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Behavioral Release Condition Score of Bull and Bonnethead Sharks as a Coarse Indicator of Stress

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ABSTRACT

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Capture and handling stress can induce acidosis and sometimes mortality in sharks. To approximate physiological condition after capture, fisheries researchers may use a behavioral health assessment at release. The goal of this study was to assess the efficacy of the behavioral release condition score (BRCS) in estimating the physiological stress response. The score was tested against changes in acid-base, blood gas, and metabolite analytes (pH, partial pressure of CO_2, and lactate) and factors known to influence those analytes (species, capture and handling time, and water temperature) among wild-caught bull (*Carcharhinus leucas*) and bonnethead (*Sphyrna tiburo*) sharks. After gill net capture, sharks were processed for tagging, morphometrics, and blood sampling. Blood was sampled immediately before release. At release, a BRCS was assigned as good, fair, poor, or moribund. BRCS was modeled as a response to changes in blood analytes and putative stressors using ordinal logistic regression (OLR). Effects of significant main factors were further explored graphically and in chi-square tests or (multivariate) analyses of variance (MANOVAs/ANOVAs). Linear discriminant analyses with cross-validation were used to assess the ability of those factors to discriminate among BRCS on a case-by-case basis. The OLR models suggest that BRCS responds in species-specific ways to all three blood analytes and putative stressors. However, the broad overlaps in ranges of these parameters among BRCS lend prediction of BRCS by either of these two sets of predictors to be challenging to utilize. Given the coarse relationship of BRCS to acid–base status in these species, further investigation of this and other behavioral assessment methods is recommended.

ADDITIONAL INDEX WORDS: *Acidosis, behavior, blood gas, capture, handling, shark physiology*.

INTRODUCTION

Understanding the connection between capture and handling, stress response, and morbidity and mortality of sharks after release is valuable for developing best conservation practices for these important apex predators. Sharks are sensitive to acute capture and handling stress, which can result in physiological acidosis in the blood and tissues and can lead to death in some cases (Cliff and Thurman, 1984; Hyatt *et al*., 2012; Skomal and Mandelman, 2012). This presents significant challenges in commercial fisheries, in catch-and-release recreational fishing, to scientists studying sharks in the wild and in human care, and to veterinarians caring for sharks in public aquaria.

Sharks have a low capacity for aerobic metabolism compared with higher vertebrates and quickly shift to anaerobic metabolism when caught or handled (Brill *et al*., 2008; Cliff and Thurman, 1984; Hoffmayer and Parsons, 2001; Mandelman and Skomal, 2009; Manire *et al*., 2001). Sharks will naturally use anaerobic muscle activity for short bursts of speed, such as in hunting or predator evasion, but response to prolonged capture and handling stress can exacerbate anaerobic metabolism, often leading to exhaustion. The adaptive components of the stress response are mediated endocrinologically via the secretion of catecholamines and glucocorticoid hormones, often considered as indices of a primary stress response (Sumpter, 1997). Together, these hormones cause a suite of changes in the physiology of the animal, considered as secondary stress indices. These include changes in the matrix of osmotic, acid–base, blood gas, and biochemical components of the blood (McDonald and Milligan, 1997). Tertiary stress indices include measures such as behavioral changes (Schreck, Olla, and Davis, 1997), and depression in growth, weight gain, fecundity, and survival of offspring (Pankhurst and Van Der Kraak, 1997). This homeostatic disruption can result in a decrease in blood pH secondary to an overreliance on glycolysis beyond a steady state, with excessive production of protons (H^+). In response, lactate production increases under these cellular conditions to prevent pyruvate accumulation and supply the nicotinamide adenine dinucleotide (oxidized) needed for glycolysis. Increased lactate production coincides with cellular acidosis and is a good indirect marker for cellular metabolic conditions that induce metabolic acidosis (Cliff and Thurman, 1984; Hoffmayer and Parsons, 2001; Holeton and Heisler, 1983; Mandelman and Skomal, 2009; Manire *et al*., 2001; Robergs, Ghiasvand, and Parker, 2004; Skomal and Mandelman, 2012).

In addition, many sharks are obligate ram ventilators. In times of capture stress or entanglement in a net, their

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ventilation is depressed or even stopped, leading to an increase in carbon dioxide in the blood (partial pressure of CO_2 [pCO_2]), which can depress the blood pH further, thus producing a respiratory acidosis (Mandelman and Skomal, 2009; Skomal and Mandelman, 2012). Sharks that fight and are unable to swim are more severely affected, with a mixed metabolic and respiratory acidosis (Mandelman and Farrington, 2007a,b; Mandelman and Skomal, 2009; Manire *et al*., 2001). There is still debate about whether this is a major contributing factor to the high rates of mortality some sharks experience after capture and handling (Frick, Walker, and Reina, 2012; Mandelman and Skomal, 2009; Wood, Turner, and Graham, 1983). The change in blood gases, acid–base balance, and biochemistry is a secondary stress response in sharks and can be used as a relative correlate to overall physiological condition (Skomal and Mandelman, 2012). Measurement of blood gas, acid–base status, and biochemical analytes, collectively referred to as blood gas analysis for simplicity, includes pH, partial pressure of oxygen (pO_2), pCO_2, bicarbonate (HCO_3^-), and lactate concentrations ([lac]). Blood gas analysis has become the standard for assessing the physiological stress response in shark fisheries research and veterinary medicine (Gallagher *et al*., 2010; Hadfield, Whitaker, and Clayton, 2007). This is a benchmark because of the well-understood mechanisms previously described, and also due to the lack of commercially available assays for the primary stress hormone in sharks,1α-hydroxycorticosterone (Nunez and Trant, 1999). Although there are many physiological indicators of stress in animals, blood gas analysis is one of the better tools, as it has been extensively studied in sharks.

Predicting postrelease mortality or survival to improve fisheries management strategies has been the topic of numerous studies (e.g., Campana, Joyce, and Manning, 2009; Carruthers, Schneider, and Neilson, 2009; Cooke *et al*., 2008; Frick, Reina, and Walker, 2010; Frick, Walker, and Reina, 2010; Gallagher *et al*., 2014; Hueter and Manire, 1994; Hueter *et al*., 2006; Mandelman and Farrington, 2007a,b; Manire *et al*., 2001; Moyes *et al*., 2006; Musyl *et al*., 2009; Renshaw *et al*., 2012; Sepulveda *et al*., 2015; Skomal, 2007). Many of the above researchers recommend the development of better predictive models on the basis of physiological consequences of capture and handling stress to estimate postrelease delayed mortality. Before predictive models for mortality can be established, a better understanding of how stress physiology relates to behavioral, physiological, and biochemical indices is warranted. The evaluation of behavior postcapture and handling and how it may relate to physiological changes can be a starting point to build upon. Some scientists have invoked the scoring of swimming behavior upon release after a stressful event as a behavioral method of assessing condition (Afonso and Hazin, 2014; Hueter and Manire, 1994; Hueter *et al*., 2006; Manire *et al*., 2001). The behavioral release condition score (BRCS) is usually given as good, fair, poor, or moribund; or a related scheme. One premise behind the BRCS is that it may relate to severity of acidosis developed during capture and handling stress. It is hypothesized that if a shark becomes overwhelmed by stress and develops acidosis, then the shark's swimming behavior could be affected upon release. As an example, a presumed minimally stressed shark with no evidence of acidosis would likely be released with a BRCS of good, whereas a highly stressed shark with severe acidosis would be released with a BRCS of poor, or even moribund. However, the BRCS of a shark as a correlate of its physiological condition has not been validated. Corresponding changes in blood gas analysis to the BRCS would help validate the use of BRCS to estimate the stress response to capture and handling. This method holds merit in ecological risk assessments, veterinary care, and husbandry of sharks, where evaluating behavioral condition would be easier, less expensive, and less invasive than blood gas analysis or other physiological diagnostics.

The objective of this study was to assess the reliability of BRCS to (1) correspond with blood gas analysis analytes of pH, pCO_2, and [lac] in assessing the severity of the secondary stress response of wild bull *(Carcharhinus leucas)* and bonnethead *(Sphyrna tiburo)* sharks to capture and handling, and (2) respond to factors known to affect the acid–base status, such as species, capture and handling time (Hyatt *et al*., 2012), and temperature (Heisler, Neumann, and Holeton, 1980).

METHODS

This study was conducted as part of a larger project evaluating blood gas analysis to assess capture and handling stress of carcharhiniform sharks in three bays (Faka Union, Fakahatchee, and Pumpkin bays) within the Ten Thousand Islands off the SW coast of Florida from 2007 to 2011 (Hyatt *et al*., 2012). This project was performed in collaboration with an ongoing shark population assessment for waterway mitigation under previously established sampling protocols (Shirley *et al*., 2005; Steiner, Michel, and O'Donnell, 2007). Each bay was sampled one evening per month, from 2 hours before until 2 hours after sunset. Bay temperature (°C) was measured once per evening with a handheld multimeter (YSI 85, Yellow Springs Corp., Yellow Springs, Ohio, U.S.A.).

One gill net was set in between two baited longlines as described in Hyatt *et al*. (2012). Gill nets and longlines were monitored continuously from a houseboat anchored nearby to allow rapid removal of sharks after capture. Baited longlines were hypothesized to draw sharks into the gill net. Movement of surface floats on the gill net indicated shark capture; subsequently, workers boarded a tethered 7.6-m outboard motorized mullet skiff to retrieve the shark an average of 6.5 (± 5.0 standard deviation [SD]) min after capture. The shark was brought onto the skiff and placed in a 1.5-m-diam plastic pool for monitoring, processing, and assisted swimming, if needed. An initial blood sample (approximately 1 mL) was obtained via caudal venipuncture using a heparin-washed 3-mL syringe and a 22-gauge 19 mm (0.75 in) or 38 mm (1.5 in) needle (depending on shark size). After blood draw, body weight, morphological measurements, and estimated life stage were collected. Each shark was tagged with a nylon-head, plastic barb tag (Hallprint Pty. Ltd., Victor Harbor, Australia) inserted just ventral to the dorsal fin, such that the tag head was anchored in the cartilage and connective tissue below the fin. These procedures were conducted for other studies, but as they putatively affect the physiological stress response during the handling period, are reported here.

Before release, a second blood draw was obtained as described previously. Blood gas analysis on the second blood

draw was conducted with the i-STAT portable clinical analyzer (Abaxis, Union City, California, U.S.A.) utilizing an i-STAT CG4+ disposable cartridge measuring pH, [lac] (mmol/L), and pCO_2 (mm Hg). Other analytes measured by the CG4+ cartridge were not considered as they are not appropriate for shark physiology. As the i-STAT is automatically thermostatted to 37°C for humans, pH and pCO_2 were body temperature corrected by the i-STAT on the basis of measured water temperature as the assumed body temperature input into the analyzer (Abbott Point of Care Inc., 2013a,b). Blood gas analysis performed on the i-STAT has been validated for use in some sharks at a given temperature to provide reliable results similar to a benchtop analyzer, particularly pH and [lac] (Gallagher *et al*., 2010; Harter *et al*., 2015). Harter *et al*. (2015) did not evaluate [lac], but found pH to be relatively accurate, although not so pO_2 or pCO_2. However, since the temperature corrections of the i-STAT are based on mammalian conversion factors and constants, the corrections for sharks may not be considered absolute, but can be considered relative corrections allowing for practical application and ease of use in the field setting (Brooks *et al*., 2012; Mandelman and Skomal, 2009). Being in a field setting, it is nearly impossible to establish nonstressed blood gas, acid–base, and metabolite analytes from which to compare results to know whether the levels obtained are indeed due to homeostatic disruption. However, Hyatt *et al*. (2012) developed "relative reference ranges" of *C. leucas* and *S. tiburo* handled in the same way.

Capture and handling time was recorded as the time (min) from initial observation of a shark getting caught in a gill net to the time of the second blood collection. If, during any time, a shark that was on the boat was not active, the shark was ram ventilated over the side of the boat while at an idle speed. Once released away from the study site so as not to be recaptured, a BRCS, in accordance with similar schemes described by Hueter and Manire (1994) and Manire *et al*. (2001), was assigned as good (swims off with vigor), fair (slowly swims away), poor (no swimming and sinks), or moribund (nonresponsive or dead in boat). This classification was assigned only by one experienced assessor (P.M.O.) who did not conduct blood analyses and was not aware of blood gas analysis results at the time of release.

Only data collected from sharks caught in gill nets were used in statistical analyses. Descriptive statistics were calculated to quantitatively characterize physiological and behavioral stress response metrics, as well as a set of putative stressors.

As BRCS is a measure constructed on an ordinal scale, ordinal logistic regression (OLR) procedures were chosen to explore the effects of species, blood gas analysis parameters, and putative stressors on BRCS as a behavioral response metric. Two models were fitted to address the stated objectives: (1) BRCS as a response to species and derangements in blood gas analysis, specifically, pH, pCO_2, and [lac]; and (2) BRCS as a response to factors known to influence blood gas analysis, specifically, species, capture and handling time, and temperature. For both models, the BRCS was numerically classified as 4 (good), 3 (fair), 2 (poor), and 1 (moribund), with 4 designated as the reference event. Species was modeled as a factor and the remaining predictors were modeled as covariates. Fully saturated models were initially tested with three link functions: logit, normit, and gompit. The gompit link function was chosen to use for further evaluation of models as this function yielded the highest (>0.05) p-values in both Pearson and deviance goodness-of-fit tests. Manual backward stepwise regression procedures were implemented, removing highest-order interactions with highest p-values sequentially above a cutoff p-value of 0.10. Final OLR models were reduced to:

$$y = s + p + o + l + s \times p + s \times o + s \times l + p \times l + o \times l + s \times p \times l + e, \quad (1)$$

where y = BRCS, s = species (*C. leucas* or *S. tiburo*), p = pH, o = pCO_2, l = [lac], and e = error; and

$$y = s + c + t + c \times t + e, \quad (2)$$

where y = BRCS, s = species (*C. leucas* or *S. tiburo*), c = capture and handling time, t = temperature, and e = error.

For each species, significant main factors from optimized models were visualized *post priori* among BRCS as histograms (for species) and box plots (for all other covariate factors). Additionally, confirmatory inferential statistical tests were run on significant main factors, as advised by Moya-Laraño and Wise (2007). A chi-square test was run to confirm associations of BRCS distributions with species. Multivariate analyses of variance (MANOVAs) were run to confirm differences among BRCS in (1) blood gas analysis parameters and (2) putative stressors determined to be significant via OLR. Parameter distributions were compared among BRCS for both species in a general linear model that collapsed species and BRCS into one factor with six levels: BRCS 1, 3, and 4 in *C. leucas* and BRCS 2, 3, and 4 in *S. tiburo* (other BRCS were omitted because of insufficient sample sizes). The Wilks' criterion was assessed for significance. Parameters included in significant MANOVAs were subsequently analyzed in one-way ANOVAs using type III sums of squares and Tukey's tests for discrimination among levels. Factors were collapsed as described above to compare distributions among BRCS, and also to compare distributions between species released at equal BRCS. Finally, significant main factors from optimized OLR models were assessed for their ability to predict BRCS using linear discriminant analyses (DA), after Wright, Goodmand, and Cameron (2010). Linear DA assumes equal covariance matrices among BRCS groups. The cross-validation technique was used in linear DA to compensate for optimistic apparent error rates.

Analytical methods were executed using Microsoft Excel (v. 2007, Microsoft Corp., Redmond, Washington, U.S.A.) and Minitab (v. 16.2.3., Minitab, Inc., State College, Pennsylvania, U.S.A.) software.

RESULTS

Seventy-six *C. leucas* and 110 *S. tiburo* were captured in gill nets and assessed between April 2007 and December 2011. Mean ± SD (interquartile [IQ] range) bay temperature during shark capture was 28.5°C ± 3.7°C (IQ range: 26.5°C to 31.9°C). Sharks endured a mean capture and handling time of 14.7 ± 5.6 (IQ range: 11 to 18) min. *Carcharhinus leucas* were scored with a median BRCS of 4 (good) at release (IQ range: 3–4), whereas *S. tiburo* were scored with a median BRCS of 3 (fair) at release (IQ range: 3–4). Blood gas analysis parameters of both species upon release are reported in Table 1.

Table 1. *Prerelease blood gas analysis parameters of* C. leucas *and* S. tiburo *as measured by the i-STAT CG4+. Values are mean ± SD in the first row, interquartile range within parentheses in the second row.*

Parameter	*C. leucas* ($n = 72$)	*S. tiburo* ($n = 103$)
pH[a]	6.989 ± 0.184 (6.908–7.119)	7.120 ± 0.211 (7.024–7.267)
pCO_2[a] (mm Hg)	10.4 ± 5.1 (7.1–12.1)	10.0 ± 4.2 (6.9–11.8)
[lac] (mmol/L)	10.25 ± 3.79 (7.24–13.70)	8.47 ± 4.08 (5.27–11.12)

[a] Temperature corrected.

Both optimized OLR models met criteria for goodness of fit in Pearson and deviance tests ($p \geq 0.374$). All effects included in optimized models represented significant predictors ($p < 0.05$), with the exception of the $o \times l$ interaction in Equation (1) ($p = 0.071$) and the $c \times t$ interaction in Equation (2) ($p = 0.061$, Table 2).

Chi-square test confirmed a difference in the distribution of BRCS between species ($\chi^2 = 12.892$, df = 3, $p = 0.005$); *i.e. C. leucas* sharks tended to be released with higher BRCS than *S. tiburo* sharks (Figure 1). Evaluation of box plots of blood gas analysis among BRCS confirms results of Equation (1). In both species, BRCS generally appears to decline with declining pH (p), increasing pCO_2 (o), and [lac] (l, Figure 2). However, there is broad overlap in the ranges of blood gas analysis parameters among scores. In contrast, evaluation of box plots of putative stressors demonstrates only slight trends, if any, among BRCS: as BRCS declines, capture and handling times and temperatures suggest slight increases in trends (Figure 3). As with blood gas analysis parameters, there is broad overlap in the ranges of putative stressors among BRCS.

The two species responded with different BRCS for given distributions in the three blood parameters (pH, pCO_2, and [lac]) overall (Wilks' $F_{15,\ 439} = 5.643$, $p < 0.001$). One-way ANOVA confirmed differences in mean pH among some BRCS across both species ($F_{5,\ 161} = 14.12$, $p < 0.001$). Tukey's tests revealed that *S. tiburo* sharks released with a BRCS of 4 had significantly higher pH values than *C. leucas* (7.242 ± 0.035, mean ± standard error [SE]), and *C. leucas* sharks released with a BRCS of 1 had significantly lower pH values (6.666 ± 0.073) than sharks of either species released with other tested BRCS (at other mean pH values ranging from 6.978 to 7.072). One-way ANOVA confirmed differences in mean pCO_2 among some BRCS across both species ($F_{5,\ 165} = 5.00$, $p < 0.001$). Tukey's tests revealed that *C. leucas* sharks released with a BRCS of 1 had significantly higher mean pCO_2 (17.53 ± 4.27 mm Hg) than *C. leucas* sharks released with a BRCS of 4 or *S. tiburo* sharks released with any BRCS (at mean pCO_2 ranging from 8.77 to 11.61). One-way ANOVA confirmed differences in mean [lac] among some BRCS across both species ($F_{5,\ 165} = 9.36$, $p < 0.001$). Tukey's tests revealed that *C. leucas* sharks released with a BRCS of 1 had significantly higher mean [lac] (14.73 ± 0.97 mmol/L) than *C. leucas* sharks released with a BRCS of 4 (9.40 ± 0.48 mmol/L) and *S. tiburo* sharks released with a BRCS of 3 or 4 (mean [lac] ranging from 6.25 to 9.26 mmol/L). Also, *S. tiburo* sharks released with a BRCS of 4 had significantly lower mean [lac] (6.25 ± 0.42 mmol/L) than all other sharks (mean [lac] ranging from 9.25 to 14.73 mmol/L, Figure 2).

Table 2. *Results of ordinal logistic regression of BRCS in response to blood gas analysis parameters and putative stressors. s = species, $o = pCO_2$ (mm Hg), l = [lac] (mmol/L), c = capture and handling time (min), t = temperature (°C). Coefficients are listed with standard errors (in parentheses). Significant* p*-values (<0.05) are in bold.*

Predictor	Coefficient	Z	p
Blood Gas Analysis			
Intercept 1	−85.488 (28.96)	−2.95	**0.003**
Intercept 2	−84.052 (28.957)	−2.90	**0.004**
Intercept 3	−81.988 (28.924)	−2.83	**0.005**
s	132.922 (31.791)	4.18	**<0.001**
p	10.777 (3.950)	2.73	**0.006**
o	0.349 (0.131)	2.67	**0.007**
l	4.789 (2.122)	2.26	**0.024**
$s \times p$	−18.148 (4.399)	−4.13	**<0.001**
$s \times o$	−0.229 (0.075)	−3.06	**0.002**
$s \times l$	−-6.947 (2.284)	−3.04	**0.002**
$p \times l$	−-0.634 (0.299)	−2.12	**0.034**
$o \times l$	−0.015 (0.008)	−1.80	0.071
$s \times p \times l$	0.967 (0.327)	2.96	**0.003**
Putative stressors			
Intercept 1	−13.345 (3.659)	−3.65	**<0.001**
Intercept 2	−11.199 (3.597)	−3.11	**0.002**
Intercept 3	−9.329 (3.58)	−2.61	**0.009**
s	0.861 (0.294)	2.93	**0.003**
c	0.413 (0.210)	1.97	**0.049**
t	0.271 (0.117)	2.31	**0.021**
$c \times t$	−0.013 (0.007)	−1.87	0.061

The two species also responded with different BRCS for given distributions in temperature and capture and handling time overall (Wilks' $F_{10,\ 276} = 3.454$, $p < 0.001$). One-way ANOVA confirmed differences in mean temperature at which sharks were captured among some BRCS across both species ($F_{5,\ 175} = 7.42$, $p < 0.001$). Tukey's tests revealed that *C. leucas* sharks released with a BRCS of 4 were captured at a higher mean temperature (30.9°C ± 0.5°C) than *S. tiburo* sharks released with a BRCS of 3 or 4 (captured at mean temperatures ranging from 27.5 to 28.3°C); and *S. tiburo* sharks released with a BRCS of 4 were captured at a lower mean temperature (27.5°C ± 0.4°C) than any *C. leucas* sharks (captured at mean temperatures ranging from 30.2 to 32.2°C). One-way ANOVA found no differences in capture and handling time among BRCS for either species ($F_{5,\ 139} = 1.16$, $p = 0.334$).

Linear DA revealed the disadvantages of the broad overlap in measures among scores. The procedure classified sharks into BRCS categories on the basis of information from blood gas analysis parameters correctly an average of 53.6% of the time for *C. leucas* and 45.9% of the time for *S. tiburo* (Table 3). Sharks with a BRCS of 4 were more likely to be classified correctly (69 and 75% of the time, respectively) than sharks with any lower BRCS (< 35% of the time). Putative stressors functioned as even poorer predictors for BRCS in linear DA; sharks were classified into BRCS categories on the basis of information from putative stressors an average of 44.6% of the time for *C. leucas* and 46.6% of the time for *S. tiburo* (Table 4). No BRCS category yielded a percentage of correct classifica-

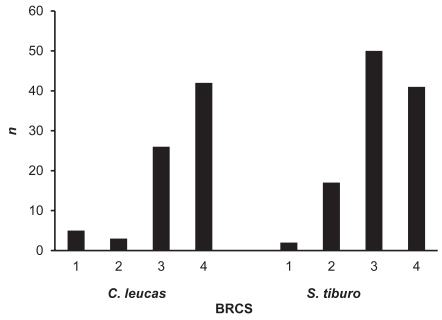

Figure 1. Histogram of BRCS among captured and handled *C. leucas* and *S. tiburo*.

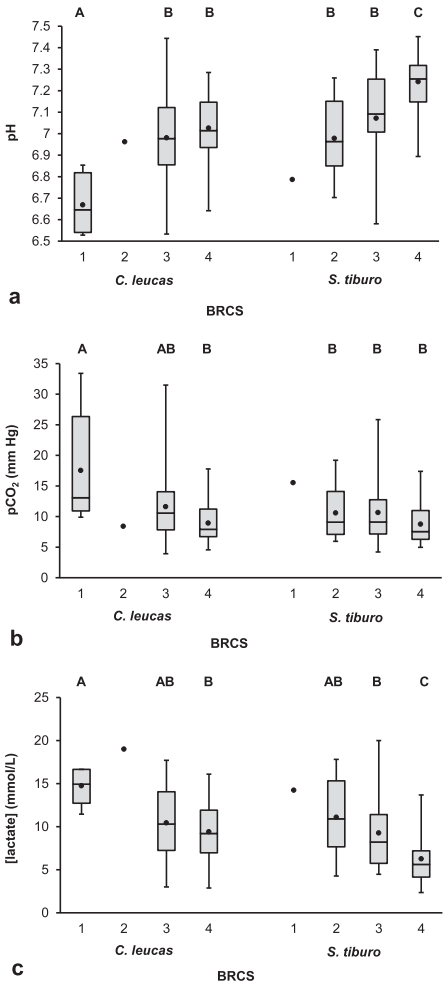

Figure 2. Box plots of blood gas analysis parameters among BRCS for *C. leucas* and *S. tiburo*. Some BRCS did not contain sufficient sample sizes to construct box plots; means are only represented in those categories (as closed circles). Means that do not share a letter above box plots are significantly different as determined by ANOVA and Tukey's test. (a) pH (temperature corrected). (b) pCO_2 (temperature corrected). (c) [lac].

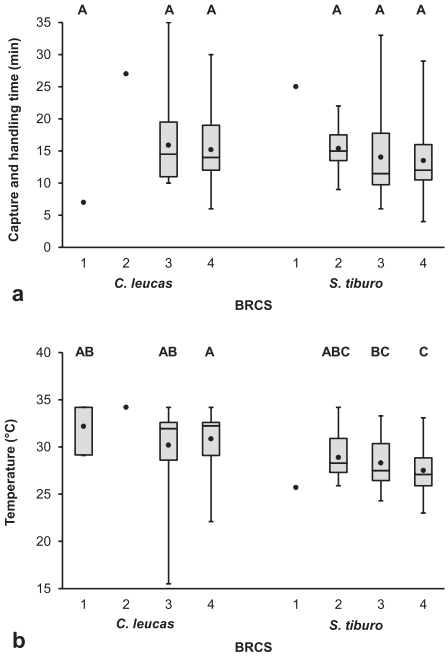

Figure 3. Box plots of putative stressors among BRCS for *C. leucas* and *S. tiburo*. Some BRCS did not contain sufficient sample sizes to construct box plots; means are only represented in those categories (as closed circles). Means that do not share a letter above box plots are significantly different as determined by ANOVA and Tukey's test. (a) Capture and handling time. (b) Temperature.

tions greater than 52.8% for *C. leucas*, although *S. tiburo* sharks with a BRCS of 4 were more likely to be correctly classified (at a success rate of 67.6%) than *S. tiburo* sharks with a lower BRCS (≤46.2%).

DISCUSSION

The present study suggests that BRCS may be considered as a coarse assessment of acid–base status in *C. leucas* and *S. tiburo*. Initially, OLR proved to be a powerful assessment of BRCS as a measure constructed on an ordinal scale. In this case, for two species of sharks whose local populations have been extensively sampled, the optimized OLR models constructed suggest that BRCS declines predictably and in species-specific ways to changes in blood gas analysis and variations in the putative stressors of capture and handling time and temperature. However, graphical assessment of results and confirmatory tests provide additional insight. There are broad overlaps in the ranges of measurements across BRCS, resulting in many adjacent BRCS with statistically equivalent distributions in blood gas analysis parameters and putative stressor measures as evidenced by ANOVAs. In addition, the broad overlaps of measurement ranges across BRCS resulted in low discriminative ability by linear DA. These broad overlaps may be indicative of BRCS being too subjective or too coarse of a health assessment tool, especially when sharks are starting to appear exhausted. This translates to a poor predictive ability of BRCS to inform acid–base status

Table 3. *Linear discriminant analysis with cross-validation: predictions of BRCS in* C. leucas *and* S. tiburo *on the basis of information from blood gas analytes: pH, pCO_2 (mm Hg), and [lac] (mmol/L). Some BRCS categories omitted from analysis because of insufficient sample size.*

	True BRCS					
	C. leucas			*S. tiburo*		
Predicted BRCS	1	3	4	2	3	4
1	0	4	3			
2				7	17	2
3	2	8	10	5	8	8
4	2	11	29	3	18	30
Total *n*	4	23	42	15	43	40
n Correct	0	8	29	7	8	30
Proportion Correct	0.000	0.348	0.690	0.467	0.186	0.750
Total Proportion Correct	0.536			0.459		

Table 4. *Linear discriminant analysis with cross-validation: predictions of BRCS in* C. leucas *and* S. tiburo *on the basis of information from putative stressors: capture and handling time (min) and temperature (°C). Some BRCS categories omitted from analysis because of insufficient sample size.*

	True BRCS				
	C. leucas		*S. tiburo*		
Predicted BRCS	3	4	2	3	4
2			6	15	6
3	6	17	3	10	6
4	14	19	4	13	25
Total *n*	20	36	13	38	37
n Correct	6	19	6	10	25
Proportion Correct	0.300	0.528	0.462	0.263	0.676
Total Proportion Correct	0.446		0.466		

or suggest levels of stressors to which sharks are exposed, on a case-by-case basis, as demonstrated by the results of linear DA.

Furthermore, BRCS is a behavioral measure that is species specific as it relates to blood gas analysis. As an example, *S. tiburo* sharks released with a BRCS of 4 demonstrate significantly higher pH values than *C. leucas* sharks released with the same BRCS. Similarly, and inverse to pH, *S. tiburo* sharks released with a BRCS of 4 demonstrate significantly lower [lac] than *C. leucas* sharks released with the same BRCS. In speculation, perhaps *C. leucas* can tolerate higher [lac] in muscles without causing muscle rigidity and reduction in swimming capacity, thus affecting their behavior, as might develop in the more sensitive *S. tiburo* at the same [lac]. Combining the knowledge of the mechanism whereby a rise in [lac] contributes to a drop in pH as a result of metabolic acidosis with the closely correlated inverse patterns between pH and [lac] described here, and the significant two-way and three-way interaction effects (with species) in the OLR model, highlight how the behavioral response corresponds in species-specific ways with aberrations in blood pH, which are in turn driven by aberrations in [lac].

In contrast, pCO_2 only differentiated between BRCS in *C. leucas*, but only between scores of 1 and 4. For *S. tiburo*, there is no discernment in pCO_2 distributions among sharks released with different BRCS. As increasing pCO_2 in the face of decreasing pH is an indicator of respiratory acidosis, the relative lack of differences in this measure among BRCS may indicate that respiratory acidosis did not develop in most sharks caught. However, recent work by Harter *et al*. (2015) found pCO_2 as measured by the i-STAT in sharks to not be as accurate as previously believed. Caution should thus be taken in interpreting pCO_2 results in this study.

The longer the duration of a stressful event, the more profound alterations occur in blood gas analysis and other blood constituents (Mandelman and Skomal, 2009; Manire et al., 2001; Wood, Turner, and Graham, 1983). In contrast, ANOVA suggests, contrary to OLR, that capture and handling time doesn't contribute significantly to variation in BRCS. Graphical assessment of results also suggest small, if any, differences in the distributions of capture and handling time among BRCS. The lack of significance in the present study may be due to the short durations of capture and handling time. Perhaps if the sharks were entangled in the gill net longer, kept on the boat longer, and handled more, then there may have been significant differences in capture and handling times across BRCS.

Graphical assessment of results and confirmatory tests also detail the species-specific pattern of behavioral response as measured by the relationship between BRCS and water temperature. As sharks were caught in increasing water temperatures, BRCS trended downward. Elevated temperature can cause more severe acidosis and increased mortality in sharks, as their metabolism will be increased, leading to a lower anaerobic threshold and higher propensity to develop acidemia (Danylchuk *et al*., 2014; Dickson *et al*., 1993). The differences in temperature distributions between species outlined in the results may be an artifact of a capture bias; *C. leucas* sharks tended to be captured in waters of higher temperature (30.8°C ± 0.4°C mean ± SE) than *S. tiburo* sharks (28.1°C ± 0.2°C; t test: $t = 5.75$, $df = 122$, $p < 0.001$), regardless of BRCS at release. Another explanation may be that when sharks were released in good condition within the above-mentioned temperature ranges, the difference in ranges may be attributed to the ideal water temperature for that species. Furthermore, *C. leucas* may be able to tolerate warmer water temperatures than *S. tiburo*. Within species, Tukey's tests suggested that temperatures across BRCS were statistically equivalent.

A large body of work has demonstrated the use of blood gas analysis as a good indicator of capture and handling stress (Cliff and Thurman, 1984; Frick, Walker, and Reina, 2012; Gallagher *et al*., 2014; Hoffmayer and Parsons, 2001; Hyatt *et al*., 2012; Mandelman and Farrington, 2007b; Mandelman and Skomal, 2009; Skomal, 2006). Knowing that pH will decrease, and pCO_2 and [lac] will rise in the face of physiological stress, leading to respiratory and metabolic acidosis, and that the more severe the stressful event is, the more that pH, pCO_2, and [lac] shift, blood gas analysis may serve as a reliable marker of capture and handling stress to compare against other assessment methods. However, no work has been performed to validate in sharks how acid–base status may relate to mortality. Thus blood gas analysis, at this time, cannot be used to predict mortality. In a field setting, the i-STAT portable clinical analyzer is an acceptable tool in assessing blood gas analysis. It is portable, relatively inexpensive compared with benchtop units, and can provide results within minutes,

eliminating the need to store and transport blood to a laboratory. The i-STAT does have its limitations for use in sharks, however, as results obtained from blood gas analysis appear to only be reliably accurate for pH and [lac]. Other analytes may be clinically irrelevant. In addition, there are ongoing debates over proper temperature correction. The temperature corrections utilized in this study were calculated using the equations programmed into the i-STAT and are described by Ashwood, Kost, and Kenny (1983). Other studies have utilized a different temperature correction equation for pH developed by Heisler, Neumann, and Holeton (1980) that was based on larger spotted dogfish (*Scyliorhinus stellaris*) acid–base physiology (Brooks *et al*., 2012; Cicia *et al*., 2012; Frick, Walker, and Reina, 2012; Gallagher *et al*., 2010, 2014; Mandelman and Skomal, 2009). As species differences in temperature correction factors are likely (Gallagher *et al*., 2010), and a comparison of the two equations is clinically very similar, we chose to use the equation programmed into the i-STAT as it has not been determined what equation is actually more accurate, particularly for *C. leucas* and *S. tiburo*. Temperature corrections in the same studies for pCO_2 utilized the same equation as the present study that was programmed into the i-STAT. Species-specific temperature correction factors need to be developed to increase the accuracy of the i-STAT for use in the field to assess the blood gas and acid–base physiology of sharks.

BRCS have been used by fisheries scientists to assess the health of an animal after capture and handling, and to attempt to predict postcapture delayed mortality (Hueter and Manire, 1994; Hueter *et al*., 2006; Manire *et al*., 2001; Suski *et al*., 2007). Hueter *et al*. (2006) indicated that postrelease mortality as predicted by tag return data increased as BRCS deteriorated, suggesting that the shark's physiological status after capture and handling was correlated with behavioral condition. The study of Hueter *et al*. (2006) featured a large sample of sharks (*S. tiburo* and blacktip [*Carcharhinus limbatus*]) that were under stressful conditions for a much longer period of time than in the present study. However, that study did not have any physiological measures to corroborate the behavioral conditions they observed. Using a model based on BRCS and recapture or tag collection data to predict mortality relies on the assumption that shark behavior correlates with physiological condition. However, the results of the present study do not support the assumption of behavior correlating with physiological condition. On the basis of results from the present study, BRCS should not be used as a proxy for an assessment of the magnitude of the physiological stress response endured by a shark during capture and handling until the discrepancies among BRCS, stress physiology, and postrelease mortality have been resolved.

This study implemented blood gas analysis as a physiological indicator of the secondary stress response in sharks, yet it is not the only means. Other studies have evaluated hematological and other plasma biochemical indices as indicators of the stress response in sharks such as hematocrit, glucose, magnesium, potassium and blood urea nitrogen with promising results (Brooks *et al*., 2012; Frick, Walker, and Reina, 2012; Heberer *et al*., 2010; Hoffmayer, Hendon, and Parsons, 2012; Marshall *et al*., 2012; Moyes *et al*., 2006). On the other hand, Manire *et al*. (2001) evaluated various serum biochemical constituents and hematocrit in a relatively small sample of *S. tiburo* sharks against BRCS and found only a few constituents to differ among some, but not all, scores. Although the present study suggests that BRCS is a coarse estimator of postcapture health as measured by blood gas analysis, it may prove to be a better correlate of other biochemical constituents; this avenue also merits further investigation.

The BRCS is a crude, subjective assessment of swimming behavior at release. In fisheries research, the behavioral condition of fish can be used to predict delayed mortality in bycatch events (Davis, 2007) using a different index based on reflex action mortality predictors, which is a method that involves checking for the presence or absence of natural fish reflexes (Davis and Schreck, 2005; Raby *et al*., 2012). This has been used successfully for different species of fishes (Campbell *et al*., 2010; Davis, 2007, 2010; Davis and Ottmar, 2006; Davis and Schreck, 2005; Humborstad, Davis, and Lokkeborg, 2009; Raby *et al*., 2012), and has recently shown promise in sharks (Braccini, Van Rijn, and Frick, 2012; Danylchuk *et al*., 2014; Gallagher *et al*., 2014). Reflex impairment may merit further testing and validation for use in sharks as an alternative behavioral assessment method by comparing its response against abnormalities in blood gas analysis.

Future research endeavors may look to advances in technology. Perhaps monitoring these animals with pop-up satellite tags (Campana, Joyce, and Manning, 2009; Gallagher *et al*., 2014; Moyes *et al*., 2006; Musyl *et al*., 2009; Sepulveda *et al*., 2015) or accelerometer tags (Whitney *et al*., 2007) may provide information about mortality events to characterize the relationship of mortality to BRCS and blood gas analysis. This could lead to the development of blood gas analysis and BRCS ranges that help predict postcapture mortality.

CONCLUSION

In conclusion, BRCS serves as a coarse estimator of the physiological condition of *C. leucas* and *S. tiburo* postcapture and handling, but responds poorly to putative stressors. Further investigation of BRCS in response to other biochemical markers, as well as the evaluation of alternative behavioral assessment methods, are warranted. Until then, however, blood gas analysis is suggested to be a more accurate indicator of physiological stress due to capture and handling, and should be monitored when feasible to make informed health management decisions.

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