactive oxygen species (ROS) that may

exceed their ability to counteract the potentially damaging effects of these compounds (Birben *et al.*, 2012). ROS are generally produced during normal metabolic processes, both through enzymatic pathways including NADPH oxidases and non-enzymatic pathways for instance in the mitochondrial electron transport chain (Turrens, 2003; Birben *et al.*, 2012). The mitochondrial electron transport chain has been shown to leak electrons to oxygen through multiple redox areas along the chain and is the primary source of the ROS superoxide anion ($O_2^{-\bullet}$) within bodily tissues (Turrens, 2003). $O_2^{-\bullet}$ is produced at a number of sites on the mitochondria, including the matrix, the outer membrane and both sides of the inner membrane, and it has been found that some of the $O_2^{-\bullet}$ from the intermembrane area can travel into the cytoplasm through voltage-dependent channels (Turrens, 2003). ROS can also be intentionally produced for a number of functions including signaling between cells (Nikolaidis *et al*., 2012; Wadley *et al.,* 2016; Gaschler and Stockwell, 2017). Myocytes, leukocytes, and endothelial cells are all known to produce ROS during exercise, an example being the production of $O_2^{-\bullet}$ from many enzymatic sources within these cells (Wadley *et al.*, 2016). The rate of ROS production is controlled by the intensity of action and increases when electron flow slows down or when the concentration of oxygen increases (Turrens, 2003). ROS can increase during exercise in other vertebrates, but under normal circumstances cells are able to protect themselves against oxidative stress by using naturally produced antioxidants and antioxidant enzymes (Nikolaidis *et al*., 2012; Turrens, 2003). If the increase in production of ROS exceeds the rate that cells can produce antioxidant protectants, macromolecular damage can occur (Nikolaidis *et al*., 2012; Turrens, 2003). Reactive species can accumulate within cells, and cause oxidative damage to macromolecules such as lipids, proteins, and DNA (Gaschler and Stockwell, 2017). $O_2^{-\bullet}$ can also undergo dismutation to form hydrogen peroxide (H_2O_2), which can in turn reduce to water or can partially be reduced to

the hydroxyl radical ($OH^{\bullet}$) through the Fenton reaction in which H_2O_2 reacts with reduced active metal ions like iron or copper (Birben *et al.*, 2012). $OH^{\bullet}$ has a very small reaction volume and tends to react with nearby biomolecules at the site of its generation. $OH^{\bullet}$ does not have antioxidant defenses due to its highly unstable nature and small reaction volume, and therefore may cause severe damage to biomolecules (Turrens, 2003; Birben *et al.*, 2012, Cadet and Wagner, 2013). ROS may also be expelled into the surrounding tissues by passive diffusion of neutral ROS, or by enzymes embedded in the plasma membrane (Wadley *et al.,* 2016). During capture, elasmobranchs struggle and may perform muscle damaging exercise, which can result in increased levels of ROS (López-Cruz *et al.,* 2010). Therefore, the capture process is likely to result in an increase in the production of ROS and oxidative stress (Renshaw *et al*., 2012; López-Cruz *et al.,* 2010).

ROS are generally difficult to measure in vertebrate plasma due to their inherent instability (Bogdanov *et al*., 1999). ROS have also been found to increase during periods of moderate hypoxia, which may be linked to nitric oxide inhibiting cytochrome oxidase when oxygen is at a low concentration (Turrens, 2003). Occurrences of fluctuation from concentrations of low oxygen to high oxygen allows ROS to build up and may eventually overwhelm antioxidant defenses, resulting in macromolecular damage (Wadley *et al.,* 2016). An example of this is the process of lipid peroxidation (LPO), which occurs when a free radical binds to a phospholipid that is a part of the plasma membrane. This new radical-lipid molecule is not stable, so it will quickly react with other lipids to attempt to increase stability (Gaschler and Stockwell, 2017). The production of intermediate radicals will continue in a chain reaction until a product eventually reacts with another radical, producing a non-radical compound and terminating the reaction (Gaschler and Stockwell, 2017). This chain reaction weakens the lipids

of the cell membrane and can cause cell death, as well as produces measurable reactive aldehydes as end products, including malondialdehyde (MDA), a commonly used indicator of the process (Birben *et al.*, 2012). Due to the instability of ROS and the inherit difficulty to measure them, the current study chose to measure the products of ROS damage to macromolecules such as MDA.

DNA oxidation is another consequence resulting from increased ROS levels. DNA oxidation can damage the deoxyribose and nitrogenous bases, causing potential strand breakage as well as the formation of DNA adducts, or a portion of DNA bound to a reactive oxygen species that renders it unavailable for transcription and ultimately replication (Aust and Eveleigh, 1999). These adducts may be cleaved from DNA, causing potential mutation if an incorrect base is replaced in the empty spot (Aust and Eveleigh, 1999). During exposure to oxidative factors, 8-hydroxy-2'-deoxyguanosine (8-OHdG) will form from the cleavage of ROS-bound guanine from DNA (Park *et al.,* 2001). The formation of DNA adducts presents major problems in conjunction with but not limited to the mutation of DNA, inability to replicate DNA, the formation of cancerous growths due to damaged DNA, and even cell-death (Park *et al*., 2001; Birben *et al.*, 2012; Valavanidis *et al*., 2009). Quantification of 8-OHdG has been known to correlate with oxidative stress and has been used to screen for cancer and other diseases in humans (Valavanidis *et al*., 2009; Nikolaidis *et al*., 2012). For this reason, the study hypothesized that using increased concentrations of 8-OHdG may provide information about capture related mortality.

Protein carbonylation is typically measurable in vertebrate blood plasma and has been shown to increase during muscle damaging exercise because of protein oxidation due to interactions with ROS (Bloomer *et al.,* 2007; Wadley *et al.,* 2016). Protein carbonylation can

lead to reduced protein function and the eventual degradation of the affected protein. If protein carbonylation occurs in excess, it can lead to cell death if enough proteins are affected (Wadley *et al.,* 2016). Many types of cells produce ROS in response to exercise, which makes their proteins and other macromolecules vulnerable to oxidation. These cells may also expel these ROS via processes discussed previously. Through cellular extrusion of ROS, proteins in blood plasma are also susceptible to oxidation and ultimately protein carbonyl formation (Wadley *et al.,* 2016). Protein carbonyls (PC) have been used as biomarkers of many human diseases resulting from inflammation and ROS formation, not limited to Alzheimer's disease, cystic fibrosis, and acute respiratory distress syndrome (Dalle-Donne *et al*., 2003). Due to its usefulness as a biomarker in human health, measurement of PCs may also provide important information about the biochemical changes that can arise from increased ROS in sharks.

In order to test macromolecular damage by means of a non-invasive test allowing animals to survive or perish solely due to capture, this study collected and analyzed blood for indicators of oxidative stress-induced damage. During capture, it was hypothesized that molecular damage such as lipid peroxidation would occur as oxidative stress occurred, therefore causing an increase in the presence of MDA within the plasma of the animal (Renshaw *et al*., 2012). In this study, MDA was quantified from the plasma as a proxy of lipid peroxidation as it was proposed to be a potentially useful indicator of capture stress in sharks in a previous study by Renshaw *et al*. (2012). Increased levels of ROS due to capture was also expected to damage DNA, resulting in an increase in the measurable amounts of 8-OHdG from the plasma of sharks, similar to the use of 8-OHdG as a biomarker of cancer and human diseases (Valavanidis *et al*., 2009; Nikolaidis *et al*., 2012). PCs have been previously measured as biomarkers of cellular damage and disease due

to increased ROS and therefore were also selected as a potential biomarker of post-release mortality (Dalle-Donne *et al*., 2003).

The potential use of cell damage markers for assessing post-release mortality in sharks

Free floating DNA fragments and nucleotides, or cell-free DNA (cfDNA), originating from apoptosis or necrosis of tissues can often be found circulating through the blood plasma of an organism (Chang *et al.,* 2003). An increase in cfDNA from blood plasma of patients has been reported during clinical disorders including cancer and autoimmune disorders, which are known to damage organs and cause cell death (Swarup and Rajeswari, 2007). Studies have shown that cfDNA will increase specifically in response to oxidative stress (Guan *et al.,* 2017; Breitbach *et al.,* 2012). Large amounts of physical activity and physical exhaustion are known to correlate with physical and metabolic muscular damage as well as the damage to DNA caused by oxidative stress, which in turn can be responsible for an increase in cfDNA (Breitbach *et al.,* 2012). Haller *et al.* (2017) argued that cfDNA is a reliable marker for monitoring aerobic exercise intensity and duration, and in their testing discovered that cfDNA will steadily increase during exercise even when secondary markers like lactate did not. Andreatta *et al*. (2018) found that cfDNA increased in response to high intensity exercise and that it was able to be measured immediately after exercise. In agreement with the previously mentioned study, Andreatta *et al*., (2018) also found that cfDNA was correlated to exercise intensity whereas lactate levels were not. Measurement of cfDNA via plasma has been shown to be a non-invasive tool to diagnose and monitor the progression of diseases as well as oxidative stress (Swarup and Rajeswari, 2007; Breitbach *et al.,* 2012). Based on these points, we hypothesized that elasmobranchs may exhibit signs of increased cfDNA corresponding to the amount of time the animal struggles on the hook. Testing cfDNA as an indicator of stress in elasmobranchs during capture would be a novel

approach to examining the physiological changes during capture and may provide insight into the occurrence of post-release mortality.

Use of indicators of rhabdomyolysis for assessing post-release mortality in sharks

Muscle damage is characterized as a weakening of sarcomeres, which results in membrane disruption and eventual breakdown of damaged muscle causing the release of muscle proteins in the blood, for instance creatine kinase, lactate dehydrogenase and myoglobin (Çakir-Atabek *et al*., 2019). The term rhabdomyolysis is used to describe this breakdown and release of muscular contents and can be quantified via circulating muscle proteins in the bloodstream (Çakir-Atabek *et al*., 2019; Watanabe *et al*., 2019). Myoglobin (Mb) is a cytoplasmic hemoprotein that is expressed in cardiac myocytes and oxidative myofibers in skeletal muscle and contains iron, allowing for the reversible binding of ligands, namely oxygen (Garry and Mammen, 2007). A number of studies have used Mb as a biomarker of muscle damage, as it is not typically detected in the blood during normal conditions and has shown to increase following muscle damaging exercise (Rodenburg *et al.*, 1993; Balnave and Thompson, 1993; Féasson *et al.*, 2002; Peake *et al.*, 2005). Balnave and Thompson (1993) suggest that Mb is small and may exit muscle cells via small lesions in the sarcolemma following damaging exercise, which allows it to accumulate in detectable quantities in the blood within an hour of muscle damage. A study by Peake *et al.* (2005) observed a significant increase in the concentration of myoglobin in the blood plasma of runners after a bout of muscle damaging exercise. Elasmobranchs experiencing capture stress may exhibit higher levels of Mb as the amount of time the animal struggles against capture increases. Testing Mb as an indicator of capture stress in elasmobranchs would be a

novel approach to examining correlations between muscle damaging exercise and post-release mortality.

Creatine kinase (CK) has been regarded as the "gold standard" in diagnosing rhabdomyolysis in human patients for diseases resulting in muscle damage such as dermatomyositis, polymyositis, and myocardial infarction (Baird *et al.*, 2012; Cervellin *et al.*, 2017; Benveniste *et al.*, 2019). CK is an enzyme found in skeletal muscle and cardiac muscle as well as the brain (Bieber and Jefferson, 2019). It has also been used to determine if muscle damage has occurred after exercise in a study done by Souza *et al.* (2017), in which CK was measured immediately after a mixed martial arts fight and again 24 hours after the fight. This study found a significant increase in CK in the fighter's blood 24 hours after the fight. Another study found that untrained men and women experienced increases in blood serum levels of CK as the volume of exercise increased, and that the most significant increase was when the highest intensity exercise was done during the shortest duration (Tiidus and Ianuzzo, 1983). This suggests that short-term high intensity of exercise may cause more muscular damage than low intensity exercise over a longer period of time. Elasmobranchs experiencing capture stress may therefore exhibit higher levels of CK as the amount of time the animal struggles against capture increases.

Use of ketone body measurements in assessing post-release mortality in sharks

In vertebrates, plasma concentrations of glucose are often measured as an indicator of the stress response because they usually increase in response to the primary stress response. During the onset of exercise in most vertebrates, glycogenolysis will occur within muscle tissue. This causes the breakdown of muscle glycogen stores, which produces glucose that can be used in

glycolysis and ultimately for oxidative phosphorylation to provide ATP to cells (Horton and Beisel, 1994). However, it is possible that elasmobranchs may respond differently to the stress of exercise. A study conducted by Marshall *et al.* (2012) collected samples from a variety of species of elasmobranch as part of a capture-stress study, and observed that glucose did not correlate with stress-induced mortality, and that some of the highest and lowest levels of glucose recorded were associated with expired sharks.

The lack of value in using glucose as a stress indicator in elasmobranchs may be due to differences in energy utilization in sharks. For example, prior studies have indicated that carbohydrates may not serve as a significant aerobic fuel based on low levels of carbohydrates in their diet and low plasma glucose concentrations (Watson and Dickson, 2001; Zammit and Newsholme, 1979). Instead, it has been proposed that elasmobranchs may use ketone bodies, as opposed to glucose or fatty acids, as primary fuels. This implies that elasmobranchs may rely more heavily on ketone bodies as the preferred substrate for oxidation to fuel metabolic processes. The term ketone bodies refer to acetone, acetoacetic acid (AcAc) and β-hydroxybutyric acid (β-HB) (Laffel, 1999). Previous studies have focused on measuring the amount of β-HB present, as it is the most prevalent ketone body that has been measured in elasmobranch blood (Zammit, 1981; deRoos, 1994; Watson and Dickson, 2001). Other studies have found that AcAc is difficult to quantify as it readily decarboxylates into β-HB and acetone, and that AcAc must be converted into β-HB before oxidation within the target tissue (Watson and Dickson, 2001; Laffel, 1999). Acetone is created from this spontaneous decarboxylation of AcAc, but it does not dissociate to yield hydrogen ions, and will be excreted via respiration (Laffel, 1999). In a study by Beis *et al.* (1980) it was found that enzyme activity measured in muscle homogenates suggest that ketone bodies and amino acids are the primary fuels involved

in aerobic elasmobranch metabolism. Another study by Ballantyne *et al.* (1992) demonstrated that the mitochondria of pelagic elasmobranchs preferred amino acids and ketones as substrates over fatty acids due to the presence of non-oxidized palmitoyl carnitine, suggesting that there was little to no metabolism of fatty acids. In most vertebrates, ketone bodies are used as alternative fuels when glucose levels are low because other energy sources, fatty acids among others, cannot cross the blood brain barrier (Laffel, 1999). In a study by Valls *et al.* (2016), it was found that elasmobranchs would mobilize hepatic lipids to use as oxidative fuel in the form of ketone bodies, and that ketone bodies were found in high concentrations under multiple conditions, akin to the ketosis of fasting mammals. In response to the stress of capture, elasmobranchs are known to mobilize energy reserves and deplete ATP, and it has been suggested that the presence of ketone bodies may play a significant role in delivering energy during the stress response (Skomal and Mandelman, 2012; Watson and Dickson, 2001). This may result in an increase of ketone bodies in the blood plasma that are used to deliver energy to essential organs (Skomal and Mandelman, 2012; Watson and Dickson, 2001). For these reasons, this study chose to also explore the use of plasma ketone body concentration as an indicator of stress and subsequent post-release mortality.

The use of the blacktip shark as study animal

The blacktip shark was used as the model species for this study due to its abundance, high commercial and recreational fishing value, as well as its moderate post-release mortality rate following capture, which contributes to its sensitivity to overfishing (Whitney *et al*., 2016; Weber, 2019). Previous studies have found a variety of mortality rates for blacktip sharks ranging from 88% at-vessel mortality captured on demersal longline to no at-vessel mortality for sharks captured on rod-and-reel, but reported a 9.7% post-release mortality rate (Morgan and

Burgess, 2007; Whitney *et al.,* 2017). Due to the issues highlighted when using secondary indicators in measuring stress related physiological damage and the wide range of mortality reported through different fisheries and fishing practices, the purpose of this study was to explore promising novel indicators that may provide more precise measurements within both the commercial and recreational fishery.

METHODOLOGY

The present study included two components. The first component focused on examining the proposed indicators of stress on sharks captured in a mock-commercial fishery setting. Biomarkers of stress previously mentioned were utilized to determine predictability of post-release mortality. Biomarkers that showed a significant difference between survival and mortality were considered validated. These validated biomarkers were then used to study stress and post-release mortality in the second component of this study. The second component focused on testing validated biomarkers of stress on animals caught in the recreational fishery. Recreationally caught fish were comprised of fish captured in boat-based fishing efforts and shore-based fishing efforts.

Component 1

The blacktip sharks used in the first component of the present study were previously captured as part of an earlier study on capture stress conducted by Whitney *et al*. (2016). In this study, bottom longlines were deployed with hook timers to determine the fight time for each animal. Lines containing 150-250 18/0 hooks were used, with soak times ranging from 2 to 15 hours. Fishing practices and locations were determined by commercial longline captains to ensure efforts mimicked commercial fishing practices. Following animal capture, blood was

collected via caudal venipuncture and stored at –80ºC for future testing. ADL tags and acoustic tags were used to determine the survival outcome for animals following release. An ADL was deployed by drilling two holes and securing it to the left side of the first dorsal fin using monofilament. Tags employed a galvanic timed release to allow tag to detach between 1 and 7 days (Whitney *et al*., 2016). The ADL was set to record tri-axial acceleration, depth, and temperature and included an AHF transmitter for recovery (Whitney *et al*., 2016). Mortality events were determined via ADL data that showed abnormal body orientation and swimming patterns as well as the cessation of body movements defined in the study done by Whitney *et al*. (2016). Plasma lactate, pH and pCO_2 were measured using a portable blood gas analyzer (VetScan i-STAT; Abaxis North America, Union City, CA) at the time of capture. Data on survivorship outcomes and secondary stress markers, along with aliquots of plasma from individual blacktip sharks used in this study, were provided by Whitney.

For this component of the study, 49 animals were captured alive and from this group, 28 survived post release while 21 expired. Of the expired sharks, 11 were female and 10 were male. Of the surviving sharks, 17 were female and 11 were male. Duration of capture was between 17 and 948 minutes (mean = 174.265 minutes). Shark fork length (FL) of animals that expired post-release ranged from 104 cm to 150 cm with an average FL of 127 cm, while sharks that survived ranged from 105 cm FL to 150 cm with an average of FL 126 cm. Differences between sexes and body size were not examined in this study.

Component 2

The blacktip sharks collected for the second component of the study were previously captured by recreational anglers as part of a study conducted by Weber (2019). Recreational

fishermen logged important parameters including time on the line, hook type, water temperature, location, and other information for the captured sharks (Weber, 2019). For the purposes of this study, recreational fishing effort was divided into two categories, boat-based fishing efforts and shore-based fishing effort, with both categories utilizing rod-and-reel to capture elasmobranchs. Boat-based practices and shore-based practices may include situations in which sharks can be injured or experience stress in addition to being partially or completely removed from water for hook removal or photography (Weber, 2019; Casselman, 2005). Post-release mortality was determined by utilizing acoustic transmitters and validated via a subset of population receiving pop-off satellite tags (PSATs) as defined by Weber (2019). Blood was collected via caudal venipuncture and was analyzed using an i-STAT portable blood analyzer for traditional indicators of stress, including pH and lactate at time of capture (Weber, 2019). The blood was then stored at –80ºC for future testing (Weber, 2019). Data on survivorship outcomes and secondary stress markers, along with aliquots of plasma, were provided by Weber.

For the second component of the study, 44 animals were obtained in which 8 individuals were deceased post-release for sharks captured by charter-boat based anglers and 7 were deceased post-release for sharks captured by shore-based anglers. All sharks captured for this study were alive at time of capture (Weber, 2019). Duration of capture varied from 3.6 and 16.9 minutes (mean = 8.032 minutes). FL for individuals experiencing mortality ranged from 87 cm to 150 cm with the average size being 128.2 cm. FL for individuals that survived ranged from 81 cm to 153 cm, with the average length being 115.55 cm. Of the expired sharks, 9 were female and 6 were male. Of the surviving sharks, 22 were female and 7 were male. Differences between sexes and body size were not examined in this study.

Biomarker Analysis

Biomarkers were quantified via a variety of routinely-employed assays, which were conducted using commercially available kits following manufacturer's instructions (Table 1). Numbers of samples analyzed per assay as well as per component differed based on sample availability, cost and sample load of assay (Table 1).

Table I Concentrations of nontraditional biomarkers were measured using commercially available assay kits following manufacturer's instructions. Number of samples run between assays differ due to sample availability, cost, and assay load. Asterisks in total number of samples analyze column denote biomarkers in which a sample pool was used to determine validity of test while preserving sample for later use. The sample pools were serially diluted to concentrations varying from 1:1 to 1:64 to determine detectability of biomarker for those particular assays.

Component I

Tested Biomarker	Assay	Total number of samples analyzed	Number of samples analyzed from surviving sharks	Number of samples analyzed from deceased sharks	Assay and Vendor
MDA	Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit	49	28	21	TBARS assay kit, Cell Biolabs, Inc., San Diego, CA
8-OHdG	8-OHdG DNA Damage ELISA Assay Kit	43	25	18	8-OHG DNA Damage ELISA assay kit, Cell Biolabs Inc., San Diego, CA
PC	Oxi-select Protein Carbonyl Fluorometric Kit	24	12	12	Oxi-select Protein Carbonyl Fluorometric kit, Cell Biolabs Inc., San Diego, CA
cf-DNA	EpiQuik Circulating Cell-Free DNA Isolation Kit	24	12	12	EpiQuik Circulating Cell-Free DNA Isolation Kit, EpiGentek, Farmingdale, NY
Ketone Bodies	β-Hydroxybutyrate (Ketone Body) Assay Kit	46	26	20	β-Hydroxybutyrate (Ketone Body) Assay Kit, Cell Biolabs Inc., San Diego, CA
Mb	Myoglobin AccuBind ELISA Kit	9*	-	-	Myoglobin AccuBind ELISA kit, Monobind Inc., Lake Forest, CA
CK	Creatine Kinase Fluorometric assay Kit	5*	-	-	Creatine Kinase Fluorometric assay kit, Cayman Chemical, Ann Arbor, MI

Component II

Tested Biomarker	Assay	Total number of samples analyzed	Number of samples analyzed from surviving sharks	Number of samples analyzed from deceased sharks	Assay and Vendor
MDA	Thiobarbituric Acid Reactive Substances (TBARS) assay	43	28	15	TBARS assay kit, Cell Biolabs, Inc., San Diego, CA
8-OHdG	8-OHdG DNA Damage ELISA assay kit	44	29	15	8-OHG DNA Damage ELISA assay kit, Cell Biolabs Inc., San Diego, CA

Data analysis

Data on plasma analytes were grouped by survivorship outcomes, as determined via electronic tagging. Mean values were compared using the Student-t-test to determine if they differed significantly for samples from both objectives. A Student-t-test was chosen for the present study as data was found to be parametric and unpaired. In addition, correlations between concentrations of plasma analytes and plasma pH, pCO_2, lactate, and time animals were on the line were analyzed using the Pearson correlation coefficient *r* for samples in first and second components. Pearson correlation coefficient *r* was chosen for this study as the data was found to be parametric and also to determine if there was a relationship between the nontraditional indicators and traditionally used indicators.

RESULTS

Component 1

Levels of MDA were quantified in 49 sharks (28 survived, 21 expired) and ranged from 0.106 nmol/mL to 7.768 nmol/mL for surviving sharks (mean ± SD = 3.562 ±0.217) and from 1.29 nmol/mL to 7.223 nmol/mL for expired sharks (mean ± SD = 3.538 ±0.305). No significant difference was found between surviving or expired sharks for MDA (Figure 1a). No significant correlations were found between concentrations of MDA and lactate (Figure 2a), pH (Figure 3a), pCO_2 (Figure 4a) and length of time on the line (Figure 5a).

Levels of 8-OHdG were quantified in 43 sharks (25 survived, 18 expired) and ranged from 705.08 pg/mL to 4540.7 pg/mL in surviving sharks (mean ± SD = 1901.9 ±150.6) and from 923.1 pg/mL to 3722.8 pg/mL in expired sharks (mean ± SD = 2432.0 ±154.8). A significant difference was found between surviving and expired sharks for 8-OHdG in this portion of this study (Figure 1b). Significant correlations were found between concentrations of 8-OHdG and

pH (Figure 3b), as well as 8-OHdG and length of time on the line (Figure 5b) but were not found between 8-OHdG and lactate (Figure 2b) or pCO_2 (Figure 4b).

Levels of cfDNA were quantified in 24 sharks (12 survived, 12 expired) and ranged from 152 ng/100 µL to 373 ng/100 µL in surviving sharks (mean ± SD = 250.5 ±47.08) and from 67 ng/100 µL to 589 ng/100 µL in expired sharks (mean ± SD = 256.8 ±22.50). No significant difference was found between surviving or expired sharks for cfDNA (Figure 1c). No significant correlations were found between concentrations of cfDNA and lactate (Figure 2c), pH (Figure 3c), pCO_2 (Figure 4c) and length of time on the line (Figure 5c).

Levels of ketone bodies were quantified in 46 sharks (26 survived, 20 expired) and ranged from 8.026 µM to 233.4 µM to in surviving sharks (mean ± SD = 40.29 ±0.889) and from 8.092 µM to 44.753 µM to in expired sharks (mean ± SD = 21.91 ±0.818). No significant difference was found between surviving or expired sharks for ketone bodies (Figure 1d). Significant correlations were found between concentrations of ketone bodies and lactate (Figure 2d), pH (Figure 3d), pCO_2 (Figure 4d), and length of time on the line (Figure 5d)

This study attempted to quantify and compare levels of PC, MB, and CK in the blood but these were all found to be below detectable levels using the assays described in Table 1.

Component 2

For the second component of the present study, levels of MDA were quantified in 43 sharks (28 survived, 15 expired) and ranged from 1.02 nmol/mL to 10.268 nmol/mL for surviving sharks (mean ± SD = 3.022 ±0.081) and from 0.82 nmol/mL to 13.644 nmol/mL for expired sharks (mean ± SD = 3.477 ±0.160). No significant difference was found between surviving or expired sharks for MDA (Figure 6a). A significant correlation was found between

concentrations of MDA and pH (Figure 8a) but was not found between MDA and lactate (Figure 7a), pCO_2 (Figure 9a) and length of time on the line (Figure 10a).

Levels of 8-OHdG were quantified in 44 sharks (29 survived, 15 expired) and ranged from 21.031 pg/mL to 4560.295 pg/mL in surviving sharks (mean ± SD = 1913.14 ±409.54) and from 294.401 pg/mL to 4160.684 pg/mL in expired sharks (mean ± SD = 1905.302 ±370.25). No significant relationship was found between surviving and expired sharks for 8-OHdG in this portion of the study (Figure 6b). No Significant correlations were found between concentrations of 8-OHdG and lactate (Figure 7b), pH (Figure 8b), pCO_2 (Figure 9b), or length of time on the line (Figure 10b).

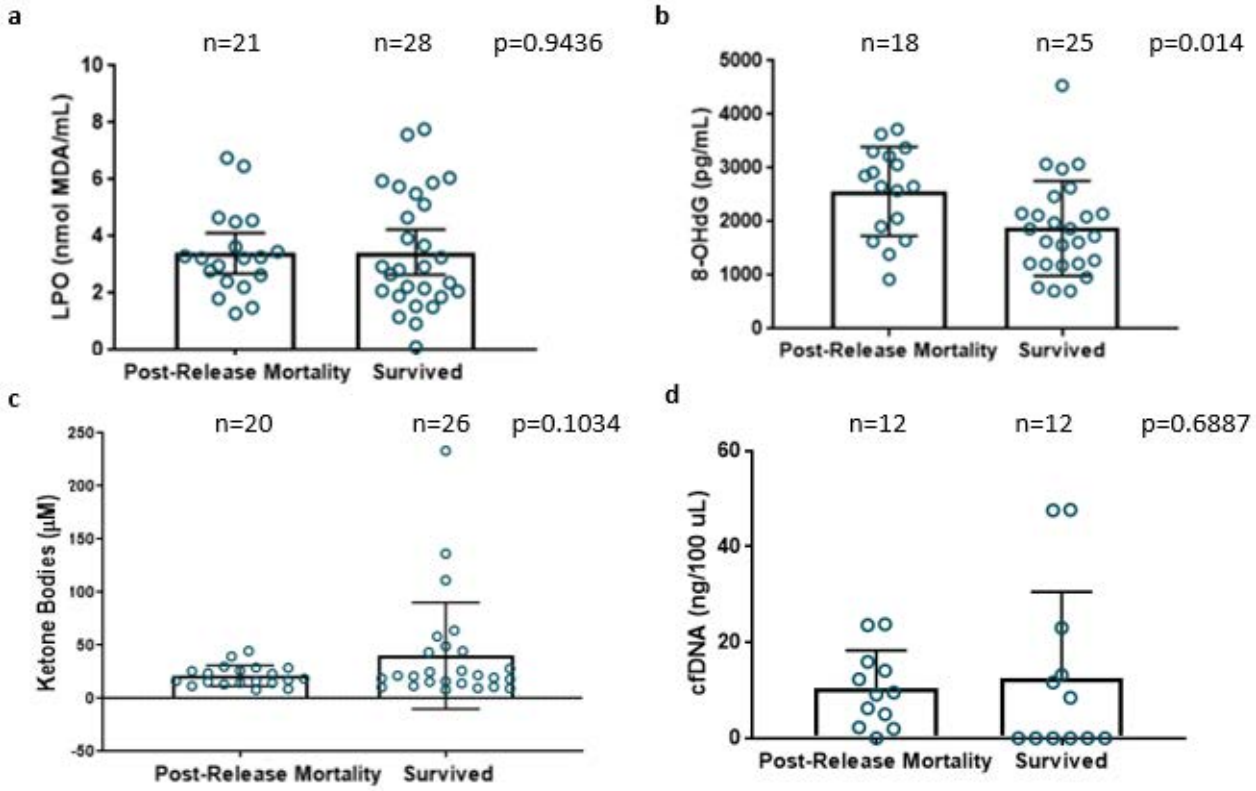

Figure 1. Plasma concentrations of a) malondialdehyde (MDA), b) 8-deoxy-2-hydroxyguanosine (8-OHdG), c) ketone bodies, and d) cell-free DNA (cfDNA) in blacktip sharks (*Carcharhinus limbatus*) that experienced post-release mortality or survived following capture in commercial bottom longline gear. Open circles represent individual values, whereas bars represent means ±SD. Sample size is shown above bar. P-values represent results of Student t-tests, which were used to determine if significant differences were observed between study groups.

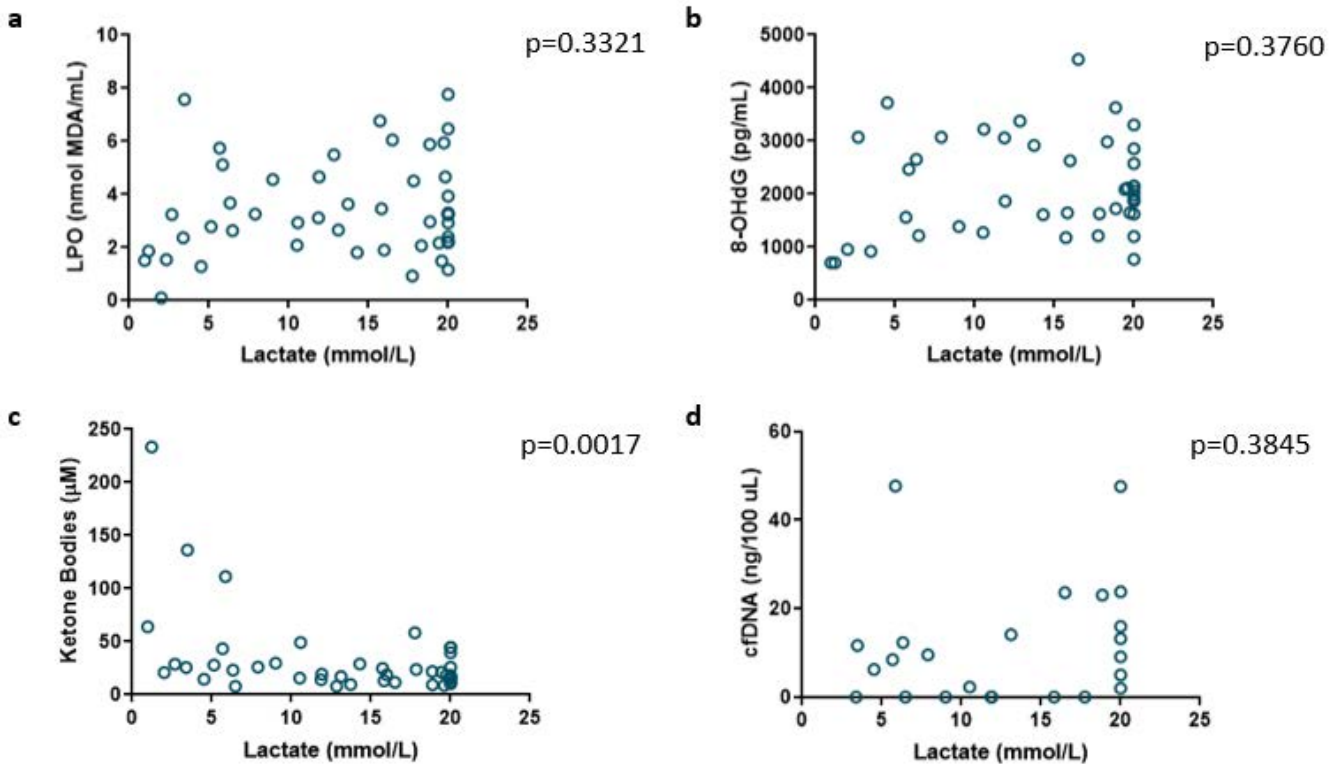

Figure 2. Plasma concentrations of a) malondialdehyde (MDA), b) 8-deoxy-2-hydroxyguanosine (8-OHdG), c) ketone bodies, and d) cell-free DNA (cfDNA) in blacktip sharks (*Carcharhinus limbatus*) compared to lactate (nmol/mL). Open circles represent individuals in which lactate and tested biomarker were both measured. P-values represent results of Pearson correlation coefficient *r*, which were used to determine if significant correlations were observed between study groups.

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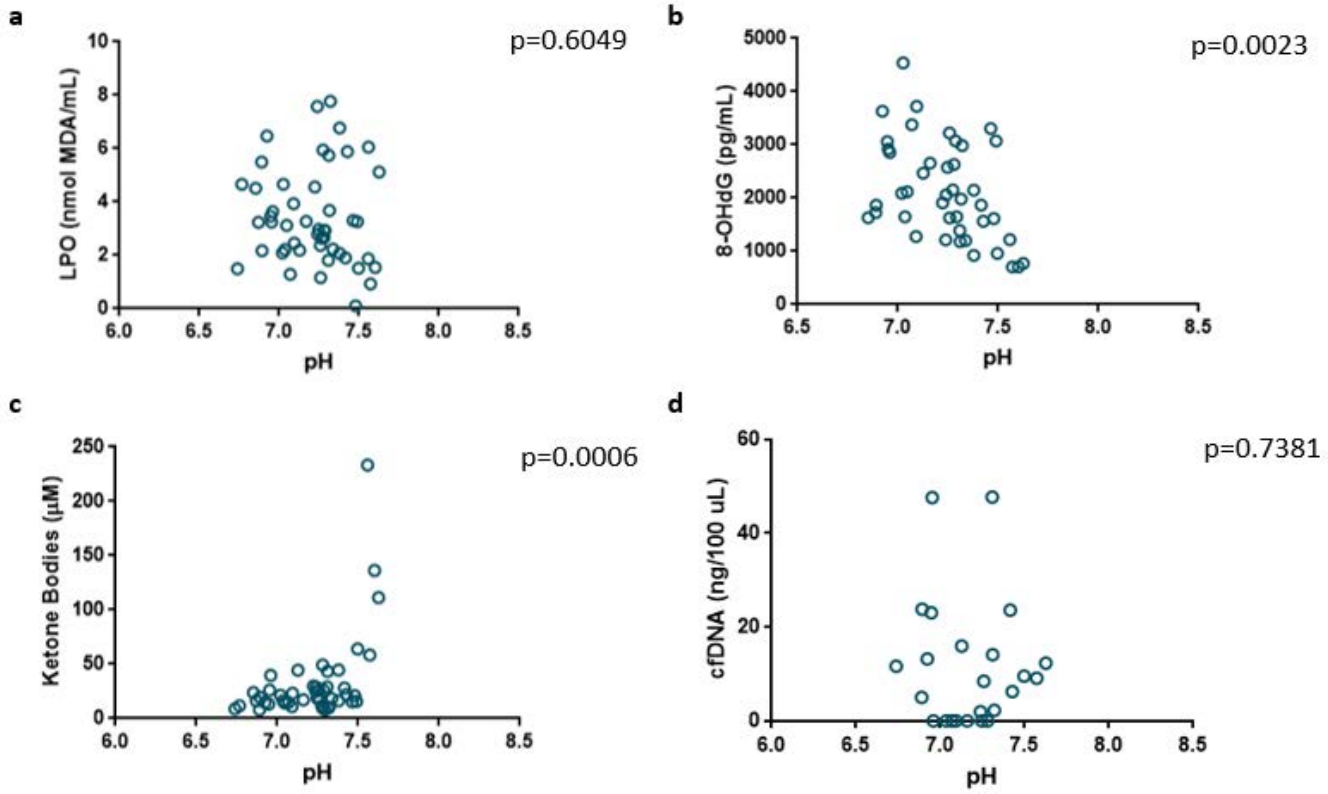

Figure 3. Plasma concentrations of a) malondialdehyde (MDA), b) 8-deoxy-2-hydroxyguanosine (8-OHdG), c) ketone bodies, and d) cell-free DNA (cfDNA) in blacktip sharks (*Carcharhinus limbatus*) compared to pH. Open circles represent individuals in which pH and tested biomarker were both measured. P-values represent results of Pearson correlation coefficient *r*, which were used to determine if significant correlations were observed between study groups.

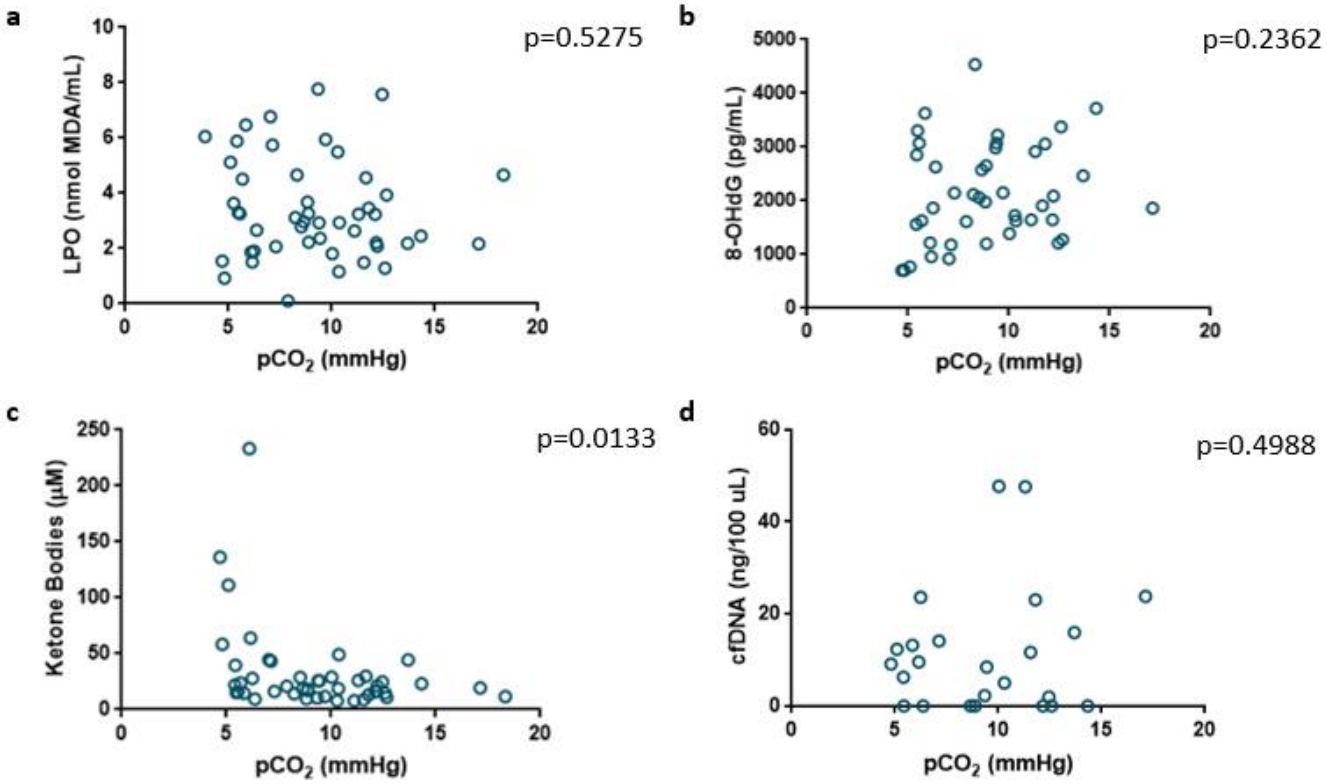

Figure 4. Plasma concentrations of a) malondialdehyde (MDA), b) 8-deoxy-2-hydroxyguanosine (8-OHdG), c) ketone bodies, and d) cell-free DNA (cfDNA) in blacktip sharks (*Carcharhinus limbatus*) compared to pCO_2 (mmHg). Open circles represent individuals in which pCO_2 and tested biomarker were both measured. P-values represent results of Pearson correlation coefficient *r*, which were used to determine if significant correlations were observed between study groups.

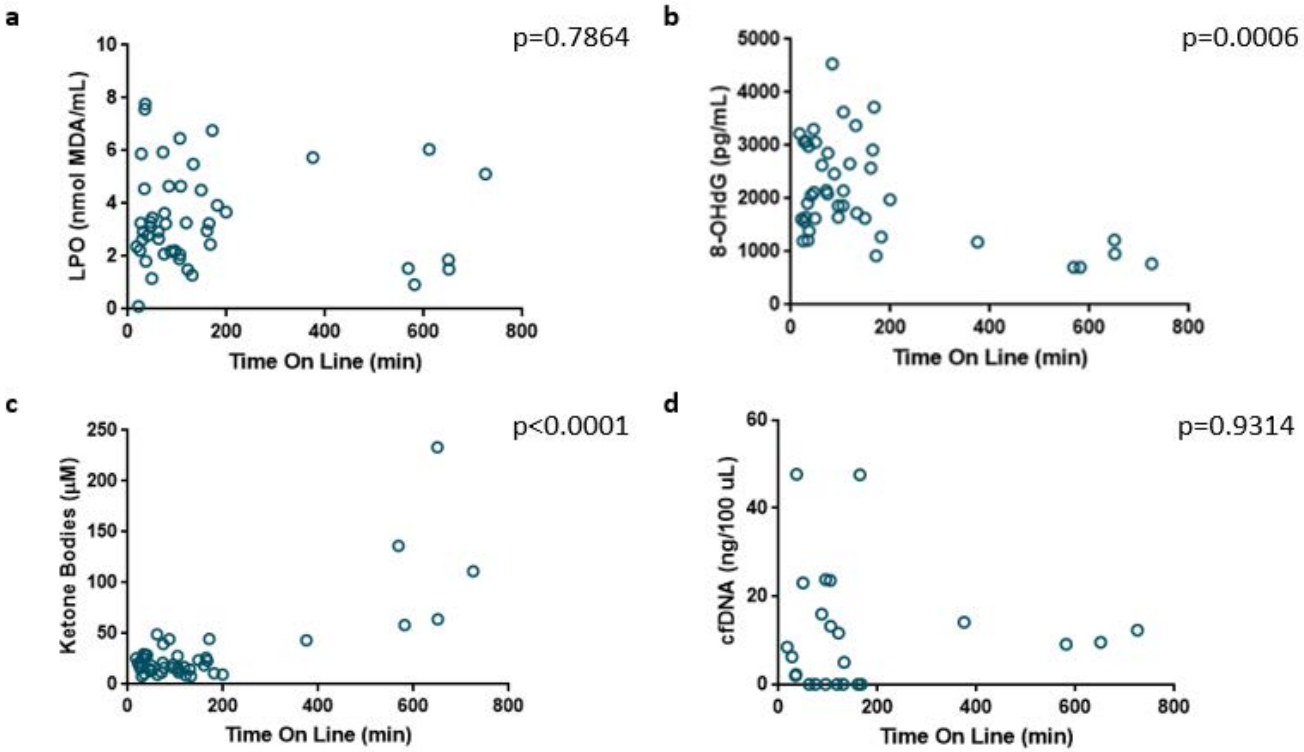

Figure 5. Plasma concentrations of a) malondialdehyde (MDA), b) 8-deoxy-2-hydroxyguanosine (8-OHdG), c) ketone bodies, and d) cell-free DNA (cfDNA) in blacktip sharks (*Carcharhinus limbatus*) compared to the time animals spent fighting on the line (minutes). Open circles represent individuals in which fight time and tested biomarker were both measured. P-values represent results of Pearson correlation coefficient *r*, which were used to determine if significant correlations were observed between study groups.

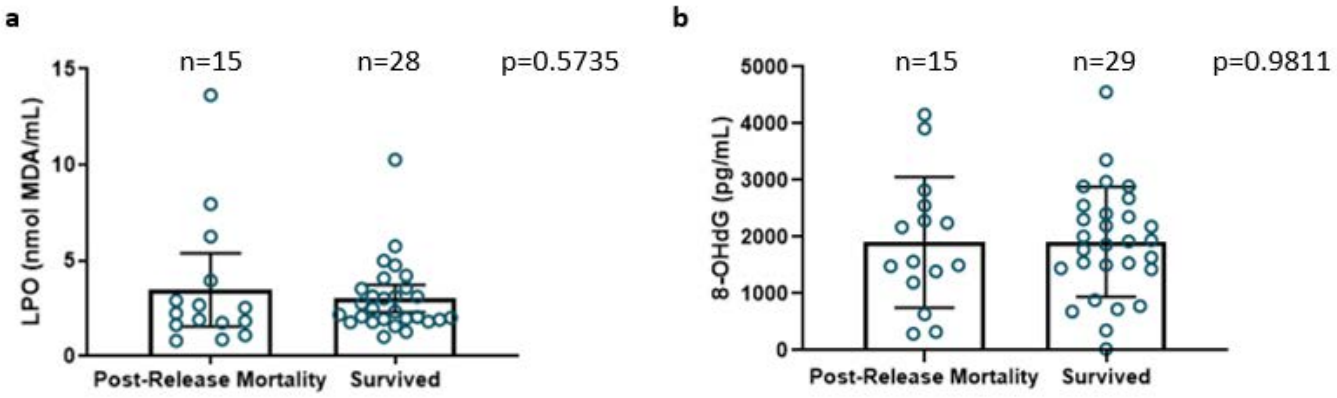

Figure 6. Plasma concentrations of a) malondialdehyde (MDA), and b) 8-deoxy-2-hydroxyguanosine (8-OHdG) in blacktip sharks (*Carcharhinus limbatus*) that experienced post-release mortality or survived following capture in recreational fishing gear. Open circles represent individual values, whereas bars represent means ±SD. Sample size is shown above bar. P-values represent results of Student t-tests, which were used to determine if significant differences were observed between study groups.

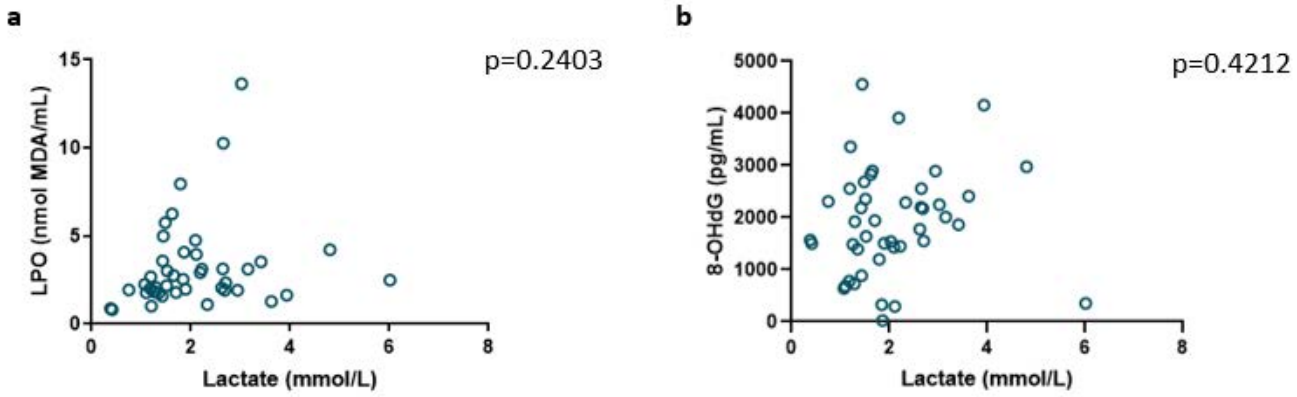

Figure 7. Plasma concentrations of a) malondialdehyde (MDA), and b) 8-deoxy-2-hydroxyguanosine (8-OHdG) in blacktip sharks (*Carcharhinus limbatus*) compared to lactate (nmol/mL). Open circles represent individuals in which lactate and tested biomarker were both measured. P-values represent results of Pearson correlation coefficient *r*, which were used to determine if significant correlations were observed between study groups.

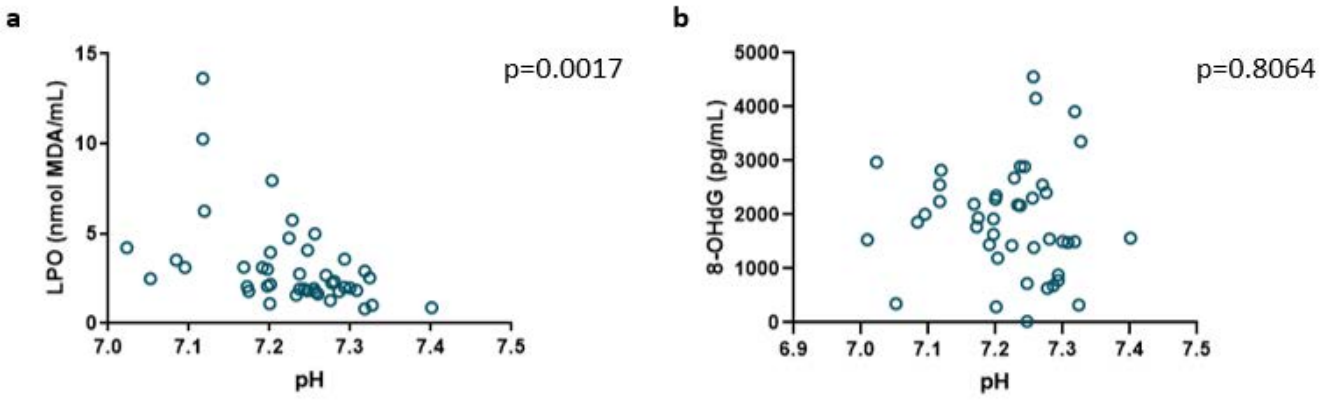

Figure 8. Plasma concentrations of a) malondialdehyde (MDA), and b) 8-deoxy-2-hydroxyguanosine (8-OHdG) in blacktip sharks (*Carcharhinus limbatus*) compared to pH. Open circles represent individuals in which pH and tested biomarker were both measured. P-values represent results of Pearson correlation coefficient *r*, which were used to determine if significant correlations were observed between study groups.

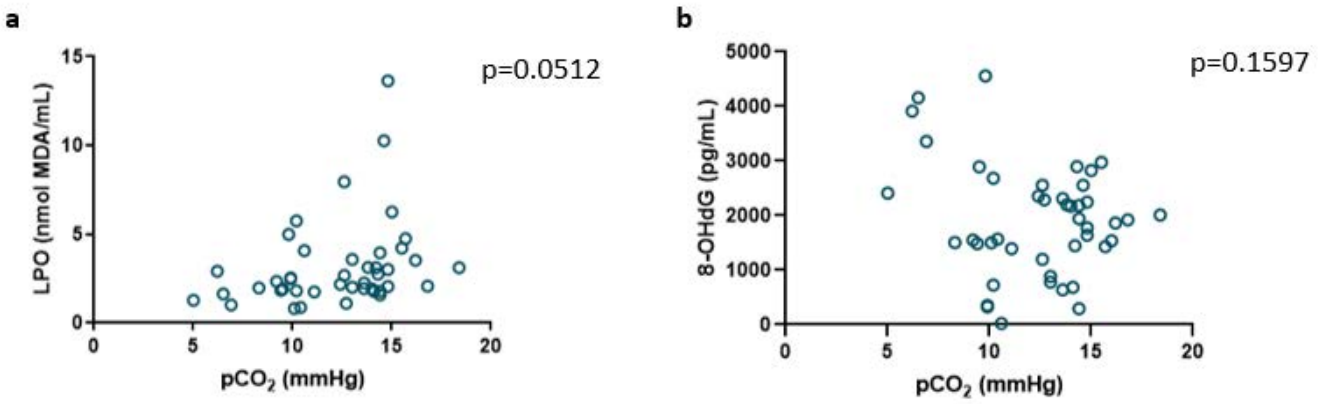

Figure 9. Plasma concentrations of a) malondialdehyde (MDA), and b) 8-deoxy-2-hydroxyguanosine (8-OHdG) in blacktip sharks (*Carcharhinus limbatus*) compared to pCO_2 (mmHg). Open circles represent individuals in which pCO_2 and tested biomarker were both measured. P-values represent results of Pearson correlation coefficient *r*, which were used to determine if significant correlations were observed between study groups.

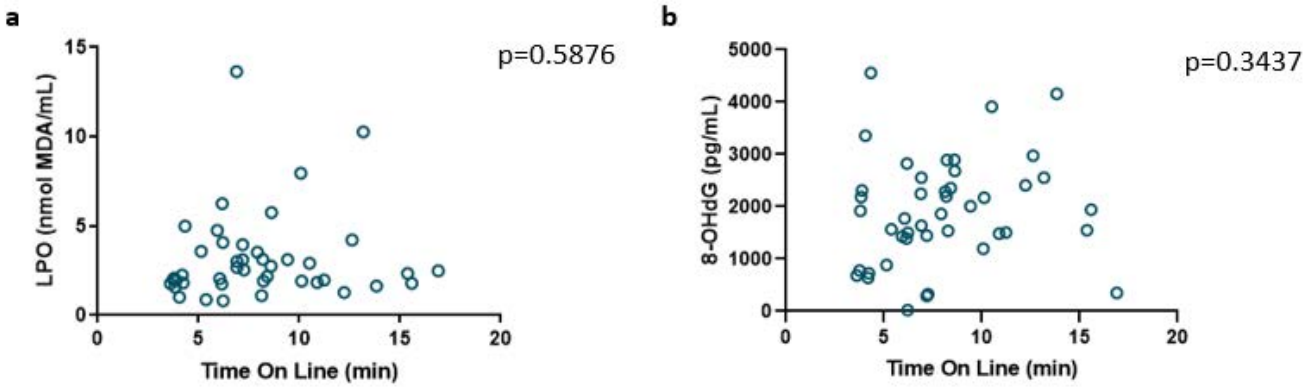

Figure 10. Plasma concentrations of a) malondialdehyde (MDA), and b) 8-deoxy-2-hydroxyguanosine (8-OHdG) in blacktip sharks (*Carcharhinus limbatus*) compared to the time animals spent fighting on the line (minutes). Open circles represent individuals in which fight time and tested biomarker were both measured. P-values represent results of Pearson correlation coefficient *r*, which were used to determine if significant correlations were observed between study groups.

DISCUSSION

Data from the first component of the present study suggested that of the tested indicators, 8-OHdG may have some value as an indicator. These results showed a significant difference between 8-OHdG in animals that survived versus those that experienced post-release mortality. 8-OHdG was also significantly correlated with some of the traditionally used secondary indicators of stress. In contrast, data from the second component did not show differences in plasma 8-OHdG levels related to survivorship, and plasma 8-OHdG did not correlate with any of the traditional indicators as well. Among the factors that differ between the first and second components, one of the greatest differences was the length of time of capture. The animals captured in the mock-commercial setting were struggling on the line for an average of 174.265 min, compared to the recreationally captured fish that were only fighting for an average of 8.032 min. Therefore, the shortened time of capture in the recreationally caught fish may have prevented 8-OHdG from accumulating to a significant amount when blood was drawn. In support of this, a study done by Çakir-Atabek *et al.* (2019) quantified 8-OHdG as an indicator of oxidative stress and found that 8-OHdG did not show any significant difference over the observation period following muscle damaging exercise. This study measured 8-OHdG over time points including pre-exercise, immediately after, 24, 48 and 96 h after, and did not find any significant difference in the level of DNA oxidation but reported the highest value of 8-OHdG measured 48 h after exercise. Another study (Kabasakalis *et al.*, 2014) found a significant increase in 8-OHdG in the blood of swimmers immediately after exercise that was sustained for an hour after exercise, and believe that the relative stability in 8-OHdG level was most likely contributed to DNA repair mechanisms. This study supports what was found in our study as the elasmobranchs that were fighting for a short period of time may have had relatively similar and stable values between surviving and non-surviving individuals that could have been due to DNA

repair mechanisms, whereas the sharks that were fighting for much longer experienced prolonged stress in which the level of damaged DNA may have exceeded the DNA repair mechanisms and contributed to the significant difference between survival outcomes.

The present study also chose to explore lipid peroxidation as an indicator of post-release mortality based on the recommendation made by Renshaw *et al.* (2012), in which the researchers suggest that lactate alone may not be a good predictor of shark mortality due to capture and that other measures including lipid peroxidation may provide useful information of stress and damage during capture in elasmobranchs. A study by Moflehi *et al*. (2012) tested for levels of MDA utilizing the TBARS procedure from the blood of 51 sedentary male humans following a single bout of aerobic exercise. This study compared the biomarker between the control group (no exercise) and 3 treatment groups of low, moderate and high intensity exercise. A significant increase in MDA was observed when compared between the 3 test groups that performed exercise and the control group but was not found between the different levels of intensity in the groups who underwent exercise. This suggests that lipid peroxidation and muscle damage may occur after a single session of exercise in which the participants did not have time to acclimate to the mechanical stresses associated with varying levels of intense exercise. The present study's results contradict this as individuals that presumably performed muscle damaging exercise and underwent the stress response to the point of mortality had statistically similar MDA levels to the levels measured in individuals that survived. This was true for animals caught in both the first and second components. Further, MDA did not correlate with any of the traditional indicators in component one, and MDA only correlated with pH for component two. A study by Bloomer and Cole (2009) tested for lactate, PCs and MDA in the blood of human males following acute exercise. In this study, levels of lactate did not correlate with PCs or

MDA. The researchers report that they utilized a method other than the TBARS method to measure MDA, which could have been the reason that their results differ from other stress studies cited by the authors. A study done by Halliwell and Chirico (1993) found that the TBARS method rarely measures the original available amount of MDA of the sample, and that the MDA measured is created due to the decomposition of lipid peroxides during the acid-heating stage. This suggests that samples that are more lipid rich may display higher levels of MDA despite perhaps not having generated as much MDA during the stress event. This can also be true for samples that are less lipid rich showing lower levels of MDA, despite perhaps having initially higher levels of MDA produced during the stress event. Therefore, the levels of MDA occurring in the samples of the current study may have been confounded by the limitations of testing. It appeared that lipid peroxidation may be taking place at some capacity for there to be correlation with some of the traditional markers in second component, but future research may be required.

A review done by Baraibar *et al.* (2013) indicates that PCs are the most commonly used marker of protein oxidation, which was a large part of the rationale for testing these molecules as a marker of stress in the current study. Baraibar *et al.* (2013) mentioned that there are multiple ways to measure PCs, but that the methods utilizing mass spectrometry and fluorescent probes similar to the one utilized in our study are more sensitive and able to detect lower abundances of carbonylated proteins. Our inability to detect PCs in blacktip shark plasma could be because PCs in elasmobranchs do not end up in the bloodstream in high enough quantities to be detectable. A study done by Barrera-García *et al.* (2012) measured PCs in blue sharks (*Prionace glauca*) skeletal muscle taken from the posterior area of the head. In this study, PCs were able to be quantified and were found to differ between stages of maturity and sex.

Other studies that quantified PCs in elasmobranchs did not use blood as the vehicle of this indicator, but instead tested for the presence of PCs largely in muscle, liver or other organs (Lopes *et al.*, 2018; Barrera-García *et al.*, 2012; Vélez-Alavez *et al.*, 2013). Another potential issue is that PCs may take a longer time to accumulate in the blood than the amount of time that was experienced during capture. Çakir-Atabek *et al.* (2019) quantified PC formation in humans as indicators of oxidative stress markers and found that PCs took several days (96 h) to show a significant increase in the blood serum following exercise. This suggests that protein carbonylation may not be a suitable marker for stress in short-term stress situations.

The present study also explored the use of cfDNA as an indicator of post-release mortality based on successful use of cfDNA to track cellular damage following rigorous exercise in humans. This was done based on recommendations made by Haller *et al.* (2017), Andreatta *et al*. (2018) and Swarup and Rajeswari, (2007). Our study measured cfDNA from blood, which was consistent with previously established methodology (Haller *et al.*, 2017; Andreatta *et al*., 2018; Swarup and Rajeswari, 2007; Breitbach *et al.*, 2012). Earlier studies focused on exercise damage in humans, however, it was hoped that sharks may display a similar pattern of increased cfDNA in muscle damaging exercise. The results of the present study showed that cfDNA did not differ in relation to survival outcomes or correlated with any of the secondary stress markers. While the measurement of cfDNA was a novel approach to quantify stress, it did not provide the insight into the elasmobranch stress response and mortality that was hoped. Because of this, cfDNA may not be an effective measurement or predictive tool for post-release mortality in elasmobranchs.

Myoglobin was chosen as an indicator of the present study due to its frequent application in medicine as a biomarker of muscle injury as a consequence of myocardial

infarction and also skeletal muscle injury (Apple *et al.*, 1999; Vassallo *et al.,* 2009; Carretón *et al.*, 2011). Myoglobin is quickly expelled during cellular injury of the muscle damaging event, and is measurable in the blood (Apple *et al.*, 1999; Carretón *et al.*, 2011). Results from the plasma of the samples tested in the current study found Mb to be undetectable. There may be several reasons for this, one being the loss of Mb before the blood samples were collected. Myoglobin is a small protein that is cleared from the blood stream rapidly and excreted in urine, and urine is a common vector when measuring levels of Mb during clinical diagnostics (Donald *et al.*, 1977). Therefore myoglobinuria, or the passing of Mb in urine, may have contributed to this protein being undetectable in the plasma samples tested. Myoglobin has been measured in elasmobranchs in other studies primarily from muscle. One such study was done by Fisher and Thompson (1979) to determine its amino acid sequence of Mb directly from muscle fibers. Therefore, blood may not be a reliable vehicle of testing for Mb in elasmobranchs, and future research may be needed.

The present study attempted to determine if CK would be a useful indicator of post-release mortality. CK concentrations were found to be undetectable in the present study, which is consistent with other studies that measure CK immediately after the exercise or stress occurs. A study done by Çakir-Atabek *et al.* (2019) found that CK levels peaked 48 h after a bout of eccentric or muscle damaging exercise. They also found that CK had variable expression, and that there was a large individual variability in CK activity among individuals. Çakir-Atabek *et al.* (2019) also found no significant relationship between the measured oxidative stress biomarkers and CK levels, but that oxidative stress biomarkers were significantly correlated with reduced muscle performance. Another study by Andreatta *et al.* (2018) found that CK did increase after intense exercise but only after 24 h had passed suggesting that this marker may not be the best

suited to determine if there is muscle damage early after the damage occurs. Another study obtained data that supports the notion that CK may not be reliably observable immediately following exercise. In this study, Souza *et al.* (2017) measured CK immediately after a mixed martial arts fight and again 24 h after the fight. They found a significant increase in CK in the fighter's blood 24 h after the fight but not immediately afterwards. Many of the studies measuring CK from blood-plasma are human exercise focused studies. Several studies have been conducted on elasmobranchs in which CK was quantified, and only one of the available studies measured its presence from blood. A study by Merly *et al*. (2019) measured heavy metals and trace elements as well as other factors including CK from the blood of White Sharks (*Carcharodon carcharias*). This study was able to measure CK from the blood of 28 *C. carcharias*, and found that the level of copper in the blood correlated to several enzymes including CK. This study reported that shark body condition was not correlated with any heavy metal except for the positive correlation between copper and several enzymes including CK, in which they suggest that copper is positively impacting shark body condition as it is a co-factor in the formation of anti-oxidative enzymes (Merly *et al*., 2019). Otway (2020) measured a multitude of biomarkers from Shortfin Mako Sharks (*Isurus oxyrinchus*) following capture. In this study, CK was quantified from the urine of 18 sharks and the average level of CK was found to be below the reference level in Sand Tiger Sharks (*Carcharias taurus*) provided by the author. A study by Treberg *et al*. (2003) compared levels of enzymes including CK in related squaloids that occupied different habitats, *Centroscyllium fabricii* (deep-sea) and *Squalus acanthias* (shallow), to determine differences in energy metabolism found in white muscle, red muscle and heart tissue after capture. CK was quantified from muscle tissue and was found to be significantly higher in the heart and red muscle of *C. fabricii*, but for white muscle tissue, the

level of CK in *C. fabricii* was much closer to the level found in *S. acanthias*. In the discussed studies, human trials revealed that CK may take a longer duration of time to build up to a measurable quantity in the blood. In the discussed elasmobranch studies, the strongest association of CK was measurement from muscle tissue. Therefore, measurement of CK in capture related stress studies may be better quantified from muscle tissue rather than blood.

Watson and Dickson (2001) examined the use of ketone bodies in elasmobranch metabolism and found evidence that they may be relying on ketones more so than fatty acids. This was the reason our study decided to look at the use of ketones as a biomarker of stress due to the inconsistency in glucose responses to stress in past studies. Blood glucose levels have shown to vary widely in elasmobranchs during capture stress (Marshall *et al.*, 2012). The current study was attempting to quantify levels of ketone bodies in the blood to determine if this test would be predictive of post-release mortality. Watson and Dickson (2001), explored the possibility of ketones as primary fuel for elasmobranch metabolism. Ketone bodies have also been tested as a biomarker of stress in Atlantic stingray *Hypanus sabinus* exposed to air for extended periods of time (Lambert *et al*. 2018). In this study, *H. sabinus* were exposed to air for 30 minutes and then allowed to recover for 48 hours. During this time, blood was taken at different time points during the air exposure as well as after the recovery period, and several biomarkers were measured to determine stress. Among the measured biomarkers, ketone bodies (βHB) were quantified and were not found to significantly change throughout the experiment or recovery period. This study did however find changes in blood chemistry, including signs of blood acidosis via elevated levels of lactate and a decrease in pH. Lambert *et al*. (2018) did note that while the average level of ketone bodies did not significantly change throughout the duration of their experiment, individual ketone levels did show some differences. These results were

consistent with what was found in the present study, as ketone bodies were found to correlate with some of the secondary stress markers but ketone body levels did not correlate with survival outcome. Therefore, future studies on the relationship between ketone bodies and stress are recommended to discern their importance in the stress-response of elasmobranchs.

Due to the inconclusive accuracy of secondary indicators in measuring stress related physiological damage and the wide range of mortality reported through different fisheries and fishing practices, our study aimed to elucidate novel indicators that may provide more precise measurements within both the commercial and recreational fishery. The 8-OHdG biomarker correlated with mortality in the mock-commercial setting, but it did not prove to be reliable for the recreationally caught blacktip sharks. This may be due to the differences in fishing style, length of time of capture, or other factors such as DNA repair. Other indicators explored in the present study were not consistently informative about the stress response or post-release mortality in elasmobranchs. Ketone bodies did correlate with many of the traditionally studied biomarkers but not with survival outcome. This suggests that ketone bodies may be mobilized during the stress response as was previously suggested by Lambert *et al*. (2018). Future studies are warranted to determine if there are other more reliable nontraditional indicators that correlate with mortality on a more consistent basis.

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VITAE

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Education

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2012-2016	B.S. Coastal Biology	University of North Florida, Jacksonville, Florida
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Academic Positions

University of North Florida (UNF):

2019-Present	Academic Advisor for the College of Arts and Sciences
2017-2019	Graduate Laboratory Teaching Assistant
2018	REU Graduate Assistant
2017	Graduate Field Teaching Assistant (UNF/ Florida Institute of Oceanography)
2016-2018	Graduate Research Assistant
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Research Grants & Travel Grants

2018-2019	OSRP Research Grant, **UNF**	$1,698.12
2018	UNF Blacktip Research Grant, **UNF**	$2,962.08
2017	SCDNR Blacktip Grant, **SC Dept. Natural Resources**	$4,443.12
2017	UNF Travel Grant, **UNF/American Elasmobranch Society**	$500.00
2015	SCDNR Hammerhead Grant, **SC Dept. Natural Resources**	$1,200.00
2012-2016	UNF Institutional Grant, **UNF**	$6,344.00

Scholarships & Awards

2012-2016	Jacksonville Commitment Scholarship, **UNF**	$22,516.00
2012-2016	Florida Medallion Scholarship, **Florida**	$8,718.00
2012-2016	Florida First Generation Scholarship, **Florida**	$6,500.00

First-authored Research Presentations (* denotes poster presentation)

2019	*Joint Meeting of Ichthyologists & Herpetologists (JMIH)
2019	Southern Division of American Fisheries Society
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2018	*UNF SOARS
2017	*JMIH
2017	*UNF SOARS
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Teaching & Mentoring Experience

University of North Florida

2019	General Biology II Lab
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Florida Institute of Oceanography and UNF collaboration

2017	Field Studies in Marine Biology

Professional Affiliations

2018-2019 Southern Division of the American Fisheries Society
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