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Hematological, serum, and plasma chemical constituents in pantropical spotted dolphins (*Stenella attenuata*) following chase, encirclement, and tagging

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ABSTRACT

Hematology, serum chemistry, and plasma hormones were evaluated in 72 pantropical spotted dolphins (*Stenella attenuata attenuata*) from the eastern tropical Pacific in an attempt to define the degree of stress associated with chase and encirclement by a tuna purse seiner, and are here reported for the first time for this species. Dolphins had high levels of dopamine and moderately elevated levels of enzymes indicative of the expected muscle damage following exertion of the chase. The length of time between the start of the capture operation and blood sampling correlated with increases in platelet and white blood cell counts and mean cell hemoglobin concentration, while the length of time between net tie-down and blood sampling influenced platelet, white blood cell, and eosinophil counts. Ten dolphins recaptured 1–3 d after their first capture had significantly lower serum creatinine kinase, thyroid (T4) and globulin levels compared to values in dolphins sampled at nominal first capture. Although small sample sizes and large individual variation limit interpretation, these data indicate a stress response occurred in all dolphins, but the extent of the response is within the expected range for adaptive responses previously measured in limited numbers of wild mammals.

Key words: pantropical spotted dolphin, *Stenella attenuata attenuata*, stress, tagging, hematology, hormones, serum chemistry, catecholamines, capture myopathy, eastern tropical Pacific Ocean.

Tuna fishermen have used the association between tuna and dolphins to fish in the eastern tropical Pacific Ocean (ETP) for more than five decades (Perrin 1969, Allen 1985, National Research Council 1992). Some dolphin stocks, particularly the northeastern offshore pantropical spotted dolphin (*Stenella attenuata attenuata*) and the eastern spinner dolphin (*S. longirostris orientalis*), were depleted by high historical levels of incidental mortality in tuna purse seines (Wade 1995). Changes in the fishery during the last few decades have greatly reduced the observed annual mortality of dolphins from hundreds of thousands in the 1970s to about 1,000 in recent years (Inter-American Tropical Tuna Commission 2009). Gerrodette and Forcada (2005) reported that population recovery has not been observed as expected following this reduction in mortality (although see Gerrodette *et al.* 2008), and concerns about other potential impacts of the fishery, such as stress during chase, have been raised (*e.g.*, Curry 1999). To address this concern, a series of studies, referred to as the Chase Encirclement Stress Studies (CHESS), were undertaken to provide data on

physiological indicators of stress in chased and captured dolphins, and, if possible, to estimate a range of consequences for individual dolphins' movement, survival, and reproduction. CHESS attempted to evaluate the degree of acute (*i.e.,* rapid onset, short duration) physiological stress represented by a single chase and capture event and undertook to recapture individual dolphins to establish the potential for chronic and/or cumulative effects on various health indices. Diving behaviors and movements (Scott and Chivers 2009), heat flux (Westgate *et al.* 2007), and a variety of blood parameters were investigated; here changes in blood parameters are reported.

The mammalian stress response evolved as an adaptive response essential to survival that is well conserved amongst different species, yet if overactivated, it can be detrimental and even result in death (Selye 1936). A stressor can be physical or psychological, acute or chronic, yet all mammalian responses have a common pathway (Sapolsky 1992, Romero *et al.* 2009). Corticotropin-releasing hormone (CRH) is released from the hypothalamus in response to a perceived stressor. CRH acts as a neurotransmitter initiating catecholamine release and also activates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex to release glucocorticoids (cortisol and corticosterone). Glucocorticoids and catecholamines (epinephrine and norepinephrine) cause changes in organ function, such as gluconeogenesis, mobilization of neutrophil blood cells, and increased heart rate and muscle activity. They also modulate thyroid gland activity to enhance mobilization of energy stores by cortisol inhibition of thyrotropin secretion from the anterior pituitary (St. Aubin and Dierauf 2001). Changes in blood constituents typical of a mammalian stress response are thus elevations of catecholamines (epinephrine, norepinephrine, dopamine), ACTH, cortisol, aldosterone, glucose, and total white blood cells, and decreases in some thyroid hormones (St. Aubin and Dierauf 2001).

A substantial literature exists on blood constituents as indicators of the stress response in cetaceans (Thomson and Geraci 1986; Orlov *et al.* 1988; St. Aubin and Geraci 1988, 1989, 1992; Ortiz and Worthy 2000). Following the general relationships in other mammals, the stress response in marine mammals can be tracked by monitoring the activity of the pituitary, adrenal, and thyroid glands and the effects of their secretions on other aspects of homeostasis (St. Aubin and Dierauf 2001). Changes associated with stress and disease typically are recognized against baseline data that have been published for many species of small cetaceans (Ridgway *et al.* 1970; Koopman *et al.* 1995, 1999; St. Aubin *et al.* 1996; Bossart *et al.* 2001). Studies on stress in free ranging wildlife are challenging because the activities necessary to collect blood from which to measure stress responses generally cause stress themselves, confounding the results. Most hematological and serum chemical studies on wildlife are based on single samples representing a "snapshot" of the individual's condition (Bossart *et al*. 2001), with the perturbations inherent in sample collection viewed as an unavoidable, but sometimes controllable, variable. However, there are no such blood data for pantropical spotted dolphins. The current study therefore had three objectives: (1) develop hematology and serum chemistry data for free-ranging pantropical spotted dolphins, (2) examine these data for evidence of acute responses to the stress of chase and encirclement, and (3) evaluate findings in repeatedly captured dolphins for evidence of additive changes that would signal an inability to recover from the stress represented by each capture. To meet the first objective, a standard suite of blood parameters used in veterinary clinical practice to detect disease was evaluated (Hall *et al*. 2010). For the second and third objectives, analyses compared select blood parameters in spotted dolphins caught presumably

for the first time to those in dolphins known to have been recaptured based on the presence of a tag applied at first capture. These comparisons included a subset of blood parameters known to be altered during the stress response in other mammals (St. Aubin and Dierauf 2001).

METHODS

Dolphin Capture, Tagging, and Sampling

Dolphins were captured using a chartered tuna seiner working with the NOAA research ship *McArthur* from 18 August through 26 September 2001, about 200–750 km south of the southern coast of Mexico. Pantropical spotted dolphin herds, sometimes accompanied by eastern spinner dolphins or bottlenose dolphins (*Tursiops truncatus*), were usually sighted by the seiner's helicopter. When the seiner was correctly positioned, the net was deployed ("let go") in an attempt to encircle the dolphins. Dolphins were encircled, the bottom of the net was pursed, and two-thirds of the net was rolled on deck to limit movements of dolphins within the net circle. Individual dolphins swimming inside the net circle were captured by a team of swimmers and placed in partially flooded rafts tied to the cork line of the net. Calves were not targeted for sampling. Total body length and sex of each dolphin were recorded prior to sampling blood. Samples were collected variably from the tail flukes or the ventral caudal peduncle using 19- or 21-gauge needles directly into Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) treated with either Na-heparin or EDTA (ethylenediaminetetraacetic acid), or into untreated tubes containing separation gel for harvesting serum. Sample tubes were placed immediately on cold packs until processing in the shipboard laboratory within 1 h of collection. While dolphins were being processed in the rafts, swimmers captured other dolphins to attach visual tags (see below) without lifting the dolphins into the raft. Sampling was terminated when it was judged that the net was beginning to collapse (usually due to currents) or because dolphins swimming inside the net circle were becoming agitated. Once the last dolphin was sampled and returned to the water inside the net, the entire group was released using the normal back-down procedure (Coe and Sousa 1972).

Individual dolphins were tagged with either a radio tag or visual tag or a satellite-linked tag in support of a separate study (Scott and Chivers 2009). Visual tags were attached through the trailing edge of the dorsal fin (Fig. 1A, "roto tag": Dufflex sheep/goat model with an antibacterial coating on the pin, Destron Fearing, South St. Paul, MN). Small radio tags (<20 g in air) were low-power transmitters encased in a hydrodynamic polyethylene plastic capsule (Fig. 1B, "bullet tag": Trac Pak, Fort Walton Beach, FL) and attached with a single Dufflex tag pin from which the tag flaps had been cut away to the rear edge of the dorsal fin. Larger radio tags had a two-point attachment to the front edge of the dorsal fin and consisted of a radio transmitter (usually high-power) combined with a data logger that collected data for studies on dive behavior, swim speed, and heat flux ("thermal tags," Westgate *et al.* 2007; "TDR tags," Scott and Chivers 2009; Fig. 1C).

There were three categories of tagged dolphins. Focal dolphins were sampled and deployed with large radio tags to relocate groups on subsequent days. Focal dolphins were resampled when recaptured but these samples were considered separately during the analyses to avoid potential confounding of the results if these tags influenced

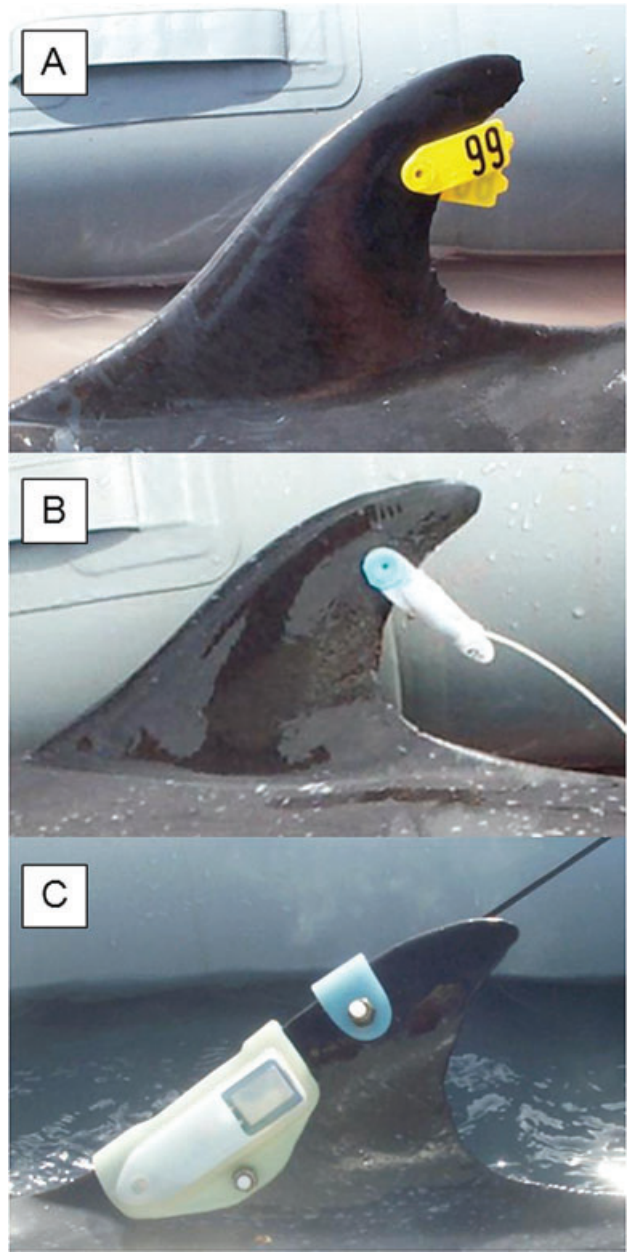

Figure 1. Types of tags attached to pantropical spotted dolphins, *Stenella attenuata attenuata*, during this study. Small tags were attached along the rear edge of the dorsal fin and included visual tags (A) and bullet tags (B). Large tags had a two-point attachment along the leading edge of the dorsal fin, as shown with the TDR tag (C).

stress parameters in subsequent blood samples. Dolphins that were sampled in the raft and released with a small tag (either a visual or bullet tag) had the highest priority for recapture to obtain a second blood sample. Dolphins that were tagged with small visual tags in the water (without an initial blood sample) were also targeted for recapture to obtain a recapture blood sample after a known period of time since the initial capture.

Sample Processing

Heparinized and EDTA blood samples were centrifuged for 5 min at 2,500 rpm, and plasma samples were frozen at −80°C, typically within 30 min after returning to the shipboard laboratory. Clotted samples were centrifuged and sera aliquotted and frozen during the ensuing 2 h. Concurrent with the processing of plasma and serum, EDTA-treated blood was analyzed for hematological constituents using an ABCDiff semiautomated analyzer (Heska Corp., Fort Collins, CO). Specimens were placed on a tube rocker to ensure mixing prior to analysis. All determinations were replicated and accepted only if results for white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), and platelet count (PLT) showed no more than 5% difference between a pair of runs. Rarely, a third determination was needed to resolve a questionable result, which was usually a case of operator error, and the outlier reading was ignored. Results are reported as the mean of the duplicate determinations. Blood smears were prepared in triplicate and stored for later differential cell counts at the Diagnostic Laboratory of the College of Veterinary Medicine at Cornell University, Ithaca, New York. Microhematocrits were determined in duplicate using standard techniques as a means of verifying the performance of the automated hematology analyzer. Paired microhematocrit tubes yielded measures that were identical or differed by only one percent (*e.g.*, 46% and 47% as duplicate hematocrits). Results showed a highly significant correlation to those reported by the analyzer ($r^2 = 0.76$, $P < 0.001$). Fibrinogen was determined in duplicate using the heat precipitation method of Millar *et al.* (1971).

Serum samples were analyzed for 28 constituents (electrolytes, metabolites, enzymes) at the Diagnostic Laboratory of the College of Veterinary Medicine at Cornell University, using a Hitachi 917 multichannel auto-analyzer (Roche Diagnostics Corp., Indianapolis, IN). Enzymes tested included alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (AP), amylase, creatinine kinase (CK), gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH). The samples were run together as a batch and were ultra-centrifuged prior to analysis to clear the samples of chylomicra and residual fibrin. Six aliquots of a pooled sample of serum were submitted blind to the laboratory and interspersed within the batch of samples to establish the confidence in the results of single determinations on the test samples. Coefficients of variation (CV) for the 32 determinations had a median value of 1.35%, with three constituents showing CVs in the range of 5%–10% (bicarbonate, creatinine, and uric acid) and one (anion gap) at 11.36%. Hormones were analyzed using commercially available kits following the manufacturer's instructions (Table 1).

Comparative Data

Due to the absence of published data for certain critical stress-associated constituents in blood from any cetacean species, samples were obtained for analysis of

Table 1. Hormonal analysis methodology, laboratory or kit supplier and intraassay (pooled sample) coefficients of variation (CV). Suppliers: 1 = Diagnostic Products Corp.; 2 = Nichols Institute Diagnostics, San Juan Capistrano, CA; 3 = Polymedco (Serono), Cortland Manor, NY; 4 = ARUP, Salt Lake City, UT.

Hormone	Method	Supplier	CV
Adrenocorticotropic hormone (ACTH)	Chemiluminescent enzyme immunoassay (Immulite)	1	2.0%
Aldosterone	Solid phase ^{125}I radioimmunoassay (Coat-a-count)	1	4.9%
Cortisol	Chemiluminescent enzyme immunoassay (Immulite)	1	9.9%
Total thyroxin (T4)	Solid phase ^{125}I radioimmunoassay (Coat-a-count). Sample volume doubled; 10 min incubation after addition to Ab tube; additional standard of 0.5 μg/dL	1	7.9%
Total triiodothyronine (T3)	Solid phase ^{125}I radioimmunoassay (Coat-a-count)	1	17.4%
Free thyroxin (fT4)	Equilibrium dialysis Solid phase ^{125}I radioimmunoassay	2	8.4%
Reverse triiodothyronine (rT3)	Double antibody ^{125}I radioimmunoassay	3	17.7%
Epinephrine	High performance liquid chromatography with electrochemical detection (detection limit = 10 pg/mL)	4	10.4%
Norepinephrine	High performance liquid chromatography with electrochemical detection	4	6.9%
Dopamine	High performance liquid chromatography with electrochemical detection (detection limit = 20 pg/mL)	4	8.9%
Testosterone	Solid phase ^{125}I radioimmunoassay (Coat-a-count)	1	7.7%
Progesterone	Solid phase ^{125}I radioimmunoassay (Coat-a-count)	1	4.8%
Estradiol	Solid phase ^{125}I radioimmunoassay (Coat-a-count). Ethel ether extraction; ^{3}H-estradiol used to test extraction efficiency and determine correction factor	1	13.6%

ACTH and cortisol from 26 free-ranging bottlenose dolphins captured and released in Sarasota Bay, Florida, in June, 2001, as described in Wells *et al.* (2004). Blood collection and handling procedures were virtually identical to those used for this study, and the samples were analyzed at the same laboratories. The principal differences between the two sets of data were that the bottlenose dolphins experienced little or no chase prior to encirclement, and virtually all the samples were collected within 60 min of deployment of the net.

Statistical Analysis

Hematology and serum chemistry data for the spotted dolphins sampled in this study were summarized using simple descriptive statistics (mean, standard deviation, median, and range). Differences between sexes were examined using standard analysis of variance (ANOVA) tests corrected for multiple testing. Acute stress responses were examined relative to two key events of capture and handling: (1) the time between the initiation of the chase (arrival of the tuna vessel's helicopter over the herd) and (2) the time between "tie-down" and sample collection. Tie-down is when two-thirds of the net has been recovered on board the purse seiner and secured; during standard fishing operations, this is when the backdown procedure to release dolphins is normally performed. In our study, swimmers entered the pursed net to capture the dolphins at tie-down (introducing a new stressor). Changes in blood constituents known to be altered during the stress response in other mammals (catecholamines, ACTH, cortisol, aldosterone, glucose, total white blood cells, and thyroid hormones; St. Aubin and Dierauf 2001) were evaluated relative to the time between these two events and blood sample collection, using generalized linear models (GLM) in the R software package (version 2.11.0; R Development Core Team 2010). The time interval (in minutes) was the predictor variable, and blood constituent values were the response variables.

Effects of repeated capture were evaluated using permutation tests (Efron and Tibshirani 1993), in which the means and cumulative distributions of blood constituents were compared between dolphins sampled at nominal first-capture and dolphins sampled at recapture. Permutation tests are free of many of the assumptions associated with parametric tests, and provide a simple method for evaluating significance of patterns within the data. In this study, we created 10,000 permutation samples by randomly shuffling all samples to reassign dolphins as nominal first-captures or recaptures. We then computed the mean value of the permuted "second captures" to provide a distribution of expected values in recaptured dolphins if all samples were drawn from a single population and there was no difference between captures. We also computed a Kolmogorov-Smirnov (KS) statistic comparing the distributions of blood constituent values at nominal first-capture and recapture.

In both permutations, subsets of the data for male and female dolphin samples were permuted separately and then recombined into a single data set. This ensured that the permuted data sets retained the actual sample sizes of each sex and avoided potential confounding effects of differences between sexes. The achieved significance level (ASL; defined as the probability of obtaining an outcome at least as extreme as the actual value) of the permutation test was estimated from the percentiles of the distribution of permuted means and KS statistics. An ASL of $P = 0.05$ provides reasonably strong evidence of a significant difference between nominal first capture and recapture, while $P = 0.01$ provides very strong evidence (Efron and Tibshirani 1993). Results are presented as histograms showing the permuted distributions of

Table 2. Summary of captured, tagged, and sampled dolphins in the 27 sets made during Chase Encirclement Stress Studies (CHESS) 2001. Sets 13—19 were made when the research vessel returned to port for reprovisioning and included only visual-tagging operations without blood sampling. Sat. = satellite, Recapt. = recaptured dolphin.

	With partial or complete blood sample						Number of dolphins	Without blood sample				Total number of
Set no.	Sat. tag	Visual tag	Bullet tag	TDR tag	Thermal tag	Recapt.	blood sampled	Sat. tag	Visual tag	TDR tag	Recapt.	dolphins processed
1	1	1		1			3					3
2		4	3	1			8			1		9
3			2			1	3	1				4
4											1	1
6	1	3	1	1			6					6
7	1	3				1	5		2			7
8		5				1	6		9			15
9		1					1		3			4
11				1		1	2					2
12						2	2					2
13									13			13
14									21			21
15									6			6
16									13			13
17									13			13
18									17			17
19									27			27
20				1			1					1
21		3		1		1	5		25			30
22		2				2	4		3			7
23	1	2	2		1		6		15			21
24		3				5	8					8
25	1	3			1		5		14			19
27		2	1	1		3	7					7
All	5	32	9	7	2	17	72	1	181	1	1	256

means and KS statistics relative to the observed mean and KS statistic for each blood constituent.

Results

Nominal First Capture Data

Blood was obtained from 72 individual dolphins, 55 of which were untagged and presumed to have been captured for the first time during the course of this study (Table 2). It is possible, however, that we had recently chased and encircled some of the latter individuals as well, because typically only a small minority of the dolphins could be sampled and tagged during a set and multiple sets occurred within the same general region. During 27 completed sets, the mean number of dolphins chased was about 700 (range 3–2,500), the average number of dolphins captured was 59 (range 0–298), while the number of dolphins handled and sampled during each set ranged from 1 to 9, with an additional 0–27 dolphins visual tagged without blood sampling (Table 2). Nine focal dolphins were radio tagged and tracked to allow the group to be recaptured (Westgate *et al.* 2007, Scott and Chivers 2009). The majority of the blood samples were collected from docile dolphins lying in the rafts, although occasionally the dolphins struggled, contributing to insufficient blood draws to support all of the intended analyses. If time was limited, blood tubes for plasma collection were

Table 3. Hematological data from 51 pantropical spotted dolphins, *Stenella attenuata attenuata*, sampled at nominal first capture during Chase Encirclement Stress Studies (CHESS) 2001.

	Units	Mean	SD	Median	Range
White blood cells (WBC)	10^3 cells/mm^3	10.1	2.5	9.7	6.0–18.6
Neutrophils (NEUT)	cells/mm^3	5,144	1,440	5,031	2,448–8,265
Bands	cells/mm^3	2	14	0	0–97
Lymphocytes (LYMPH)	cells/mm^3	2,462	1,305	2,142	213–7,440
Monocytes (MONO)	cells/mm^3	316	169	291	0–836
Eosinophils (EOSIN)	cells/mm^3	2,050	964	1,944	424–5,580
Basophils (BASO)	cells/mm^3	78	96	61	0–506
Platelets (PLT)	cells/mm^3	152	44	143	17–263
Mean platelet volume	fm^3	11.1	0.9	11.2	9.4–13.3
Red blood cells (RBC)	10^6 cells/mm^3	4.7	0.3	4.7	4.0–5.6
Hemoglobin (HGB)	g/dL	16.6	0.8	16.7	15.3–18.7
Hematocrit (HCT)	%	46.8	2.3	47.2	42.1–51.9
Mean cell hemoglobin (MCH)	pg	35.8	1.9	36.0	30.3–41.3
Mean cell hemoglobin concentration (MCHC)	g/dL	35.6	0.6	35.6	34.5–37.4
Mean cell volume (MCV)	fm^3	100.5	4.6	101	85.5–110.5

prioritized as these would allow analysis of constituents most likely to be affected by a stress response. The dolphins were in the rafts for 4–18 min before being released.

At nominal first capture, hematology data were obtained from 51 dolphins (Table 3), and serum chemistry and hormone values were obtained from 53 individuals (Table 4, 5). Not all constituents could be examined for all individuals because of occasional incomplete blood draws caused by handling logistics. Significant differences among sexes were identified only for three thyroid hormones that were greater in males ($n = 26$) than females ($n = 25$): reverse triodothyronine (rT3, with mean and standard errors of 1.68 ± 0.08 *vs.* 1.21 ± 0.07 ng/dL for males and females, respectively, $P < 0.01$), total thyroxin (T4, 7.69 ± 0.34 *vs.* 5.58 ± 0.30 ng/dL, $P < 0.01$), and free thyroxin (fT4, 3.61 ± 0.18 *vs.* 2.52 ± 0.14 ng/dL, $P < 0.01$). One female dolphin (D012) had an elevated progesterone level of 18 ng/mL, typical of pregnancy (Robeck *et al.* 1998).

Acute Effects of Nominal First Capture

The time of blood collection relative to the beginning of fishing operations varied because of the variability in such factors as the time and distance required to approach a dolphin herd prior to let go, the amount of net that had to be let out to encircle the herd and then retrieved, the occurrence of a variety of difficulties in retrieving the net and keeping the net circle from collapsing, and the number of dolphins captured and processed. The beginning of the chase, when the helicopter first circled above the dolphins, was considered the time at which dolphins could first be stressed by a specific capture operation. Time between first appearance of the helicopter and blood sampling was positively correlated with platelet and WBC counts and mean cell hemoglobin concentrations (MCHC; Fig. 2). The net tie-down, when backdown procedures are normally initiated, was the time at which swimmers entered the water

Table 4. Serum chemistry data from pantropical spotted dolphins, *Stenella attenuata attenuata*, at nominal first capture. *n* = sample size.

Constituent	Units	*n*	Mean	SD	Median	Range
Sodium (Na)	mEq/L	53	154.7	2.9	154	150–171
Potassium (K)	mEq/L	53	4.0	0.7	4.0	2.8–6.7
Chloride	mEq/L	53	119.2	3.1	120	113–128
Na:K		53	39.4	6.0	39	23–55
Bicarbonate	mEq/L	53	22.2	3.4	22	10–29
Calcium	mg/dL	53	8.9	0.5	8.8	7.7–10
Phosphorous	mg/dL	53	5.7	1.5	5.6	3.1–9.0
Iron	ug/dL	53	123.5	38.3	121	48–227
Magnesium	mEq/L	53	1.6	0.2	1.6	1.3–2.2
Glucose	mg/dL	53	1, 365.8	24.1	135	92–215
Urea	mg/dL	53	67.8	12.2	70	44–91
Uric Acid	mg/dL	53	0.9	0.6	0.8	0.2–3.9
Creatinine	mg/dL	53	0.8	0.2	0.8	0.4–1.5
Direct bilirubin	mg/dL	53	0.10	0.03	0.10	0.00–0.20
Indirect bilirubin	mg/dL	53	0.08	0.07	0.10	0.00–0.30
Total bilirubin	mg/dL	53	0.17	0.07	0.20	0.10–0.40
Cholesterol	mg/dL	53	231.5	52.7	227	129–338
Triglycerides	mg/dL	53	197.4	134.4	166	32–506
Total Protein	g/dL	53	7.2	0.6	7.0	6.0–9.0
Albumin (A)	g/dL	53	3.7	0.2	3.8	3.0–4.0
Globulin (G)	g/dL	53	3.5	0.6	3.4	2.7–5.3
A:G		53	1.1	0.2	1.0	0.7–1.4
Fibrinogen (FIB)	mg/dL	48	369.3	119.5	351.5	204.9–936.4
Alkaline phosphatase (AP)	U/L	53	377.0	191.6	357	75–935
Alanine transaminase (ALT)	U/L	53	127.0	40.3	118	61–258
Aspartate transaminase (AST)	U/L	53	334.0	66.7	331	182–520
Amylase	U/L	53	1.04	0.28	1.00	1.00–3.00
Creatinine kinase (CK)	U/L	53	262.9	89.3	258	127–560
Gamma glutamyl transferase (GGT)	U/L	53	30.5	4.2	30	22–39
Lactate dehydrogenase (LDH)	U/L	53	619.0	128.2	612	450–1,308

to capture dolphins. Time between tie-down and blood sampling was positively correlated with platelet, WBC, and eosinophil counts, but not with MCHC (Fig. 2). Cortisol levels did not show any trends with time (Fig 2).

Effects of Recapture and Tagging

Ten dolphins fitted with small tags (nine visual tags and one bullet tag) were recaptured and sampled (Table 6). Two of these dolphins had blood sampled when initially captured and when recaptured; the other eight dolphins were tagged on first capture while restrained in the water alongside an inflatable raft and had blood sampled only when recaptured. Six of the 10 dolphins were recaptured after one day, one was recaptured after two days, and three after three days (Table 6). All dolphins recaptured with tags of any type were blood sampled if possible. Six of the nine focal dolphins fitted with larger tags with radio transmitter/data-logger combinations (*e.g.*, Fig. 1C) were recaptured and resampled (Table 6). Two dolphins were sampled

Table 5. Blood hormone data from pantropical spotted dolphins, *Stenella attenuata attenuata*, at first capture. *n* = sample size; See Tables 1–2 for definitions of abbreviated constituent names.

Constituent	Units	*n*	Mean	SD	Median	Range
ACTH	pg/mL	51	462.8	306.2	356.0	90.1–1,532.0
Cortisol	ug/dL	53	5.04	1.25	4.99	2.57–7.95
Aldosterone (ALDO)	pg/mL	53	131.7	74.3	124.3	5.0–305.5
Epinephrine (EPI)	pg/mL	37	181.0	265.6	118.5	38.0–1,641.0
Norepinephrine (NOREPI)	pg/mL	37	852.5	506.9	682.0	270–2,410
Dopamine (DOPA)	pg/mL	37	136.3	68.4	130.0	46–429
Total T4	ug/dL	51	6.66	2.01	6.16	3.23–10.87
Total T3	ng/mL	51	1.05	0.29	1.00	0.60–1.99
Free T4	ng/mL	51	3.08	1.05	2.95	1.55–6.20
Reverse T3	ng/mL	51	1.45	0.47	1.40	0.53–2.73
rT3/T3		51	1.45	0.57	1.28	0.65–2.93
Testosterone	ng/mL	26	5.5	8.9	1.0	0.02–35.1
Estradiol	pg/mL	23	16.4	4.2	16.9	8.6–24.2
Progesterone	ng/mL	24	1.6	4.0	0.4	0.17–18.1

on the day following application of the tag, two were sampled after two days, one after three days, and one of the dolphins sampled after two days (D029) was sampled again six days after the initial capture (Table 6).

Hematology data from the 10 recaptured dolphins with small tags were compared to the population sampled at first handling. For the purposes of this analysis, the first-capture blood samples from the two resampled dolphins with small tags (D067 and D034) were excluded from the baseline population to maintain the discreteness of the two groups. The sex composition of the recaptured group (3 females, 7 males) differed from that of the first capture sample to which it was compared (26 females, 25 males), but the permutation test accounted for this difference. Based on the permutation

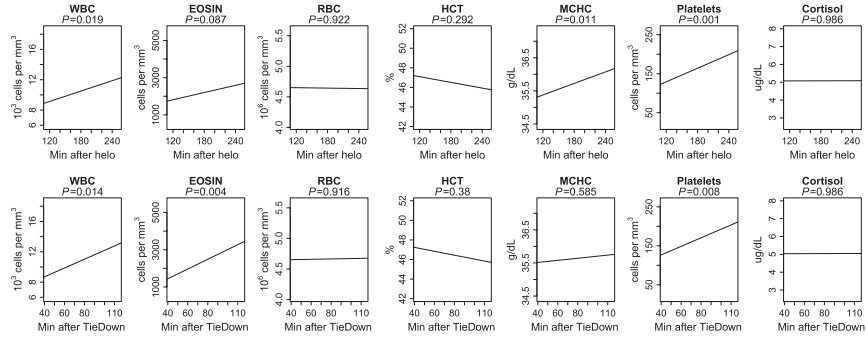

Figure 2. Results of the regression analysis examining acute effects of capture in select blood constituents relative to initiation of chase, when the helicopter arrived over the dolphins (top row), and relative to "tie-down," when swimmers entered the water and handling began (bottom row). See Table 3 for blood constituent abbreviations.

Table 6. Hematology parameters in pantropical spotted dolphins, *Stenella attenuata attenuata*, recaptured after tagging. See Tables 3–4 for definitions of parameter abbreviations and measurement units.

Tag type	ID	Day	WBC	RBC	HGB	HCT	PLT	MCV	MCH	MCHC	FIB	NEUT	Bands	EOSIN	MONO	LYMPH	BASO
Bullet	D067	0	8.5	4.39	15.8	44.3	133	101	35.95	35.7	426	3,910	0	2,635	340	1,615	0
Bullet	D067	1	8.1	4.31	15.25	43.4	138	101	35.45	35.2	345	4,455	81	1,701	405	1,458	0
Visual	D034	0	12.9	4.68	17.05	47.4	220	102	36.55	36.1	391	7,611	0	3,096	387	1,806	0
Visual	D034	2	12.1	4.69	17.2	48.0	232	103	36.7	35.9	401	6,266	0	2,892	241	2,410	241
Visual	D193	1	7.5	4.11	15.45	44.1	89	107	37.7	35.2	243	3,725	0	1,788	223	1,713	0
Visual	D203	1	7.1	4.64	17.85	51.0	161	110	38.45	35.0	251	4,615	0	710	355	1,349	71
Visual	D209	1	6.2	4.63	16.45	47.3	114	102	35.55	34.8	280	3,286	0	806	124	1,984	0
Visual	D215	1	9.7	4.67	15.25	43.7	117	93	32.65	35.0	312	6,658	0	1,445	193	1,254	98
Visual	D242	1	12.5	4.28	15.75	44.3	78	104	36.85	35.7	175	7,125	0	3,125	375	1,875	0
Visual	D244	3	15.5	4.59	16.65	46.9	125	102	36.3	35.6	324	11,278	0	1,694	463	2,016	0
Visual	D245	3	11	4.58	17.2	48.6	125	106	37.6	35.4	360	7,590	0	1,199	327	1,744	109
Visual	D257	3	9.7	4.61	16.6	46.6	115	101	35.95	35.6	315	5,432	0	2,231	388	1,552	97
TDR	D019	0	10.6	4.75	16.6	47.5	136	100	35.0	35.0	455	7,950	0	424	0	2,226	0
TDR	D019	2	16.4	4.46	15.8	44.3	74	99	35.45	35.7	498	14,552	0	654	164	981	0
TDR	D029	2	28.7	4.27	15.05	41.8	267	98	35.25	36.1	437	25,212	0	1,146	860	1,433	0
TDR	D029	6	26	4.26	15.15	41.0	131	96	35.55	37.0	775	20,800	0	1,690	1,690	1,820	0
TDR	D042	0	11.4	5.33	17.2	49.4	165	93	32.35	34.9	347	5,675	0	1,249	227	4,200	0
TDR	D042	1	12.1	4.56	15.35	42.3	143	93	33.7	36.3	352	11,374	0	121	0	605	0
TDR	D047	0	9.7	4.9	16.05	46.5	190	95	32.8	34.6	516	6,080	97	1,255	579	1,544	97
TDR	D047	2[a]															
Thermal	D063	0	9.5	4.49	16.35	46.2	175	103	36.3	35.4	302	5,605	0	1,710	95	1,995	95
Thermal	D063	1	14.4	4.47	16	46.0	163	103	35.85	34.8	280	11,624	0	1,148	431	1,005	144
Thermal	D227	0	8.9	4.85	17.05	48.4	93	100	35.15	35.2	372	7,031	0	801	267	801	0
Thermal	D227	3	13.7	4.54	16.5	45.7	81	101	36.25	36.1	527	11,603	0	683	410	956	0

[a]No blood sample obtained.

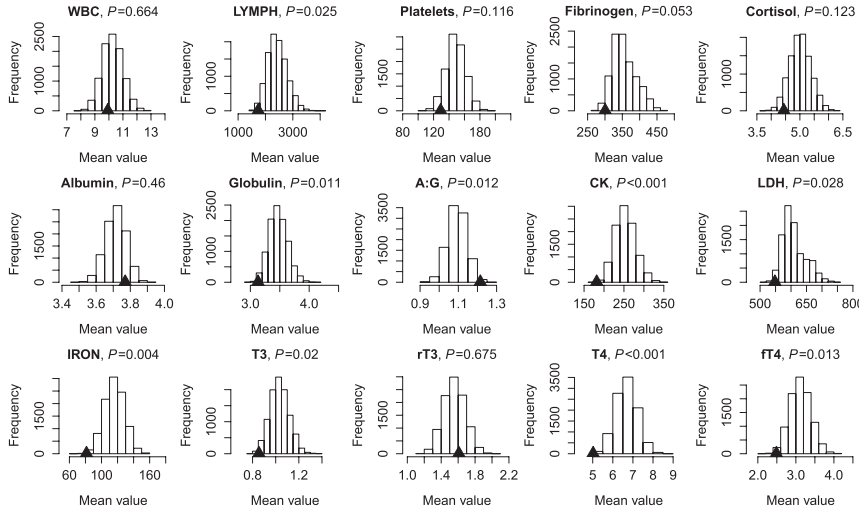

Figure 3. Results of the permutation tests examining differences in mean values of select blood constituents for nominal first capture and recapture. The small triangle indicates the observed mean value for 10 recaptured dolphins; the histogram represents the expected range of mean values if there were no difference between dolphins at nominal first-capture and recapture. See Tables 3–5 for blood constituent abbreviations.

tests examining differences in mean values of stress-related blood constituents for dolphins at nominal first capture *vs*. recapture, there were significantly lower mean lymphocytes, fibrinogen, globulin, CK, LDH, iron, T3, T4, and fT4 and significantly greater albumin:globulin (A:G) ratios in recaptured dolphins (Fig. 3). Cumulative distributions of the blood constituents among sampled dolphins, as measured by the KS statistic, revealed significant differences in the distributions of CK, A:G, T4, and platelets (Fig. 4). None of the stress hormones (aldosterone, cortisol, epinephrine, dopamine, ACTH or norepinephrine) differed significantly in mean values or the distribution of values between nominal first capture and recapture.

Due to the small number of samples available from recaptured dolphins and the small number of repeat samples from dolphins with larger tags, the effects of tag size and attachment method on blood parameters was not evaluated statistically. The hematology data (Table 6) for one dolphin (D029) with a large tag revealed an elevated WBC two days after tagging (28,650/mm^3), which decreased slightly by six days post tagging but was still high at 26,000/mm^3. However, no WBC data were obtained during this dolphin's initial capture, so it is unknown whether the elevated WBC was associated with the tag or with a pre-existing condition in this dolphin.

Comparative Data

Mean (and standard deviation, SD) of the ACTH levels in 26 bottlenose dolphins captured in Sarasota was 246.2 (SD = 219.3) pg/mL, compared with the mean value of 462.8 (SD = 306.2) pg/mL in the 51 spotted dolphins sampled in this study.

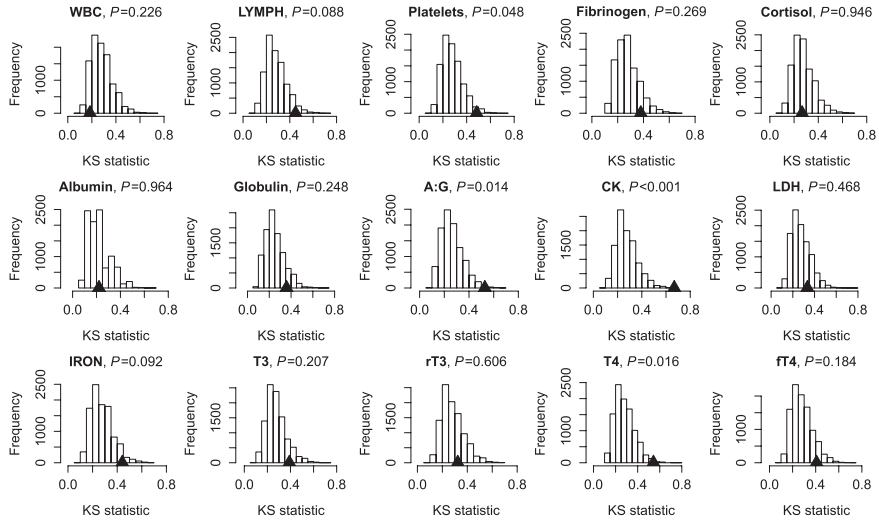

Figure 4. Results of the permutation tests examining differences in cumulative distributions of select blood constituent values for nominal first capture and recapture. The small triangle indicates the Kolmogorov-Smirnov (KS) statistic for the observed data; the histogram represents the expected range of KS statistics if there were no difference between dolphins at nominal first-capture and recapture. See Tables 3–5 for blood constituent abbreviations.

Discussion

This analysis of blood samples collected from pelagic spotted dolphins has provided significant new data on hematological and serum chemical constituents for this species in the wild and contributed new information for hormones such as ACTH and the catecholamines. Most blood data available to date from small cetaceans have been developed from individuals under human care and thus possibly influenced by changes in diet and husbandry, with the notable exception of data for bottlenose dolphins from inshore populations (Bossart *et al.* 2001, Hall *et al*. 2007). Although the blood data presented in this study are the first available for wild spotted dolphins, they are presumably influenced by the effects of capture and restraint, unlike data available for some captive species with individuals trained to allow blood sampling following voluntary presentation of a fluke. In this study, a further confounding factor was that the time since the last capture, either by our research operations or by the purse-seine fishery (see Archer *et al.* 2010), was not known for most of the sampled dolphins except for the recaptured tagged dolphins. The difficulties inherent in successfully capturing any individual (*e.g.*, weather, dolphin evasion, identifying tagged individuals in herds of several hundred dolphins) and the lack of group cohesion from one day to the next interfered with our ability to repeatedly resample individual dolphins and thereby address effects of recapture.

Furthermore, the logistics involved in collecting samples were such that blood could not be collected any sooner than 1.6 h, and as much as 4 h, after the initial disturbance caused by helicopter overflight. However, this temporal heterogeneity afforded an opportunity to determine whether any of the perturbations presumed to be indicative of a stress response were developing within this sampling window.

For some of the constituents examined, such as cortisol, glucose, and the various leucocytes, this time interval falls within the period of expected maximal effect of an acute stress response in mammals (Sapolsky 1992). It might be expected that dolphins subjected to the continuous stress of confinement and gradual constriction of the space around them would express these changes to an increasing degree over time. In fact, few such associations were detected statistically, and several of those that were detected ran counter to the anticipated direction of change.

Glucocorticoids

The most obvious indications of an acute stress response in these spotted dolphins are the levels of ACTH, catecholamines, cortisol, and glucose. Stimulation with exogenous ACTH produces elevations in cortisol that peak after 1–2 h in bottlenose dolphins and beluga whales (*Delphinapterus leucas*; Thomson and Geraci 1986, St. Aubin and Geraci 1990), which corresponds to the average time between "let go" and blood sampling in this study. Hyperglycemia is a generally accepted consequence of elevated cortisol levels and results from suppression of insulin and stimulation of gluconeogenesis in the liver (Orth and Kovaks 1998). Cortisol values were higher than most other reported values for odontocetes including bottlenose dolphins captured by net encirclement (St. Aubin *et al.* 1996, St. Aubin and Dierauf 2001), with the exception of those in harbor porpoises (*Phocoena phocoena*) sampled after periods of 1–3 d of entrapment in fishing weirs and then captured by seine net (Koopman *et al.* 1995), and Yangtze finless porpoises (*Neophocaena asiaeorientalis*) sampled after a boat chase and net capture (Hao *et al*. 2009).

Glucose levels in these pantropical spotted dolphins were elevated above those reported for most odontocetes, which typically average approximately 110 mg/dL (Bossart *et al.* 2001). As further evidence of stress-associated hyperglycemia in odontocetes, levels in weir-impounded harbor porpoises averaged nearly 200 mg/dL (Koopman *et al.* 1995) and Yangtze finless porpoises sampled after chase and capture had glucose levels that correlated with the time out of water before sampling (Hao *et al.* 2009).

Although circulating levels of ACTH in the spotted dolphins were markedly higher than those recorded for captive belugas trained to present their flukes for sampling (6.6 ± 5.8 pg/mL; Schmitt *et al.* 2010), and about double those for bottlenose dolphins caught, sampled and released in Sarasota Bay in support of this study, the dynamics of this hormone in odontocetes have not yet been determined. However, elevations are detected in humans within 10–30 min after stimulation by exogenous corticotropin releasing hormone or intense exercise and then diminish as cortisol levels rise (Orth and Kovacs 1998, Singh *et al.* 1999). The apparent persistence of significantly elevated levels of ACTH in these spotted dolphins implies that either confinement within the net constituted an on-going source of acute stress (although the dolphins did not appear to be agitated prior to capture attempts by the swimmers), or, more likely, chasing by swimmers, handling, and sampling for this study caused restimulation of the hypothalamic-pituitary axis for these dolphins.

There was also no consistent temporal change in the levels of aldosterone. This is perplexing given the consistency of the response of this hormone to stimulation by exogenous ACTH in cetaceans (Thomson and Geraci 1986, St. Aubin and Geraci 1990). It has been hypothesized that the release of aldosterone as part of the stress response in marine mammals is an adaptation to maintain fluid and electrolyte

balance under duress (Geraci 1972, St. Aubin and Geraci 1986, St. Aubin *et al*. 1996, St. Aubin and Dierauf 2001). Repeated stimulation of the adrenal cortex by ACTH can lead to a state known as "aldosterone escape," in which the *zona glomerulosa* shows a diminished response, so preventing continued loss of sodium through the kidneys when aldosterone levels are elevated for prolonged periods (Turban *et al.* 2003). If this phenomenon, which is well documented in humans, occurs in dolphins, it could explain the lack of consistent temporal changes in aldosterone levels observed in this study.

Catecholamines

Stimulation of the adrenal medulla is typically one of the first events in the mammalian stress response. The release of catecholamines, principally epinephrine (EPI), has a host of cardiovascular, visceral, and metabolic effects that contribute to a general state of preparedness to flee or fight (Young and Landsberg 1998). Other catecholamines, such as norepinephrine (NOREPI) and dopamine (DOPA), are primarily neurotransmitters, and their appearance in circulation typically reflects enhanced neurological activity. As for some of the other constituents analyzed in this study, published data for catecholamine levels in odontocetes are meager. Thomas *et al.* (1990) reported levels of 0–101 pg/mL for EPI and 160–604 pg/mL for NOREPI in captive beluga whales, both resting and subjected to high amplitude playbacks of drilling rig noise. Romano *et al.* (2004) reported levels of 17–95 pg/mL for EPI, 37–114 pg/mL for DOPA and 461–1,450 pg/mL for NOREPI in a beluga whale exposed to impulsive sounds by a water gun. By contrast, free-ranging beluga whales captured after a 5–15 min chase had levels averaging 634 and 1,423 pg/mL for EPI and NOREPI, respectively (St. Aubin and Geraci, unpublished data). Levels for the spotted dolphins showed particularly large variation, with mean levels of EPI and NOREPI lower than for the wild-caught beluga whales but maximum values higher than for the same wild beluga whales. The findings in spotted dolphins are therefore indicative of an ongoing adrenergic stress response that is lower than responses measured in some belugas and consistent with the consequences of exertion.

The persistent elevations of the catecholamines in the spotted dolphins are noteworthy. These substances are usually characterized by rapid clearance from the bloodstream (Young and Landsberg 1998), and levels of NOREPI would have had sufficient time to normalize if the chase had been the principal stimulus for release of this neurotransmitter. The presence of the sampling rafts and activities of the swimmers could well explain almost instantaneous and repeated spikes throughout the sampling period. Dolphins in the seine net were continually swimming but not exerting themselves to the same degree as during the chase (Westgate *et al.* 2007). The precise role of DOPA, whether as part of a neural or endocrine-based system, has not been resolved, in part because resting levels are typically very low in the blood (Young and Landsberg 1998). The marked and prolonged elevations of this substance in the spotted dolphins are therefore enigmatic at this time.

White Blood Cell Counts

Total white blood cell counts (WBC) for the spotted dolphins were generally higher than values reported for a variety of other species of captive odontocetes, but typical of samples drawn from free-ranging marine mammals (Bossart *et al.* 2001,

Hall *et al.* 2007). In some of the untagged spotted dolphins, total WBC counts exceeded 15,000 cells/μL, levels that are associated with active inflammation in captive bottlenose dolphins (Reidarson *et al.* 1998), however other markers of inflammation such as fibrinogen were not elevated. Blood cellular changes characteristic of the stress response are termed the "stress leucogram," which consists of depressions of eosinophil and lymphocyte counts (eosinopenia and lymphopenia) that are offset by increased numbers of circulating neutrophils (neutrophilia) and result in a net elevation in total white blood cell count (Thomson and Geraci 1986; St. Aubin and Geraci 1989, 1990). Thus the temporal pattern expected was that lymphocyte and eosinophil counts, and levels of T3 and fT4, would decrease during the sampling window, but later samples in fact had higher values than earlier samples. Eosinophils in fact increased with time since the tie-down, contrary to the expected decrease in eosinophils with stress reported in most domestic mammals (St. Aubin and Dierauf 2001). Interestingly, an increase in platelet counts was observed in the first captures, with both time since helicopter overflight and time since tie-down correlating significantly with platelet counts. Although not typically considered to play a role in the stress response, these blood corpuscles important in the clotting pathway have been demonstrated to increase following emotional stress in humans, potentially contributing to the link between stress and coronary heart disease (Levine *et al*. 1985).

Enzymes

A suite of enzymes are found in muscle tissue and released into the circulation following excessive exertion with lysis of muscle fibers. At extreme levels, capture myopathy may occur, which in some species has fatal consequences (Spraker 1993). In cetaceans, damage to the cardiac muscle in addition to skeletal muscle has been observed and is common in stranded odontocetes, presumably due to the stress of stranding (Turnbull and Cowan 1998, Herráez *et al.* 2007). Elevated blood levels of CK and AST have been documented in stranded striped dolphins (*Stenella coeruleoalba*) and were presumed to be a consequence of skeletal muscle damage (Gales 1992). Mean levels of CK, AST, and LDH at nominal first capture were all elevated relative to reported values for several species of captive odontocetes (Bossart *et al.* 2001), but were similar to those reported for net-impounded harbor porpoises (Koopman *et al.* 1995) and lower than levels in stranded dolphins (Gales 1992). Thus, these data suggest some ongoing muscle damage was associated with the stress of capture. Interestingly, CK and globulin levels were lower in the dolphins recaptured after tagging than in the nominal first capture group. This could potentially be due to the half lives of CK and globulin being several days, and the possibility that dolphins had actually been caught prior to the first captures in events leading to greater muscle damage than the capture event at which they were blood sampled.

Conclusions

In conclusion, the three objectives of this study: (1) to develop hematology and serum chemistry data for free-ranging pantropical spotted dolphins; (2) to examine these data for evidence of acute responses to the stress of chase and encirclement; and (3) to evaluate findings in repeatedly captured dolphins for evidence of additive

changes that would signal an inability to recover from the stress during capture were achieved, but were limited by considerable logistic difficulties resulting in small sample sizes of repeatedly sampled dolphins. Detailed analyses of discrete parameters suggest that chase and encirclement of dolphins by a tuna purse seiner results in a measurable stress response typical of odontocetes. The response is characterized by elevated blood catecholamine, cortisol, and ACTH levels, as well as a mild elevation of enzymes released from muscle following exertion. Although the considerable individual variation in most parameters limited interpretation, the magnitude of the stress response was generally greater than that observed in bottlenose dolphins known to survive following sampling during live-capture-release operations. Enzyme levels reflecting muscle damage were lower than those measured in mass stranded odontocetes that likely had acute muscle damage. There were remarkably few changes in blood parameters in recaptured dolphins, and stress hormones in particular showed no differences between the nominal first capture and recapture groups. The long-term consequences, however, of the acute activation of the stress response during capture on survival and reproduction cannot be determined from these data. Future studies to evaluate stress in free-living dolphins could incorporate novel, less invasive methods, such as genomics and proteinomics on skin biopsies (Mancia *et al.* 2008), to evaluate chronic, long-term, effects of stress on reproduction and survival of individuals and populations.

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